



The University of
Nottingham

**Immune Responses to Hepatitis C Virus (HCV):
Investigation of the Role of L-ficolin
and Anti-E1E2.**

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Abstract

Hepatitis C virus (HCV) causes acute and chronic liver diseases in humans. Its two envelope proteins, E1 and E2, are heavily glycosylated. They interact with host cell receptors and provide a target for host immune recognition. The host virus interactions determine the pathogenesis and outcome of HCV infection.

L-ficolin is a soluble pattern recognition molecule of importance in innate immune defence against microorganisms. It activates the lectin complement pathway upon binding to carbohydrate recognition patterns on microorganisms. It was hypothesised that L-ficolin could interact with HCV glycoproteins. Both recombinant and serum derived L-ficolin were investigated for binding to the envelope glycoprotein E1E2 of HCV. Specific, dose-dependent binding of L-ficolin to HCV glycoprotein E1E2 was observed. The interaction between L-ficolin and HCV particles in infected sera was also demonstrated. Binding of L-ficolin to HCV pseudoparticles expressing E1E2 glycoproteins resulted in neutralisation of virus infectivity. The serum L-ficolin level was significantly higher in patients with mild HCV liver fibrosis compared to patients with severe HCV liver fibrosis. These results suggest a potential protective effect of L-ficolin, as an innate immune defence, against HCV infection.

To study the role of anti-HCV E1 and E2 (anti-E1E2) in HCV disease, the levels of anti-E1E2 antibodies were evaluated in 230 sera of patients with chronic hepatitis C by enzyme-linked immunosorbent assay. The antigens used were recombinant HCV glycoproteins derived from genotype 1 (H77c) and genotype 3 (UKN3A1.28). Seroreactivity was greater when sera were tested against antigen derived from their homologous genotype than against heterologous antigen. The seroreactivity was inversely proportional to the viral load and to the degree of liver fibrosis. These results demonstrate that seroreactivity against E1E2 depends upon the genotypic origin of the E1E2 antigens and the infecting genotype, and suggest a possible protective effect of anti-E1E2 against disease progression.

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Abbreviations

aa	Amino acid
AP	Alkaline phosphatase
APCs	Antigen presenting cells
BGH	Bovine growth hormone
Bp	Base pair
BSA	Bovine serum albumin
C1q	Subunit of C1 enzyme complex
C2	Complement component 2
C3	Complement component 3
C4	Complement component 4
C5	Complement component 5
CDC	Centers for disease control and prevention
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CTL	Cytotoxic T lymphocyte
C-terminus	Carboxy-terminus
CV	Column volume
CysNAc	N Acetyl L-Cysteine
DC	Dendritic cells
DC-SIGN	DC-Specific ICAM-3 grabbing non-integrin
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic reticulum
ESLD	End stage liver disease
EVR	Early virologic response
FBS	Foetal bovine serum
GlcNAc	N-acetyl glucosamine
GNA	<i>Galanthus nivalis</i> agglutinin
gp	Glycoprotein
H77c	Genotype 1 HCV strain

HAI	Histological activity index
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	HCV cell culture
HCVpp	HCV pseudoparticle
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HLA	Human leucocytic antigen
HPA	Health Protection Agency
HRP	Horse radish peroxidase
Huh 7	Human hepatoma cells
HVR	Hypervariable region
IC50	Half maximal inhibition concentration
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IMAC	Immobilised metal affinity chromatography
IMAGE	Integrated Molecular Analysis of Genomes and their Expression
INF	Interferon
IRES	Internal ribosome entry site
IVDU	Intravenous drug use
JVH1	Japanese fulminant HCV strain-1
kDa	Kilo Dalton
LDL	Low density lipoprotein
L-SIGN	Liver/Lymph specific ICAM-3 grabbing integrin
MASP	MBL associated serine protease
MBL	Mannan binding lectin
MHC	Major histocompatibility complex
min	minute
MLV	Murine leukaemia virus
MSM	Men who have sex with men
NCR	Non coding region
NEAA	Non essential amino acids
NHCV	Non HCV

NK	Natural killer
NS	Non structural
N-terminus	Amino-terminus
OAS	2'5'-oligoadenylate synthetase
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
p	Probability value
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PKR	dsRNA-dependent Protein kinase R
pNPP	p-Nitrophenyl phosphate
R/NR	Relapsers/Non responders
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
RVR	Rapid virologic response
SDS	Sodium dodecyl sulphate
sec	Second
sMAP	Small MBL associated protein
SNP	Single nucleotide polymorphism
SR-B1	Scavenger receptor class B, number 1
SVR	Sustained virologic response
TAE	Tris acetate EDTA buffer
TBS	Tris buffered saline
Tc	T cytotoxic cell
Th	T helper cell
TMB	3,3',5,5'-tetramethylbenzidine
UKN2B1.1	Genotype 2 HCV strain
UKN3A1.28	Genotype 3 HCV strain
VLDL	Very low-density lipoprotein

1. Introduction

1.1. Hepatitis C Virus

The Hepatitis C virus (HCV) was first recognised in 1989 as a parenterally transmitted agent responsible for most transfusion-acquired non-A, non-B hepatitis (NANBH) cases. It was identified by Choo et al., who cloned a complementary DNA (cDNA) of the virus from a patient diagnosed with NANBH [1]. The virus was termed Hepatitis C virus (HCV), and since then it has become a target for painstaking research. HCV infection is a significant global health problem due to viral persistence and progression to chronic hepatitis, leading to cirrhosis and increased risk of hepatocellular carcinoma (HCC). It is also the leading factor for liver transplantation worldwide [2].

1.1.1. Epidemiology of HCV Infection

World Health Organisation (WHO) estimates that about 170 million people (3% of world population) are infected with HCV around the world [3]. Epidemic proportions of HCV infection have been reached and more than one million new cases of infection are reported annually, worldwide [4]. The prevalence of HCV in the UK is less than 1% and it varies widely in different parts of the world, ranging from 0.003% in Sweden up to around 20% in highly endemic areas such as Egypt [5, 6]. This variation partly reflects different modes of HCV transmission. The high prevalence rate reported from Egypt is attributed to the use of non-sterile

needles during the Egyptian parenteral antischistosomal therapy mass-treatment campaigns in the 1960s-70s [5]. Some worrying data suggest that the silent epidemic of HCV will continue to remain a major health hazard in the developing world, at least in the near future [6].

HCV is transmitted primarily by parenteral routes such as blood transfusion, intravenous drug use (IVDU), tattoos, contaminated medical equipments, and rarely by sexual and vertical routes. Although the incidence of transfusion-associated HCV infection in developed countries has been reduced since the 1990s, due to improved blood donor screening [3, 7], injecting drug use accounts for most newly acquired HCV infections [8, 9]. Blood transfusions from unscreened donors and unsafe therapeutic procedures are still the main modes of transmission in developing countries [6]. Centers for disease control and prevention (CDC) reported that the risk of HCV transmission per needle-stick exposure in health care workers is 1.8% [10]. Sexual transmission of HCV can occur, especially in long-term partners of HCV-infected patients. However, this could be partly due to sharing of sharp kits such as razors and toothbrushes [11, 12]. HCV infection has also been reported in HIV-positive men who have sex with men (MSM) [13] and even, although at a lower prevalence, in MSM who are not injecting drug users [14]. The rate of perinatal transmission, which is the commonest cause of transmission of HCV infection to children, is 4-7% per

pregnancy in women with detectable viraemia [15]. In all cases, a greater risk of transmission is associated with HIV co-infection.

HCV infection is diagnosed serologically by detection of anti-HCV antibodies to structural and non-structural proteins or by detection of HCV ribonucleic acid (RNA) in the serum [16].

Cumulative laboratory reports of HCV infection in the UK show rises in HCV-related disease (including hospital admissions, transplants and deaths), as well as newly confirmed cases (figure 1.1) [17]. Between 1992 and 2007, a total of 62,238 laboratory-confirmed diagnoses of hepatitis C infection from England were reported. About 69% of reported cases were men and 51% of cases were amongst individuals aged between 25 and 39 years. The major risk factor for acquisition of infection is injecting drug use, accounting for 93% of cases with known risk factor.

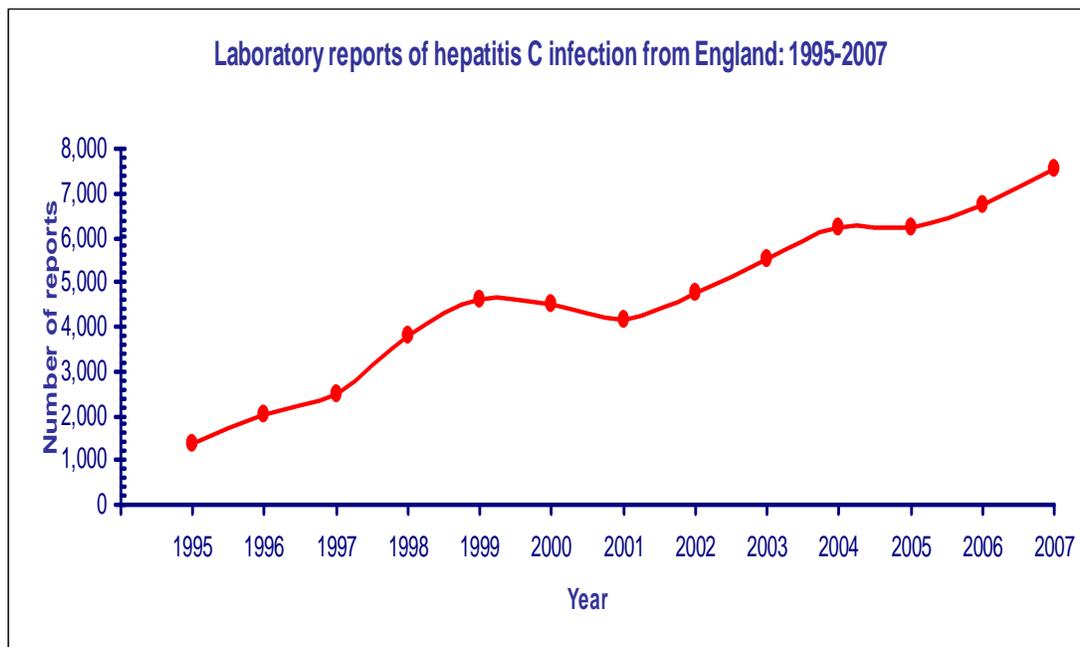


Figure 1.1: Laboratory reports of hepatitis C infection from England.

The Health Protection Agency Centre for Infections receives, electronically, confirmed laboratory cases of hepatitis C. A laboratory confirmed case is an individual with a positive test for hepatitis C antibody (anti-HCV) or detection of hepatitis C viral ribonucleic acid (RNA) in the serum. For infants aged less than one year, who may acquire maternal anti-HCV antibodies, diagnosis needs further confirmation by hepatitis C RNA testing or by a subsequent positive anti-HCV test in the second year of life to be included. This graph shows a consistent rise in the number of newly-acquired HCV infection, suggesting an increased disease burden on health care services. Adapted from HPA, 2008.

1.1.2. Natural History of HCV Infection

HCV is an important cause of acute and chronic liver disease in humans (figure 1.2). In most cases, acute HCV infection is mild and asymptomatic. It is estimated that only 10-15% of cases present with an acute hepatitis, manifested clinically as jaundice, while 10-20% present with non specific symptoms including malaise, anorexia, abdominal pain and fatigue [18, 19].

Although 15-25% of cases spontaneously resolve the infection by clearing the virus within 2-6 months, infection persists in up to 80% of infected individuals. Persistent HCV infection may progress over decades, resulting in a broad spectrum of clinical outcomes including chronic hepatitis and cirrhosis. Cirrhosis results in a decompensated end stage liver disease (ESLD) and is a clear risk factor for the development of hepatocellular carcinoma (HCC) [2, 20-22].

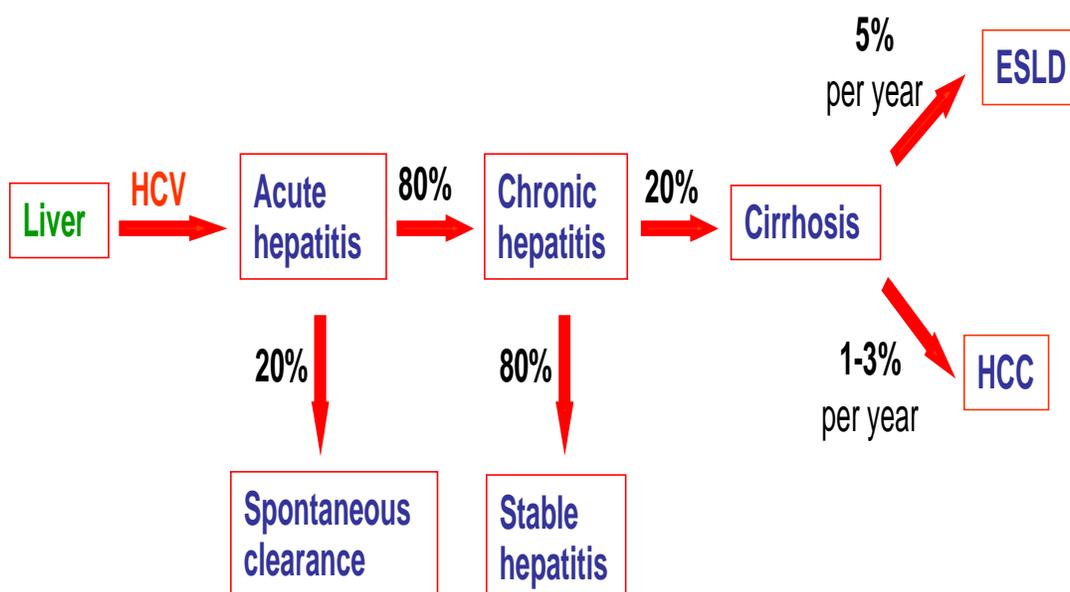


Figure 1.2: HCV disease progression.

This diagram shows person-to-person variability in HCV outcome. The HCV disease is usually insidious and subclinical. Acute HCV infection generally causes a mild course of disease, and patients are usually asymptomatic. However, 80% of patients who contract acute HCV infection will develop persistent infection, which often lasts for decades. Chronically infected patients have the risk of serious complications of liver disease, such as liver failure and hepatocellular carcinoma (HCC). The progression rate is accelerated with alcohol consumption, HIV or HBV co-infection. Adapted from Qureshi, 2007.

The estimated risk for decompensation is about 5% per year in cirrhotic patients [23] and the 5-year survival rate for decompensated cirrhotics is less than 50% [24]. About 3% of patients with cirrhosis will develop HCC per year [25]. Thus, it is estimated that HCV is accountable for about 27% of cases of cirrhosis and 25% of reported instances of hepatocellular carcinoma, worldwide [26].

As HCV is usually fatal only when it leads to cirrhosis, estimating the rate of fibrosis progression is an important parameter to evaluate the vulnerability of chronic HCV-infected patients. The exact pathogenesis of HCV disease and factors that may influence viral clearance are not fully understood [27, 28]. However, several factors have been clearly shown to be associated with the fibrosis progression rate, including older age at infection, male gender, consumption of alcohol, duration of infection, and human immunodeficiency virus (HIV) co-infection [29-31].

Poyand and colleagues evaluated the progression of liver fibrosis in 2235 untreated patients with chronic hepatitis C (figure 1.3). Using the median rate of fibrosis progression, they reported that rapid progression from infection to cirrhosis was strongly associated with three independent host factors: male sex, age at infection older than 40 years, and daily alcohol consumption of 50 g or more. The median estimated duration of infection for progression to cirrhosis was 30 years in intermediate progressors, ranging from 13 years in men infected after

the age of 40 (rapid progressors) to 42 years in women who did not drink alcohol and were infected before the age of 40 (slow progressors). There was no association between HCV genotype and fibrosis progression [23].

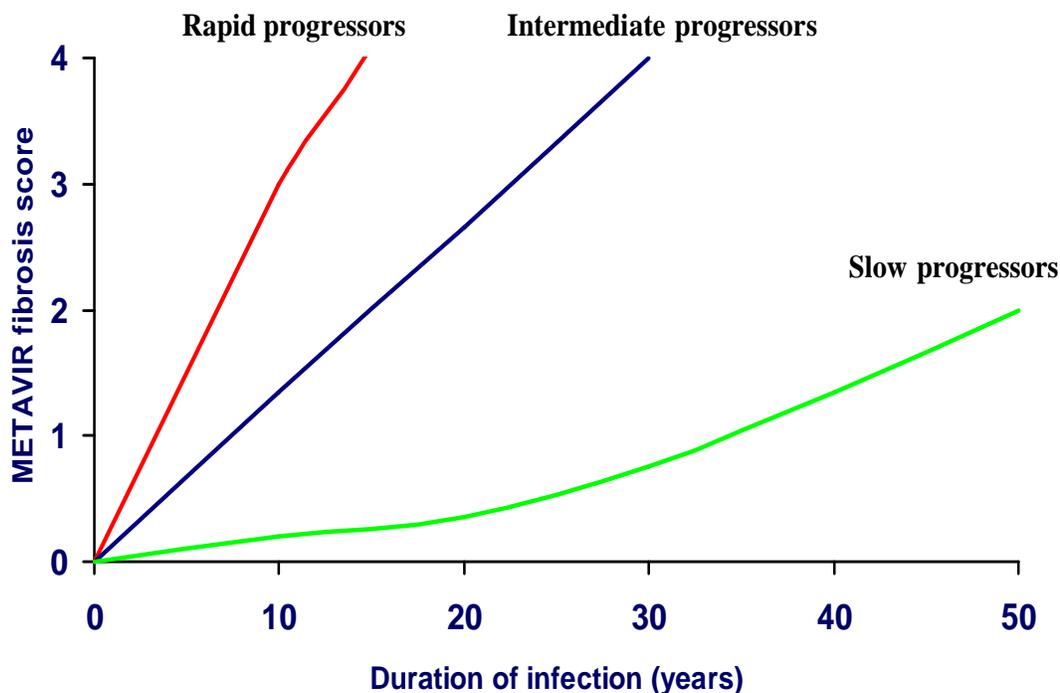


Figure 1.3: Modeling of liver fibrosis in chronic hepatitis C.

The diagram shows the rate of fibrosis progression per year [the ratio between fibrosis stage in METAVIR units (F0 = no fibrosis, F4 = cirrhosis) and the duration of infection, in years]. In untreated patients with chronic hepatitis C, the median expected time to cirrhosis was 30 years (intermediate progressors). However, 33% of patients had an expected median time to cirrhosis of less than 20 years (rapid progressors), and 31% will only progress to cirrhosis after more than 50 years, if ever (slow progressors). Adapted from Poynard et al., 1997.

The outcome of HCV infection may be related to genetically determined variation in the efficiency of presentation of viral peptides to T cells. Several human

leucocytic antigen (HLA) alleles have been found associated with HCV disease prognosis. Susceptibility to HCV infections was associated with HLA A*28, A*29, B*14, DR7 in Egyptians [32]. HLA A11-C*04 was found to be associated with HCV persistence in Ireland [33], and HLA DRB1*15-DQB1*0602 were associated with increased risk for a higher viral load and more severe HCV disease in Irish viraemic females [34]. In contrast, certain class II alleles of HLA; DQB1*0301 and DRB1*1101 were more associated with viral clearance in a cohort of Irish women [35].

One study showed that HCV-infected patients were at increased, though non-significant, risk of mortality from liver disease in the first decade of infection [36]. However, a significant, three-fold, increase in the death rate of patients infected with hepatitis C compared to that observed in an age- and sex-matched population was detected. A worse prognosis was associated with increased disease severity, in contrast to an improved outcome that was encountered in responders to treatment [37]. The increased risk of fibrosis progression with older age [38], along with the poor prognosis (a higher rate of HCC and/or decompensation and a lower survival without liver transplantation) associated with severe liver fibrosis (Ishak stage ≥ 4) secondary to hepatitis C [39], suggest a future increase in HCV-related disease burden.

Although HCV is a hepatotropic virus, its pathology is not only restricted to the liver. About 30 to 40% of patients with chronic hepatitis C may present with an extrahepatic manifestation [40], which include B-lymphoproliferative disorder, essential mixed cryoglobulinemia, glomerulonephritis, porphyria cutanea tarda and lichen planus [41-43].

1.1.3. Disease Severity

Scoring systems that utilise the concepts of grading and staging to evaluate liver biopsies from patients with chronic hepatitis have been described by Knodell [44], Ishak [45] and the METAVIR cooperative study group [46]. Staging measures the fibrosis and indicates the structural progression of the disease (table 1.1), while grading expresses the intensity of necroinflammatory activity (table 1.2). Staging and grading provide a way of recording the histological characteristics representing severity and progression of chronic hepatitis. Semi-quantification of grade and stage provides a numerical index of histological activity, summarised as minimal, mild, moderate or severe chronic hepatitis, thus it can enable comparison across different series of patients. However, the application of such grading and staging in a single biopsy is limited by potential sampling error, as they may depend on the sample size and quality. Thus, a higher degree of accuracy can be accomplished using multiple biopsies.

Table 1.1: Ishak staging.

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

Histological features included in staging [45].

Table 1.2: Modified histological activity index (HAI) grading.

	Necroinflammatory changes	Score
A.	Periportal or periseptal interface hepatitis (piecemeal necrosis)	
	Absent	0
	Mild (focal, few portal areas)	1
	Mild/moderate (focal, most portal areas)	2
	Moderate (continuous around 50% of tracts or septa)	3
	Severe (continuous around >50% of tracts or septa)	4
B.	Confluent necrosis	
	Absent	0
	Focal confluent necrosis	1
	Zone 3 necrosis in some areas	2
	Zone 3 necrosis in most areas	3
	Zone 3 necrosis + occasional portal-central (P-C) bridging	4
	Zone 3 necrosis + multiple P-C bridging	5
	Panacinar or multiacinar necrosis	6
C.	Focal (spotty) lytic necrosis, apoptosis and focal inflammation	
	Absent	0
	One focus or less per 10X objective	1
	Two to four foci per 10X objective	2
	Five to ten foci per 10X objective	3
	More than ten foci per 10X objective	4
D.	Portal inflammation	
	None	0
	Mild, some or all portal areas	1
	Moderate, some or all portal areas	2
	Moderate/marked, all portal areas	3
	Marked, all portal areas	4

Necroinflammatory features included in grading [45].

1.1.4. Genome Organisation

HCV is a small (50 nm), enveloped, RNA virus. It is classified as the sole member of the genus Hepacivirus that belongs to the *Flaviviridae* family [47], which also includes flaviviruses and pestiviruses [48]. The HCV genome is a positive-sense single-stranded RNA molecule, approximately 9.6 kb in length. It contains a single open reading frame (ORF) that encodes a single polyprotein of about 3,000 amino acids (figure 1.4). The ORF is flanked by 5' and 3' noncoding regions (NCR) of approximately 341 and 230 nucleotides in length, respectively. Both 5' and 3' NCR have highly conserved RNA structures essential for polyprotein translation and genome replication [20]. The 5' NCR directs the translation of the HCV ORF by acting as an internal ribosome entry site (IRES) that permits the binding of ribosomes proximal to the start of the ORF and directs the translation process [49]. The 3' NCR is important for replication in vivo and it encodes a variable sequence, poly U tract and a conserved 98 nucleotide sequence [50].

The polyprotein precursor is cleaved by viral and host encoded proteases at the endoplasmic reticulum membrane to form the mature viral proteins. These proteins can be classified into structural and nonstructural proteins. The structural proteins, at the amino (N)-terminus, which include the core protein (C), and the envelope glycoproteins E1 and E2 are processed by host signal peptidases. The structural proteins are separated from the nonstructural proteins by the short membrane peptide (P7). The nonstructural (NS) proteins, at the C-terminus, include NS2,

NS3, NS4A, NS4B, NS5A and NS5B. They are cleaved by virus-encoded proteases [20, 51].

The core protein (191 aa; 21 kDa) which is the major constituent of the nucleocapsid is the first cleavage product of the polyprotein [52]. It is a multifunctional protein essential for viral replication maturation as well as pathogenesis. It has been shown to modify cellular processes leading to the induction of hepatocellular carcinoma [53, 54]. Protein F is a newly discovered protein with unknown function, produced by ribosomal frame shift mutation around codon 11 of core protein [55]. The envelope glycoproteins E1 and E2 are glycosylated transmembrane proteins that interact with cellular receptors mediating viral entry and represent a target for host immune recognition. E1 and E2 are discussed in more detail in section 1.1.6.1.

The P7 protein is a small (63 aa; 7 kDa) hydrophobic protein, believed to be a viroporin, which is known to enhance membrane permeability. It can form an ion channel in artificial lipid bilayers. The non-structural viral proteins are not incorporated into viral particles but are required for RNA replication [56]. NS2 (217 aa; 21 kDa) and the amino terminal domain of NS3 encode a viral protease catalysing cleavage of the NS2/NS3 site [57]. NS3 (631 aa ; 68 kDa) has two functions; the amino terminus acts as a serine protease catalysing cleavage of NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B, while the carboxy

terminus displays NTP-ase and helicase properties essential for genome replication and translation [58-60]. NS3 may also function by interfering with host cell signal transduction [61].

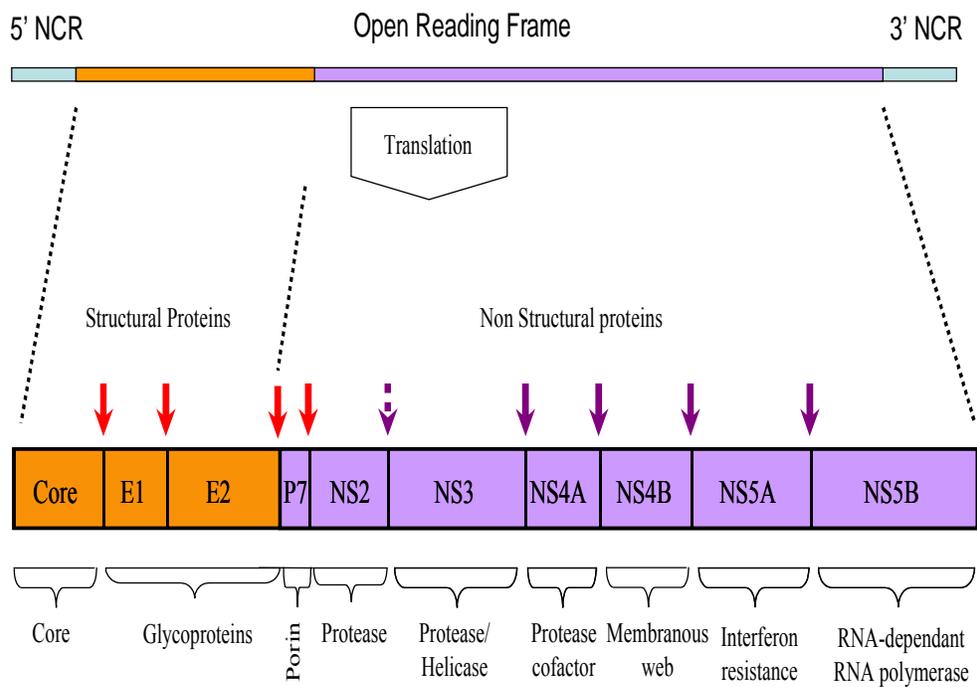


Figure 1.4: HCV genome organisation and polyprotein product.

Schematic diagram representing the open reading frame in the HCV genome flanked by the 5' and 3' noncoding regions (NCR). It also shows the position and the main functions of the structural and non structural proteins within the polyprotein. The polyprotein of 3000 amino acids is translated as one single polypeptide, which is then co- and post-translationally processed by viral and cellular proteases. The arrows point at cleavage sites for host signal peptidase (↓), NS2 protease (↓), and NS2/NS3 protease (↓) during polyprotein processing. Adapted from Suzuki et al., 2007.

NS4A (54aa; 6 kDa) is a cofactor of the NS3 protease [51, 60]. NS4B (261 aa; 27 kDa) induces the formation of a specialised membranous web within which viral RNA replicates [62]. NS5A (448 aa; 56 kDa) may be involved in RNA replication and contain the interferon (INF) sensitivity determining region (ISDR) which is thought to influence the resistance to the antiviral effects of IFN. NS5B (591 aa; 65 kDa) has been identified as the virally encoded RNA-dependent RNA polymerase, the catalytic core of the replicative machinery [50].

1.1.5. Genotypes

HCV genome shows exceptional sequence variation because of the lack of proofreading activity of the NS5B RNA-dependent RNA polymerase, resulting in misincorporation of nucleotides during RNA replication [20, 63]. According to the phylogenetic analysis of nucleotide sequences, HCV is classified into 6 major genotypes (1-6) based on evaluation of complete genome sequences and subgenomic regions. The genotypes differ in their nucleotide sequences by 31-34%, and in their amino acid sequences by ~30%. Each genotype is subsequently subdivided into more closely associated subtypes numbered alphabetically (a, b, c, ...) [47, 64]. Moreover, such high mutation rate of the RNA genome of HCV results in the presence of multiple isolates, called quasispecies, in the same patient [65]. The hypervariable region HVR1 of E2 is the most variable region of the genome [66], followed by the p7 gene, and the C-terminal region of NS5A [67],

while both the 5' and 3' NCR as well as the core have highly conserved RNA structures [20, 66].

The HCV genotypes vary in geographical and epidemiological distributions [47]. The commonest HCV genotypes in Europe and USA are 1a, 1b, 2a, 2b and 3a, type 4 genotypes are common in Northern Africa and the Middle East, while type 5 is common in South Africa and type 6 in Hong Kong [68, 69]. The commonest HCV genotypes in the UK are 3a, 1a, and 1b [70].

The effect of differences between the genotypes of HCV on clinical course and disease association has been investigated with varying conclusions. For example, the correlation between the infecting HCV genotype and disease severity is still debatable. While some studies show that infection with HCV genotype 1b viruses may be associated of more severe liver disease with development of HCC [71-74], other studies found no specific link between cirrhosis or HCC and infection with genotype 1b [69, 75-77]. On the contrary, a higher rate of viral clearance was observed with genotype 1 infection [78]. So, it seems that in order to use HCV genotyping more effectively in the clinical settings, further studies are required.

However, a significant variation between genotypes in rates of response to treatment with interferon has been observed with reduced viral clearance in genotype 1 infected patients compared with genotypes 2 or 3 [79-81]. Also, virally

induced steatosis linked with liver fibrosis is specifically associated with infection by genotype 3 [82-84]. The underlying mechanism for the observed differences between genotype is not clear. However, it may be a result of differences in sequence of structural components such as E1 and E2 glycoproteins and replicative ability of the virus [85].

1.1.6. Structure of HCV Virion

Due to the absence of a competent cell culture system and the limited amount of virus that can be recovered from serum, the structural analyses of HCV virions are limited. After production of infectious HCV particles in cell culture systems [86, 87], it was recently shown, by cryo-electron microscopy (cryo EM), that HCV virions have a spherical shape with a diameter of approximately 50 to 55 nm [88], confirming previous results [89, 90].

It is believed to have a classical icosahedral scaffold (figure 1.5) in which its two envelope glycoproteins, E1 and E2, are anchored to the host cell-derived double-layer lipid envelope. Underneath the membrane is the nucleocapsid that is composed of multiple molecules of the core protein, forming an internal icosahedral viral coat encapsidating the genomic RNA [91, 92]. A spherical structure, suggesting the nucleocapsid that harbors the viral genome, with a diameter of approximately 30-35 nm has been observed [87]. The core particles

exhibit six-fold symmetry and the hexagon side length is approximately 20 nm [92].

HCV virions exist in the blood of infected individuals in various forms: free circulating virions [93], virions bound to very low-density lipoproteins (VLDL) [94], virions bound to low-density lipoproteins (LDL) [95], and virions complexed with immunoglobulins [96]. The association of a major portion of circulating virions with LDL and/or VLDL may facilitate the viral entry to hepatocytes via the LDL receptor [97] and protects the virus against neutralisation by HCV-specific antibodies.

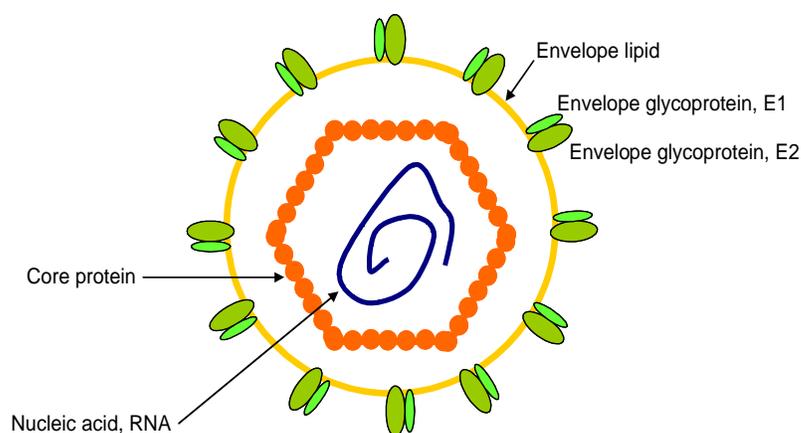


Figure 1.5: Structure of HCV virion:

Schematic diagram showing the icosahedral symmetry of HCV nucleocapsid. HCV RNA is encapsidated in the viral core particle, encoded by the core gene. The nucleocapsid is surrounded by a lipid bilayer envelope studded with complexes of two envelope glycoproteins E1 and E2, as heterodimer.

1.1.6.1. HCV Glycoproteins

The two envelope glycoproteins, E1 (192 aa; 35 kDa) and E2 (363 aa; 72 kDa) are type-I transmembrane glycoproteins, with N-terminal ectodomains of 160 and 334 amino acids respectively, and a short C-terminal transmembrane domain of approximately 30 amino acids (figure 1.6). The ectodomains of E1 and E2 contain numerous proline and highly conserved cysteine residues that may form 4 and 9 intramolecular disulphide bonds respectively. These proteins are expressed as a noncovalent E1E2 heterodimer, in the viral envelope. E1 extends from aa 192-383 and E2 extends from aa 384-746 of the polyprotein [20, 98, 99].

E1 and E2 are highly glycosylated proteins, containing up to 5 and 11 N-glycosylation sites respectively (figure 1.6). N-linked glycosylation supports the correct folding of newly synthesised peptides in the endoplasmic reticulum (ER). Both E1 and E2 influence each other's folding, assisted by host chaperones [99, 100]. Among the 11 identified potential glycosylation sites of E2, 9 sites are strongly conserved. However, there is variant glycosylation pattern across genotypes; the site at aa 476 is less conserved in genotype 1 and absent in genotype 5. Also, the glycosylation site at 540 does not exist in genotypes 3 and 6 [98]. This altered glycosylation pattern across genotypes may affect protein production, folding and the epitopes that interact with cellular receptors and the immune system.

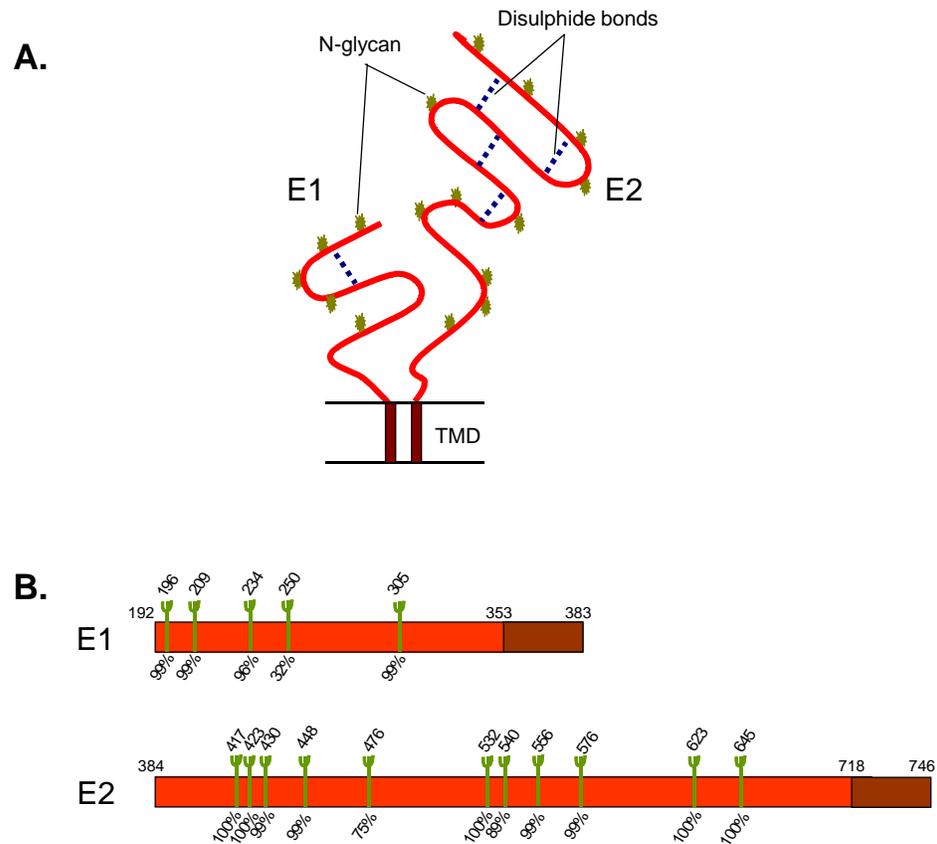


Figure 1.6: E1E2 heterodimer.

A: Schematic diagram representing the viral glycoproteins E1 (35 kDa) and E2 (72 kDa) in the form of non-covalent heterodimers. It shows the N-linked glycosylation sites on the N-terminal ectodomains. Both E1 and E2 have short C-terminal transmembrane domains (TMD) of approximately 30 amino acids. B: The potential glycosylation sites in E1 and E2. The position of the glycosylation sites are indicated above the boxes corresponding to E1 and E2 proteins, while the percentages of conservation of the glycosylation sites are indicated below each site. Adapted from Op De Beeck et al., 2001.

E1 and E2 play crucial roles at different steps of the HCV replication cycle. They mediate viral entry by interacting with cellular receptors CD81, scavenger receptor class B member 1 (SR-B1) and claudin 1, essential for host-cell entry [101-104] and inducing fusion with the host-cell membrane. They are also thought to play a

role in viral particle assembly [105]. They also provide a target for immune recognition [56, 106].

E1 and E2 are encoded by E1 and E2 genomic regions, which are characterised by a high variability; especially E2 that contains two hypervariable regions (HVRs). When HVRs are subject to immune pressure, they lead to the formation of escape mutants (quasispecies) [107, 108]. Increasing complexity of quasispecies is associated with failure of the immune system to clear the virus and with a relatively low response to interferon treatment [109, 110].

1.1.7. Replication

1.1.7.1. Model systems

Investigation of the HCV life cycle, understanding of HCV pathogenesis and development of novel antiviral treatments were hampered by absence of a satisfactory cell culture system and small animal models. The development of HCV replicon model in a human liver hepatoma cell line (Huh-7) [56] was a major breakthrough that offered the opportunity to investigate the molecular biology of HCV infection. Another important milestone was achieved when a robust HCV cell culture (HCVcc) system, that allowed a relatively efficient amplification of HCV, was developed [87]. The replicon model was derived from JFH-1, an HCV genotype-2a isolate from a Japanese patient with fulminant hepatitis, and capable

of producing infectious virions. Subsequent work resulted in improved productivity of infectious virus [111].

Another approach to the viral entry into cells was the generation of infectious HCV pseudotype particles (HCVpp) that were assembled by displaying functional HCV E1 and E2 glycoproteins onto retroviral and lentiviral core particles [106]. The presence of a marker gene, such as a green fluorescent protein marker gene, within these HCVpp allowed consistent and swift determination of infectivity mediated by the HCV glycoproteins. Alongside in-vitro replication systems progress, small animal models of infection are being developed including immunotolerised rat model [112] and transgenic mice [113].

1.1.7.2. HCV receptors

The first identified putative HCV receptor involved in HCV entry was CD81 [114]. CD81, a member of the tetraspanins, is a widely expressed multifunctional cell surface protein that is involved in cell adhesion, cell activation and signal transduction [115]. Its role in HCV entry has been confirmed with HCVpp and HCVcc studies [116-118]. Although recombinant CD81 and anti-CD81 antibodies have been reported to neutralise infection in tissue culture [86, 87], CD81 expression alone is not enough for cell entry of HCVpp as some CD81-positive cell lines can not be infected [102], suggesting that CD81 may act as a post-attachment entry co-receptor.

The human scavenger receptor class B type I (SR-BI), concerned with cellular lipometabolism, has been identified as a putative receptor recognised by E2 and involved in HCV entry [119, 120]. The enhancement of HCVpp infectivity by high-density lipoprotein (HDL) which is a ligand to SR-BI suggested a modulating effect of SR-BI to HCV entry [121, 122]. LDL receptors can also mediate viral entry by binding to lipoprotein that has been shown to associate with HCV particles in serum [97]. A recently recognised co-receptor of HCV is the tight junction component claudin-1, that mediates a late step viral entry [104].

Other molecules, including calcium-dependent (C-type) lectins such as liver/lymph node-specific intracellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (L-SIGN) and Dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN), that interact with recombinant HCV envelope proteins have been studied as potential HCV receptors [123, 124]. Their possible role, as they are not expressed on hepatocytes, is mediated by the capture and transmission of HCV to hepatocytes [125, 126].

1.1.7.3. Life cycle

The first step in the viral life cycle (figure 1.7) is the viral interaction, via the envelope glycoproteins, with attachment and entry receptors [127]. Entry of HCV particles to host cells via receptor-mediated endocytosis, followed by viral particle fusion with endosome membrane which is catalysed by a reduction in pH in the

late endosome. Then, after uncoating, viral RNA is released into the cytoplasm [128]. The positive-sense RNA is directly translated via an internal ribosome entry site (IRES)-mediated process in the cytoplasm. [129]. The IRES allows translation of the RNA in a cap-independent manner and also leads to shutting down the translation process in the host cell so that the cell's translational machinery will operate to translate viral mRNA. The polyprotein is processed by cellular and viral proteases to produce mature structural and nonstructural proteins which remain associated with the membrane of the endoplasmic reticulum (ER) [130].

Complementary negative-sense RNA is synthesised by NS3-5B replicase and acts as a template for the production of positive stranded RNA. The presence of negative stranded HCV RNA indicates replication of the positive stranded RNA genome [131]. HCV forms a membrane-associated replication complex, the membranous web, composed of viral proteins, replicating RNA and altered cellular membranes [62, 132]. Positive stranded RNA associates with core proteins to form the nucleocapsid [133]. The virus particles acquire the viral envelope by budding from the ER. Viral particles then travel through the Golgi apparatus before release at the cell surface [50, 134].

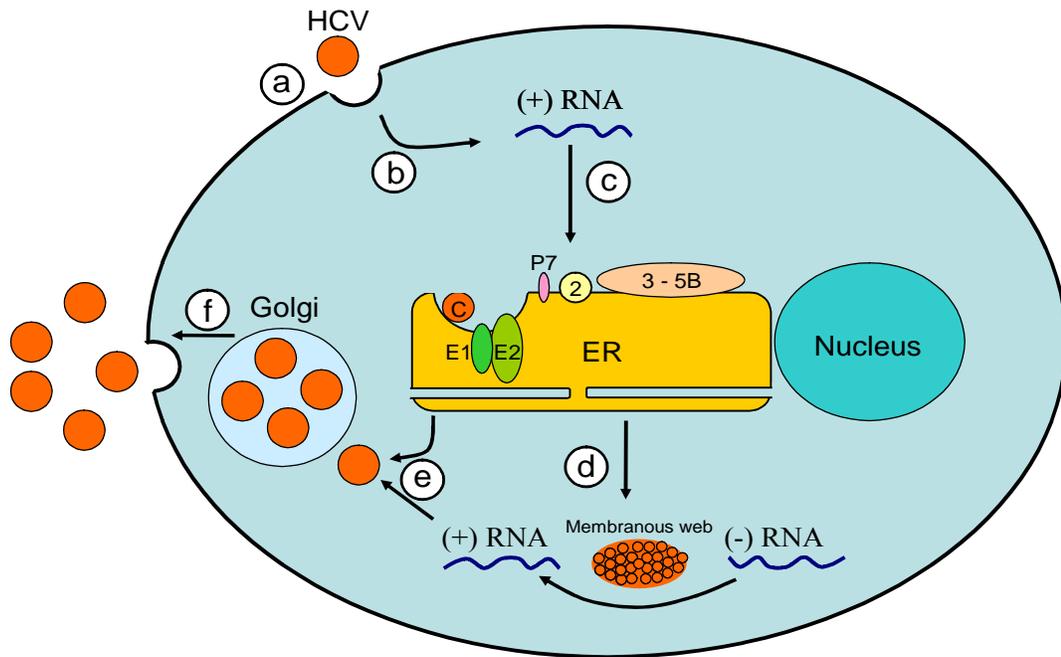


Figure 1.7: HCV replication cycle.

Schematic model of the HCV replication cycle. After virus binding and internalisation (a), the plus-strand RNA genome (+) RNA is uncoated and liberated into the cytoplasm (b), followed by IRES-mediated translation and polyprotein processing (c). Viral proteins remain tightly associated with membranes of the endoplasmic reticulum (ER). Minus-strand RNA (-) is synthesised by the NS3-5B replicase complex, acting as template for production of (+) RNA. Packaging and assembly (e) of (+) RNA via encapsidation with the structural proteins. Mature virus particles are enveloped by budding into the lumen of the ER and virus particles transit through the Golgi apparatus before release (f). IRES-mediated translation and polyprotein processing, as well as membranous web formation and RNA replication, which are illustrated as separate steps for simplicity, might occur in a tightly coupled fashion. Redrawn from Moradpour et al., 2007.

1.1.8. Immune Response

The host immune response is a really important factor in viral clearance. Immune responses to viral infection can be divided into innate and adaptive responses.

Although the innate immune responses are less mature than the adaptive immune

responses, they represent the first line of defence before the adaptive responses eventually come into play and moreover they have a significant effect on the development of the clonal immune response [135]. Innate responses are mediated by complement, antigen presenting cells (APCs) such as dendritic cells and macrophages, and natural killer cells. Adaptive responses are mediated by B lymphocytes, antibody-producing plasma cells, CD4⁺ T lymphocytes (helper T cells; Th), and CD8⁺ T lymphocytes (cytotoxic T cells; Tc). These adaptive responses have the properties of memory, antigen specificity and diversity [136]. The evolution and prognosis of HCV infection is influenced by the interactions between the virus and host immune responses in the first weeks after exposure [137].

1.1.8.1. Innate (Non-Specific) Immune response

The innate immunity provides a rapid antimicrobial host defence and facilitates the initiation of the adaptive immune response [138]. There is growing proof for involvement of innate immune responses and interference to HCV infection. It is seen in all individuals, regardless of whether they control the virus or develop chronic infection [55, 139]. Natural killer (NK) cells and NK-T cells, present in high proportions in the liver, provide a rapid immune response to HCV, mediating unrestricted Major histocompatibility (MHC) cytolysis of virus-infected cells [140]. NK cells also suppress HCV replication in hepatic cells through an interferon- γ mediated mechanism [141]. Dendritic cells (DCs) are professional

APCs that present antigens as processed peptides to T cells in the context of MHC and also activate lymphocytes. Both blood and hepatic populations have been reported to be significantly higher in patients with resolved HCV infection than in patients with chronic HCV infection [140, 142].

The interferon (INF) system represents a powerful antiviral immune response in most viral infections [143]. Production of INF- α/β is induced by viral infection via stimulation of pattern-recognition receptors (PRR) such as toll-like receptor 3 (TLR3) upon binding of viral double stranded RNA (dsRNA). Stimulation of TLR3 activates intracellular signaling cascades leading to transcription of IFN- α/β genes [144]. Induction of IFN- α/β requires the activation of nuclear factor kappa B (NF- κ B) and IFN regulatory factor-3 (IRF-3) [145, 146]. The secreted IFN- α/β binds to the IFN- α/β receptor on neighbouring uninfected cells and results in the activation of related signaling pathways, known as the Jak/STAT pathways, leading to upregulation of IFN-stimulated genes [147]. The upregulated genes that encode enzymes that result in an antiviral state include dsRNA-dependent protein kinase R (PKR) [148] and 2'5'-oligoadenylate synthetase (OAS) [149]. Interferon has direct antiviral properties and also acts as a cytokine with immunomodulatory effect; enhancing expression of MHC proteins and activation of natural killer cells and macrophages [150]. Although HCV induces the production of type 1 interferon, it is resistant to its antiviral activity [151]. Interferon may also induce the expression and presentation of HCV antigens on the infected hepatocyte

surface to the immune system by stimulating the generation of immunoproteosomes [152]. The potential role of mannan binding lectin (MBL) and ficolins as innate immune mediators is described later in section 1.2.7.

1.1.8.2. Adaptive (Specific) Immune response

1.1.8.2.1. Cell-mediated immune response

Cell-mediated immune response plays an important role in pathogenesis and control of HCV infection [153]. CD4⁺ Th cells are stimulated when they recognise viral peptides bound to MHC class II antigens on the surface of APCs. They proliferate and provide helper function by producing cytokines that enhance humoral and cellular immune responses. Th1 produces IFN- γ that stimulates recruitment of neutrophil and macrophage activation and upregulates inflammatory responses. On the other hand, Th2 produces cytokines including IL-4 and IL-10 to limit Th1-mediated inflammatory responses, thus restraining inflammation and preventing excessive tissue destruction [154]. Subcutaneous administration of recombinant IL-10 improved hepatic inflammation and fibrosis scores in patients with HCV-induced fibrosis [155], without affecting viral load. Patients with potent proliferative antiviral CD4⁺ T-cell response to HCV antigens E2, NS3, NS4, and NS5 during acute infection had better resolution and favorable course of infection [156].

Cytotoxic CD8⁺ T cells (CTLs), MHC class I-restricted peptide-specific cells, represent a significant effector that contend with viral infection. CTLs migrate into infected tissues, recognize infected cells presenting viral peptides in association with MHC class I, and become activated to reduce viral replication through a combination of cytolysis and secretion of antiviral cytokines; such as INF- γ and tumor necrosis factor (TNF) [157]. HCV-specific CD8⁺T-cell activity was reported to be related to increased histological inflammatory activity and lower levels of viraemia [158]. However, the lack of ongoing CTLs selection in chronically infected patients suggests that CTLs escape occurs early during HCV infection [159]. It is possible that, during established HCV infection, insufficiently activated CD8⁺ T cells are not able to clear the viral infection, but may still be able to induce immunologically-mediated liver injury through cytotoxic effects [140]. In contrast to the strong CD4⁺ T cell response to HCV that is maintained for many years after clearance, the memory CD8⁺ T cell response may be maintained with variable efficiency [139].

Cellular immune responses appear to have a role in the virologic outcome during acute infection rooted in strong association of a persistent and multispecific antiviral CD4⁺ and CD8⁺ T cell response with HCV elimination during acute infection [139]. On the other hand, in established chronic infection HCV-specific T cell response is weak and unable to clear the virus. It provides only a minimal selection pressure for T cell escape variants, and even inactivate them through T

cell antagonism [139]. The frequency of virus-specific cytotoxic T cells in HCV-chronically infected patients is much lower than that observed during infection with other persistent viruses, such as hepatitis B virus or HIV, which may reflect an immunosuppressive effect leading to viral persistence after infection [160].

1.1.8.2.2. Humoral immune response

The humoral immunity to HCV is mediated by antibodies, which target and bind epitopes of viral proteins. The proportion of antibodies that target exposed epitopes of the viral structural proteins, such as viral envelope glycoproteins, can neutralise the virus and thus prevent or control infection [161]. It has been reported that early robust antibody response to the HVR1 of the E2 glycoprotein is associated with a self-limited infection [162], and an initial complex quasispecies evolution is associated with HCV persistence [163]. To evaluate the protective nature of antibodies, a prospective study of HCV viraemia in injection-drug users has been performed. The results showed that patients who had previously cleared HCV (anti-HCV positive and HCV RNA negative) had a significantly lower level of viraemia and higher rate of spontaneous clearance when re-infected with HCV, compared with patients infected for the first time [164]. These results suggest that clearance of HCV infection confers anti-HCV immunity and protects against persistent infection. The role of the antibodies to E1E2 (anti-E1E2) in the pathogenesis of HCV disease has been investigated however, the results are controversial [165-167].

A few studies have suggested that E1E2 antibody may be useful as a serological marker of HCV infection, and incorporation of assays for E1E2 antibody may improve the sensitivity of currently available HCV antibody assays, which use recombinant antigens from 3 nonstructural proteins (NS3, NS4, and NS5) and 1 structural antigen (core) [168, 169]. This may be helpful for detection of HCV infection and improving the performance of the assays for anti-HCV screening [170]. However, this may be applicable for highly viraemic carriers who can be efficiently screened by the detection of E1E2 antibody. A considerable proportion of low viraemic carriers may not show the E2 antibody response [171].

1.1.8.3. Evasion of the Immune Responses

Although HCV-RNA can be detected in the serum after one-to-two weeks of exposure, indicating viral replication [172], there is a reported delay of the adaptive cellular and humoral responses [137, 173] suggesting that HCV can suppress the adaptive immune response. This hypothesis is supported by the paucity of symptomatic HCV infections, knowing that clinical signs, especially jaundice, result from liver injuries mediated by T lymphocytes [173]. In addition, the high incidence of HCV persistence after infection in many patients who are otherwise immune competent suggests that this virus has evolved a mechanism to evade the host response, despite high levels of virion production and strong induction of innate immune response [151]. This immune evasion may be due to generation of viral variants during infection that could escape from immune

recognition. This strategy is in harmony with the high error rate of the viral polymerase during viral RNA replication which leads to production of HCV quasispecies [174].

Another mechanism of immune evasion may be the ability of HCV to encode one or more products that inhibit viral clearance leading to progressive or persistent viral infection [160]. *In vitro* studies have shown that NS5A and E2 can interfere with PKR which inhibit HCV translation, core protein can inhibit the IFN signalling pathway, and NS3/4A can block the accumulation of phosphorylated interferon regulatory factor-3 (IRF-3), which inhibits expression of type I IFNs and IFN-stimulated genes [55, 175].

1.1.9. Treatment

1.1.9.1. Objectives

The goals of HCV treatment are to cure HCV infection by achieving a sustained virological response (SVR), defined as the absence of detectable HCV RNA 6 months after cessation of therapy, and also to delay or prevent disease progression [176, 177].

1.1.9.2. History

Before 1998, interferon alfa monotherapy for chronic HCV resulted in a SVR rate of up to 27% [178]. After the introduction of ribavirin, a broad-spectrum antiviral

guanosine analogue, the combination therapy enhanced the SVR rate to 40% [179, 180]. Then, interferon was replaced with pegylated interferon (peginterferons) [181], adding a new milestone to the treatment of chronic hepatitis C. Pegylation of interferon, by attaching polyethylene glycol polymer, improves the drug efficiency by increasing its binding affinity for cell receptors and enhancing its half-life, resulting in more efficient antiviral effect [22]. Subsequently, the overall SVR to peginterferon/ribavirin combination therapy increased to 54-63% [80, 81, 182]. Recently, more improved forms of interferon, peginterferon and ribavirin have been developed including oral prodrugs of ribavirin; Viremagine [22] and taribavirin [183], and albuferon alfa which is a fused form of interferon $\alpha 2b$ with human serum albumin [184].

1.1.9.3. Current therapy

1.1.9.3.1. Regimen

The current recommended strategy for the management of chronic HCV infection, a combination of peginterferon plus ribavirin [177, 185] has remained stable since 2001. Recommended dosage and duration of treatment vary with viral genotype. Patients with HCV genotype 1 receive peginterferon, 180 mcg subcutaneously (SC) once-weekly plus ribavirin, 1000 or 1,200 mg/day, orally in 2 divided doses, for 48 weeks, whereas patients infected with genotype 2 or 3 receive peginterferon plus a lower dose of ribavirin, 800 mg daily, in 2 divided doses, for 24 weeks [185].

1.1.9.3.2. Response to treatment

The timing and magnitude of the virological response to antiviral therapy in HCV-infected patients is variable. These regimens lead to SVR rates of 50% for patients infected with genotype 1 viruses and of approximately 80% for those infected with either genotype 2 or 3 [80, 81, 186, 187]. A high SVR rate (97%) can be achieved when treating acute HCV infection with peginterferon for 12 months, regardless of the infecting genotype [188, 189]. However, the asymptomatic nature and the absence of a clear-cut diagnostic approach for diagnosis [190] represent obstacles for treating acute HCV infection.

1.1.9.3.3. Prediction of treatment response

On-treatment monitoring of HCV RNA is recommended especially for patients with genotype 1. One of the best predictors of end-of-treatment virological response and SVR is the rapid virologic response (RVR), defined as an undetectable HCV RNA after 4 weeks of therapy. Achievement of RVR was associated with a low rate of relapse during follow-up in HCV genotype 1 patients [191, 192]. Achievement of RVR can be used as an indicator to shorten the duration of treatment to 24 weeks in individuals with HCV genotype 1 infection or to 12–16 weeks in patients with HCV genotype 2 or 3 infection [193]. Early virologic response (EVR), defined as the achievement of negative HCV RNA or a 2-log drop in viral load compared with the baseline measure by 12 weeks, is a negative predictor of response to treatment. The failure to achieve EVR is an

indication to stop therapy. Also, patients with genotype 1 with detectable HCV RNA at week 24 will not accomplish SVR and therefore treatment should be discontinued. A promising tool for anticipation of response to treatment, even before start of treatment is the study of liver gene expression of interferon-stimulated genes which is different in non-responders and patients with a SVR [194]

1.1.9.3.4. Side effects

Interferon-related side effects include bone marrow suppression (neutropenia and thrombocytopenia), flu-like symptoms (fever, headache, arthralgia and myalgia), neuropsychological disorders (irritability, apathy and depression), and autoimmune syndromes (autoimmune thyroiditis). The main adverse effect of ribavirin is hemolytic anemia. The side effects associated with peginterferon and ribavirin may reduce adherence to therapy and may affect the management of patients with chronic hepatitis C, by either premature withdrawal of therapy or dose modifications [81, 186].

1.1.9.4. Future therapy

As the current regimen is toxic, expensive, requires prolonged compliance, and not all patients with chronic HCV infection meet the criteria for treatment [195], there is a pressing need for new HCV drug therapies. The development of an in-vitro system for neutralising HCV replication will enable screening of agents that

inhibit viral RNA replication [87, 196]. Many specifically targeted antiviral therapies for hepatitis C (STAT-C) compounds are promising and currently in development. The novel drug therapies for HCV include specific antibodies or small molecule inhibitors, blocking E1 and E2 [197, 198], protease inhibitors that inhibit the viral protease NS3/4B [199, 200], nucleoside analogue NS5B polymerase inhibitors [201, 202], non-nucleoside NS5B inhibitors that target and bind directly to the RNA-dependent RNA polymerase [203, 204], RNA interference therapies [205], and immune modulators [206, 207].

1.1.10. Prevention

1.1.10.1. Vaccines

As a result of continuous spread of HCV infection, several approaches were taken to develop vaccines to control the spread of HCV. HCV envelope proteins, E1 and E2, were the first choice in vaccine development against HCV infection. The choice of the envelope proteins as an attractive target for vaccine development is attributed to their role in receptor binding [114], mediating viral entry [208], and the presence of multiple neutralising epitopes [209, 210]. Although it is difficult to neutralise a wide range of potential infecting strains especially with the rapid production of new variants under immune selection, it could be possible to overcome that issue with antibody-based vaccines by the generation of broadly cross-reactive neutralising antibodies [211].

Experimental studies in mice with a prototype vaccine based on recombinant plasmids encoding HCV proteins induced humoral and cellular immune responses [212, 213]. A therapeutic vaccine trial for chronic hepatitis C based on the repeated administration of the HCV E1 protein revealed an increase in anti-E1 antibody levels along with decrease in total Ishak score as well as relative decreases in both Ishak fibrosis score, despite unchanged plasma HCV-RNA levels [214]. Despite some encouraging results that have been achieved by various efforts made to develop a vaccine against HCV infection [213-215], there is no effective vaccine available so far.

1.1.10.2. General measures

Due to the absence of an effective vaccine, preventive measures are directed towards reducing HCV transmission, by effective screening of blood and blood products, proper sterilisation of medical equipment, use of disposable needles, and primary prevention of illegal drug injecting [8]. The preventive measures also include educating health workers and increasing public awareness about the risk of infection from unsafe practices, counseling and testing for HCV for individuals at risk [6]. Improved identification and treatment of HCV patients with newly-acquired infection may significantly reduce onward transmission and reduce chronicity [190].

1.2. Ficolins

Ficolins are related to the collectin family of proteins, which represent major effectors of the innate immune defence in mammals [216-218]. They were first described as transforming growth factor- β 1-binding proteins on pig uterine membranes [219]. Their name, ficolins, reflects their unique structure of fibrinogen- and collagen-like domains [220]. Depending on the species, different types of ficolin family proteins have been identified; ficolins H, L, and M in humans [216, 218, 221], ficolins α and β in pigs [220], ficolins A and B in mice [222, 223], and ficolins 1, 2, 3 and 4 in frogs [224].

1.2.1. Types

In humans, three types of ficolins have been described to date (table 1.2), L-ficolin (ficolin-2, ficolin/P35 or Hucolin) [225], H-ficolin (ficolin-3 or Hakata Ag) [216], and M-ficolin (ficolin-1 or ficolin/P35-related protein) [221, 225]. L-ficolin is synthesised in the liver and found in the blood circulation [218]. Healthy adult plasma contains, on average, an L-ficolin level of 4.13 $\mu\text{g/mL}$ [217]. H-ficolin was first identified as a serum autoantigen (Hakata Antigen), recognised by antibodies in patients diagnosed with systemic lupus erythematosus and other autoimmune diseases [216]. It is synthesised in the liver by hepatocytes as well as bile duct epithelial cells and is secreted into both blood circulation and bile. It is also synthesised by ciliated bronchial and type II alveolar epithelial cells and is secreted into the bronchus and the alveolar space [226]. Therefore, it may play an

important role in both systemic and mucosal immune defence systems [227]. The mean serum concentration of H-ficolin is 15 $\mu\text{g/mL}$, with minor variations. The serum levels of H-ficolin decrease in liver disease with increasing severity of cirrhosis [228]. M-ficolin is a non-serum ficolin expressed on peripheral blood leukocytes. It might act as a phagocytic receptor or adaptor on circulating monocytes for microorganism recognition [218, 227] and it has been described recently as a secretory protein, in the cytoplasmic secretory granules of neutrophils, monocytes and type II alveolar epithelial cells [229].

1.2.2. Structure

Ficolins, like the collectins, represent a group of multimeric proteins built of identical polypeptides [230]. They are composed of four structural subunits. Each is composed of three identical polypeptides of 35-KDa linked by disulfide bonds (figure 1.8). Each polypeptide chain is formed of a short cysteine-rich NH_2 -terminal region of 24 aa with two cysteine residues followed by a collagen-like domain that consists of tandem repeats of Gly-Xaa-Yaa triplet sequences (where X and Y represent any amino acid), then a short link region (neck domain) of 12 aa and then the fibrinogen-like sugar-binding domain of 207 aa [231]. The length of the collagen-like domain in H-ficolin (33 aa; 11 Gly-Xaa-Yaa repeats) is shorter than M-ficolin (57 aa; 19 Gly-Xaa-Yaa repeats) and L-ficolin (45 aa; 15 Gly-Xaa-Yaa repeats) [232, 233]. These polypeptides form triple helices in the collagen-like

region and assemble to form a subunit comprising triple helix 'bundle-of-tulips' structures [216, 230].

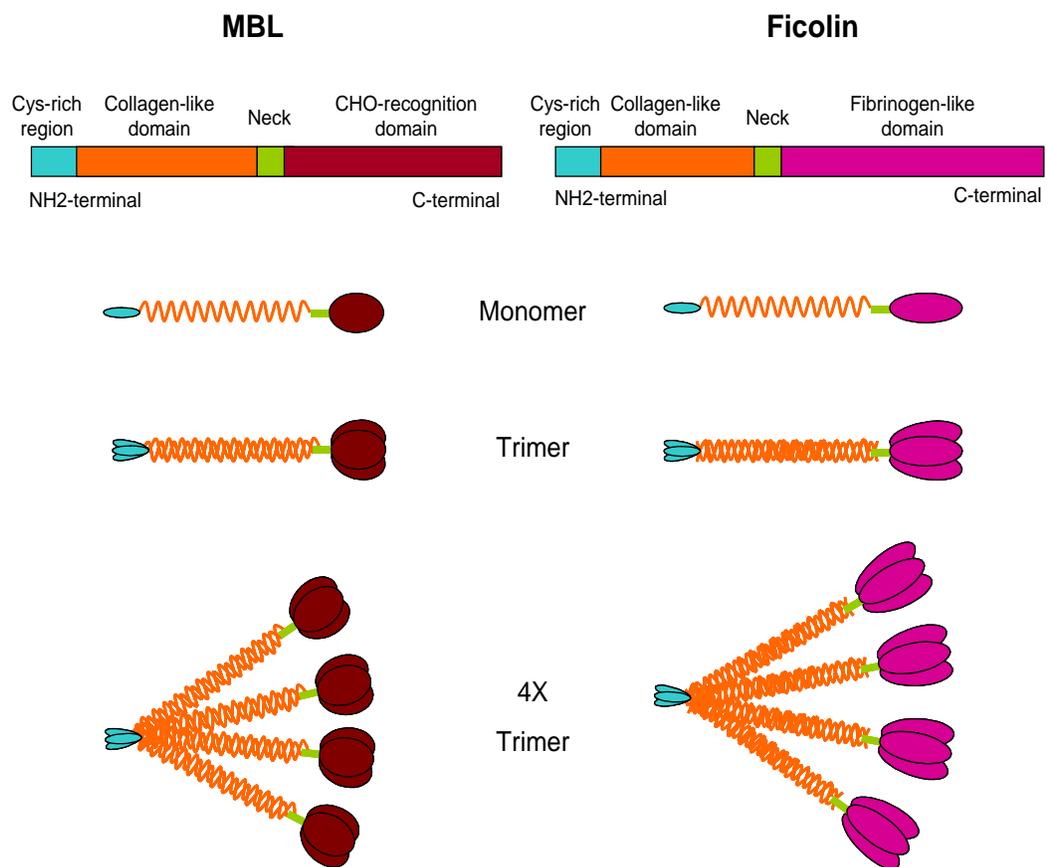


Figure 1. 8: Structural organisation of MBL and ficolins.

Schematic diagram illustrating the domain and oligomeric structure of MBL and ficolins. Both are oligomers of structural subunits, of three identical polypeptides (trimer). Each polypeptide chain (monomer) contains: a cysteine rich N-terminal domain, a collagen-like domain, a neck region, and a C-terminal carbohydrate-recognition domain in MBL and a fibrinogen-like domain in ficolins. In this figure, the monomers oligomerise into trimers, which further oligomerise into tetra-trimers (12-mer) giving an overall bouquet like structure. However, lower and higher oligomers, which are more frequent in MBL than in ficolin, have similar organization. Adapted from Fujita et al., 2004.

The collagen domains assemble the subunits into trimers, and each four trimers join together at the N-terminal regions into 12-mers [234]. This collagen-like multimeric structure is shared with the mannan-binding lectin (MBL), the complement protein C1q (the recognition molecule of the first complement component) and surfactant proteins A and D (SP-A and SP-D) [106, 235].

It has been shown that the fibrinogen-like domains at the C-terminal end of the polypeptide recognize carbohydrate patterns on the outer walls of microorganisms, with a general specificity for N-acetylglucosamine (GlcNAc) [216, 227, 236].

1.2.3. Interaction with microorganisms

The fibrinogen-like domains at the C-terminal end of the polypeptide in ficolins, like the carbohydrate recognition domains in MBL, recognize carbohydrate patterns on the outer walls of microorganisms. The ligands on microorganisms differ between each ficolin and MBL, due to the discrete sequences in their recognition domains [229]. The difference in binding properties indicates that each lectin binds a specific pathogen-associated molecular pattern [237]. Ficolins are able to bind to different types of bacteria (table 1.3) with a general specificity for N-acetylated groups [230, 237, 238]. The ability of ficolins to bind to various bacteria, may indicate that ficolins function in host surveillance against infection by recognition of the macropatterns on pathogenic microorganisms.

L-ficolin has been shown to bind *Salmonella typhimurium* strains with exposed GlcNAc [218, 239] and serotype III group B *Streptococci* [240]. In addition, L-ficolin specifically binds to lipoteichoic acid from Gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus agalactiae* [241] and also binds to *Escherichia coli* [230]. H-ficolin binds to *Aerococcus viridans* [242, 243]. The cell-associated M-ficolin was able to bind to *S. aureus* and to interact with a smooth-type strain of *S. typhimurium* [229]. The phagocytosis of *E. coli* by a human-derived monocytic cell line (U937) was inhibited by anti-M-ficolin antibody [227].

Although MBL has shown inhibition of Influenza virus hemagglutination and infectivity [244, 245] as well as binding activity to gp120 of human immunodeficiency virus (HIV) [246, 247], it is not known so far if ficolins are able to recognize viruses.

1.2.4. Functions

The collectins and ficolins cooperate with phagocytes and humoral factors such as complement to eliminate microorganisms [231].

1.2.4.1. Complement Activation

The main biological effects of MBL and ficolins are mediated through activation of the complement system, which represents the key humoral component of the

innate immune system, upon binding of MBL or ficolins to sugar structures on bacterial surfaces [248]. Like MBL, ficolins associate with MBL-associated serine proteases (MASPs) and activate complement independently of antibody through a third complement activation pathway (other than the classical and the alternative pathways) termed the lectin complement pathway [229, 242, 249, 250].

Four related proteins have been described; three MASPs; MASP-1, MASP-2 and MASP-3, with a C-terminal serine protease domain, and another small (19 kDa) MBL-associated protein; MAp19 [251]. MASP-1 is an alternative splice product of MASP-3, and MAp19 is an alternative splice variant of MASP-2 [252, 253]. The MASPs circulate in the blood complexed with MBL and also with ficolins. They bind to MBL or ficolins via the collagenous region [229, 239, 242]. MASP-1, MASP-2 and MASP-3 are homologues of the classical pathway proteases, C1r and C1s [254, 255]. MBL and L-ficolin function separately and activate the lectin complement pathway independently of each other [256].

Table 1.3: Types and characters of ficolins.

Type	Synthesis	Tissue expression	Genetic determinants	Sugar specificity	Pathogen interaction
L-ficolin	Liver (hepatocytes)	Serum (4.13 µg/mL)	On chromosome 9 (eight exons)	N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, N-acetyl-L-cysteine, N-acetylglycine, elastin, acetylcholine, 1,3-b-D-glucan, and lipoteichoic acid.	<i>S. aureus</i> ^{1,2} , <i>S. pyogenes</i> ¹ , <i>S. pneumoniae</i> ¹ , <i>S. agalactia</i> ¹ , <i>E. coli</i> ³ , and <i>S. typhimurium</i> ^{4,5}
H-ficolin	Liver (hepatocytes and bile epithelium) and type II alveolar cells	Serum (15 µg/mL), bile, bronchus, and alveolus	On chromosome 1 (eight exons)	N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, fucose, glucose, and polysaccharide A. viridans.	<i>A. viridans</i> ^{6,7} , <i>S. typhimurium</i> ⁸ , and <i>S. minnesota</i> ⁸ .
M-ficolin	Monocytes	Monocyte surface and a secretory molecule	On chromosome 9 (nine exons)	N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and Sialyl-N-acetyl lactosamine	<i>S. aureus</i> ⁹ and <i>S. typhimurium</i> ⁹ .

Although the three human ficolins; H- L- and M-ficolin share common structural and functional characteristics, this table shows the main differences in terms of synthesis, tissue expression, sugar specificity and pathogen interaction. ¹[241], ²[237], ³[230], ⁴[218], ⁵[239], ⁶[243], ⁷[242], ⁸[216], ⁹[229]

The lectin pathway (figure 1.9) of complement activation is initiated by the binding of MBL or ficolins to carbohydrate surfaces on invading pathogens [257, 258]. This binding promotes the activation of MASPs which convert from an inactive proenzyme form of a single polypeptide to an activated form with two polypeptides linked by a disulfide bond, thus acquiring proteolytic activities [259].

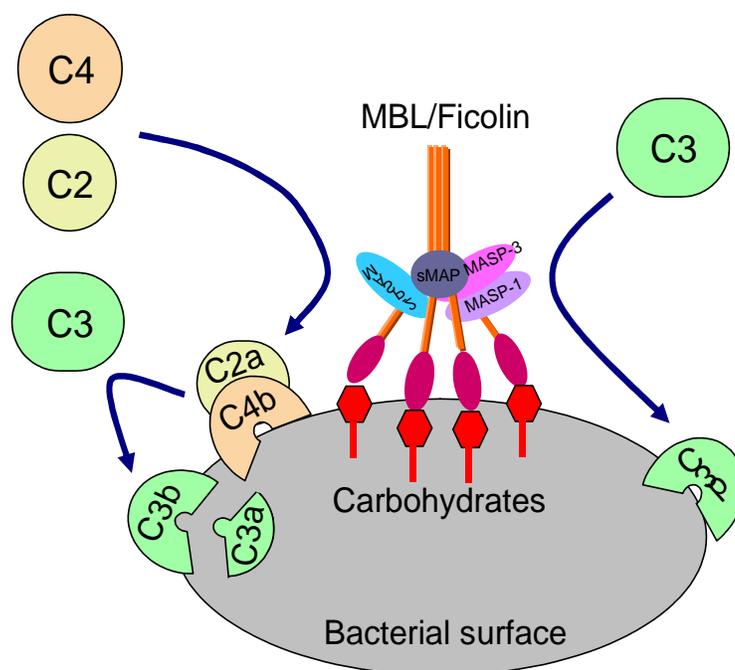


Figure 1.9: Lectin complement pathway.

The lectin pathway is activated by the binding of either mannose-binding lectin (MBL) or ficolin, in association with MBL-associated serine protease 1 (MASP-1), MASP-2, MASP-3 and small MBL-associated protein (sMAP), to carbohydrate moieties on the bacterial cell surface. MASP2, similar to C1s, is responsible for the activation of C4 and C2, which results in the production of C3 convertase (C4bC2a) as in the classical pathway. Adapted from Fujita, 2002.

The activated MASPs cleave C4 and C4b-bound C2 components of the complement cascade to generate the C3 convertase, C4b2a. Accumulation of C3 cleavage product, C3b, can result in C5 convertase activity [248, 249]. Complement activation may also result in the destruction of the target through the formation of the membrane attack complex [217, 240].

1.2.4.2. Opsonisation

Binding of ficolins, via their fibrinogen-like domains, to carbohydrate moieties on microorganisms leads to opsonisation of the microorganisms via complement activation and deposition of C3b which stimulates phagocytosis and clearance of pathogens bearing surface GlcNAc residues [217, 218, 260, 261]. Alternatively, ficolins may function directly as opsonins. This mechanism is supported by the ability of L-ficolin to enhance phagocytosis of *S. typhimurium* by neutrophils [218] and the inhibition of phagocytosis of *E. coli*, by promonocytic U937 cells, by anti-M-ficolin antibody [227]. Also, H-ficolin has been shown recently to bind to late apoptotic cells, resulting in enhanced adhesion and uptake by macrophages [262].

1.2.5. Interaction with host cells

The carbohydrate structures recognised by MBL and other collectins do not include galactose and sialic acid, the crucial sugars that are usually present in mammalian glycoproteins which also lack the presence of the specific pathogen-

associated molecular patterns (PAMP) [231]. This characteristic is supposed to be one of the main mechanisms for the ability of ficolins and MBL to differentiate between self and nonself [229]. However, aberrantly glycosylated cancer cells and apoptotic cells represent an important exception. This has been shown by the ability of MBL to bind to apoptotic bodies and enhance macropinocytosis of dead cell bodies [263] as well as the complement activation after binding of L-ficolin and H-ficolin to apoptotic cells [264]

1.2.6. Phylogeny

Collectins and ficolins, as main effectors of non adaptive immune defence, are found in all vertebrates as well as in species representing early evolutionary stages. The fibrinogen-like domain of the ficolins has been found throughout the animal kingdom, e.g. in animals, insects, sea cucumber, and horseshoe crab [265]. In humans, the genes of L-ficolin and M-ficolin are close to each other on chromosome 9 (9q34), and are 80% homologous at the amino acid level, suggesting a late duplication and divergence, whereas the gene for H-ficolin is situated on chromosome 1 (1p35.3), and has about 48% homology at the amino acid level with both L-ficolin and M-ficolin [216, 221]. The M-ficolin gene contains nine exons, whereas L-ficolin and H-ficolin genes are composed of eight exons [235]. The first exon encodes the 5'NCR, leader peptide and N-terminal amino acids; the second and the third exons encode the collagen-like domain; the fourth exon encodes the link region, and the exons five to eight encode the

fibrinogen-like domain. The eighth exon also encodes the 3'NCR. The ninth exon (M-ficolin) encodes additional segments of Gly-X-Y repeats in the collagen-like domain [221, 266].

1.2.6.1. Polymorphisms

The ficolin genes are polymorphic, particularly L-ficolin that has functional polymorphic sites that regulate both the expression as well as the function of L-ficolin, which may have pathophysiological implications for innate immunity.

L-ficolin polypeptide chains exist in at least three variants, which are named L-ficolin-A (wild-type), L-ficolin-B (Thr236Met) and L-ficolin-C (Ala258Ser). The L-ficolin genetic system is regulated during transcription through promoter and structural polymorphisms. Single nucleotide polymorphisms (SNPs) in the promoter region of the L-ficolin gene, like MBL, were associated with changes in the serum concentration [235]. Alternatively, SNPs reported in the exon 8, encoding the fibrinogen-like domain, were associated with change in GlcNAc binding capacity [267].

The association between these alleles with clinical pathophysiology and/or disease susceptibility is under investigation. Low concentrations of L-ficolin have been reported with increased frequency in children with recurrent respiratory infections [268]. The amino acid changes in L-ficolin are clustered in exon 8 encoding the

fibrinogen-like domain, which could indicate that the genetically determined variation in the L-ficolin concentration and change in binding affinity or specificity may have led to some changes in host-pathogen interactions similar to what has been previously suggested for MBL [269, 270].

1.2.7. Potential Role in HCV Disease

There is currently no literature describing interaction between ficolins and HCV. However, some studies of relationships between MBL and HCV infection report a possible correlation between MBL polymorphisms and HCV disease progression [271] and response to treatment [272, 273]. These studies differ in the cohorts used and the MBL gene mutations investigated. Polymorphisms in the promoter and structural regions of functional MBL gene (MBL2) have been reported to affect oligomer formation and circulating levels of MBL. The wild type allele of MBL is referred to as allele A. Three single point mutations in codons 52 (Arg→Cys, allele D), codon 54 (Gly→Asp, allele B), and codon 57 (Gly→Glu, allele C) of exon 1 results in amino acid substitution in the collagenous region of MBL [270, 274-277].

A Japanese population with mutation in the MBL gene had lower serum levels of MBL, which increased the possibility of disease progression from chronic inactive hepatitis to chronic active hepatitis or liver cirrhosis compared to those with the wild type allele. This result suggest that mutation in the MBL gene may be a risk

factor in the progression of hepatic injury in HCV infection [271]. Moreover, the wild type MBL allele was necessary for successful treatment of HCV with INF and possession of the homozygous B allele predicted unresponsiveness to treatment [272]. However, Kilpatrick et al., reported that the MBL concentrations were higher in HCV positive patients than in healthy controls with no significant correlation between MBL concentration and disease progression or the response to antiviral therapy in an European cohort [278].

On the other hand, the enzymatic activity of MBL/MASP-1 complexes has been found to be directly associated with the severity of fibrosis in HCV-infected patients [279]. As MASP-1 has a similar proteolytic activity to thrombin on fibrinogen and factor XIII [280-282], this result may suggest contribution of MBL/MASP-1 complexes to the pathogenesis of liver fibrosis. Thus, as L-ficolin has similar structural characteristics and interacts in a similar fashion to MBL, it seems logic to explore the potential role of L-ficolin in the pathogenesis of HCV infection.

1.3. Aims of the Work

L-ficolin, a component of innate immune system, is a serum opsonin and a pattern recognition receptor which activates the lectin complement pathway by interacting with the surface of the microorganisms. Based on the structural and functional similarities between ficolins and MBL, the main goal of this study was to investigate the potential role of L-ficolin, as a humoral innate immune molecule, in HCV disease. This study also involved evaluation of the role of anti-E1E2 antibodies, as mediators of the humoral adaptive responses, in HCV disease.

The objectives of this study included expression and purification of recombinant L-ficolin and purification of serum L-ficolin in order to employ the purified ficolin preparations in investigating whether L-ficolin could bind to HCV glycoproteins E1E2 and studying the effect of L-ficolin binding to HCV particles. To elucidate the role of L-ficolin in HCV liver disease, it was essential to evaluate the serum L-ficolin levels in patients with chronic hepatitis C infection. Another objective was to find out the association between the level of anti-E1E2 antibodies in patients with chronic hepatitis C infection and the infecting HCV genotype, viral load, disease severity, and response to treatment.

2. Production and Purification of L-ficolin

2.1. Aims

The primary goals of this part of the study were the production and purification of recombinant L-ficolin and purification of L-ficolin from human serum. Purified preparations of recombinant and serum L-ficolin were required for characterisation of the biochemical interactions with HCV glycoproteins. This section also included the development of an ELISA-based assay for quantification of purified recombinant and native L-ficolin.

2.2. Materials and Methods

2.2.1. Materials

2.2.1.1. Plasmids and Bacterial Strains

The human L-ficolin gene was amplified from an Integrated Molecular Analysis of Genomes and their Expression (IMAGE) clone pPCR-Script Amp SK [clone number BCO 69572 (Open Biosystem)]. pENTR™ TOPO®, pcDNA-DEST40 and pcDNA-DEST26 vectors were purchased from Invitrogen. Plasmids were maintained in and harvested from *Escherichia coli* TOP10 (Invitrogen). Transformed bacteria were grown in Luria Bertani (LB) broth (Sigma) overnight at 37°C.

2.2.1.2. Chemicals and Primers

All the reagents were of the highest standard commercially available as follows: bovine serum albumin (BSA), Sodium dodecyl sulphate (SDS), tetramethylethylenediamine (TEMED), ammonium persulphate (APS) Tween 20, ethidium bromide, dithiothreitol (DTT), dimethylsulfoxide (DMSO), polyethylene glycol (PEG) 4,000, Ampicillin, Kanamycin, imidazole, NaCl, CaCl₂, N-Acetyl-D-glucosamine (GlcNAc), divinyl sulfone, p-nitrophenyl phosphate substrate, Tris-Acetate EDTA (TAE) buffer and molecular biology water were all obtained from Sigma-Aldrich Company Ltd, Poole, UK. PCR reagents were obtained as follows: HotStar Taq DNA polymerase kit was obtained from QIAGEN, deoxynucleotide triphosphates (100 mM dNTPs) were obtained from Bioline. Expand High Fidelity polymerase mix and Expand High Fidelity Buffer were obtained from Roche, Pfu DNA Polymerase was purchased from Stratagene, M13 forward and M13 reverse primers were purchased from Invitrogen, and all oligonucleotides used were synthesised by Proligo and kept at a stock concentration of 100 µM. Primers were designed by Dr Alexander Tarr, using the Primer Select component of DNASTar package (Table 2.1). The underlined sequences represent the sequences for the FLAG-peptide.

Table 2.1: PCR and sequencing primers.

Primer	Sequence (5'-3')	T_m °C
Ficolin forward	CACCGGAAGAGATGGAGCTGGACAGA	65
Ficolin reverse	CTAGGCAGGTCGCACCTTCATCTCTGACAC	67
FLAG-signal antisense	<u>CTTGTCGTCGTCGTCCTTG</u> TAGTCAGCCCAGG CCATGCCAGGAAAGAGA	85
FLAG-ficolin sense	<u>GACGACGACGACAAGCTCC</u> AGGCGGCAGAC ACCTGT	80
M13 forward	TGTA AACGACGGCCAG	52
M13 reverse	CAGGAAACAGCTATGACC	54
T7 forward	TAATACGACTCACTATAGGGAGAC	55
BGH reverse	TAGAAGGCACAGTCGAGG	56

2.2.1.3. Cell Culture

Dulbecco's Modified Eagles Medium and Opti-MEM medium were purchased from GIBCO BRL, Paisley, UK. Fetal bovine serum (FBS), non-essential amino acids (NEAA), Geneticin, trypsin/EDTA, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS [135 mM NaCl, 3 mM KCl, 2 mM KH₂PO₄, pH 7.4]) were all purchased from Sigma-Aldrich Company Ltd, Poole UK. LipofectamineTM 2000 was purchased from Invitrogen.

2.2.1.4. Antibodies

Mouse monoclonal anti-human L-ficolin antibody (GN5) was purchased from Hycult Biotechnology, Penta-his antibody was purchased from QIAGEN, and Mouse monoclonal anti-FLAG antibody, M2 was purchased from Sigma.

Horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody was purchased from Dako Cytomation (Table 2.2).

Table 2.2: Antibodies used in western blot analysis:

	Primary antibody	Secondary antibody
His-tagged L-ficolin	GN5, Mouse anti human L-ficolin monoclonal antibody. Monoclonal Penta-His antibody.	Anti-mouse monoclonal horseradish peroxidase (HRP)-conjugated antibody.
FLAG-tagged L-ficolin	GN5, Mouse anti human L-ficolin monoclonal antibody. Anti-FLAG, M2 monoclonal antibody.	Anti-mouse monoclonal HRP-conjugated antibody.

2.2.2. Production of His-tagged L-ficolin

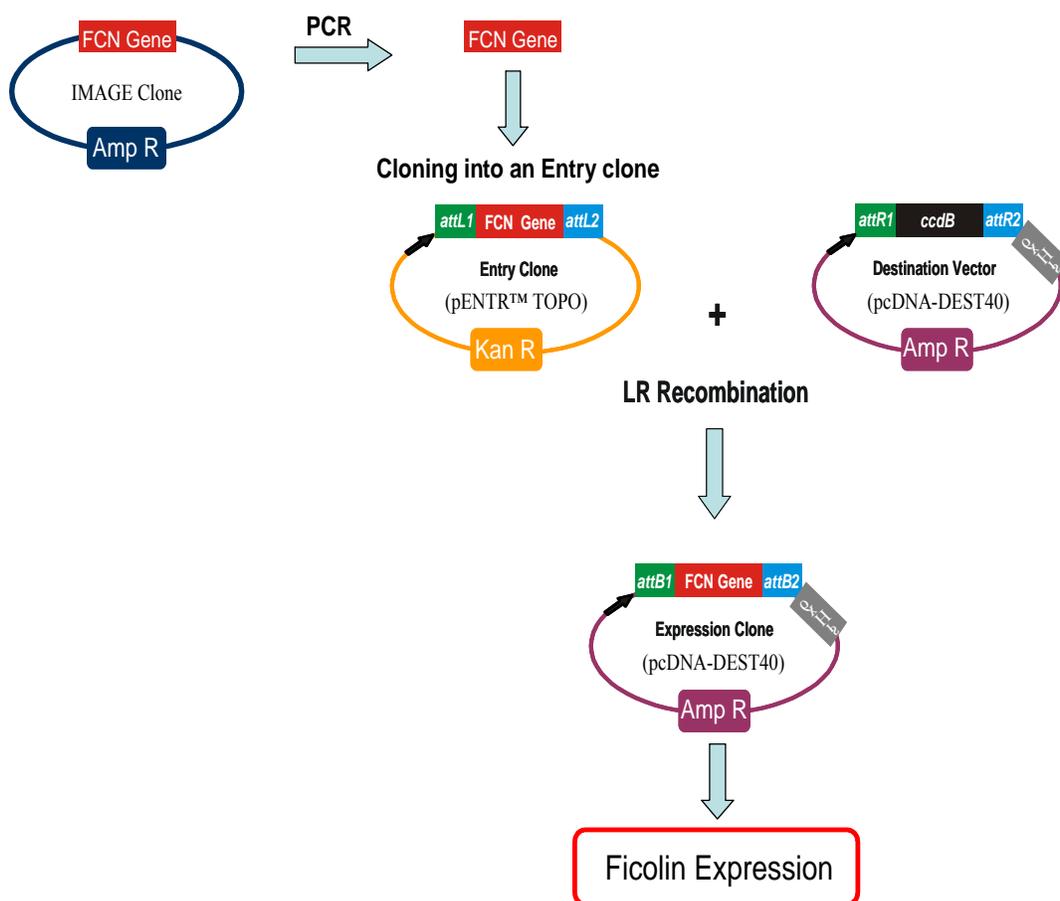


Figure 2.1: Production of recombinant His-tagged L-ficolin

In order to express His-tagged L-ficolin in mammalian cells, 2 main steps were followed. The first step was the creation of an entry clone by cloning a purified PCR product of the ficolin (FCN) gene insert, from an IMAGE clone, into an entry vector, pENTR™ TOPO® vector. The second step was the production of an expression clone by performing LR recombination between the entry clone (pENTR™ TOPO), containing the ficolin gene flanked by *attL* sites and a destination vector (pcDNA-DEST40), containing the promoter for expression of ficolin gene and C-terminal hexa-histidine (6xHis) tag (for further detection and purification of the recombinant protein). *att* site is a defined length of DNA that constitutes a recombination site. *ccdB* is a counterselectable gene that allows for negative selection of unwanted by-product plasmids after recombination.

2.2.2.1. Generating an Entry Clone

The entry clone was created by cloning a blunt-ended purified PCR product of the ficolin insert into an entry vector, pENTR™ TOPO. The cloning reaction was followed by transformation into TOP10 chemically competent *E. coli* cells and analysis of the positive transformants.

2.2.2.1.1. Miniprep extraction

IMAGE vector plasmids, carrying the full-length human L-ficolin insert, were purified using QIAprep Miniprep Spin Kit (QIAGEN). Overnight LB broth culture (3 mL) were pelleted at 5,000 x g for 10 min and plasmids were purified according to the manufacturer's instructions. Plasmid DNA was eluted in 10 mM Tris-HCl, pH 8.5. DNA was quantified using a Nanodrop spectrophotometer which converts A_{260} to concentration in ng/ μ L. The purity of DNA was also assessed, using a Nanodrop spectrophotometer, by measuring A_{260}/A_{280} ratio.

2.2.2.1.2. PCR

Purified miniprep extraction of the IMAGE vector was amplified by PCR. A typical single PCR reaction was made up with aliquots (1 μ l) of 1/100 dilution of the miniprep as a template, 0.5 μ L (10 pmol/ μ L) of both the sense primer (ficolin forward) and the antisense primer (ficolin reverse), 2.5 μ L (10X) PCR buffer, 0.2 μ L dNTPs (200 μ M each), 0.125 μ L (unit) of Expand High Fidelity taq which were added in a 25- μ l reaction volume in 0.2 mL thin-walled PCR tubes. A

negative control was set up by using the same materials as above but substituting the DNA template with 1 μ L of water. This PCR reaction was designed to amplify the whole L-ficolin encoding sequence. PCR was performed in a PTC-200 thermal cycler (Bio-Rad) with heated lid. Amplification was carried out with 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 3 min. A final extension step at 72°C for 7 min was performed.

2.2.2.1.3. Agarose gel electrophoresis

The PCR products were analysed by agarose gel electrophoresis along with a standard DNA marker and a negative control. Gel electrophoresis was conducted using 2% (w/v) agarose in TAE buffer (40 mM Tris-Acetate and 1 mM EDTA, pH 8.0), containing 0.5 μ g/mL ethidium bromide, in an electrophoresis tank containing running 1x TAE running buffer. An aliquot of PCR product (5 μ L) was mixed with 2 μ L loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol). Aliquots (5 μ L) of a standard DNA marker (Gene Ruler; Fermentas) and DNA samples to be run were loaded into each gel well. The samples were run at 90 V for 40 min. The DNA bands were visualised using UV light transilluminator and captured using the HeroLab video capture printer.

2.2.2.1.4. PCR purification

The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN), according to the manufacturer's instructions. DNA adsorbed to the

silica membrane in the presence of high salt while contaminants passed through the column. Impurities were efficiently washed away, and the pure DNA was eluted with Tris buffer and then quantified using a Nanodrop Spectrophotometer.

2.2.2.1.5. Creation of Blunt-ended DNA product

The purified PCR product was made blunt to allow the conversion of DNA fragments with 3'- and 5'-protruding ends to DNA fragments with blunt ends. Conversion of the two types of protruding ends was accomplished simultaneously by the 3'->5' exonuclease and 5'->3' polymerase activities of Pfu DNA Polymerase. Production of the blunt-ended DNA product was carried out with 7 μ L purified PCR product, 1 μ L 10X PCR buffer, 1 μ L dNTPs, and 1 μ L (2.5 units) Pfu DNA polymerase. The mixture was incubated at 72°C for 30 min.

2.2.2.1.6. Cloning

The resultant blunt-ended DNA was diluted to a concentration of 5 ng/ μ L, then ligated into a pENTR™ TOPO vector to generate an entry clone using the DNA Ligation Kit Solutions (Invitrogen). The following 6 μ L reaction was set up: 1 μ L blunt-end PCR product, 1 μ L salt solution, 3 μ L sterile water, and 1 μ L pENTR™ TOPO® vector. The mixture was incubated at 22°C for 20 min, then placed on ice for 20 min and used to transform *E. coli*.

2.2.2.1.7. Transformation

The TOPO cloning reaction was added into a vial of One Shot® TOP10 chemically competent *E. coli* cells, that had been thawed in ice, and mixed gently. The mixture was incubated on ice for 20 min. The cell suspension was heat-shocked for 30 seconds in water at 42°C, and then the tubes were immediately transferred to ice for 2 min. Then, 250 µl of S.O.C. Medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) were added and incubated at 37°C for 1 hr, with shaking. Then 150 µl of the transferred cells were spread on a pre-warmed LB agar plate (containing 50 µg/mL Kanamycin) and incubated overnight at 37°C.

2.2.2.1.8. Analysis of transformants

The positive transformants (colonies that grew on a Kanamycin-containing LB agar plate) were analysed by directional PCR and by sequencing.

Directional PCR

The positive transformants were examined by directional PCR, using 2 sets of primers; ficolin forward and M13 reverse primers, and M13 forward and ficolin reverse primers, to confirm the correct orientation of the insert. The sequence of ficolin insert was amplified by HotStar Taq polymerase kit. The PCR programme was initially started with a heat activation step at 95°C for 15 min followed by

denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 3 min. A final extension step at 72°C for 7 min was performed.

Sequencing

Colonies containing correctly orientated inserts were propagated overnight in 3 mL LB cultures, containing 50 µg/mL Kanamycin, and the transformed plasmid containing recombinant L-ficolin was purified using QiaPrep plasmid purification kit (Qiagen) and quantified using a Nanodrop Spectrophotometer. Sequencing reactions were performed using vector-specific insert-flanking primers (M13 forward or M13 reverse), to ensure maintenance of recombinant L-ficolin ORF. A sequencing reaction mix of 2 µL BigDye, 2 µL buffer (v1.1, Applied Biosystems), 1 µL of 3.2 pmol primer (M13 forward or M13 reverse), 400 ng template DNA was prepared on ice and made up to a total volume of 10 µL with ultra pure water. Samples were added to a preheated block (95°C) and subjected to the following thermal profile: [denaturation (96°C for 30 sec), annealing (50°C for 10 sec), elongation (60°C for 4 min)] for 25 cycles. Amplified ssDNA was diluted with 10 µL 18 megohm H₂O and precipitated by addition of 50 µL 100% ethanol and 2 µL 3M sodium acetate (pH 5.2). The mix was then incubated in the dark at room temperature, overnight. Samples were then centrifuged at 15,000 x g for 45 min. Supernatant was removed and the pellet was washed with 250 µL 70% ethanol. Centrifugation was repeated for 15 min, supernatant removed and 70% wash repeated. Finally, supernatant was removed and the pellets air-dried at 50°C

for 15 min. The sequencing reaction was carried out by the ABI PRISM BigDYE Terminator cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems). The resulting DNA sequences were checked for potential PCR errors using Chromas version 2.23 and aligned using MEGA3.1 software.

2.2.2.2. Generating an Expression Clone

The production of an expression clone was performed by LR recombination to shuttle the ficolin gene from the entry clone (pENTR™ TOPO), containing the ficolin gene flanked by attL sites to a destination vector (pcDNA-DEST40), containing a CMV promoter for ficolin expression.

2.2.2.2.1. Recombination reaction

The reaction was mediated by a Gateway® LR Clonase enzyme mixture (Invitrogen), which contains a blend of Int (Integrase), IHF (Integration Host Factor) and Xis (Excisionase) enzymes that catalyze the in vitro recombination between an entry clone (containing the ficolin gene flanked by attL sites) and a destination vector (containing attR sites) to generate an expression clone. LR recombination reaction was performed, after miniprep extraction of both the entry and the destination vectors, as follows: 2 µL (200 ng) entry clone, 1 µL (400 ng) destination vector, 4 µL clonase buffer, TE buffer up to 16 µL and then 4 µL LR clonase II enzyme were added. The mixture was incubated at 25°C for 1 hr, then 2 µL proteinase K was added and the mixture was incubated at 37°C for 10 min.

2.2.2.2.2. Transformation

The recombination reaction was transformed into One Shot® TOP10 chemically competent *E. coli* cells, as described in section 2.2.2.1.7. Then 150 µl of the transferred cells were spread on a pre-warmed LB agar plate, containing 100 µg/mL Ampicillin, and incubated overnight at 37°C

2.2.2.2.3. Analysis of transformants

The expression constructs in positive transformants (colonies that grew on an Ampicillin-containing LB agar plate) were verified by directional PCR (using two sets of primers; ficolin forward and BGH reverse primers, and T7 forward and ficolin reverse primers) and sequencing from both directions to confirm the presence and correct orientation of the ficolin insert, as described before in section 2.2.2.1.8.

2.2.2.2.4. Midiprep preparation

After confirmation of the ficolin sequence, a Midiprep preparation was performed prior to ficolin expression. Single colonies were transferred to 50 mL LB broth medium and raised for plasmid preparation using HP Plasmid Midiprep Kit (Sigma, Poole, UK). Overnight broth culture was pelleted at 5,000 x g for 10 min and plasmids were purified according to the manufacturer's instructions. Plasmid DNA was eluted in 10 mM Tris-HCl, pH 8.5. DNA quantification and purity was assessed using a Nanodrop spectrophotometer.

2.2.3. Production of FLAG-tagged L-ficolin

2.2.3.1. Creation of FLAG-tagged L-ficolin Construct

A recombinant amino (N)-terminal FLAG-tagged ficolin was created by inserting a FLAG peptide, composed of 8 amino acids, DYKDDDDK downstream of the signal peptide. This N-terminal FLAG tag is recognised by the monoclonal antibody M2 (Sigma), which was subsequently utilised for detection and purification of the recombinant ficolin.

2.2.3.1.1. Detection of the Cleavage Site

The recombinant L-ficolin is normally cleaved post-translationally. The cleavage site of the signal peptide was determined using SignalP-NN Prediction. The insertion site of the N-terminal FLAG tag was designed to be immediately downstream of the cleavage site (between aa 25 and 26), to overcome the effect of post-translational processing. The cleavage site was re-evaluated taking in consideration the new aa sequence after inserting the FLAG peptide.

2.2.3.1.2. Fusion PCR

After determination of the cleavage site of the signal peptide, the FLAG-tagged recombinant L-ficolin was produced by fusion PCR (figure 2.2).

2.2.3.1.2.1. The First Round PCR

In a first round PCR, 2 fragments were created; fragment A, using ficolin forward and FLAG-signal antisense primers, and fragment B, using FLAG-ficolin sense and ficolin reverse primers (figure 2.2). The PCR reaction was carried out with 5 pmol sense primer (ficolin forward/FLAG-ficolin sense) and antisense primer (FLAG-signal antisense/ficolin reverse), 0.5 U of Expand High Fidelity polymerase, and 200 μ M dNTPs in a 25- μ L reaction volume containing 1x Expand Buffer. The cycling parameters for PCR amplification consisted of 35 cycles of the following conditions: 30 sec at 94°C, 30 sec at 50°C, and 30 sec at 72°C, followed by a final step at 72°C for 7 min. PCR products were confirmed by agarose gel electrophoresis and purified using QIAQuik columns (Qiagen, UK).

2.2.3.1.2.2. The second Round PCR

In the second round PCR, the fragment A amplicon and the fragment B amplicon were used as primers and templates to each other to produce the N-terminal FLAG-tagged ficolins (figure 2.2). The amplification reaction was performed at 1:1 molar ratio, using 12 μ L (150 ng) of fragment A product and 48 μ L (1310 ng) of fragment B product in a 250 μ L reaction. The second round PCR was done using similar conditions to the first-round amplification. Resultant recombinant L-Ficolin was again confirmed by agarose gel electrophoresis.

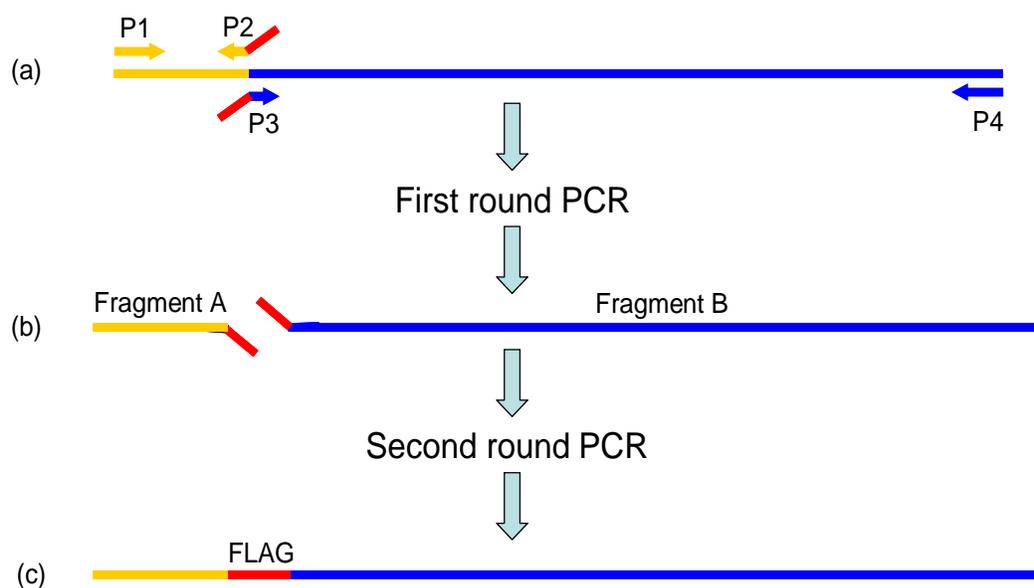


Figure 2.2: Creation of FLAG-tagged L-ficolin by fusion PCR.

A schematic diagram illustrating the creation of FLAG-tagged L-ficolin by fusion PCR. (a) two sets of primers were used in the first round PCR. P1 (ficolin forward) and P2 (FLAG signal antisense) to create fragment A (the signal peptide and part of the FLAG tag). P3 (FLAG ficolin sense) and P4 (ficolin reverse) to produce fragment B (the proper ficolin chain and a part of the flag tag). Both fragments A and B (b) were combined in the second round PCR to produce the full-length L-ficolin (c), including the FLAG tag.

2.2.3.2. Generating an Entry Clone

As described in section 2.2.2.1., the final PCR product was column purified using QIAquick PCR Purification Kit. The purified recombinant L-ficolin was blunt-ended. Then, the resulted blunt-ended DNA was diluted to a concentration of 5 ng/ μ L and ligated into the pENTRTM D-TOPO vector. The TOPO cloning reaction was transformed into TOP10 chemically competent *E. coli* cells. Finally, the correct orientation of the insert was confirmed by analysing the positive transformants by directional PCR.

2.2.3.3. Generating an Expression Clone

As described in section 2.2.2.2, the recombinant L-ficolin gene was transferred from the entry vector to the destination vector (pcDNA-DEST26TM, Invitrogen) using LR ClonaseTM II Enzyme Mix to generate an expression clone. Then, the recombinant plasmids were transformed into *E. coli* One Shot® TOP10 Competent Cells and the selection of the resistant cells was performed on Ampicillin-containing LB-agar plates (100 µg/mL). The recombinant L-ficolin insert was verified by directional PCR, using T7 forward and ficolin reverse primers and by sequencing using vector primers M13 forward and M13 reverse.

2.2.4. Cell Culture and Expression of Constructs

2.2.4.1. Cell Lines and Media

The human epithelial kidney (HEK) 293FT cell line (Invitrogen, Paisley, UK) stably transfected with pCMVSPORT6TA_g.neo plasmid was used for transfection with plasmids encoding either His-tagged or FLAG-tagged L-ficolin. The cells were thawed and growth medium was added [Dulbecco's Modified Eagles Medium with 2 mM L-glutamine (GIBCO) supplemented with 10% FBS, 0.1 mM NEAA, 500 µg/mL Geneticin (to maintain pCMVSPORT6TA_g.neo)]. Cells were maintained in 75-cm² polystyrene cell culture flasks fitted with 0.22 µm filter caps (Nunc), containing approximately 20 mL growth medium.

2.2.4.2. Cell Manipulation and Culture

2.2.4.2.1. Manipulation

All cell manipulations described in this chapter were carried out in a class II vertical laminar flow cabinet (Envair) which was cleaned with 70% v/v ethanol (in water), before and after each manipulation. All cell lines were grown in a LEEC CO₂ incubator, at a temperature of 37°C in 5% CO₂ humidified atmosphere.

2.2.4.2.2. Cell Storage

2.2.4.2.2.1. Transfer of cells from frozen storage

Frozen cells were removed from liquid nitrogen storage and defrosted rapidly in warm water. The resulting suspension was transferred to a 75-cm² polystyrene cell culture flask and 20 mL fresh growth medium were added to the cell culture flask. The growth medium was changed after approximately 24 hrs, and the cells were passaged when confluent (typically after 3 days).

2.2.4.2.2.2. Transfer of cells to frozen storage

The growth medium was removed by aspiration; the remaining cells were washed by swirling with 10 mL sterile PBS, pre-warmed to 37°C. The cell monolayer was disrupted with 5 min incubation with 2 mL 10% trypsin/EDTA in PBS, pre-warmed to 37°C. Flasks were shaken to help disrupt the cell monolayer. The resulting cell suspension was transferred to a sterile tube diluted with 8 mL fresh growth medium and centrifuged at 250 x g for 5 min. Following centrifugation, the

supernatant layer was removed and the pellet was re-suspended in 8 mL of 10% v/v DMSO in FBS. Each mL of the resulting suspension was transferred to a cryo vial suitable for storage in liquid nitrogen.

2.2.4.2.3. Cell Passage

The first part of this procedure was identical to that described in section 2.2.4.2.2. Following the centrifugation step, the supernatant was discarded and the cell pellet re-suspended in 10 mL fresh growth medium (pre-warmed to 37°C). A small amount (10 μ L) of the resulting suspension was pipetted into each chamber of a Neubauer haemocytometer, which has 25 central squares, and allowed to settle for 1 min before counting. All cells within the 5 X 5 division grids of both chambers were counted. The mean count was calculated and the numbers of cells was calculated using the following equation: Number of cells/mL = Mean cell count X dilution factor (10) X 10^4 . The suspension was diluted with fresh growth medium and poured into cell culture plates or cell culture flasks.

2.2.4.3. Transfection of HEK 293FT Cells

HEK 293FT Cells were used for transient expression of the L-ficolin constructs. Transfection was carried out using the cationic lipid reagent, LipofectamineTM 2000. Tissue culture plates (9 cm) were seeded at a density of 2×10^5 cells/mL one day prior to transfection and incubated overnight for 90% confluence at transfection. Medium was changed with Opti-MEM medium 3 hrs prior to

transfection. Two tubes A and B were prepared containing; A: 24 µg of a plasmid DNA encoding recombinant L-ficolin in 1.5 mL Opti-MEM and B: 60 µl Lipofectamine in 1.5 mL Opti-MEM. Tube B was incubated for 5 min at room temperature before addition to tube A. After mixing, the transfection solution was incubated for 20 min at room temperature to allow the DNA-Lipofectamine complexes to form. Then 3 mL of the transfection solution per plate were added drop wise to cells and mixed gently. The cells were incubated at 37°C, in 5% CO₂ incubator. Six hours after transfection, the medium was removed and replaced by Dulbecco's modified Eagle's medium and reincubated.

The supernatants containing L-ficolin were harvested 72 hrs after transfection and centrifuged at 250 x g for 5 min to remove any cell remnants before adding Protease Inhibitor Cocktail (Roche), to inhibit serine and cysteine proteases. A proportion of the supernatants was analysed for L-ficolin content by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the remainder of the supernatant was stored at -20°C.

2.2.5. SDS-PAGE, Western Blot Analysis and Silver Stain

2.2.5.1. SDS-PAGE

Separation of proteins was achieved by SDS-PAGE according to the Laemmli method [283]. The samples were electrophoresed in 10% SDS-polyacrylamide gels. In a discontinuous system, a non-restrictive large pore gel, called a stacking

gel, is layered on top of a separating gel called a resolving gel. Each gel is made with a different buffer. The stacking gel (2.5 mL per gel) and 10% polyacrylamide resolving gel (7.5 mL per gel) were prepared as follow: stacking gel [1.7 mL H₂O, 415 μ L 30% acrylamide, 315 μ L 1 M Tris (pH 6.8), 12.5 μ L 20% SDS, 8.5 μ L 30% APS and 5 μ L TEMED] and resolving gel [3 mL H₂O, 2.5 mL 30% acrylamide, 1.9 mL 1.5 M Tris (pH 8.8), 37.5 μ L 20% SDS, 50 μ L 30% APS and 11.25 μ L TEMED]. Samples were added to equal volumes of 2 times Laemmli Buffer (100 mM Tris-HCl pH 6.6, 4 % SDS, 20 % glycerol, 0.2 % Bromophenol blue). Generally, unless mentioned otherwise, the protein samples were loaded under non-reduced conditions, as a standard reaction condition. Reduced experiments are mentioned wherever used. Dithiothreitol (DTT) [40 mM] was added to 2X Laemmli Buffer for reduced samples. The Full-range Rainbow Marker Protein Standard (GE Healthcare) was used as a molecular weight standard. All samples were heated at 95°C for 5 min. and gels were run at 150V for 90 min.

2.2.5.2. Western Blot Analysis

After electrophoresis, proteins were transferred, as described by Towbin et al., 1970 [284], to Hybond ECL nitrocellulose membranes (Amersham), using a Trans-Blot Semi-Dry Transfer Cell (BioRad). Blotting paper and nitrocellulose membrane were soaked in Transfer Buffer (39 mM glycine, 48 mM tris base, 0.037 % SDS, 20 % methanol, pH 8.3) before addition to the blotter. Proteins were

transferred under a current of $1\text{mA}/\text{cm}^2$ for 1.5 hr. Membranes were blocked in 50 mL blocking solution (5 % Marvel in PBS-Tween) with gentle shaking for 1 hour or overnight. The blot was washed 3 times for 5 minutes per wash in 100 mL PBS-Tween (0.05 % Tween 20) and then incubated in primary antibody solution in PBS-Tween for 1 hour with gentle rocking. Expression of His-tagged L-ficolin was detected by either anti-L-ficolin monoclonal antibody, GN5 or penta-his monoclonal antibody. The FLAG-tagged L-ficolin was probed with either anti-L-ficolin monoclonal antibody, GN5 or anti-FLAG monoclonal antibody M2 (table 2.2). After incubation with primary antibody, washes were repeated to remove the excess antibody and membranes incubated with HRP-conjugated goat anti-mouse secondary antibody diluted in PBS-Tween for 1 hour with gentle rocking followed by three washes for 5 minutes per wash. The blots were developed using enhanced chemiluminescence detection reagents ECL-Plus (GE Healthcare, UK) for 1 min and then removed from the solution and wrapped in cling film. The membrane was put into a film case and brought to the dark room. A sheet of Kodak film (Kodak Bio Max Film) was overlaid on the blot for 2 minutes. Then the film was soaked in developer for 2 minutes, fixed in fixing solution for 2 minutes, then washed in water for 2 minutes and allowed to dry.

2.2.5.3. Silver Stain

Samples were added to equal volume of Laemmli buffer under non-reducing and reduced conditions (with 20 mM DTT), then loaded and gel electrophoresis was

performed as in section 2.2.5.1. Staining gel with Silver stain was carried out using SilverQuest stain kit (Invitrogen) according to manufacturer's instruction. After staining, the gel was dried using a Dry Ease Gel Drying System (Invitrogen).

2.2.6. Purification of Expressed Proteins

2.2.6.1. Purification of His-tagged L-ficolin

2.2.6.1.1. Immobilised Metal Affinity Chromatography

Nickel agarose gel was used for purification of the polyhistidine-tagged L-ficolin by Immobilised Metal Affinity Chromatography (IMAC) using a 1 mL HisTrap Chelating HP column (GE Healthcare). The supernatant containing L-ficolin was injected and loaded onto the column. Weakly-bound proteins were then washed off with 5 column volumes (CV) wash in Wash Buffer (20 mM sodium phosphate, 300 mM NaCl, and 20 mM imidazole pH 7.4). Then an elution step, 5 CV steps of 50 mM imidazole [0-500 mM imidazole using Elution Buffer (20 mM sodium phosphate, 300 mM NaCl, 0.5 M imidazole, pH 7.4)]. A post elution step of 5 CV of 0.5M imidazole was used to ensure removal of all proteins. The start material, flow-through, wash and eluate were analysed by western blot analysis and ELISA, and the peak fractions were kept at 4°C, for further analysis.

2.2.6.1.2. Ion Exchange Chromatography

A Mono Q 5/50 GL Tricorn column (Amersham) was equilibrated with start buffer (20 mM Tris-HCl, pH 8.0) for 10 minutes at a flow rate of 0.5 mL/min. The

sample was loaded onto the column at the same flow rate. The bound protein was then eluted in a 20 mL linear salt gradient from 50 mM to 1 M NaCl, in 20 mM Tris-HCl, pH 8.0. The column flow-through and eluates were collected in 1 mL batches and analysed for the presence of L-ficolin by western blot analysis and ELISA.

2.2.6.2. Purification of FLAG-tagged L-ficolin

FLAG-tagged L-ficolin was purified by affinity chromatography using anti-FLAG M2 affinity gel (Sigma). Prior to loading the L-ficolin sample, the affinity matrix was equilibrated with wash buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Binding of FLAG-tagged ficolin to the M2 gel was performed by incubation for 3 hrs at room temperature with gentle mixing to capture the FLAG-tagged ficolin. Contaminants were washed off the resin with wash buffer, in a column format, until the absorbance of the eluate at 280 nm was less than 0.05 versus a wash solution blank. Bound FLAG-tagged L-ficolin was eluted off the column by competitive elution with 5 CV of a solution containing 175 ng/ μ L FLAG peptide in wash buffer. One millilitre fractions were collected, and the protein-containing fractions were pooled and dialysed against TBS buffer. The purity of the expressed L-ficolin was monitored by SDS-PAGE. L-ficolin-containing fractions were stored at -20°C .

2.2.7. Purification of Serum L-ficolin

Serum ficolin was isolated from 1 litre of citrated human plasma (HD Supplies) using a method adapted from Cseh et al. [285].

2.2.7.1. Immobilisation of GlcNAc on Sepharose-4B

Sepharose-4B beads (GE Healthcare) were derivatised with GlcNAc according to Krarup et al., 2004 [286]. The beads were activated by incubation with 10% (v/v) divinyl sulfone in 0.5 M Na₂CO₃, pH 11, for 1.5 h at room temperature. Then the beads were washed with 250 mM Na₂CO₃, pH 11, and derivatised in 250 mM Na₂CO₃, pH 11 containing 10% (w/v) GlcNAc overnight at room temperature. After washing the beads with water, they were incubated with 100 mM ethanolamine, pH 9, to block residual active groups, before a final wash with TBS buffer (50 mM Tris, 150 mM NaCl, 20 mM CaCl₂, pH 7.4).

2.2.7.2. L-ficolin Purification

Plasma was coagulated by addition of 1 M CaCl₂ to a final concentration of 20 mM CaCl₂ and incubated at 37 °C for 30 min, followed by incubation at 4 °C for 3 hrs. The serum was collected from the clot by centrifugation at 3000 X g. Polyethylene glycol (PEG) 4000 was added to the serum to a final concentration of 7% (w/v) PEG. After incubation at 4 °C for 2 hrs, the PEG precipitate was separated by centrifugation for 30 min at 4000 X g at 4 °C. The pellet was dissolved in 200 mL TBS (50 mM Tris, 0.2 M NaCl, 20 mM CaCl₂, pH 7.4),

followed by incubation with GlcNAc-Sepharose-4B beads for 2 hrs at 4 °C. The matrix was washed with TBS until absorbance of flow-through at 280 nm was 0.001. Bound MBL was eluted by a mannose wash (0.1 M mannose solution in TBS), then ficolin was eluted with 0.6 M GlcNAc in TBS. Pooled ficolin fractions were dialysed against TBS.

2.2.8. Ficolin quantitation

The levels of recombinant, as well as purified serum L-ficolin were determined by ELISA, based on another assay developed by Dr Anders Krarup (MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford), using biotinylated anti L-ficolin monoclonal antibodies, GN5. A standard L-ficolin preparation was kindly provided from Dr Anders Krarup [286].

2.2.8.1. Biotinylation of anti L-ficolin (GN5)

The GN5 antibody was dialysed in 0.1M Sodium Borate Buffer (pH 8.8). N-hydroxysuccinimidobiotin (Sigma) was prepared at 10 mg/mL in dimethyl sulphoxide (DMSO). The antibody solution was mixed with biotin ester at a ratio of 2.5 mg of ester per mg of antibody. After vortexing for 5 min, the mixture was incubated at 4°C for overnight on a rotor. Then, 20 µL of 1M NH₄Cl were added per 250 µg of ester, to inactivate free biotin, and incubated for a further 10 min at room temperature. The mixture was then dialysed against PBS at 4°C for overnight on a rotor.

2.2.8.2. ELISA

MaxiSorb microtiter plate wells (Nunc, Denmark) were coated with 100 μ L of 1 mg/mL bovine serum albumin (BSA) in phosphate buffered saline (PBS) (figure 2.3). After overnight incubation at 4°C, wells were incubated for 2 hrs at room temperature with 100 μ L 0.1% acetic anhydrid in methanol, to acetylate BSA. Wells were thereafter blocked with 200 μ L of PBS, 0.05% (v/v) Tween 20 (PBS-Tween), containing 5% milk powder for 4 hrs at room temperature. The plate was washed three times (Denley Well Wash) with TBS (10 mM Tris-Cl, 140 mM NaCl, pH 7.4), containing 0.05% Tween 20 and 5 mM CaCl₂ (wash buffer). All subsequent washes were performed with this buffer. Wells were washed and incubated with 100 μ L of a source of L-ficolin, diluted in PBS-Tween, overnight at 4 °C. After this incubation, the plates were washed 3 times as before. For detection of L-ficolin, 100 μ L of mouse IgG monoclonal biotin-labelled anti-human L-ficolin antibody (GN5, 0.5 μ g/mL) were added to each well. The plates were incubated for 2 hrs at room temperature. Wells were washed and incubated for 30 min at 37 °C with a 1:5000 dilution of a streptavidin-alkaline phosphatase (streptavidin-AP) conjugate in wash buffer. Following a further three washes with TBS-Tween, binding was visualised with 100 μ L of p-nitrophenyl phosphate substrate (pNPP). The absorbance was measured at 405 nm using a Vmax plate reader (Molecular Devices) after 30 min.

A two-fold dilution series (1/6.25 to 1/400) of a pooled human serum in PBS-T were added to the wells. PBS-Tween was used as a negative control (blank). L-ficolin levels were quantified using a standard curve (figure 5.2) generated using a two-fold serial dilution of a pooled serum, containing 4 $\mu\text{g/mL}$. This sample was quantified by comparison to a standard L-ficolin preparation, as previously described [286]. Optical densities of the standard sample were used to determine standard curves on a log 10 scale and interpolated X-values were obtained, as described in section 5.3.1.

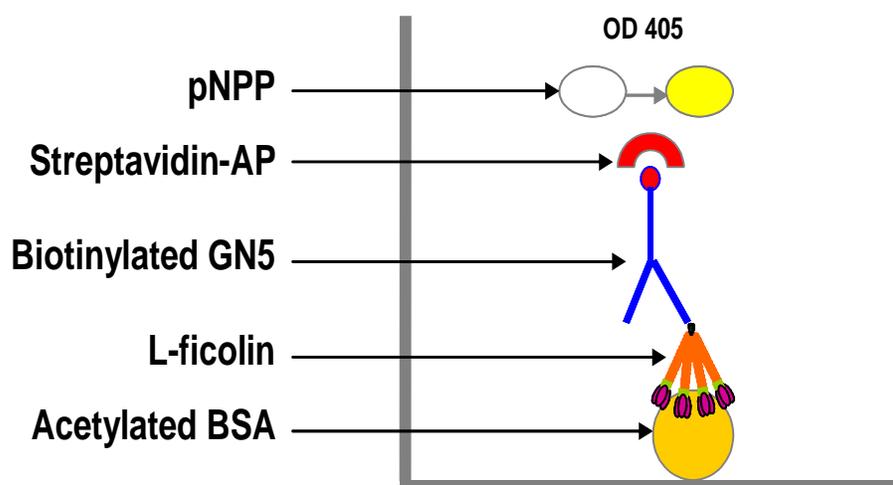


Figure 2.3: Quantitation of L-ficolin by ELISA.

A schematic diagram shows the components of the ELISA assay used to quantify recombinant and serum-derived L-ficolin. The wells were coated with BSA followed by acetic anhydride. Ficolin samples were added and the bound ficolin was detected by biotinylated monoclonal anti-L ficolin, GN5. Streptavidin-alkaline phosphatase (streptavidin-AP) conjugate was added followed by p-ntirophenyl phosphate (pNPP) substrate. The OD was determined at 405 nm.

2.3. Results

2.2.2. Production of His-tagged L-ficolin

2.2.2.1. Generating an Entry Clone

PCR was carried out for the extracted miniprep of the IMAGE vector (pPCR-Script Amp SK) containing the coding region for the full-length human L-ficolin, using ficolin forward and ficolin reverse primers with Expand High Fidelity PCR system (figure 2.4).

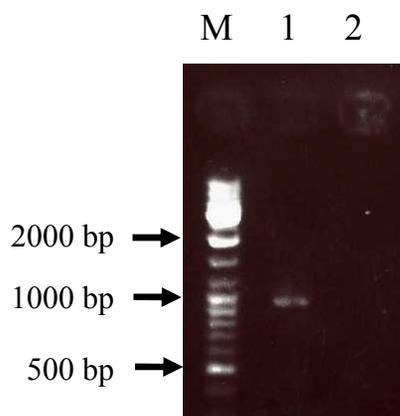


Figure 2.4: Detection of ficolin insert in IMAGE vector by PCR.

PCR amplification of the full-length human L-ficolin insert in IMAGE vector, using ficolin forward and ficolin reverse primers. The ficolin insert 942 base pair (bp), was detected in lane 1. Lane 2 is a negative control, containing no DNA template.

The PCR product was purified then blunt-ended and ligated into a pENTR vector to generate an entry clone. The cloning product was transformed into TOP10 chemically competent *E. coli* cells that were plated out onto Kanamycin-

containing LB agar plates for selection of the resistant cells as the pENTR vector carries a Kanamycin resistance gene. After overnight incubation, ten colonies were selected and screened by PCR (figure 2.5), using the vector primers; M13 forward and M13 reverse primers, to confirm the presence of the insert.

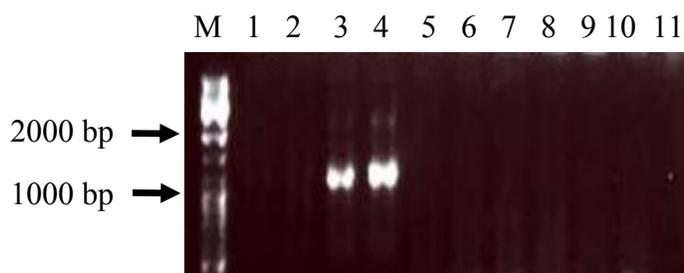


Figure 2.5: Screening of the colonies for positive transformants by directional PCR.

After transformation of the L-ficolin DNA ligated to pENTR vector into TOP10 *E. coli*, 10 colonies (1-10) were screened by PCR using M13 forward and M13 reverse primers for positive transformant colonies containing pENTR. The colonies no. 3 (lane 3) and no. 4 (lane 4) are positive transformants, containing the ficolin insert. Lane 11 represents a negative control, containing no DNA template.

Two colonies were detected by PCR (figure 2.5; lane 3 and 4) as positive transformants with ficolin-carrying pENTR, and directional PCR was carried out for them to confirm the correct orientation of the insert (figure 2.6).

Both positive transformants (colonies 3 and 4) contained the ficolin insert with correct orientation as confirmed by directional PCR (figure 2.6; A: lane 1 and 2 and B: lane 4 and 5) and by sequencing.

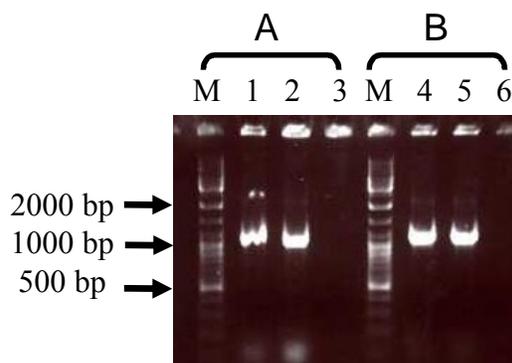


Figure 2.6: Directional PCR for the positive transformants with pENTR.

Directional PCR for positive transformant colonies (no. 3 and 4), using; A: ficolin forward and M13 reverse primers (lane 1 and 2), B: M13 forward and ficolin reverse primers (lane 4 and 5). Detection of the ficolin insert in both sets of reactions confirms the correct orientation of the insert. Lane 3 and 6 represent negative controls, containing no DNA templates.

The sequencing reactions were performed with BigDye 1.1 chemistry using vector-specific insert-flanking primers (M13 Forward and M13 Reverse primers) for plasmid DNA isolated, from the positive transformant, by miniprep extraction. The resulting DNA sequences were checked using Chromas version 2.23 and the resulting ORF checked using MEGA3.1 software. Sequence analysis and alignment against ficolin sequence database revealed a 100% match with the reference ficolin sequence (NCBI data base; accession number: NP_004099). Figure 2.7 represents the amino acid sequence of L-ficolin. A glycerol stock was made from each colony to be used as a source of entry clone in recombination reaction to generate an expression clone.

```

1  MELDRAVGVLGAATLLLSFLGMAWALQAADTCPEVKMVGL 40
41 EGSDKLTILRGCPGLPGAPGPKGEAGTNGKRGERGPPGPP 80
81 GKAGPPGPNGAPGEPQPCLTGPRTCKDLLDRGHFLSGWHT 120
121 IYLPDCRPLTVLCDMDTDGGGWTVFQRRVDGSVDFYRDWA 160
161 TYKQGFGRSLGEFWLGNDNIHALTAQGTSELRVDLVDFED 200
201 NYQFAKYRSFKVADEAEKYNLVLGAFVEGSAGDSLTFHNN 240
241 QSFSTKDQDNDLNTGNCAVMFQGAWWYKNCHVSNLNGRYL 280
281 RGTHGSFANGINWKSGKGYNYSYKVSEMKVRPA 313

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Figure 2.7: Amino acid sequence of L-ficolin.

The figure shows the amino acid sequence of L-ficolin. The different regions within the polypeptide chain are highlighted in different colours, as follows: the signal peptide (yellow), the amino terminal region (cyan), the collagen-like domain (magenta), the neck region (grey), and the fibrinogen-like domain (green). The underlined amino acid sequences refer to the potential glycosylation sites. This sequence is available in UniProtKB database, accession: Q15485.

2.2.2.1. Generating an Expression Clone

LR recombination reaction was carried out between the entry clone pENTR and the destination vector pcDNA-DEST40 to generate an expression clone. The reaction was performed using LR Clonase enzyme mixture, which contains the necessary enzymatic activity to excise the gene of interest from the entry clone and integrate it into the destination vector pcDNA-DEST40 which provides the CMV promoter for high-level, constitutive expression in mammalian systems. The destination vector has the *attR1* and *attR2* sequences and the counter selectable marker *ccdB*. Upon addition of LR Clonase, the expression clone is produced along with a by-product plasmid containing *ccdB*. The *ccdB* protein poisons DNA gyrase, causing degradation of the host chromosome and cell death. The

recombination reaction was transformed into TOP10 chemically competent *E. coli* cells. As the destination vector contains an Ampicillin resistance gene, the expression clones are selected on Ampicillin-containing plates. Thus, 150 μ L of the transformed cells were plated out on an Ampicillin-containing LB plate to select the positive transformants.

Then, positive transformants were verified by PCR (figure 2.8), using 2 sets of primers; ficolin forward and BGH reverse primers, and T7 forward and ficolin reverse primers to confirm the presence and correct orientation of the ficolin insert. All the 10 colonies contained the ficolin insert with correct orientation as confirmed by directional PCR (figure 2.8) and by sequencing. The correct ficolin insertion in all selected colonies represents high recombination efficiency. A glycerol stock was made from one colony to be used as a source of expression clone and midiprep extraction was done to be used for transfection.

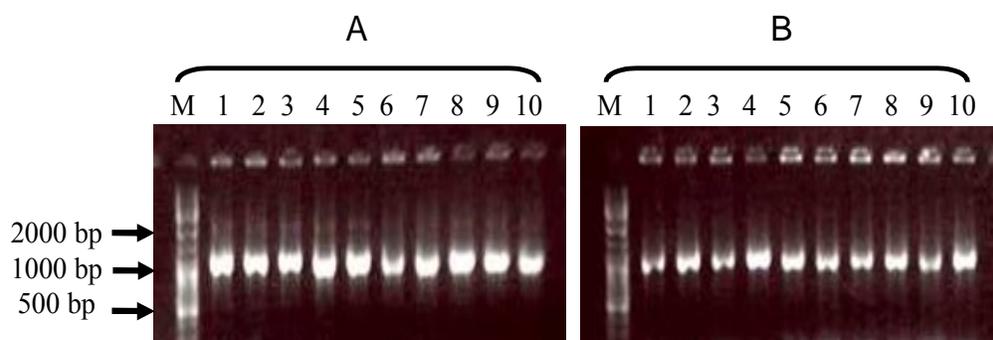


Figure 2.8: Directional PCR for the positive transformants with *pcDNA DEST40*.

Directional PCR for ten positive transformant colonies (1-10), using; A: ficolin forward and BGH reverse primers, and B: T7 forward and ficolin reverse primers. All the selected colonies showed the presence of the ficolin insert in both sets of reactions, confirming the correct orientation of the ficolin insert.

2.2.3. Production of FLAG-tagged L-ficolin

2.2.3.1. Creation of FLAG-tagged ficolin construct

Determination of the signal peptide cleavage site was performed using SignalP-NN Prediction. The cleavage site was determined to be between aa 25 and 26. Thus, the insertion site of the N-terminal FLAG tag was designed to be immediately downstream of the cleavage site. Figure 2.11 shows the sequence data of the insertion site of N-terminal FLAG peptide.

An amino-terminal FLAG-tagged L-ficolin construct was produced by insertion of the FLAG peptide downstream of the cleavage site, by fusion PCR (figure 2.9). The first round PCR products (A amplicon; lane 1 and B amplicon; lane 2) were combined in the second round to produce a full-length FLAG-tagged ficolin (lane 3).

2.2.3.2. Generation of an Entry Clone

The full-length FLAG-tagged ficolin was cloned into the pENTR™ TOPO entry vector and transformed into TOP10 *E.coli*, which were plated onto Kanamycin-containing LB agar plates for selection of positive transformants. Ten growing colonies of TOP10 *E. coli* were examined by directional PCR (figure 2.10), using; A: ficolin forward and M13 reverse primers, B: M13 forward and ficolin reverse primers. The ficolin insert was detected in the colony no. 8 in both sets of reactions.

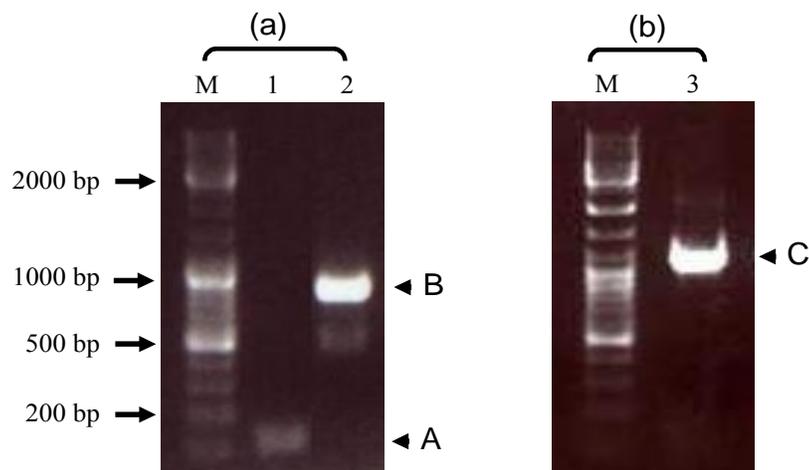


Figure 2.9: Creation of FLAG-tagged L-ficolin by fusion PCR.

Gel electrophoresis and ethidium bromide staining of the resulting PCR products. (a) First round PCR products; fragment A (lane 1), using ficolin forward and FLAG-signal antisense primers and fragment B (lane 2), using FLAG-ficolin sense and ficolin reverse primers. (b) Second round PCR product: fragment A and fragment B were purified and combined in the second round to act as primers and templates to each other to produce the final terminal FLAG-tagged ficolin, C (lane 3). The DNA size marker (M) is shown on the left, in base pairs (bp).

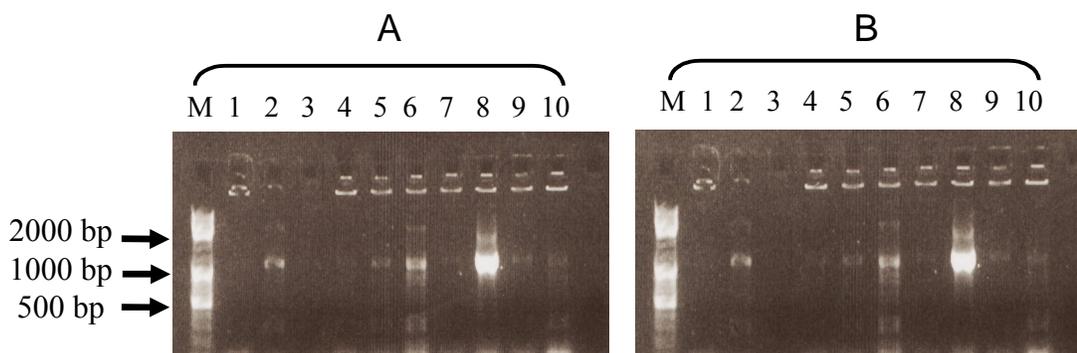


Figure 2.10: Directional PCR for the positive transformants with pENTR.

Directional PCR for 10 colonies (1-10) of transformed TOP10 *E. coli* growing on a Kanamycin-containing LB agar plate, using; A: ficolin forward and M13 reverse primers, B: M13 forward and ficolin reverse primers. The ficolin insert can be detected in the colony no. 8 in both sets of reactions, confirming the correct orientation of the insert.

Bidirectional sequence analysis was also performed to check the sequence integrity of the ficolin insert (figure 2.11), using M13 forward and M13 reverse primers. The contiguous DNA sequences were constructed from forward and reverse sequencing reactions. The chromatograms showing the sequence data generated by Chromas version 2.23 revealed the ficolin sequence including the inserted octapeptide FLAG tag, that would enable further ficolin purification, downstream of the signal peptide.

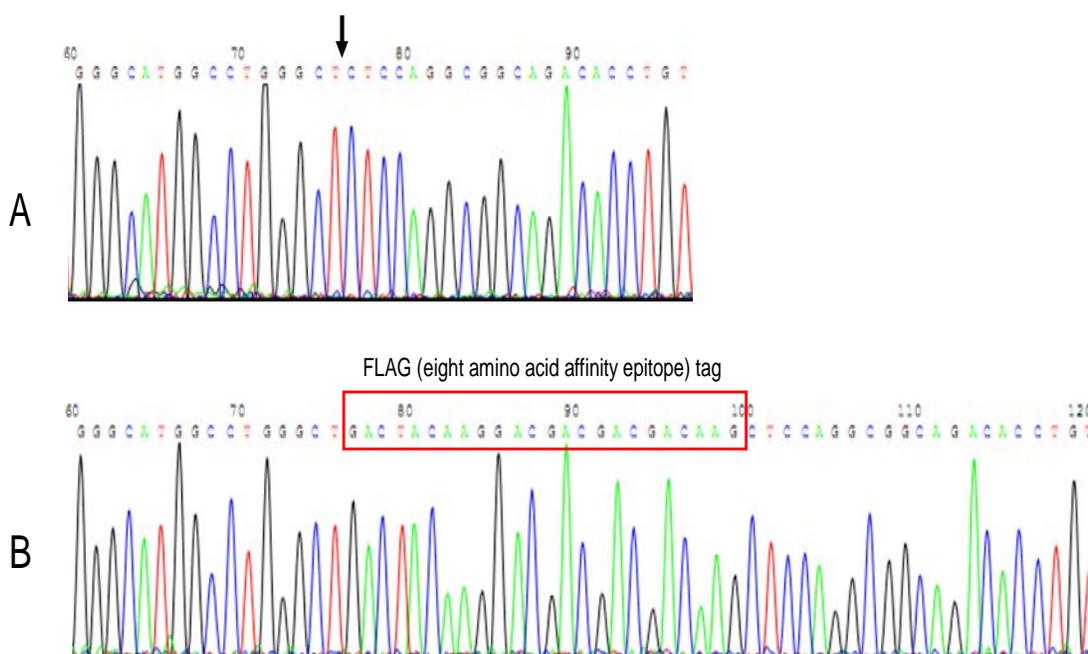


Figure 2.11: Sequence data; insertion of N-terminal FLAG peptide.

Chromatograms showing examples of sequence data generated by Chromas version 2.23. The upper chromatogram (A) depicts the sequence of the amino-terminal end of L-ficolin and the black arrow points at the insertion site of the FLAG peptide. The lower chromatogram (B) shows the sequence of the upper segment after insertion of octapeptide affinity epitope (FLAG) tag (24 bp).

2.2.3.3. Generation of an Expression Clone

The ficolin insert was subsequently transferred from the entry vector to the destination vector pcDNA-DEST26 for expression of the recombinant FLAG-tagged L-ficolin. The expression clone was also verified for the presence and orientation of the ficolin insert by directional PCR, then the ficolin sequence was confirmed by bidirectional sequence analysis.

2.2.4. Expression of L-ficolin Constructs

Recombinant L-ficolin was produced by transient transfection of His-tagged and FLAG-tagged L-ficolin into HEK 293FT cell line using Lipofectamine with serum-free media, to eliminate BSA contamination. The ficolin-containing supernatants were harvested and the ficolin was separated by SDS-PAGE using 10% polyacrylamide gels, under non-reducing and reducing conditions. Western blot was carried out for detection of the expressed ficolin.

His-tagged ficolin was detected with either 5-His primary antibody or GN5, anti L-ficolin monoclonal antibody, then anti-mouse monoclonal HRP-conjugated antibody was used as a secondary antibody. SDS-PAGE under non-reducing conditions demonstrated that recombinant L-ficolin was a mixture of monomers and oligomers (figure 2.12). Besides the monomeric band at 35 kDa, covalent oligomers ranged from dimers (70 kDa) to oligomers containing several subunits. The presence of two bands at 35 kDa may represent 2 separate polypeptides

differing in glycosylation. A significant band was observed above the 250 kDa band of the marker possibly corresponding to the 12-mer consisting of four trimeric L-ficolin oligomers. Some weaker bands can be seen above the possible 12-mer, suggesting that L-ficolin is able to form covalently-linked higher molecular weight oligomers. Under reducing conditions (figure 2.12 B), by adding DTT to a final concentration of 20 mM, the oligomers were reduced to monomers with a molecular mass of 35 kDa as a result of disruption of the disulphide bonds connecting oligomeric ficolin.

FLAG-tagged L-ficolin was analysed by western blot using M2 anti-FLAG antibody or GN5, then anti-mouse monoclonal HRP-conjugated antibody were used as a secondary antibody (figure 2.12 A). Similar to His-tagged L-ficolin (H), FLAG-tagged L-ficolin (L) separated into a ladder of bands from 35 kDa to the top of the gel, suggesting the formation of dimmers (70 kDa) and oligomers of the 35 kDa subunit. The possible L-ficolin oligomers are seen at and above 250 kDa. As a negative control, a cell culture supernatant from untransfected HEK 293FT cells (U) was also examined using the same antibodies.

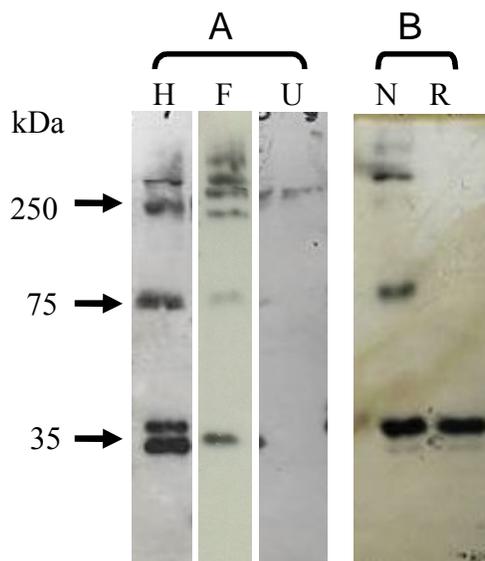


Figure 2.12: Detection of expressed L-ficolin by western blot analysis.

Both His-tagged (H) and FLAG-tagged (F) L-ficolin were produced by transfection in HEK 293FT cells using lipofectamine. Supernatant samples were loaded on 10% SDS-PAGE gels, then blotted. Panel A: Detection of L-ficolin (H and F) by western blot analysis was carried out with GN5, monoclonal anti-human L-ficolin, followed by goat anti-mouse peroxidase conjugated secondary antibody, under non-reducing conditions. Ficolin monomer is visible at 35 kDa and oligomeric forms are also visible at and above 250 kDa. Cell culture supernatant from untransfected HEK 293FT cells (U) was used as a negative control. Panel B: His-tagged L-ficolin subjected to 10% polyacrylamide gel under non-reducing (N) and reducing (R) conditions. There are 2 bands at 35 kDa which could represent 2 separate polypeptides differing in glycosylation. Disruption of disulphide bonds under reducing conditions, using DTT at a final concentration of 20 mM, was evident by disappearance of the higher oligomeric forms, which were replaced by monomeric ficolin visible at 35 kDa. Position of protein size markers are shown (in kDa)

2.2.5. Purification of Expressed L-ficolin

2.2.5.1. Purification of His-tagged L-ficolin

The expressed His-tagged L-ficolin was nickel agarose purified by metal affinity chromatography, using 20 mM imidazole in the initial binding stage and thereafter a stepwise gradient up to 500 mM to release the bound ficolin. Fractions of 1 mL were collected and the purification profiles of the recombinant L-ficolin protein were studied by SDS-PAGE and immunoblot analysis as well as ELISA. Purification of L-ficolin (figure 2.13) revealed an elution profile where some putative degradation products of the L-ficolin were seen in the 200 mM imidazole fractions, whereas the majority of the multimeric protein eluted at 250 mM imidazole, although some putative degradation was visible. L-ficolin quantitation of the different fractions was determined by ELISA, previously described in section 2.2.8.2. Protein concentrations of more than 10-12 ug purified protein/mL elution buffer was achieved using these conditions. A total of 50 ug of purified L-ficolin could be obtained from 45 mL of cell culture. The peak fractions were pooled and dialysed against PBS then further purified by Mono Q ion exchange chromatography using a linear gradient from 50 mM 1 M NaCl. The multimeric L-ficolin was eluted at 400-500 mM NaCl.

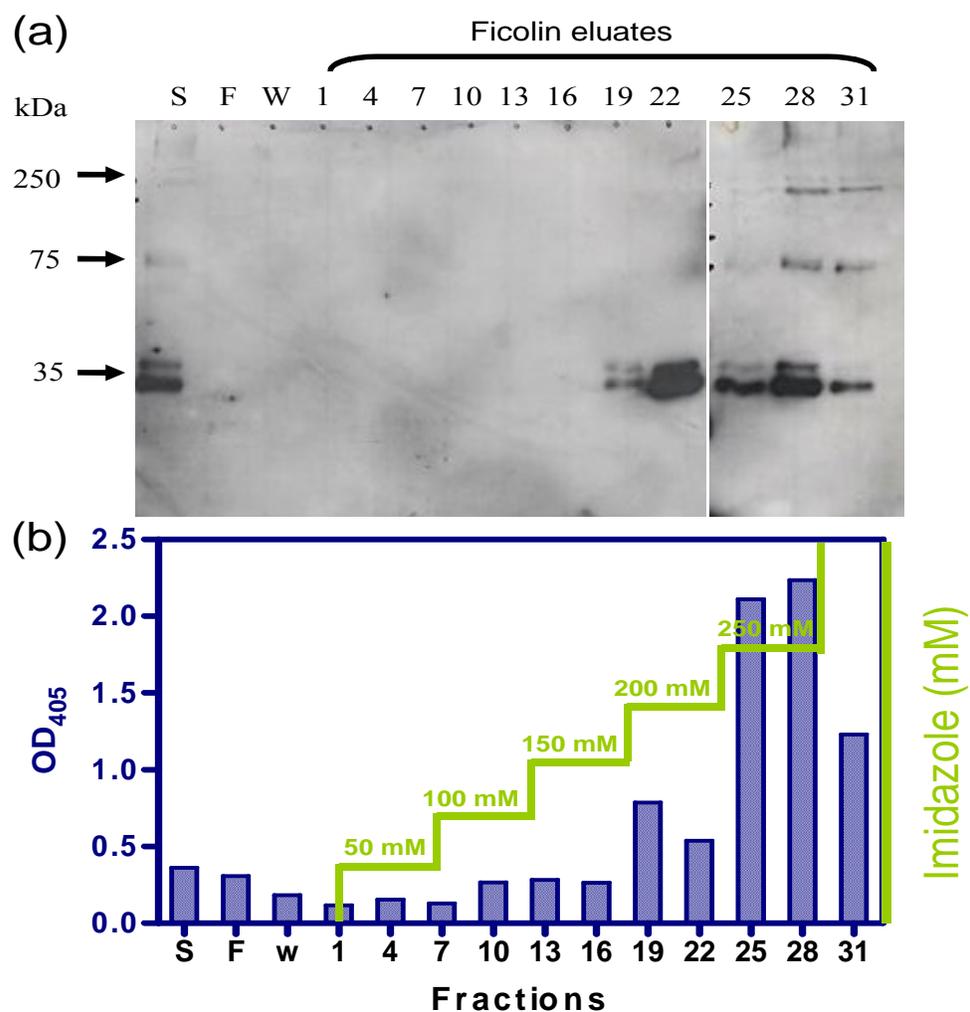


Figure 2.13: Detection of purified His-tagged ficolin.

Supernatants from 3 tissue culture plates (45 mL) were harvested and nickel agarose purified using a step gradient (50 mM rises) of imidazole. The start material (S), flow-through (F), wash (W) and the eluted fractions (1 to 34, every third fraction) were loaded on 10% SDS-PAGE gels (a) and quantified for ficolin content by ELISA, previously described in section 2.2.8.2 (b). Ficolin was detected by western blot analysis (a) using penta-His primary antibody and anti-Mouse monoclonal peroxidase conjugated secondary antibody. L-ficolin polypeptide is visible as a band ~35 kDa in fractions 19 and 22, at an imidazole concentration of 200 mM, representing monomeric L-ficolin. Another peak of eluted ficolin is detectable in fraction 25, 28 and 31, at an imidazole concentration of 250 mM, representing multimeric L-ficolin. Quantification of ficolin content in the eluted fractions by ELISA (b) revealed 2 peaks matching with those detected in western blot analysis; one lower peak in fractions 19 and 22, and a second higher peak in fractions 25, 28 and 31, containing multimeric L-ficolin.

The silver stained SDS-PAGE (figure 2.14) after ion exchange chromatography of the IMAC-purified His-tagged L-ficolin revealed that the eluted fractions showed L-ficolin protein sample of high purity.

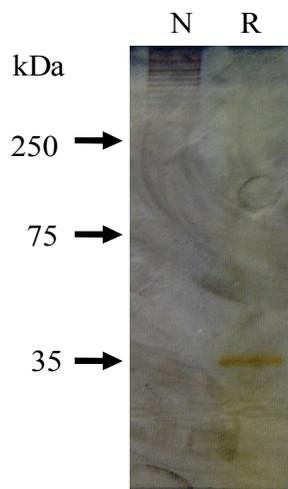


Figure 2.14: Silver stain of purified His-tagged L-ficolin.

After IMAC purification of His-tagged L-ficolin, the peak fractions were pooled and further purified by ion exchange chromatography using a linear gradient of NaCl. Silver staining of the eluted fractions was performed to check purity of the purified ficolin. By loading 100 ng of L-ficolin, the oligomeric ficolin can be seen as higher molecular weight bands under non-reducing conditions (N). The monomeric ficolin is not visible in the silver-stained gel as a result of lower sensitivity of the silver stain compared to western blot analysis. Under reducing conditions (R), the ficolin is visible as monomeric form, at 35 kDa.

2.2.5.2. Purification of FLAG-tagged L-ficolin

The FLAG-tagged L-ficolin was purified by affinity chromatography using the anti-FLAG M2 Affinity Gel, as described in section 2.2.6.2. The purified ficolin was applied to SDS-PAGE gels (10% polyacrylamide) and transferred electrophoretically to nitrocellulose membranes which were probed with either

monoclonal anti-human L-ficolin antibody GN5 or anti-FLAG monoclonal antibody, M2. The immunoblot shows a large homogenous band corresponding to the expected size of L-ficolin, above 250 kDa (figure 2.15). Some higher molecular weight complexes as well as some smaller molecular weight proteins were also visible. These were speculated to be multimeric L-ficolin complexes and breakdown products, respectively, as they are antibody reactive. Quantitation of the eluted ficolin was determined by ELISA, indicating elution of 20-25 ug of L-ficolin from 30 mL of cell culture supernatant.

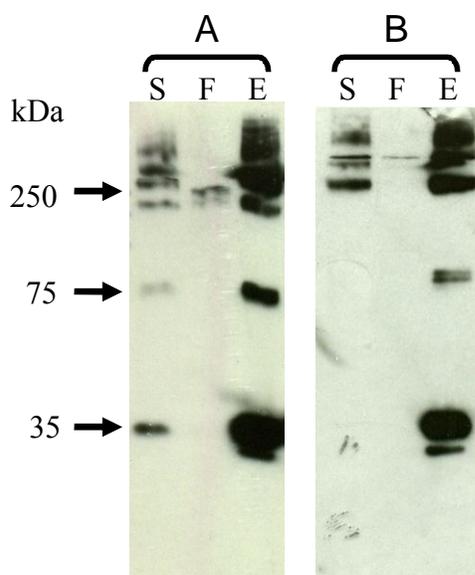


Figure 2.15: Western blot analysis of purified FLAG-tagged L-ficolin.

FLAG-tagged L-ficolin was expressed in HEK 293FT cells and purified using an anti-FLAG M2 affinity gel. The eluted L-ficolin samples were then resolved using non-reducing polyacrylamide gel electrophoresis, followed by western blot. The membrane was probed with either monoclonal anti L-ficolin antibody, GN5 (A) or with anti-FLAG monoclonal antibody, M2 (B). Each blot represents the start material (S, supernatant) in lane 1, the flow through (F) in lane 2 and the eluted L-ficolin (E) in lane 3. This SDS-PAGE analysis illustrates the monomeric (35 kDa), dimeric (70 kDa), and multimeric (above 250 kDa) forms of L-ficolin.

2.2.8. Purification of Serum L-ficolin

Serum L-ficolin was purified by a method adapted from Cseh et al. [285]. Pooled citrated human plasma was treated with CaCl_2 to remove fibrinogen. Proteins in the supernatant were precipitated with PEG and the resulting pellet was resuspended in TBS buffer containing calcium ions. The suspension was incubated with GlcNac-4B beads, then the beads were washed with TBS and with mannose solution to remove bound MBL. Little amount of L-ficolin was observed in the flow-through and mannose wash (figure 2.16), indicating that little ficolin protein is lost at this stage. Ficolin was then eluted with GlcNac and showed a banding pattern of L-ficolin on western blot analysis. Proteins of 35 kDa, 70 kDa and above 250 kDa, were observed using anti-L-ficolin antibody under non-reducing conditions, representing ficolin monomers and multimers. These oligomeric forms were replaced by a single monomeric form of 35 kDa when subjected to SDS-PAGE under reducing conditions. Using this strategy in purification of serum L-ficolin from 1 litre of human plasma resulted in a ficolin yield of 260 μg and a concentration of up to 33 $\mu\text{g}/\text{mL}$ in eluted fractions.

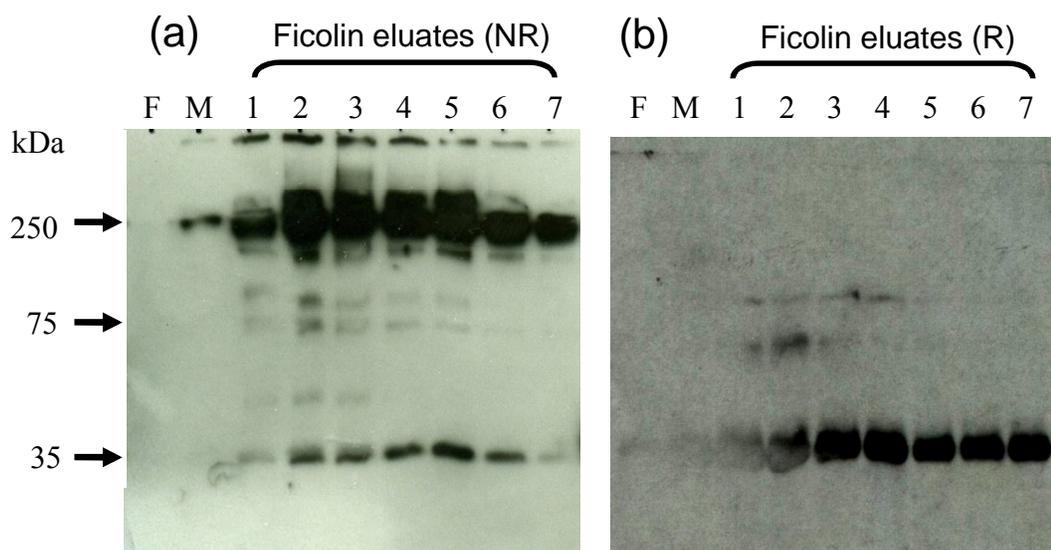


Figure 2.16: Western blot analysis of purified serum L-ficolin.

L-ficolin was obtained from human serum using GlcNAc-Sepharose beads. (a): The flow-through (F), mannose wash (M) and ficolin eluates (1-7) were loaded to 10% SDS-polyacrylamide gel, under non-reducing conditions (NR). The blot was developed using anti-L-ficolin monoclonal antibody, GN5. As expected, the eluted ficolin (1-7) showed bands at 35 kDa (monomers), 70 kDa (dimers) and at and above 250 kDa (multimers) indicating oligomeric composition. (b): the same fractions were applied to 10% SDS-polyacrylamide gel, under reducing conditions (R), the oligomers of serum L-ficolin were reduced to a single chain with a molecular mass of 35 kDa. Molecular weight markers are indicated on the left.

2.4. Discussion

HCV infection has been recognised as a major worldwide health problem, with several host and viral factors contributing to HCV disease progression [29]. HCV envelope glycoproteins, E1 and E2, provide a target for host immune recognition. L-ficolin is a soluble innate immune effector that acts as a serum opsonin and activates complement upon binding to glycosylated targets. It has been shown to react with a range of microorganisms [218, 239, 240].

When this project took place in 2005, there was no published description of any direct interaction between ficolins and HCV. However, based on some reports which demonstrated apparent links between MBL, which is structurally and functionally similar to ficolins, and HCV infection in terms of HCV disease progression [271] and response to treatment [272] as well as a direct association between the enzymatic activity of MBL/MASP-1 complexes and the severity of fibrosis in HCV patients [279], it appeared worthy to evaluate the possible role of L-ficolin, an innate immune defence, in the pathogenesis of HCV infection. The hypothesis was based on the ability of L-ficolin to interact with the HCV envelope glycoproteins E1 and E2 and that L-ficolin recognition of viral particles may play a role in HCV disease outcome.

There are three types of ficolins in humans, H-, L-, and M-ficolin. The selection of L-ficolin to investigate its activity in HCV disease was based on several reasons. The first reason was the serum availability of L-ficolin, thus its binding to HCV particles may result in either opsonic or neutralising effect, and its serum level can be measured to evaluate any potential correlation with HCV disease. The second reason was the evident polymorphism affecting either the serum level, as a result of single nucleotide polymorphisms in the promoter region of the L-ficolin gene [235], or the binding activity, as a result of single nucleotide polymorphisms in the exon 8, encoding the fibrinogen-like domain [267]. Thus, the L-ficolin polymorphism can be studied to find out the implications of the serum levels and

functional activity in the context of HCV disease. The third reason was the relatively higher scientific interest in characterisation of L-ficolin and its interaction with many microorganisms [218, 239, 240].

In order to characterise the biochemical interaction between HCV and L-ficolin, it was essential to produce recombinant L-ficolin and to purify serum L-ficolin (and also to produce HCV envelope glycoproteins E1E2) to be used in binding assays, that determine the ability of L-ficolin to bind HCV glycoproteins.

Two types of recombinant L-ficolin were produced; C-terminal His-tagged L-ficolin and N-terminal FLAG-tagged L-ficolin, using the Gateway cloning technology. The recombinant N-terminal FLAG-tagged ficolin was created by fusion PCR where the FLAG peptide was inserted downstream of the signal peptide cleavage site (between aa 25 and 26) , allowing further detection and purification of the recombinant ficolin using anti-FLAG M2 antibody. The aim of producing an N-terminal FLAG-tagged L-ficolin was to avoid the potential hindering effect of the relatively large C-terminal tag which could interfere with the ligand binding through the C-terminal fibrinogen-binding domain as well as the polymerisation of the heads in 'bunch-of-tulips' structure. However, although the N-terminal end is more flexible, it is a cysteine-rich region where the collagen domains assemble the trimers and join together to form 12-mers [234]. This oligomerisation is crucial for L-ficolin functionality in terms of ligand binding

through its C-terminal fibrinogen domain as a high-avidity binding is essential. The biological activity depends on simultaneous multiple bindings accomplished through the clustering of fibrinogen-binding domains in oligomeric structures [231]. Therefore, the oligomerisation and binding activity of recombinant L-ficolin were compared to that of native ficolin purified from serum, as a reference.

L-ficolin was also purified from human serum by passage through a GlcNAc-Sepharose matrix, using an adaptation from the method developed by Cseh et al., [285]. The binding of L-ficolin to GlcNAc-Sepharose ensured the presence of ficolin oligomers as the ficolin-GlcNAc interaction requires multiple weakly binding fibrinogen-binding domains to provide sufficient binding avidity.

The His tag and the FLAG tag facilitated the purification of expressed L-ficolin from mammalian cell culture supernatants using nickel agarose and anti-FLAG agarose, respectively. The His-tagged L-ficolin was eluted using imidazole at 200-250 mM, in agreement with Hummelshoj et al., who used a 250 mM imidazole-containing elution buffer to release the bound L-ficolin, in a one-step chromatographic purification [287]

Following purification, recombinant L-ficolin expressed in human endothelial kidney 293FT cells possessed a similar oligomerisation pattern as serum-derived L-ficolin when visualised by non-reducing SDS-PAGE, followed by western blot

analysis. Both recombinant and serum L-ficolin migrated as a 35 kDa monomer, and as higher oligomers including a ~400 kDa oligomer, in agreement with Hummelshoj et al., who reported a similar mixture of monomeric and oligomeric ficolin [287]. Under reducing conditions, the oligomers of both recombinant and serum L-ficolin were reduced to monomeric form with a molecular mass of 35 kDa. This may indicate that the tags had no significant effect on the oligomerisation of the recombinant L-ficolin polypeptides and thus would not interfere with the ficolin binding activity in subsequent binding assays. Another supportive observation in the ELISA assay was the ability of recombinant ficolin to bind acetylated BSA, which has been reported as a ligand for ficolin [288].

However, it is not possible to measure the relative quantity of monomers and multimers by western blot analysis. The three blot figures (figure 2.13 a, figure 2.15 and figure 2.16 a) revealed quite different proportions of monomers and oligomers. Generally, there was a relatively higher multimeric contents in serum-derived L-ficolin (figure 2.16 a) than in recombinant FLAG-tagged L-ficolin (figure 2.15) and His-tagged L-ficolin (figure 2.13 a). The oligomerisation pattern of recombinant L-ficolin could be further investigated by gel filtration, sucrose density gradient ultracentrifugation, and mass spectrometry [287].

The ELISA assay described in section 2.2.9.2 provided a good tool to evaluate not only the ficolin concentration, but also the oligomerisation pattern of the expressed

L-ficolin as the binding activity to acetylated BSA is directly related to the presence of correctly oligomerised multimeric ficolin.

In this part of the study, a successful production of two types of recombinant L-ficolin was achieved as evident by purification of multimeric His-tagged and FLAG-tagged L-ficolin which were comparable to purified serum ficolin in terms of oligomerisation and reactivity in ELISA (figure 3.3 b). Having said that, this part of the study was challenged by many obstacles that led to a significant delay to achieve such results. For example, the occurrence of a point deletion mutation, during production of the FLAG-tagged L-ficolin, which led to a premature termination of the L-ficolin polypeptide. This missing base was retained by site directed mutagenesis. Another obstacle during expression of the His-tagged L-ficolin there was a difficulty in obtaining a multimeric L-ficolin which was attributed to the presence of C-terminal tagging that could interfere with polymerisation. However, some modifications in the post-expression manipulation such as addition of protease inhibitors to the harvested cell culture supernatant and omitting the addition of Iodoacetamide resulted in better oligomerisation results.

3. L-ficolin and HCV Glycoprotein Interaction

3.1. Aims

In this part of the study viral glycoproteins E1E2 and soluble E2 (sE2) were produced and used with purified L-ficolin preparations to characterise the interaction of L-ficolin with HCV glycoproteins. Purified L-ficolin preparations were also used to pull down HCV particles from serum and to evaluate the neutralising activity of L-ficolin to HCV infectivity in a pseudoparticle infectivity assay.

3.2. Materials and Methods

3.2.1. Materials

3.2.1.1. Plasmids and Bacterial Strains

Clones of E1E2 and soluble E2₆₆₁ (sE2) were previously developed by Dr Alexander Tarr. Full length E1E2₁₇₀₋₇₄₆ constructs (representing amino acid residues 170 to 746) and soluble E2₆₆₁ (sE2) constructs [aa 363-661, including a C-terminal 6xHis-tag) of genotype 1 isolate H77c were previously cloned into pcDNA3.1 (Invitrogen).

3.2.1.2. Reagents and Antibodies

N Acetyl d-Glucosamine (GlcNAc), N Acetyl L-Cysteine (CysNAc), D-Mannose, TMB (3,3',5,5'-tetramethylbenzidine) were all obtained from Sigma-Aldrich

Company Ltd, Poole, UK. M-280 tosyl-activated Dynabeads® were purchased from Dynal Biotech ASA, Oslo, Norway. Polyethylenimine (Exgen 500) was purchased from Fermentas. Viral RNA Extraction Kit was purchased from QIAGEN. Luciferase assay system (luciferase substrate and lysis buffer), hexamer oligonucleotides and murine leukemia virus (MLV) RT enzyme were purchased from Promega. The primers for 5' noncoding region (NCR) (Table 3.1) were synthesised by Proligo. Two Mouse monoclonal antibodies against HCV glycoprotein E2 (ALP98 and AP33) were developed and kindly supplied by Dr Arvind Patel (MRC Virology Unit, Glasgow).

Table 3.1: PCR primers for 5' NCR.

Primer	Sequence (5'-3')	Tm °C	Product size (bp)
NCR outer sense	AGCGTCTAGCCATGGCGT	58	265
NCR outer anti-sense	GCACGGCTCACGAGACCT	60	265
NCR inner sense	GTGGTCTGCGGAACCGG	58	174
NCR inner anti-sense	GGGCACTCGGAAGCACCC	62	174

3.2.2. HCV Glycoprotein Expression

Viral glycoproteins (E1E2₁₇₀₋₇₄₆ or sE2₃₆₃₋₆₆₁) were subcloned into the eukaryotic expression vector pcDNA3.1m prior to transfection into HEK 293FT. Plasmids were purified using HP Plasmid Midiprep Kit, as described in section 2.2.2.2.4. Transfections of HEK 293FT cells were performed using Lipofectamine, as described before in section 2.2.4.3.

The cell-associated E1E2 was harvested 72 hrs after transfection by lysing the cells with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 20 mM Iodoacetamide, pH 7.6). To each plate, 1.5 mL lysis buffer were added and mixed, then the lysates were clarified by centrifugation at 8,000 x g for 5 min. The supernatant was used as a source of E1E2 after analysing a proportion for E1E2 content by SDS-PAGE (10% polyacrylamide gel) and the remainder of the supernatant was stored frozen at -72°C. Alternatively, for sE2, the supernatants were harvested 72 hrs post-transfection and iodoacetamide was added to a final concentration of 20 mM, to prevent oligomerisation of monomeric E2. Iodoacetamide is an alkylating sulfhydryl reagent that binds covalently with cysteine, thus interfere with disulfide bonds formation and further protein aggregation. The supernatants were then centrifuged at 250 x g for 5 min to remove any cell remnants. A proportion of the supernatants was analysed for sE2 content by 10% SDS-polyacrylamide gels and the rest of the supernatant was stored at -20°C for further purification.

3.2.3. SDS-PAGE and Western Blot Analysis

The expressed viral glycoproteins E1E2 and sE2 were characterised by separation using gel electrophoresis on 10% SDS polyacrylamide gels, as previously described in section 2.2.5.1. Western immunoblot was used to identify the expressed E1E2 and sE2. After samples were separated on 10% SDS polyacrylamide gels, they were transferred to Hybond ECL nitrocellulose membranes, as described earlier in section 2.2.5.2. Non-specific binding was blocked with 50 mL blocking solution (5 % Marvel in PBS-Tween) with gentle shaking for 1 hour at room temperature. The membranes were then incubated with monoclonal antibody against E2, ALP98 [289] (1 µg/mL, in PBS-Tween) for 1 hr, followed by HRP-conjugated goat anti-mouse secondary antibody (1 µg/mL, in PBS-Tween) for 1 hr. The membranes were washed 3 times for 5 min in PBS-Tween between each step. The proteins were then visualised using the ECL system, as described earlier in section 2.2.5.2.

3.2.4. Nickel Agarose Purification of sE2

His-tagged sE2 glycoprotein were nickel agarose purified by metal affinity chromatography using a 1 mL HisTrap HP column, as described for His-tagged L-ficolin purification in section 2.2.6.1. The sE2 glycoproteins were bound to the nickel agarose, washed with buffer containing 20 mM imidazole and eluted with 500 mM elution buffer, using 5 CV steps of 50 mM imidazole (0-500 mM imidazole). Start material, flow-through, and eluates were stored at 4°C for

analysis of the peak fractions using SDS-PAGE electrophoresis and quantification using Nanodrop spectrophotometer at 280 nm.

3.2.5. L-Ficolin and HCV Glycoprotein Binding

The binding ability of L-ficolin to HCV glycoproteins (E1E2 and sE2) was first examined by an ELISA binding assay (figure 3.1).

3.2.5.1. L-Ficolin and HCV ELISA Binding Assay

Maxisorp plates were coated (100 μ L/well) with 0.5 μ g/mL of monoclonal anti-L-ficolin antibody GN5 in PBS and incubated at 4 $^{\circ}$ C overnight. Wells were blocked with PBS-Tween, 5% milk, 4 hrs at room temperature. After washing three times with wash buffer (PBS-Tween, pH 7.4), wells were incubated with 100 μ L per well L-ficolin (5 μ g/mL) overnight at 4 $^{\circ}$ C. L-ficolin was previously quantified as described in section 2.2.8. After washing, 100 μ L per well of either lysates containing HCV glycoproteins E1E2 derived from genotype 1 (H77c), diluted 1/10 in PBS-Tween or sE2 (4 μ g/mL) in PBS-Tween were added. Two-fold serial dilutions of E1E2 (from 1/10 to 1/320) or sE2 (4 μ g/mL to 0.25 μ g/mL) in PBS-Tween were used to evaluate the dose-dependent pattern of binding interaction and to determine the dynamic range of binding interaction. A cell lysate from untransfected 293FT cells was included as negative control. The plates were incubated overnight at 4 $^{\circ}$ C. After washing, 100 μ L per well biotinylated monoclonal anti-E2 antibody AP33 at 1 μ g/mL were added and incubated for 1 h

at room temperature. After washing three times, wells were incubated for 30 min at 37°C with 100 μ L of 0.5 μ g/mL HRP-conjugated streptavidin in PBS-Tween. After a further three washes with PBS-Tween, 100 μ L/well of 3,3',5,5'-tetramethylbenzidine (TMB) were added and the absorbance was measured at 620 nm after 15 min.

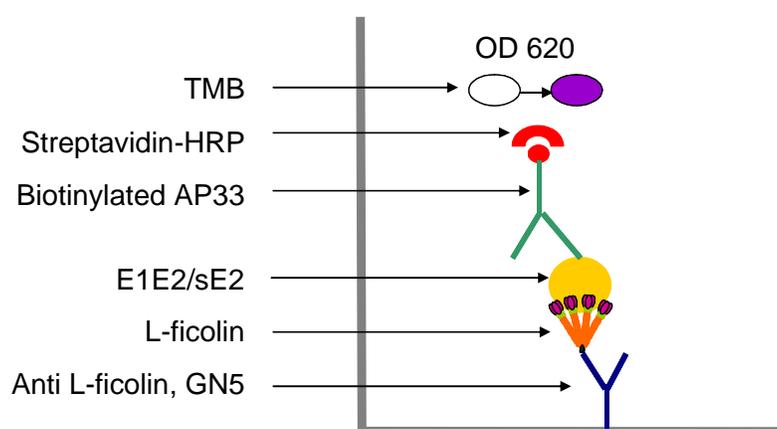


Figure 3.1: L-ficolin and HCV glycoproteins ELISA binding assay.

A schematic diagram representing the components used in ELISA to determine whether binding of L-ficolin to HCV glycoproteins (E1E2 and sE2) was possible.

3.2.5.2. L-ficolin and HCV Binding Inhibition

Inhibition of L-ficolin binding to HCV glycoproteins by competitors was performed as described in section 3.2.5.1, with an additional step after L-ficolin incubation. The extra step involved adding 100 μ L/well PBS containing either GlcNAc (0.1, 1, 10, 100, or 1000 mM), CysNAc (0.1, 1, 10, 100, or 1000 mM), D-Mannose (0.1, 1, 10, 100, or 1000 mM), or no competitor. After 1 hr incubation

at room temperature, 100 μL /well sE2 (1 $\mu\text{g}/\text{mL}$) were added and binding of ficolin was detected spectrophotically as described in section 3.2.5.1.

3.2.6. L-ficolin Binding to Serum-Associated HCV Particles

Another technique to evaluate the L-ficolin/HCV binding interaction, by testing the ability of L-ficolin to pull down HCV particles from serum, was approached. Briefly, M-280 tosyl-activated Dynabeads, coated with L-ficolin were used to pull down HCV particles from serum. The HCV virions were then detected by reverse transcription PCR (RT-PCR).

3.2.6.1. HCV Particles Pull-Down Experiment

M-280 tosyl-activated Dynabeads (10 μL) were coated with monoclonal anti-L-ficolin antibody, GN5, at a concentration of 3 $\mu\text{g}/10^7$ beads, according to the manufacturer's instructions. After washing, the coated beads were then incubated in a blocking buffer (0.2 M Tris with 0.1% BSA, pH 8.5), to block free tosyl-groups. Recombinant L-ficolin diluted in PBS at a molar concentration (assuming that 420 kDa is the molecular mass of L-ficolin) equivalent to that of the antibody was added to the GN5-coated beads and incubated for 2 hrs at room temperature. These ficolin-GN5-coated beads were then used to bind viral E1E2 glycoproteins and pull down HCV particles from HCV-infected patient's serum containing HCV at 6,141,619 IU/mL. The beads (10 μL) were incubated with 1 mL of serum, diluted 1/50 in PBS-Tween. Alternatively, the diluted serum was spiked with 5

$\mu\text{g/mL}$ recombinant L-ficolin and incubated for 2 hrs at room temperature to allow L-ficolin binding to HCV particles, before mixing with GN5-coated beads for 2 hrs at room temperature. GN5-coated beads were used also to pull down HCV particles. A competition assay was also performed, by incubating L-ficolin-GN5-coated beads with 0.6 M CysNAc for 1 hr before mixing with sera. In each case, immunocomplexes were collected via magnetic separation and washed 5 times with PBS. Pellets were then resuspended in 140 μL PBS to allow recovery of HCV RNA from the immunoprecipitate. Non-coated beads (not coated with either GN5 or L-ficolin) were incubated with serum under the same conditions as a negative control.

3.2.6.2. Detection of HCV by RT-PCR

HCV RNA was extracted from 140 μL of the immunoprecipitate using a Viral RNA Extraction Kit, according to the manufacturer's instructions. Annealing mix was prepared by adding 2.5 μL 5X RT buffer to 10 μL extracted RNA. The mix was incubated at 70°C for 5 min, then reverse transcribed by adding 12.5 μL of extension mix (2.5 μL 5X RT buffer, 0.5 μL random hexamer oligonucleotides, 2.4 mM of each dNTP, 0.5 μL MLV RT enzyme, and 5.5 μL water), to generate the complementary DNA (cDNA). The mix was incubated at room temperature for 10 min, and then incubated at 37°C for 1 hr. The resulting cDNA was amplified by nested PCR of the 5' noncoding region (5' NCR). The first-round PCR was performed as follows: 2 μL of cDNA were added to 23 μL of mixture containing 5

pmol of primer NCR outer sense and NCR outer anti-sense, 200 μ M dNTPs and 0.625 U of Hot Star Taq polymerase in 1x PCR buffer. One microlitre of the first-round product was then used as a template in second-round reactions using the primers NCR inner sense and NCR inner anti-sense. The first round and second round nested PCR cycles included 1 cycle of 95°C for 15 min; 35 cycles of (94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min), then a final 7-minute extension at 72°C. RT-PCR of HCV positive serum was included as a positive control for the extraction and PCR.

3.2.7. HCV pseudo-particles (HCVpp) Neutralisation Assay

3.2.7.1. HCVpp Production

HCVpp (retroviral particles pseudotyped with HCV glycoproteins) were generated by Dr Alexander Tarr. They were produced by co-transfection of expression plasmids encoding the full-length E1E2 glycoproteins from either HCV type 1a (H77c) or type 2b (UKN2B1.1), the CMV-Gag-Pol MLV packaging construct, and firefly luciferase reporter gene, as a gene marker, under the control of the human cytomegalovirus promoter, into the producer cell line, HEK 293-T cells. Briefly, 2 μ g of each plasmid were transfected into 1.2×10^6 293-T cells, seeded the day before in 9-cm plates, using Polyethylenimine (Exgen 500) as a gene-delivering agent. It interacts with DNA to form small, diffusible, readily endocytosed complexes. Transfections were followed, after 6 hrs, by changing the medium (10 mL per plate) to fresh DMEM containing 10% FBS. Supernatants containing the

pseudo-particles were harvested 72 hrs post-transfection and filtered through 0.45 μm filters. Purified supernatants were gently loaded onto 1 mL of a 20% sucrose cushion, followed by ultracentrifugation in Beckman SW40 ultracentrifuge (at 40,000 rpm, for 2 hrs at 4°C). Pseudoviral particles pellets were suspended in 500 μL PBS.

3.2.7.2. Infectivity Neutralisation Assay

The infectivity assay was performed using the Human hepatoma cell line, Huh7. Cells were plated on 48-well plate, at a density of 4×10^4 cells per well the day before infection. The infection was performed using 50 μL of HCVpp with 200 μL fresh DMEM media in each well. For neutralisation assay, 50 μL of HCVpp were incubated with 50 μL of a serial dilution of purified recombinant ficolin (at a final concentration from 6 $\mu\text{g}/\text{mL}$ to 0.1875 $\mu\text{g}/\text{mL}$, in PBS), or PBS as a positive control, for 30 min at 37°C before adding to Huh7 cells. On the following day, another 250 μL media were added. After 72 hrs of transfection, the ficolin-mediated neutralisation of HCVpp was evaluated by luciferase assay.

3.2.7.3. Luciferase Assay

The media were removed and the cells were lysed by adding 50 μL of Glo-lysis buffer. The plates were kept for 15 min on a rocking platform until the cells were lysed. The cell monolayer was scraped and removed with the buffer solution to a 0.5-mL Eppendorf tube. The tubes were vortexed for 10 sec, then centrifuged at

12,000 x g for 2 min. From each tube, 35 μ L were transferred to a cuvette followed by 90 μ L luciferin substrate. The luciferase activity was measured immediately and the OD was determined at 420 nm, using Turner TD-20 luminometer.

3.3. Results

3.3.1. Expression of HCV glycoproteins

Clones representing the entire E1 and E2 coding regions (aa170-746) or a soluble fragment of the ectodomain of the E2 protein (aa 363-661; sE2₆₆₁) were both expressed in HEK 293FT cells. Cell lysates were used as a source of E1E2, while the C-terminal His-tagged sE2 was purified from the culture supernatants by metal affinity chromatography.

3.3.2. Purification of sE2

Metal affinity chromatography, using 20 mM imidazole in the initial binding stage followed by a step elution of imidazole (50 mM rises from 0 to 500 mM imidazole), was used to purify the expressed His-tagged E2₆₆₁ and enable separation of monomer and aggregated material. Fractions of 1 mL were collected and the eluted fractions were studied by SDS-PAGE and western blot analysis (Figure 3.2). Monomeric E2 was eluted at 150 mM imidazole and the majority of the aggregated material was eluted at 200 mM imidazole. Quantification of

purified E2₆₆₁ was performed by measuring the protein content at A₂₈₀ using a Nanodrop spectrophotometer.

3.3.3. Confirmation of Expressed Glycoproteins

Both the E1E2 lysate and the sE2 eluates were analysed by western blot analysis using monoclonal anti-E2 antibody ALP98. This revealed E2 monomers (70 kDa) of E1E2 and sE2 (figure 3.2). E2 monomers represent correctly-folded E2. Disulfide-linked aggregates may represent misfolded complexes [290]. The E1E2 heterodimers (105 kDa) were dissociated by the denaturing nature of the gel revealing the E2 monomers (70 kDa), which were detected with monoclonal anti-E2 antibody ALP98. These protein models were used as surrogates of the surface of HCV virions in ficolin binding assays.

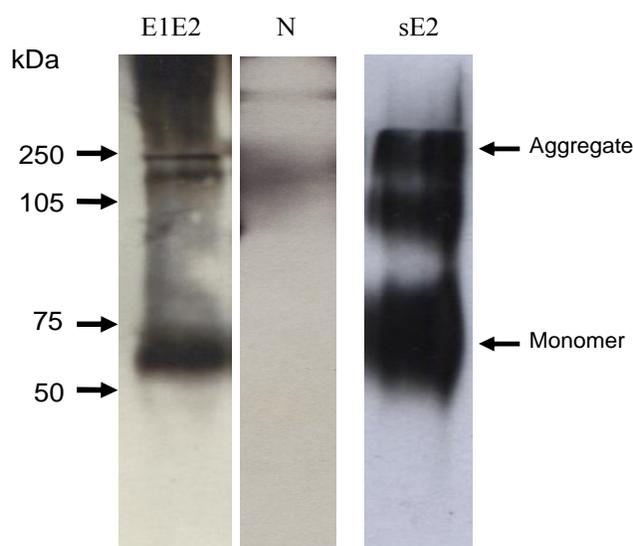


Figure 3.2: SDS-PAGE analysis of HCV glycoproteins E1E2 and sE2.

HEK 293FT cells were transfected with a plasmid encoding viral glycoproteins E1E2 or soluble E2 of genotype 1 (H77c). E1E2 lysates and IMAC-purified sE2 were analysed by SDS-PAGE. The immunoblot analysis was performed using monoclonal anti-E2 antibody (ALP98). The blots show bands relating to E2 monomers (70 kDa) of E1E2 and sE2 and aggregates. Untransfected 293FT cell lysate was used as a negative control (N). Positions of protein size markers are shown (in kDa) on the left.

3.3.4. L-ficolin Interaction with HCV Glycoproteins

The binding of L-ficolin to HCV glycoproteins (E1E2 and sE2) was assessed in ELISA. Two-fold serial dilution series of E1E2 from cell lysate (1/10 to 1/320) or purified sE2 (4 ug/mL to 0.25 ug/mL) was incubated with L-ficolin, captured by monoclonal anti-L-ficolin antibody, GN5. A dose-dependent binding of both E1E2 and sE2 to L-ficolin was observed (figure 3.3). The specificity of this binding interaction was shown by low background when mock-transfected HEK 293FT cellular proteins were used. This is evidence that HCV glycoproteins possess Pathogen Associated Molecular Patterns (PAMPs) recognised by L-ficolin.

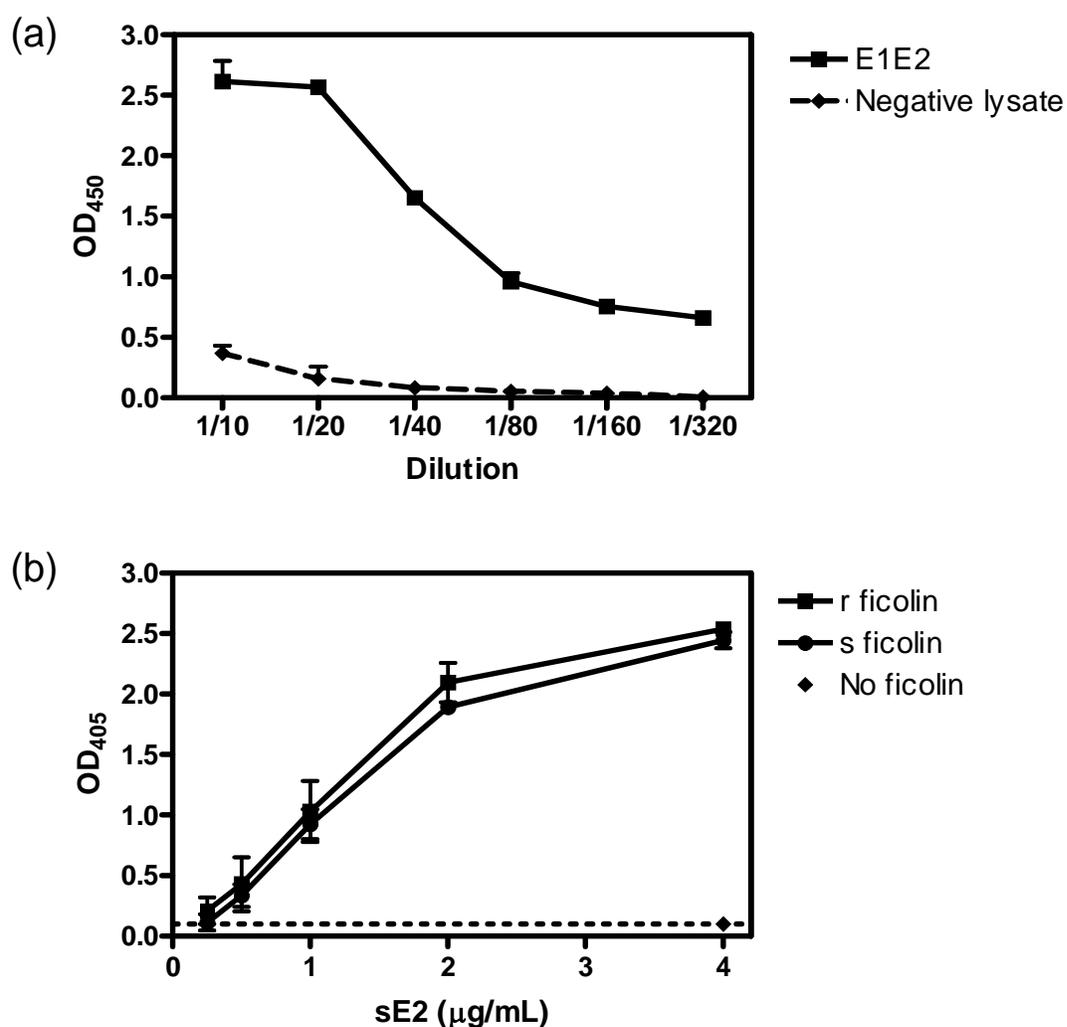


Figure 3.3: L-ficolin and HCV glycoproteins binding assay.

The ability of L-ficolin to bind to HCV glycoproteins (E1E2 and sE2) was evaluated by ELISA, using L-ficolin (5 μg/mL), that was captured by anti L-ficolin antibody (GN5), and a serial dilution of E1E2 or sE2 was added. Bound E1E2 and sE2 were detected by monoclonal anti-E2 antibody, AP33, at OD₄₀₅. (a) E1E2 binding to L-ficolin was directly related to E1E2 concentration in the cell lysate. A negative control lysate from untransfected cells was used to show the specificity of interaction. (b) A dose-dependent pattern of binding interaction between sE2 and both recombinant ficolin (r ficolin) and purified serum ficolin (s ficolin), illustrating a close level of both recombinant ficolin and serum ficolin (at the same concentration). The dotted line represents the OD of a negative control reaction in which no ficolin was added, to exclude the possibility of non specific binding between sE2 (at a concentration of 4 μg/mL) and GN5.

To investigate the ability of known L-ficolin ligands to interfere with the interaction between L-ficolin and HCV glycoproteins, L-ficolin was pre-incubated with varying concentrations of either ficolin ligands (GlcNAc or CysNAc), or a control sugar specific only for MBL (D-Mannose) for 1 hr prior to addition of sE2 to the ficolin-containing microtitre plate (figure 3.4).

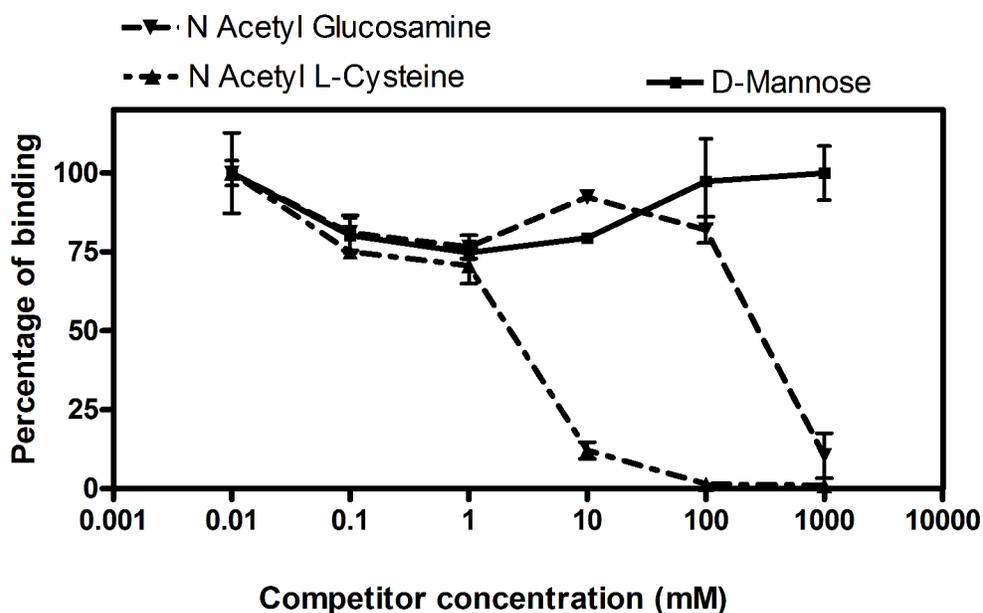


Figure 3.4: Effect of competitors on the binding of L-ficolin to sE2.

The effect of pre-incubating L-ficolin with the indicated concentrations of 2 L-ficolin ligands, GlcNAc and CysNAc; or a non-ficolin ligand, D-Mannose, prior to adding purified sE2. sE2 binding to L-ficolin was examined and percentage binding was evaluated compared to a binding assay without inhibitor. Both GlcNAc and CysNAc inhibited binding of L-ficolin, with IC_{50} values of 260, and 1.8 mM, respectively. D-Mannose did not inhibit the interaction at a concentration of 1M.

The effect of these ligands on binding to sE2 was compared to uninhibited binding of recombinant L-ficolin. Both CysNAc and GlcNAc inhibited the binding

interaction between recombinant L-ficolin and the sE2 protein. The inhibition of binding of L-ficolin to sE2 was dose-dependent (figure 3.4). The concentration that resulted in 50% binding inhibition (IC_{50}) was 1.8 mM and 260 mM of CysNAc and GlcNAc respectively. Even at a concentration of 1 M, D-Mannose had no effect on the binding interaction, demonstrating a specific inhibition of the ligand binding domain of L-ficolin.

3.3.5. L-ficolin binding to HCV Virions

To further confirm the ability of L-ficolin to bind HCV glycoproteins, the ability of purified L-ficolin to bind to HCV particles in patients' sera was evaluated. L-ficolin-GN5-coated magnetic beads were incubated with sera from patients with chronic HCV infection, isolating authentic HCV particles. In a parallel experiment, the patient's serum was spiked with recombinant L-ficolin at a concentration of 5 $\mu\text{g}/\text{mL}$ and GN5-coated magnetic beads used to pull down the immune complexes. Complexes containing L-ficolin were also isolated with GN5-coated magnetic beads. HCV RNA associated with Dynabeads after immunoprecipitation was detected by RT-PCR of the 5' NCR (figure 3.5 a). The resulting PCR products of GN5-coated Dynabeads incubated with serum previously spiked with recombinant L-ficolin (lane 4) and recombinant L-ficolin-GN5-coated Dynabeads incubated with serum (lane 5) were of the same size as the positive control (HCV RNA positive serum; lane 2).

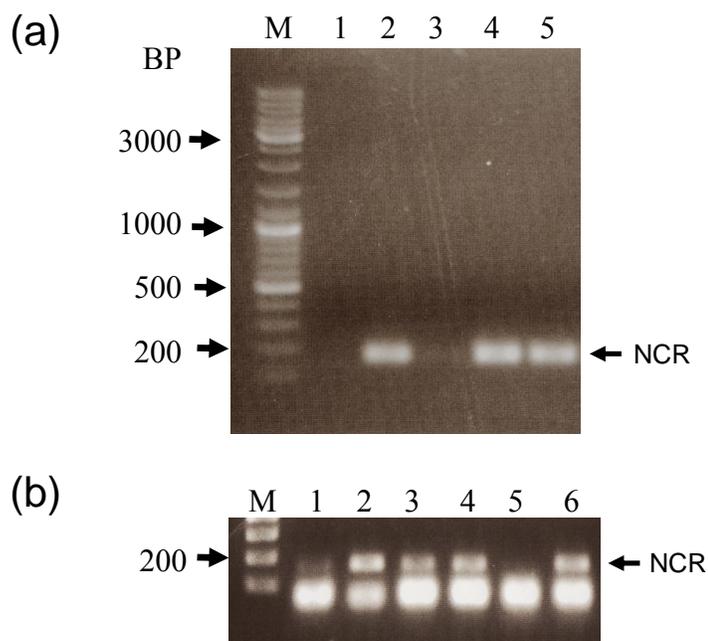


Figure 3.5: Detection of HCV RNA by RT-PCR.

(a) HCV RNA was recovered from the L-ficolin-GN5-coated Dynabeads that had been incubated with serum from HCV-infected patient (RNA-positive), by RT-PCR. The PCR products were detected by agarose gel electrophoresis. Positive bands are detectable from the following immunocomplexes: recombinant L-ficolin-GN5-coated Dynabeads incubated with serum (lane 5) and GN5-coated Dynabeads incubated with serum previously spiked with recombinant L-ficolin (lane 4) which provided a result similar to that detected from RNA positive serum (lane 2). Lane 3 represents the non-coated beads (not coated with either GN5 or L-ficolin) and lane 1 represents the RT-PCR negative control.

(b) Effect of CysNAc on L-ficolin binding to HCV virions was performed by pre-incubating L-ficolin-GN5-coated beads with 0.6M CysNAc before mixing with serum. A lower yield of HCV RNA was detected in CysNAc-treated L-ficolin-GN5-coated Dynabeads (lane 1) compared to non-treated L-ficolin-GN5-coated Dynabeads (lane 2) and that detected from RNA positive serum (lane 6). A similar yield was observed using either GN5-coated Dynabeads incubated with serum (lane 3), or GN5-coated Dynabeads incubated with serum previously spiked with recombinant L-ficolin (lane 4). Lane 5 represents the non-coated beads.

Viral RNA was not detected when using uncoated beads (not coated with either GN5 or L-ficolin), which were used as a negative control, demonstrating the requirement for L-ficolin to mediate immunoprecipitation. This fact was further confirmed by the ability of GN5-coated beads to immunoprecipitate HCV RNA (figure 3.5 b; lane 3). CysNAc, known to interact strongly with L-ficolin, was pre-incubated with L-ficolin-GN5-coated beads, at 0.6M, prior to mixing with serum to inhibit the L-ficolin binding to HCV virions. As expected, CysNAc, which was shown to inhibit L-ficolin binding to sE2, reduced the ability of L-ficolin to bind to HCV virions (figure 3.5 b; lane 1).

3.3.6. HCVpp Neutralisation Assay

A retroviral-based pseudoparticle assay was performed to examine the neutralising effect of L-ficolin on HCVpp infectivity in Huh7 cells. A serial dilution of L-ficolin from 6 $\mu\text{g/mL}$ to 0.1875 $\mu\text{g/mL}$, in triplicates, was pre-incubated with HCVpp for 30 min at 37°C. The HCVpp display E1E2 glycoproteins from two HCV genotypes, type 1a (H77c) or type 2b (UKN2B1.1) and contain a luciferase gene as a marker. The neutralising activity was expressed as percentage of inhibition compared to a control HCVpp which was not incubated with L-ficolin. Measurement of the luciferase activity demonstrated a reduction in the infectivity of two pseudoparticles expressing E1E2 glycoproteins from two HCV genotypes, type 1a (H77c) or type 2b (UKN2B1.1). The infectivity was inhibited to different degrees by L-ficolin in a dose-dependent pattern (figure 3.6).

The concentration of L-ficolin required to achieve 50% inhibition of infection (IC_{50}) for type 1a (H77c) and type 2b (UKN2B1.1) pseudoparticles was 1.5 and 0.7 $\mu\text{g}/\text{mL}$, respectively.

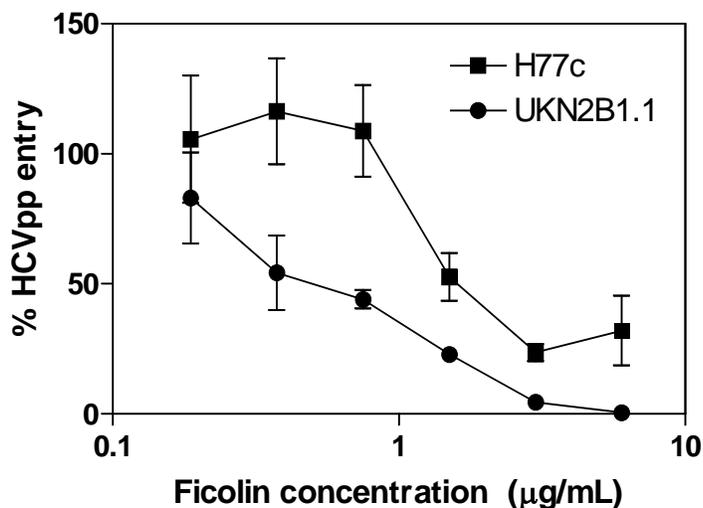


Figure 3.6: Neutralisation of HCV pseudoparticles by L-ficolin.

The L-ficolin was pre-incubated with HCVpp for 30 min at 37°C in 2-fold serial dilution down from 6 $\mu\text{g}/\text{mL}$ to 0.1875 $\mu\text{g}/\text{mL}$ and then the HCVpp were used to infect the Huh-7 cells. Inhibition of infectivity was determined by reduction in the luciferase activity. Infectivity of the HCVpp derived from 2 genotypes type 1a (H77c) or type 2b (UKN2B1.1) was inhibited in a dose-dependent manner. L-ficolin completely abrogated infection of UKN2B1.1 at a concentration of 6 $\mu\text{g}/\text{mL}$.

3.4. Discussion

L-ficolin, through its fibrinogen-like domains, recognises carbohydrate patterns on the outer walls of microorganisms, with a general specificity for N-acetylated groups [236]. The binding of ficolins to sugar structures leads to complement activation and deposition of C3b and thus results in phagocytosis and clearance of

pathogens [217, 218]. In such a way, L-ficolin has been suggested to have a role as an innate immune mediator against a range of infectious diseases [218, 239, 240]. HCV possesses two envelope glycoproteins; E1 and E2 expressed as non-covalent E1E2 heterodimers in the viral envelope [98]. These are glycosylated transmembrane proteins, containing up to 5 and 11 N-linked glycosylation sites respectively [20]. As that pattern of glycans might serve as ligands for L-ficolin, the potential ability of L-ficolin to interact with HCV glycoproteins and the potential binding implications, in terms of neutralising infectivity, were investigated.

Two recombinant glycoprotein constructs of the HCV genotype 1a infectious clone H77c (E1E2 and sE2) were expressed for analysis of the interaction between L-ficolin and HCV. The expressed E1E2 proteins were further evaluated for function using a retroviral pseudoparticle model of infectivity [291]. Using an ELISA assay, binding curves were created in order to demonstrate the ability of purified recombinant L-ficolin to bind to E1E2 and sE2 in a dose-dependent manner. Further characterisation of L-ficolin binding to HCV glycoproteins was achieved by sugar competition assays to confirm binding via sugar residues. Consistent with previous reports demonstrating binding of L-ficolin to GlcNAc [218] and CysNAc [286], this study demonstrated that recombinant L-ficolin binding to HCV glycoprotein E2 (sE2) could be specifically inhibited by pre-incubation of L-ficolin with either GlcNAc or CysNAc (figure 3.4). The ability of

these ficolin ligands to compete with the binding of L-ficolin to a soluble form of E2 suggests that the binding is mediated by the L-ficolin fibrinogen-like domain and the N-linked glycans of E1 and E2. However, the inhibitory effect of CycNac ($IC_{50} = 260$) is much greater than the inhibitory effect of GlcNAc ($IC_{50} = 1.8$ mM), in contrast to Krarup et al., [286] who reported a higher inhibitory effect of GlcNAc on L-ficolin binding to *A. viridans* or *S. pneumoniae* than that of CycNac. Thus, the marked reduction of L-ficolin to E2 using 100 mM CycNac could be a result of its reducing effect that dissociates the multimeric ficolin. This possible effect could be excluded by alkylating the sulfhydryl group of CycNac before incubation with L-ficolin.

To determine if the interaction between L-ficolin and HCV glycoproteins extended to authentic viral particles, the ability of L-ficolin to associate with HCV particles from infected patients' sera was next demonstrated. HCV particles containing viral RNA were immunoprecipitated with either L-ficolin-anti L-ficolin GN5-coated beads or even anti L-ficolin GN5-coated beads, confirming not only the L-ficolin/HCV glycoproteins binding interaction observed by ELISA, but also the ability of L-ficolin to form complexes with the circulating viral particles. This *ex vivo* interaction demonstrates that the HCV particles circulating during infection must present glycans with GlcNAc moieties in the correct conformation to be accessible to ficolins [238] and suggests that circulating HCV may form complexes with this soluble innate effector molecule *in vivo*.

As the HCV glycoproteins mediate viral entry by interacting with multiple cellular receptors [102-104], the potential function of L-ficolin in HCV infection was addressed by analysing the ability of L-ficolin to inhibit virus infectivity of Huh7 cells by HCV pseudo-particles. The inhibition of infectivity was evaluated by a luciferase assay. Interestingly, binding of L-ficolin to HCV glycoproteins resulted in neutralisation of virus infectivity in this *in vitro* model, using HCVpp expressing E1E2 glycoproteins from HCV genotype 1a (H77c) and 2b (UKN2B1.1). These two clones are able to form functional glycoproteins, previously determined by the pseudotype infectivity assay [101]. The neutralisation was almost 100% for genotype 2b-derived pseudoparticles at a concentration of 6 $\mu\text{g/mL}$, which is slightly higher than the reported average physiological level of L-ficolin, 4.13 $\mu\text{g/mL}$ [217]. Thus, L-ficolin binding to HCV glycoproteins may have a blocking effect on entry of virions into host cells, which is mediated by binding of E1E2 to cellular receptors [102-104]. Blocking this interaction might have a clinically relevant neutralising effect, suggesting a ficolin activity as a biotherapeutic. The variation in the magnitude of neutralisation of infectivity between the 2 clones of HCV may be related to the genotypic differences in glycosylation sites. E1 glycoprotein of genotype 2b (UKN2B1.1) has an additional glycosylation site at aa 300, that is absent in genotype 1a (H77c).

This neutralising effect is in agreement with a recent study which revealed the ability of porcine ficolin alpha to bind and neutralise porcine reproductive and

respiratory syndrome virus (PRRSV) evident by reducing cytopathic effect of PRRSV and inhibiting replication of infectious viral particles, determined by plaque assay [292].

As the main biological effects of the ficolins are mediated through complement activation upon binding to sugar structures on bacterial surfaces [248], L-ficolin binding to HCV might activate the complement system via L-ficolin associated MASPs. In such a pathway, L-ficolin may assist in viral clearance through activation of serine proteases that activate the lectin complement pathway. MASP-1 activation is another potential functional consequence of L-ficolin binding to HCV glycoprotein. MASP-1 has a thrombin like spectrum of activity on fibrinogen and factor XIII [280-282], thus, stimulates fibrin deposition and supports the development of fibrosis during HCV diseases. L-ficolin/MASP-1 activation may demonstrate the ability of L-ficolin to play a role in chronic HCV infection, by contributing to the pathogenesis of liver fibrosis.

4. Role of Anti-E1E2 in HCV Liver Disease

4.1. Aims

HCV envelope glycoproteins, E1 and E2, associate as a non-covalent heterodimers [20]. They are thought to play crucial roles at different steps of the HCV replication cycle. They are essential for host-cell entry, binding to cellular receptors and inducing fusion with the host-cell membrane [101, 102]. The humoral immunity to HCV is mediated by antibodies which bind viral proteins including E1 and E2, leading to the clearance of circulating virus particles. However, this may be essentially ineffective despite evidence for neutralising antibody response, possibly due to rapid selection of antibody escape mutants [139].

The aim of this section was to investigate the role of antibodies to HCV glycoproteins E1 and E2 (anti-E1E2) in HCV disease by evaluating the serum levels of anti-E1E2 in sera of patients with chronic HCV infection. The anti-E1E2 assay was performed using unique recombinant HCV glycoproteins, derived from genotype 1 (H77c) and genotype 3 (UKN3A1.28), as antigens. The assay was employed to find out the correlation between the levels of anti-E1E2 and viral load, viral genotype, disease severity and responsiveness to treatment. The seroreactivity to E1E2 was estimated in 230 sera from 180 patients with chronic

HCV infection obtained from the Trent HCV cohort study. Production of HCV glycoproteins E1E2 was required for use as antigens in ELISA assays.

4.2. Materials and Methods

4.2.1. Plasmid Constructs

Full length E1E2₁₇₀₋₇₄₆ constructs (aa 170-746) from HCV genotype 1 strain H77c and genotype 3 strain UKN3A1.28 were generated and cloned into pcDNA3.1 (Invitrogen) by Dr Alexander Tarr as previously described [101]. Briefly, viral RNA was extracted from the sera of patients with different genotypes of HCV and virus-specific cDNA sequences of the viral glycoproteins was made using genotype-specific primers. Nested PCR was used to generate PCR products corresponding to the sequence including amino acids 170-764 of the E1E2 polyprotein. cDNA was sequenced using Big Dye and ABI Prism 3100 sequencer and the sequences were analysed using ClustalX, MEGA version 2.2, against reference strains; genotype 1a (AF011752) and 3a (D17763) [101]. Fragments were then cloned into pcDNA3.1, for E1E2 expression. The quality of the expressed E1E2 proteins was previously evaluated with retroviral pseudoparticle infectivity, in which both types conferred infectivity [101, 293].

4.2.2. Reagents and Antibodies

Maxisorp ELISA plates were purchased from Nunc, Kamstrup, Denmark. GNA lectin, carbonate-bicarbonate buffer, phosphate-buffered saline (PBS),

p-nitrophenyl phosphate (pNPP) substrate, normal human serum, and alkaline phosphatase-conjugated anti-human IgG antibody were purchased from Sigma-Aldrich Company Ltd, Poole, UK. Mouse anti-E2 monoclonal antibody, ALP98 was developed and kindly supplied by Dr Arvind Patel (MRC Virology Unit, Glasgow). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody was purchased from Dako Cytomation.

4.2.3. Cell culture and Glycoprotein Expression

Transfection of plasmids encoding the viral glycoproteins E1E2₁₇₀₋₇₄₆ of genotype 1 (H77c) and genotype 3 (UKN3A1.28) was carried out in the HEK 293FT cell line (Invitrogen) stably transfected with pCMVSPORT6TA_g.neo plasmid. The cells were cultured in Dulbecco's Modified Eagles Medium with glutamine (GIBCO) supplemented with 10% fetal calf serum and non-essential amino acids at 37°C, in a 5% CO₂ incubator. Transfection was carried out using the cationic lipid reagent, Lipofectamine 2000, as previously described in section 2.2.4.3. The growth medium was changed 24 hrs after transfection. E1E2 glycoproteins were harvested 72 hrs post-transfection by lysing the cells with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 20 mM Iodoacetamide, pH 7.6). To each plate, 1.5 mL lysis buffer were added and mixed, then the lysates were clarified by centrifugation at 8,000 x g for 5 min. The supernatant was used as a source of E1E2 after analysing a proportion for E1E2 content by SDS-PAGE (10% polyacrylamide gel) and the remainder of the supernatant was stored at -72°C.

4.2.4. Confirmation of Expressed Protein

Separation of proteins was achieved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to nitrocellulose using a Trans-Blot Semi-Dry Transfer Cell (Bio Rad). Expression of E2 protein was detected by western blot analysis using mouse anti-E2 monoclonal antibody ALP98 primary antibody (1 µg/mL in PBS-Tween), and horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1 µg/mL in PBS-Tween). Western blots were developed using enhanced chemiluminescence detection reagents ECL-Plus and exposure to a photographic film, as previously described in section 2.2.5.2.

4.2.5. Patients and Samples

The levels of anti-HCV E1 and E2 antibodies were evaluated in 230 sera from 180 patients with chronic hepatitis C obtained from the Trent HCV Cohort. All samples were collected prior to treatment, except in the sub-study examining the effect of treatment on anti-E1E2 reactivity. This cohort includes HCV positive patients from five centres within the Trent region [294]. All patients are defined as chronically infected with HCV by testing positive for HCV antibody using a third generation immunoassay (ELISA, Ortho Vitros ECI) and for HCV RNA using a commercially available genome amplification technique (Roche Amplicor). HCV genotypes are determined using the Bayer line probe assay (Bayer HealthCare LLC). Liver biopsies are assessed by a single pathologist, and scored according to

the criteria of Ishak et al [45]. In a sub-study examining a possible correlation between anti-E1E2 reactivity and liver disease activity, sera were specifically chosen from patients with either mild (defined as Ishak fibrosis stage 0-2) or severe (Ishak stage 5-6) disease. Sera were taken within 6 months of biopsy, or in an interbiopsy period where both pre- and post-sample biopsies had the same stage. Response to treatment was defined on the basis of serum HCV-RNA clearance by PCR. Sustained virologic response was defined as the absence of detectable HCV RNA 6 months after cessation of therapy (peg-interferon plus ribavirin). Serum samples were stored at -72°C .

4.2.6. Anti-E1E2 Assay

The level of anti-E1E2 in patients' sera was determined using an ELISA-based assay (figure 4.1). Maxisorp ELISA plate wells were coated ($50\ \mu\text{L}/\text{well}$) with $5\ \mu\text{g}/\text{mL}$ of *Galanthus nivalis* agglutinin (GNA) [211] in carbonate-bicarbonate buffer (pH 8.9) for 2 hrs at 37°C . Wells were blocked with blocking buffer [phosphate-buffered saline (PBS) 0.05 % (v/v) Tween-20, 5% milk powder] ($200\ \mu\text{L}/\text{well}$) overnight at 4°C , then washed 3 times with wash buffer [PBS 0.05% Tween 20 in, pH 7.4], using the Denly WellWash plate washer. Lysates containing HCV glycoproteins E1E2 derived from genotype 1 (H77c) or genotype 3 (UKN3A1.28) ($50\ \mu\text{L}/\text{well}$) were diluted 1/80 in PBS-Tween-Milk prior to addition to the plates and incubated for 2 hrs at room temperature. After washing, $50\ \mu\text{L}$ serum diluted 1/50 in PBS-Tween-Milk was added and incubated for 2 hrs

at room temperature. After washing 3 times, 50 μ L of anti-human IgG-alkaline phosphatase (anti-human IgG-AP) conjugate, diluted 1/1,000 in TBS supplemented with 0.05% Tween 20 and 5% milk, was added for 1 hr at room temperature. Following a further 3 washes, with TBS-Tween, binding was visualised with 50 μ L of p-nitrophenyl phosphate (pNPP) substrate. The OD at 405 nm was determined using a Vmax plate reader (Molecular Devices) after 30 min. Un-transfected 293FT cell lysates and normal human serum were included as negative controls. Manipulation of patient sera during the ELISA assay, including washing steps, was carried out in a class I biological safety cabinet (LEEC) which was cleaned with 70% v/v ethanol and 2% Trigene, before and after each manipulation.

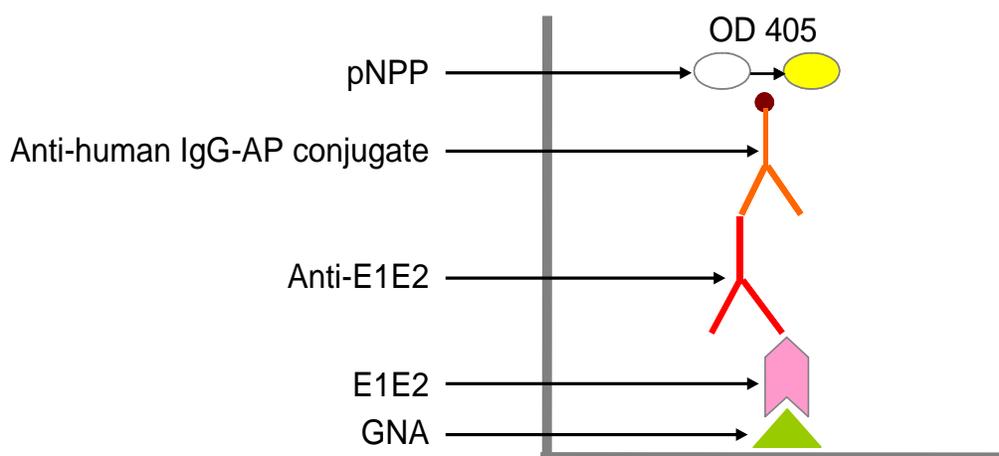


Figure 4.1: Evaluation of anti-E1E2 antibodies by ELISA.

A schematic diagram representing the components used in ELISA to evaluate the levels of anti-E1E2 in sera from patients with chronic hepatitis C. Two full length E1E2 constructs from HCV genotype 1 strain H77c and genotype 3 strain UKN3A1.28 generated and expressed in HEK 293FT cells, were used as antigens.

4.2.7. Statistical analysis

Statistical analyses were performed using Prism version 4.03 (Graph Pad, San Diego, CA, USA). Comparison between groups was carried out by Mann Whitney test or t test. The association between seroreactivity to E1E2 and viral load was analysed using the linear regression test. In all tests, a probability level (p) of 0.05 or smaller denoted a statistical significance.

4.3. Results

4.3.1. Confirmation of Expressed E1E2

HEK 293FT cells were used for transfection of the plasmids encoding the genotype 1 (H77c) and genotype 3 (UKN3A1.28) viral glycoproteins E1E2, using Lipofectamine. The expressed HCV glycoproteins were separated by SDS-PAGE (10% polyacrylamide gel) and detected by western blot analysis using monoclonal anti-E2 primary antibody, ALP98 and goat anti-mouse HRP-conjugated secondary antibody.

The western blot showed several bands indicating the presence of E2 monomers (70 kDa) and oligomers of H77c and UKN3A1.28 (figure 4.2). The monomer is considered to represent correctly folded E2. As expected, the E1E2 heterodimers (105 kDa) were dissociated by the denaturing nature of the gel, and the E2 monomer (70 kDa) was detected using an anti-E2 antibody (ALP98) that

recognises an epitope identical in both genotype 1 and 3 proteins (amino acids 644-651; referenced to H77c).

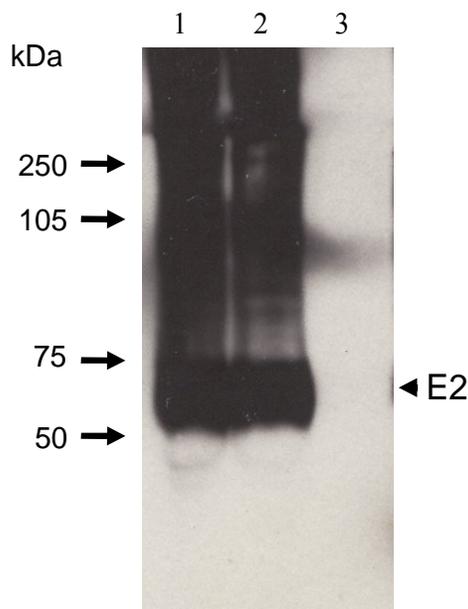


Figure 4.2: Detection of monomeric E1E2 in lysate by western blot.

The proteins were subjected to 10% SDS-PAGE; and after transfer, immunoblot analysis was performed using monoclonal antibody ALP98. Antibody binding was detected with HRP-conjugated anti-mouse immunoglobulin as a secondary antibody. Bands were visualised using the ECL plus system. The blot showed two bands relating to E2 monomers of H77c (lane 1) and UKN3A1.28 (lane 2). Untransfected 293FT cell lysate was loaded in lane 3 as a negative control. The position of E2 monomer (70 kDa) is shown with an arrow to the right.

4.3.2. Anti-E1E2 Assay

The seroreactivity of the tested sera (diluted 1/50 in PBS-Tween) to H77c and to UKN3A1.28, at OD 405, as an indication for the levels of anti-E1E2, was evaluated by ELISA in 230 sera from 180 patients with chronic hepatitis C. The H77c and UKN3A1.28 antigens used in this assay represented two functional E1E2 glycoproteins from genotype 1 and genotype 3 viruses, respectively. The

reactivity of each serum to E1E2 was determined after exclusion of the background reactivity to untransfected HEK 293TF cell lysate (figure 4.3).

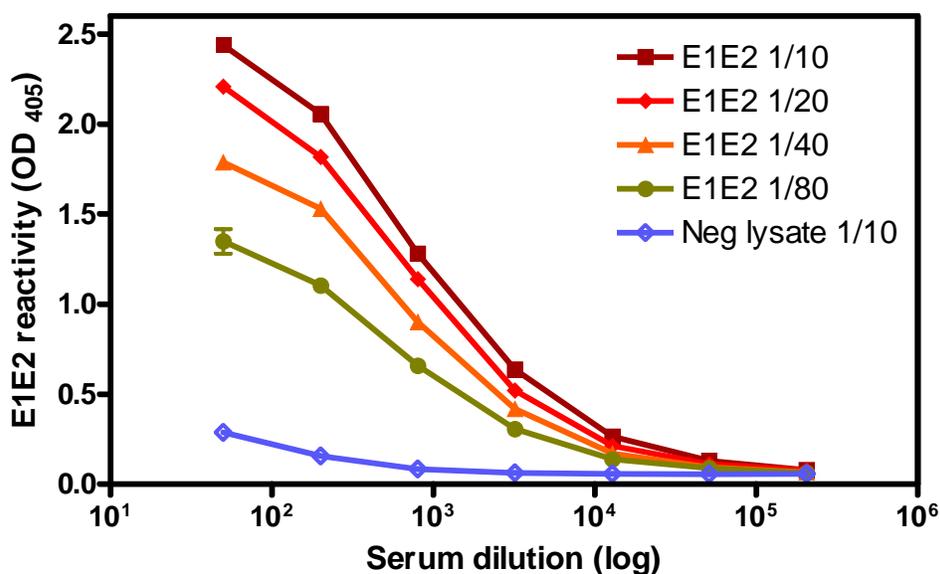


Figure 4.3: Serum reactivity to E1E2.

The serum reactivity to E1E2 was determined at different concentrations of serum and E1E2 (expressed in HEK 293 cells). A set of 4-fold serial dilution (from 1/50 to 1/204,800 in PBS-Tween) of the serum (from HCV genotype 1 infected patient) was examined for reactivity to another 2-fold series of E1E2 dilution (from 1/10 to 1/80, in PBS-Tween). The background was determined by evaluating the serum reactivity to an untransfected HEK cell lysate. The seroreactivity was considered as the signal above the background. Further evaluation of the reactivity was performed using sera, diluted 1/50, to E1E2, diluted 1/80.

In order to determine the pattern of anti-E1E2 reactivity over time, the antibody reactivity to E1E2 in 10 patients over 2-5 years was evaluated. The levels of reactivity in each patient were fairly constant and did not show variability (figure 4.4), suggesting that the level of reactivity can be considered as a true

representative for the level of anti-E1E2 during the course of chronic HCV disease.

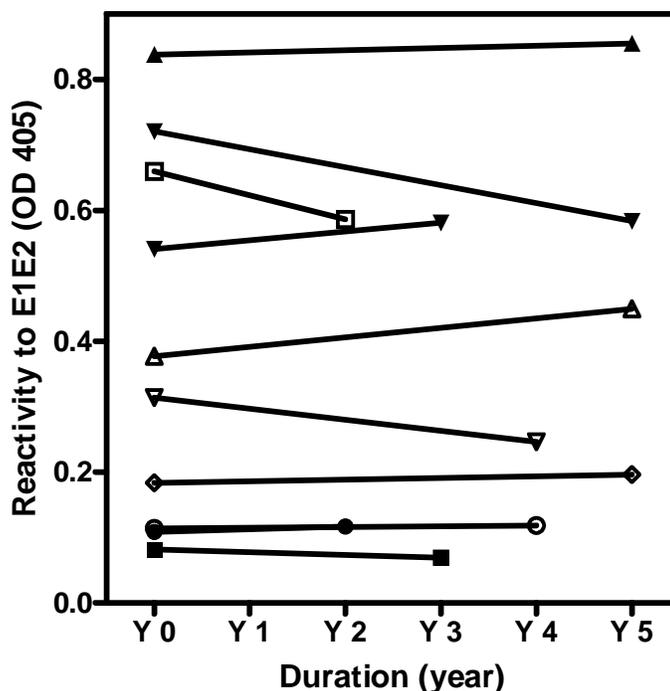


Figure 4.4: Pattern of anti-E1E2 reactivity over time.

The seroreactivity of 10 sera to E1E2 was examined over a time period of 2-5 years, from the start point (Y0), to year 2 (Y2) through year 5 (Y5). The reactivity of the examined sera showed little or no variation over the time period, indicating that the seroreactivity at a single time point can represent the level of anti-E1E2 during the course of chronic HCV disease.

4.3.3. Correlation with HCV Genotype

The reactivity of 67 sera (36 sera from patients infected with HCV genotype 1 and 31 sera from patients infected with HCV genotype 3) was assessed by comparing the reactivity of each serum to both HCV E1E2 derived from genotype 1 (H77c)

and genotype 3 (UKN3A1.28). The results revealed that the reactivity of sera from patients infected with HCV genotype 1 was significantly higher when tested against H77c E1E2 (Median: 1.002, Range: 0.0005-2.848) than against UKN3A1.28 E1E2 (Median: 0.715, Range: 0.062-2.035), $p=0.045$ (figure 4.5 A). Similarly, the reactivity of sera from patients infected with HCV genotype 3 was significantly higher when tested against UKN3A1.28 E1E2 (Median: 1.661, Range: 0.0115-2.401) than against H77c E1E2 (Median: 0.8535, Range: 0.048-2.456), $p=0.0007$ (figure 4.5 B).

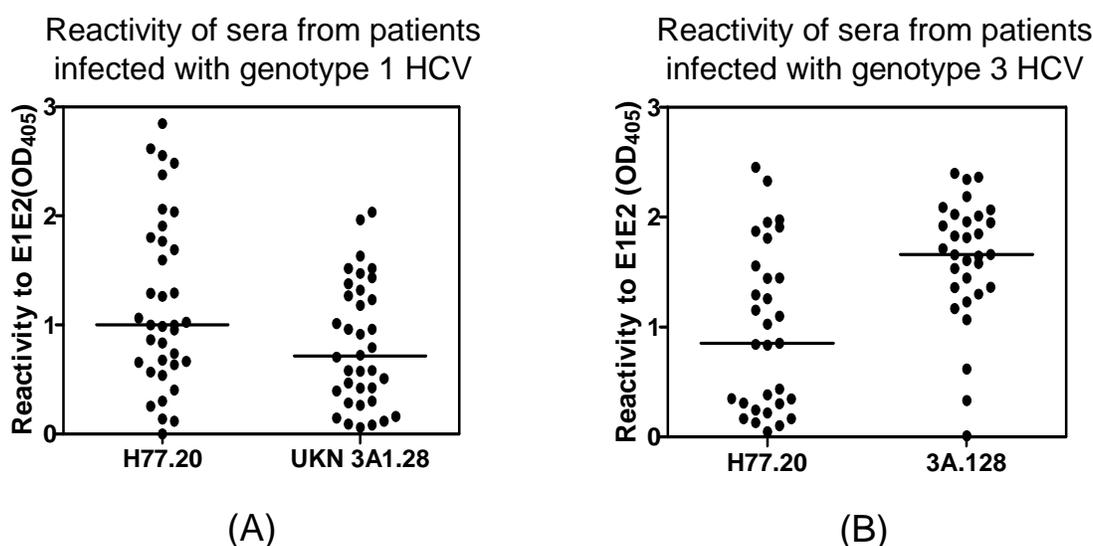


Figure 4.5: Comparison of reactivity of sera to H77c and UKN3A1.28.

The reactivity (signal above background) of 36 sera of patients infected with HCV genotype 1 and 31 sera of patients infected with HCV genotype 3 (all sera were diluted 1/50) to either H77c or UKN3A1.28 was compared, at OD₄₀₅, as an indication for the levels of anti-E1E2. The reactivity of sera infected with HCV genotype 1 (A) was significantly higher to H77c than to UKN3A1.28 ($p=0.045$). The reactivity of sera infected with HCV genotype 3 (B) was significantly higher to UKN3A1.28 than to H77c ($p=0.0007$). The horizontal lines represent the median values.

Furthermore, the reactivity of sera of patients infected with HCV genotype 3 against their homologous E1E2 construct, UKN3A1.28, was significantly higher than that of sera from patients infected with HCV genotype 1 against homologous E1E2 construct, H77c, ($p=0.01$).

To confirm these results, the ratio of reactivity of each serum to H77c and UKN3A1.28 was calculated (figure 4.6). For the sera derived from patients infected with genotype 1, the average ratio of reactivity to H77c/ UKN3A1.28 was 2.07 and for the sera derived from patients infected with genotype 3, the average ratio of reactivity to H77c/ UKN3A1.28 was 0.716, $p<0.0001$.

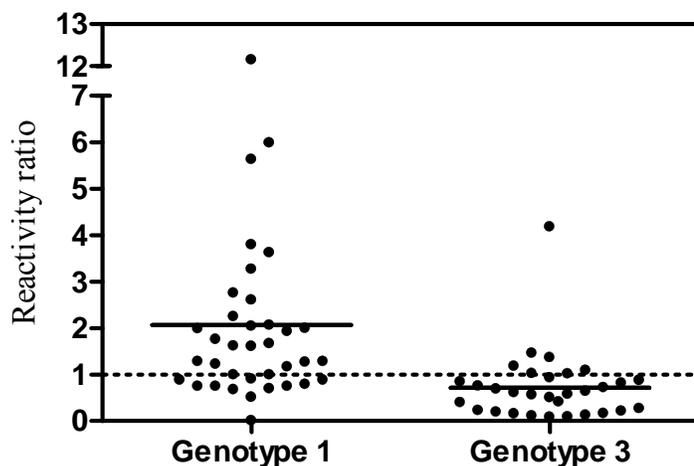


Figure 4.6: Ratio of reactivity of sera to and UKN3A1.28.

The ratio of reactivity of each serum to H77c and UKN3A1.28. The ratio of reactivity of sera derived from patients infected with genotype 1 was >1 (mean; 2.07), while the ratio of reactivity of sera derived from patients infected with genotype 3 was <1 (mean; 0.716) ($p<0.0001$). The horizontal lines represent the mean values.

4.3.4. Correlation with Viral Load

The reactivity of 54 sera (34 from patients infected with genotype 1 and 20 from patients infected with genotype 3) to E1E2 (both H77c and UKN3A1.28) was compared with the viral load (range from 600 - 850,000 IU/mL) of each serum. The seroreactivity of all sera infected with either genotype 1 or 3 to the corresponding E1E2 construct (H77c or UKN3A1.28) was plotted against the viral load of each serum. The seroreactivity of the tested sera was inversely proportional to the viral load (figure 4.7). The deviation of the slope from zero was significant ($p=0.0361$).

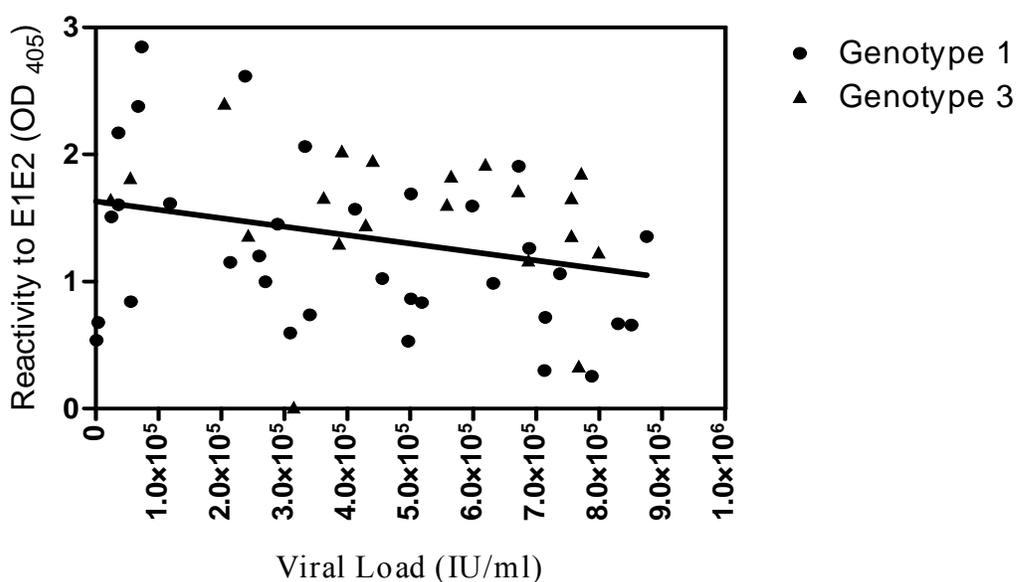


Figure 4.7: Correlation between reactivity to E1E2 and viral load.

The reactivity of each serum to E1E2, at OD₄₀₅, is plotted against viral load in 54 sera infected with either genotype 1 or 3 (tested against corresponding E1E2 construct). The reactivity to E1E2 was higher in patients with lower viral load ($p=0.0361$).

4.3.5. Correlation with Liver Disease Severity

To investigate the correlation between the level of anti-E1E2 and the degree of liver fibrosis, the reactivity of 28 sera from patients infected with HCV genotype 1 (17 patients with mild liver fibrosis and 11 patients with severe liver fibrosis) to H77c was examined. Also, the reactivity of 23 sera of patients infected with HCV genotype 3 (13 patients with mild liver fibrosis and 10 patients with severe liver fibrosis) to UKN3A1.28 was examined (figure 4.8). The overall reactivity of the tested sera against their homologous E1E2 proteins was significantly higher in patients with mild liver fibrosis (Median: 1.719, Range: 0.901-2.305) than in patients with severe liver fibrosis (Median: 1.325, Range: 0.827-2.182) ($p=0.025$).

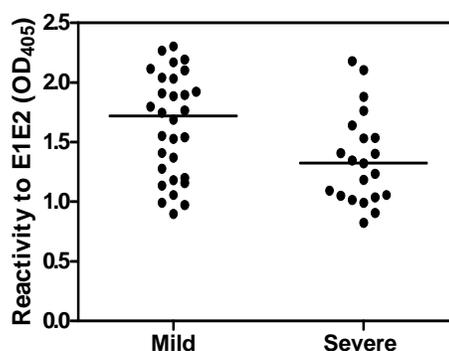


Figure 4.8: *Correlation between reactivity to E1E2 and degree of liver fibrosis.*

The reactivity of 28 sera of patients infected with HCV genotype 1 to H77c and of 23 sera of patients infected with HCV genotype 3 to 3A1.28, at OD₄₀₅, is shown in relation to the degree of liver fibrosis, mild or severe. Patients with a mild degree of liver fibrosis showed a significantly higher level of seroreactivity compared to patients with patients with a severe degree of liver fibrosis ($p=0.025$). The horizontal lines represent the median values.

As the above data utilised the OD reading of individual sera all tested at the same dilution (1/50), a further experimental assay of anti-E1E2 titre was performed to validate the findings (figure 4.9). The anti-E1E2 titre was determined by testing sera at serial 10-fold dilutions and the titre defined was assigned as the reciprocal of the last dilution at which the OD value was greater than the OD of serum against negative lysate.

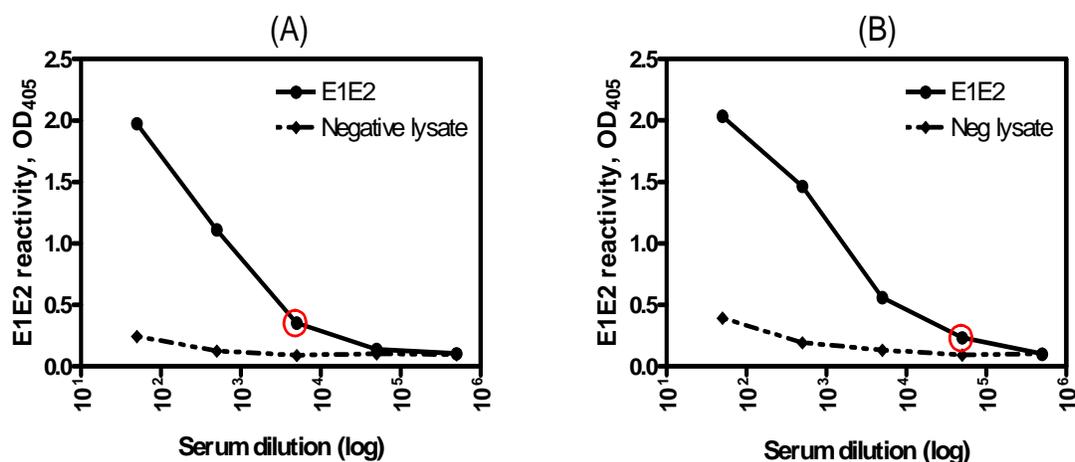


Figure 4.9: Quantitation of anti-E1E2 antibodies.

The antibody titre of the tested sera was determined by evaluating the anti-E1E2 reactivity of a 10-fold serial dilution series of sera (from 1/50 to 1/500,000) against their homologous E1E2 proteins. The anti-E1E2 titre was determined as the reciprocal of the last dilution at which the OD value was greater than the OD of serum against negative lysate. The titration of anti-E1E2 antibodies in 2 sera (A and B) with a similar OD, demonstrates a different anti-E1E2 titre (marked by the red circle).

The relationship between the OD value at a single dilution, and the titre of anti-E1E2 reactivity was evaluated. By comparing sera from both groups having a similar level of reactivity, there was a trend of a higher anti-E1E2 titre in sera from

patients with mild HCV liver disease compared to sera from patients with severe HCV liver disease (figure 4.10 A).

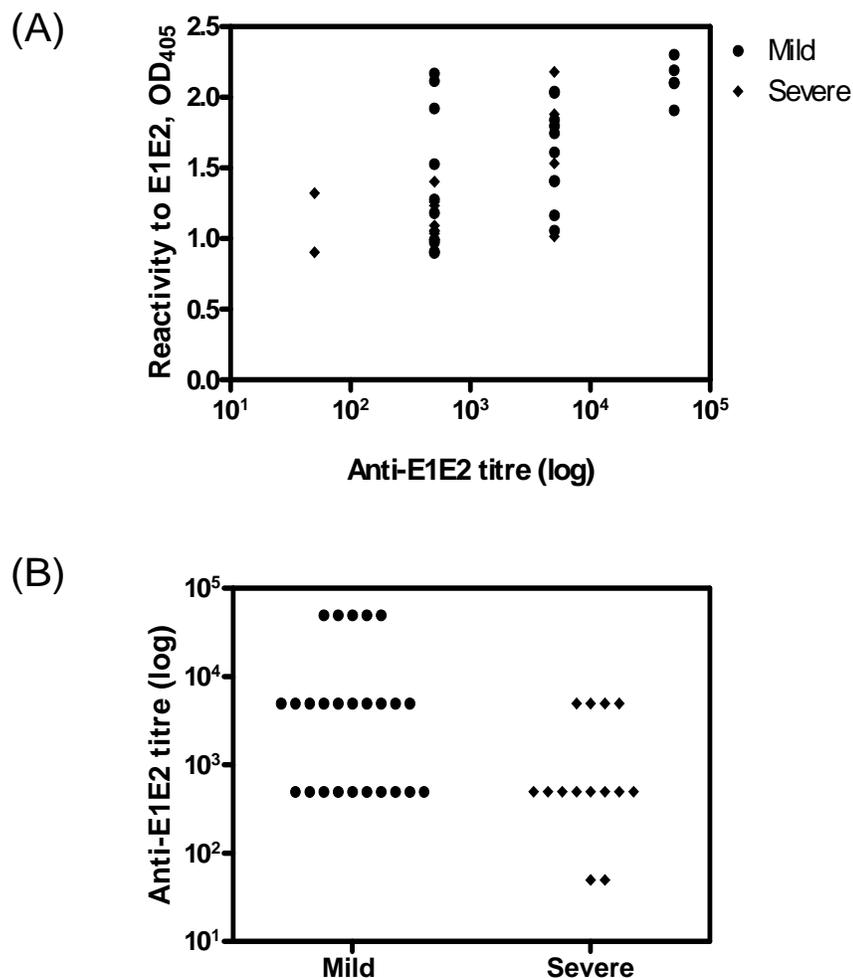


Figure 4.10: Correlation between anti-E1E2 antibodies titres and degree of liver fibrosis.

A: The correlation between the reactivity of 25 sera of patients with a mild degree of liver fibrosis and of 14 sera of patients with a severe degree of liver fibrosis to homologous E1E2, at OD₄₀₅, and the anti-E1E2 titre of each serum. B: The antibody titre of 25 sera of patients with a mild degree of liver fibrosis and of 14 sera of patients with a severe degree of liver fibrosis is shown in relation to the degree of liver fibrosis. The titre of the tested sera against their homologous E1E2 proteins was significantly higher in patients with mild liver fibrosis than in patients with severe liver fibrosis ($p=0.0278$).

The antibody titre of tested sera was evaluated in relation to the degree of liver fibrosis. Lower anti-E1E2 reactivity, when expressed as a titre, correlated with more severe liver disease (figure 4.10 B) and showed statistical significance ($p=0.0278$), confirming the result obtained previously using the OD value at a single dilution.

Several factors are known to be associated with liver disease severity in chronic HCV infection, including male sex, age, and duration of infection. The patients in this study with severe HCV liver disease were on average 11 years older than those with mild disease. No association between age at infection, duration of infection or sex and anti-E1E2 reactivity was found (figure 4.11).

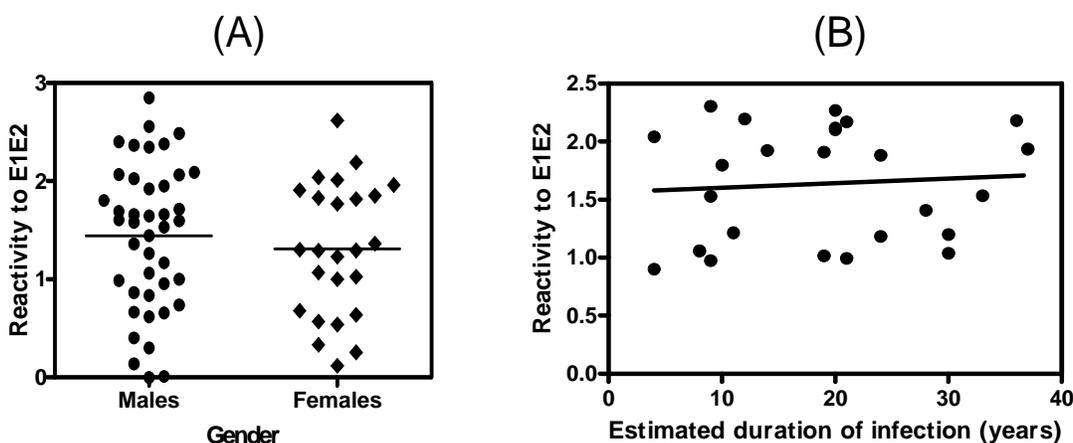


Figure 4.11: Correlation between anti-E1E2 reactivity and confounding factors.

Male gender, older age at infection and duration of infection are factors shown to be associated with the rate of fibrosis progression. The correlation between anti-E1E2 reactivity and confounding factors, gender (A) and estimated duration of infection (B), was evaluated. Anti-E1E2 reactivity showed no significant association with either male gender or estimated duration of infection.

The sera of 7 patients who progressed from mild to severe fibrosis were also examined, by comparing the seroreactivity of their sera in the severe stage with the seroreactivity of their sera whilst in the mild stage. The results showed no significant difference between the seroreactivity between the two stages (figure 4.12).

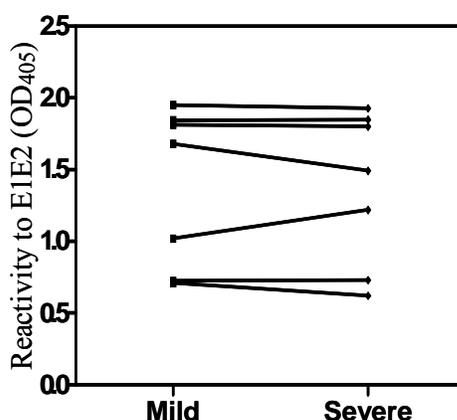


Figure 4.12: Retrospective evaluation of the anti-E1E2 reactivity.

Retrospective evaluation of the anti-E1E2 reactivity in 7 patients who progressed from mild to severe fibrosis. By comparing their seroreactivity in the severe stage with their seroreactivity whilst in the mild stage, the level of seroreactivity showed minimal variation in both stages.

4.3.6. Correlation with Response to Treatment

In order to investigate the relationship between anti-E1E2 reactivity and response to therapy, A retrospective assay of the anti-E1E2 level in 50 patients treated with standard doses of pegylated interferon and ribavirin combination therapy; 26 patients infected with HCV genotype 1 and 24 patients infected with HCV genotype 3 was performed. These patients were classified into two groups; 25

sustained viral responders (SVR) and 25 relapsers and non responders (R/NR). Two samples were examined from each patient; the first sample was taken before treatment and the second one was taken six months after termination of treatment. The pre-treatment level of reactivity against homologous E1E2 proteins was significantly higher in SVR than in R/NR ($p=0.0256$). The seroreactivity of the tested sera in SVR was slightly reduced after treatment. However, this reduction in the reactivity after treatment was not significant in either group of patients (figure 4.13).

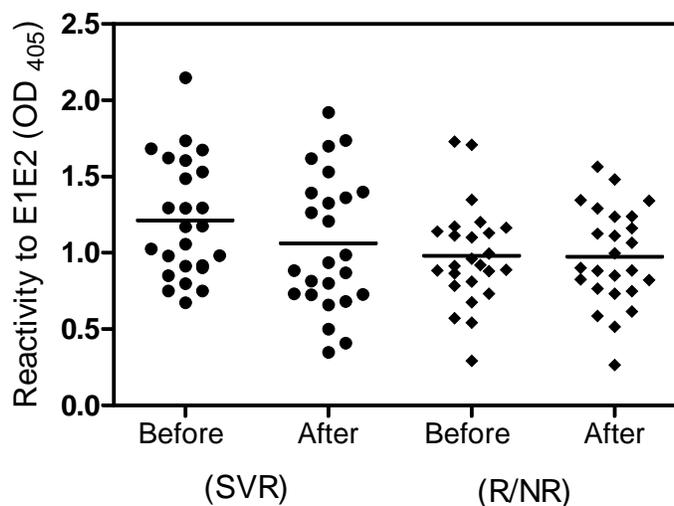


Figure 4.13: Correlation between reactivity to E1E2 and responsiveness to treatment.

The reactivity of each serum to its homologous E1E2, at OD₄₀₅, as an indication for the level of anti-E1E2 is shown before and after treatment for sustained viral responders (SVR) and relapsers/non-responders (R/NR). Although the post-treatment reduction of the anti-E1E2 level was not significant in either group, the pre-treatment anti-E1E2 level was significantly higher in sustained responders ($p=0.0256$).

4.4. Discussion

The role of the antibodies to E1E2 (anti-E1E2) in the pathogenesis of HCV disease has been investigated, however, the results are controversial [165, 166]. In this part of the study, the level of anti-E1E2 antibodies were evaluated in chronic HCV-infected patients. Uniquely, the anti-E1E2 antibodies were evaluated using E1E2 proteins derived from two genotypes (1 and 3) that conferred infectivity in pseudoparticle infectivity assay [101].

The reactivity of each serum was assessed to E1E2 proteins from two different genotypes. The reactivity of the tested sera was significantly higher to their homologous E1E2 than to heterologous E1E2. This higher reactivity may suggest the presence of genotype-specific antigens within E1E2, assuming that the various E1E2 preparations have a similar proportion of native and denatured aggregates which might affect epitope exposure and reactivity. By comparing the level of anti-E1E2 to the infecting genotype, the level of anti-E1E2 was significantly lower in patients infected with genotype 1 than in patients infected with genotype 3. This is in contrast to Zampino et al. (2004) who evaluated the level of anti-E1E2 against antigens derived from a pool of epitopes of different HCV genotypes and reported a similarity in the level of E1E2 antibodies among patients infected with diverse HCV genotypes. The ability of sera from patients infected with genotypes 1, 2, and 3 to neutralise to the same extent the binding of recombinant E2 derived from genotype 1 has also been reported [208].

The results showed a significant correlation between higher anti-E1E2 levels and lower viral load (figure 4.7). This result suggests a potential protective effect of anti-E1E2 against HCV replication, in agreement with Mehta et al. (2002), who reported a lower level of viraemia in anti-HCV positive intravenous drug users, who previously cleared HCV, when re-infected with HCV. However, other studies reported either the absence of significant correlation between anti-E1E2 levels and viral load [165, 166], or even a positive correlation between antibodies to HCV envelope proteins with hepatitis C viraemia [295], where it was suggested that the production of antibodies to HCV envelope protein is driven by HCV replication. In light of the recent report describing detection of a 5' end subgenome of HCV terminating at nucleotide 384 in patients' plasma and liver tissues [296], viral load results must be interpreted cautiously as they may not give a true value for full length viral genome.

The anti-E1E2 antibody level was assessed before and after treatment. The results showed a non-significant reduction in the anti-E1E2 level in sustained viral responders after treatment. The pre-treatment level of anti-E1E2 was significantly higher in sustained viral responders. These results are in agreement with Depraetere et al. (2000) and Zampino et al. (2004) who reported that the anti-E1E2 levels tend to be higher in patients with a long-term or a transient response to interferon treatment than in patients who were absolute non-responders, and their level tended to be significantly lower after treatment in long-term responders to

therapy with HCV clearance than that observed in relapsers and non-responders [165, 166].

When exploring a possible correlation between anti-E1E2 reactivity and liver disease severity, selected sera were deliberately selected from patients with the extremes of disease activity. No samples from patients with Ishak 3-4 were tested, due to limited number. Significantly higher median values of seroreactivity to E1E2 were associated with patients with a mild degree of liver fibrosis (Ishak fibrosis stage: 0-2) compared to patients with severe fibrosis (Ishak fibrosis stage: 5-6). This was true whether the anti-E1E2 reactivity was expressed as an optical density value at a single dilution or as a titre, which was used instead of the reactivity to discriminate between sera with similar reactivity, at 1/50 dilution, but with different antibodies titres. However, there was overlap in anti-E1E2 reactivity between the 2 selected groups of patients, and it is not possible to define a cut-off value of anti-E1E2 reactivity which differentiates the 2 groups. The relationship of confounding factors that are likely to be associated with disease progression was analysed to exclude the possibility of independent association with disease severity. In 7 patients, the level of anti-E1E2 did not change during progression from mild to severe liver disease, suggesting that the lower level of anti-E1E2 in patients with severe liver disease is not a consequence of liver disease.

These data may implicate a modulating effect of anti-E1E2 on HCV disease prognosis i.e. a high level of anti-E1E2 is associated with protection against HCV liver disease progression. This finding would support studies reporting the ability of E1E2 to elicit production of neutralising antibodies against the virus [85, 208, 297, 298] and the consideration of E1 or E2 as a major candidate for an anti-HCV vaccine [211, 214, 297, 299]. However, to elicit a protective antibody response, vaccines will require a rational design, targeting production of specific neutralizing antibodies against conformation-sensitive epitopes.

5. Evaluation of Serum L-ficolin levels

5.1. Aim

The main goal in this section was to evaluate the potential role of L-ficolin in HCV infection by estimating the serum L-ficolin levels in patients with chronic HCV infection. Correlations of L-ficolin levels with viral genotype, viral load, response to therapy, and severity of liver disease were investigated.

5.2. Materials and Methods

5.2.1. Materials

Bovine serum albumin (BSA) and acetic anhydride were obtained from Sigma-Aldrich Company Ltd, Poole, UK. Mouse monoclonal anti-human L-ficolin antibody (GN5) was purchased from Hycult Biotechnology.

5.2.2. Patients

The serum levels of L-ficolin were examined in 160 patients with chronic hepatitis C obtained from the HCV Trent Study Cohort. All sera were positive for HCV antibody and for HCV RNA with determined HCV genotypes. Liver biopsies were assessed and scored according to the criteria of Ishak et al. [45]. In a sub-study examining a possible correlation between L-ficolin serum level and liver disease activity, as previously described in section 4.2.5, sera were specifically chosen from patients with either mild (defined as Ishak fibrosis stage 0–2) or severe (Ishak

stage 5–6) disease. Samples from 30 blood donors were used as healthy controls. A further control population of 32 patients with alcoholic liver disease or non-alcoholic fatty liver disease was also analysed. Serum samples were stored at -72°C .

5.2.3. ELISA Assay

An ELISA assay was employed to examine the ficolin levels in serum samples from the HCV-infected chronic hepatitis patients and controls, as previously described in section 2.2.9.2 (figure 5.1). In brief, MaxiSorb microtiter plates were coated with BSA, which was then acetylated by 0.1% acetic anhydride in methanol. Serum samples, diluted 1/50 in PBS-Tween (in duplicates), were incubated overnight at 4°C and then the serum ficolin was detected by mouse IgG monoclonal biotin-labelled anti-human L-ficolin antibody, GN5. GN5 antibody was biotinylated as previously described in section 2.2.9.1. The plates were washed 3 times with wash buffer after each step, using the Denly WellWash plate washer. A streptavidin-alkaline phosphatase conjugate and p-nitrophenyl phosphate (pNPP) substrate were used and the OD was measured at 405 nm. L-ficolin level was determined using a standard curve with a 2-fold serial dilution of a pooled human serum of known L-ficolin concentration ($4\ \mu\text{g}/\text{mL}$), as a reference. Manipulation of patient sera during the ELISA assay, including washing steps, was carried out in a class I biological safety cabinet (LEEC) which was cleaned with 70% v/v ethanol and 2% Trigene, before and after each manipulation.

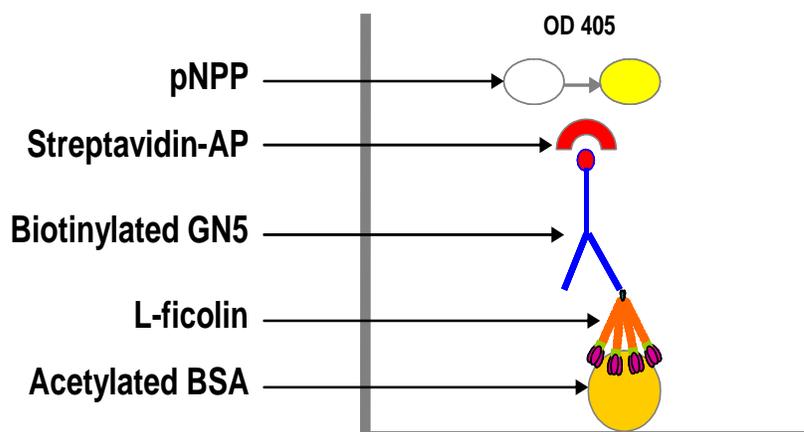


Figure 5.1: Evaluation of serum L-ficolin by ELISA.

A schematic diagram shows the components of the ELISA assay used to quantify serum L-ficolin. The wells were coated with BSA followed by acetic anhydride. Serum samples (diluted 1/50, in PBS-Tween) were added and the bound ficolin was detected by biotinylated monoclonal anti-L ficolin, GN5. Streptavidin-alkaline phosphatase (streptavidin-AP) conjugate was added followed by p-ntirophenyl Phosphate (pNPP) substrate. The OD was determined at 405 nm.

5.2.4. Statistical analysis

Statistical analyses were performed using Graphpad prism version 4.03 (Graph Pad, San Diego, CA, USA). Optical densities of the standard sample were used to determine standard curves on a log 10 scale and interpolated X-values were obtained. Comparison between groups (non-normally distributed continuous variables) was performed using the Mann-Whitney U-test. The genotype variation of serum L-ficolin was analysed by a one-way ANOVA test. The correlation between L-ficolin level and viral load was analysed using the linear regression model. SPSS statistical software (version 14.0, Chicago, IL, USA) was used for binary logistic regression analysis, to assess the effect of age as a confounding factor. In all tests, a p value of <0.05 was considered statistically significant.

5.3. Results

5.3.1. Determination of Serum L-ficolin Level

The level of L-ficolin in serum samples from 160 patients with chronic hepatitis C infection, 30 healthy blood donors and 32 patients with non-HCV liver disease (NHCV) was examined, using ELISA-based assay. A standard curve was created using a two-fold serial dilution of a pooled serum (containing 4 $\mu\text{g/mL}$ L-ficolin), starting from 1/6.25 down to 1/400, which corresponded to L-ficolin concentration of 0.64 $\mu\text{g/mL}$ to 0.01 $\mu\text{g/mL}$ (figure 5.2).

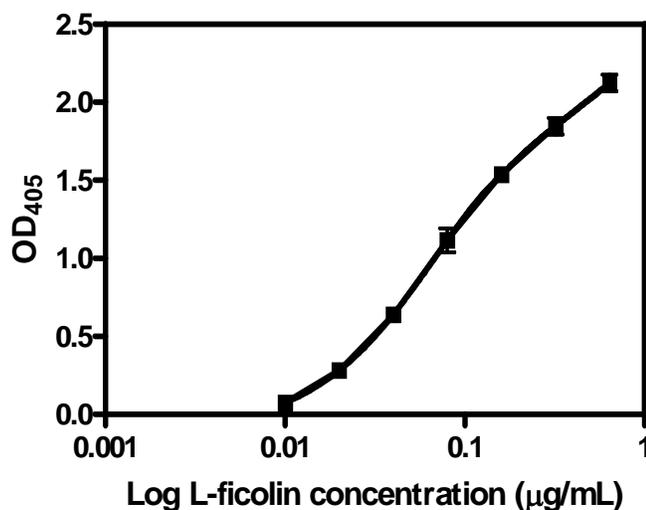


Figure 5.2: A standard curve used for determination of serum L-ficolin concentration.

The serum level of L-ficolin was determined by ELISA and calculated using a standard curve. A 2-fold serial dilution of a pooled serum (4 $\mu\text{g/mL}$), from 1/6.25 down to 1/400 was used and the OD₄₀₅ of each dilution was plotted against the corresponding ficolin concentration (at a logarithmic scale), to create a standard curve. The figure is an example of a standard curve used for further determination of serum L-ficolin level, based on the OD₄₀₅ of each serum in the ELISA assay.

The optical density values, at 405nm, were plotted against a logarithmic scale of ficolin concentration. To overcome the intra assay variation, the standard and the serum samples were assayed in duplicates.

5.3.2. Correlation with Demographic Characters

The correlation between serum L-ficolin level and either gender or age was examined in 115 patients. The median L-ficolin level in the male patient samples (n = 76) was 3.62 $\mu\text{g/mL}$ (range: 0.50-12.52 $\mu\text{g/mL}$) and the median L-ficolin level in the female patient samples (n = 39) was 4.11 $\mu\text{g/mL}$ (range: 1.36-9.15 $\mu\text{g/mL}$) (figure 5.3). There was no statistically significant difference in L-ficolin levels between the genders (p = 0.37).

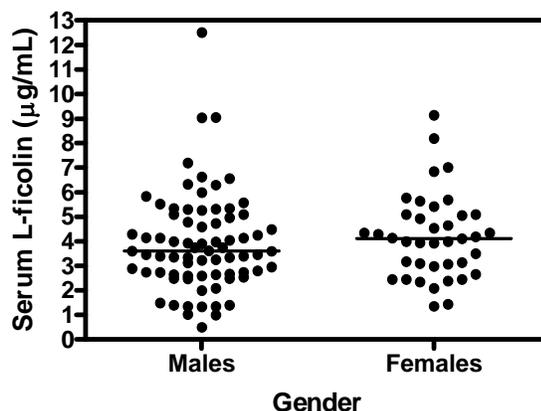


Figure 5.3: Gender correlation with L-ficolin levels.

L-ficolin level in the male and female HCV-infected patients. The difference in L-ficolin levels was not statistically significant (p = 0.37).

The effect of age of HCV-infected patients on the serum level of L-ficolin was investigated (figure 5.4). The age of the studied patients ranged from 22 to 78

years (median = 45 years). There was a tendency toward a reduction in serum L-ficolin level in older age, and the linear regression analysis showed a significant association ($p = 0.01$).

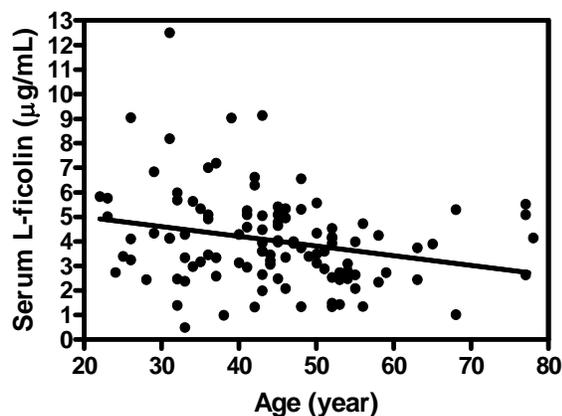


Figure 5.4: Age correlation with L-ficolin levels.

Correlation between the age of HCV-infected patients and serum L-ficolin level showed statistically significant association ($p = 0.01$). The older the age of HCV-infected patients, the lower the serum L-ficolin level.

5.3.3. Correlation with Viral Genotype

The correlation between the viral genotype and serum L-ficolin level was examined in 115 patients; 48 patients with HCV genotype 1 infection, 55 patients with HCV genotype 3 infection, 8 patients HCV genotype 2 infection and 4 patients with HCV genotype 4 infection (figure 5.5). Genotypes 1 and 3 represent the two major genotype groups, while the number of patients infected with HCV genotype 2 or 4 is relatively low. The median L-ficolin level in the HCV genotype 1 infected patients was 3.95 $\mu\text{g/mL}$ (range: 1.01-9.05 $\mu\text{g/mL}$). The median L-ficolin level in the HCV genotype 3 infected patients was 3.67 $\mu\text{g/mL}$ (range:

0.50-9.15 $\mu\text{g/mL}$). The median L-ficolin level in the HCV genotype 2 infected patients was 4.85 $\mu\text{g/mL}$ (range: 1.02-12.52 $\mu\text{g/mL}$). The median L-ficolin level in the HCV genotype 4 infected patients was 4.58 $\mu\text{g/mL}$ (range: 2.75-7.2 $\mu\text{g/mL}$). There was no statistically significant difference in L-ficolin levels seen between the genotype groups ($p = 0.23$).

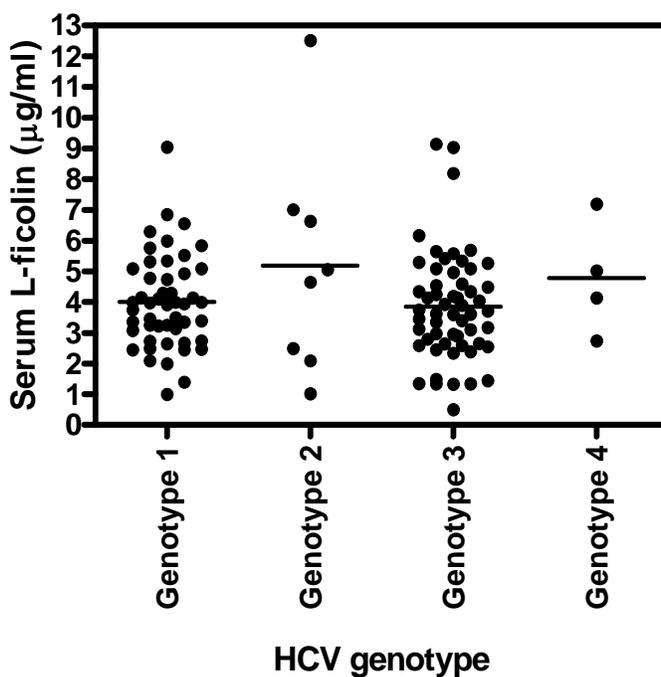


Figure 5.5: Correlation between serum L-ficolin levels and HCV genotype.

L-ficolin level in association with the infecting HCV genotype. There was no significant association between the infecting HCV genotype and serum L-ficolin levels ($p = 0.23$).

5.3.4. Correlation with Viral Load

The serum level of L-ficolin in 115 sera was plotted against the viral load of each serum and a linear regression analysis was obtained. There was no significant

correlation between the level of serum L-ficolin and viral load, $p= 0.95$ (figure 5.6). The serum L-ficolin level in the tested sera was variable in patients with similar viral loads. The variation extended throughout the range of viral load (1,871-874,457 IU/mL).

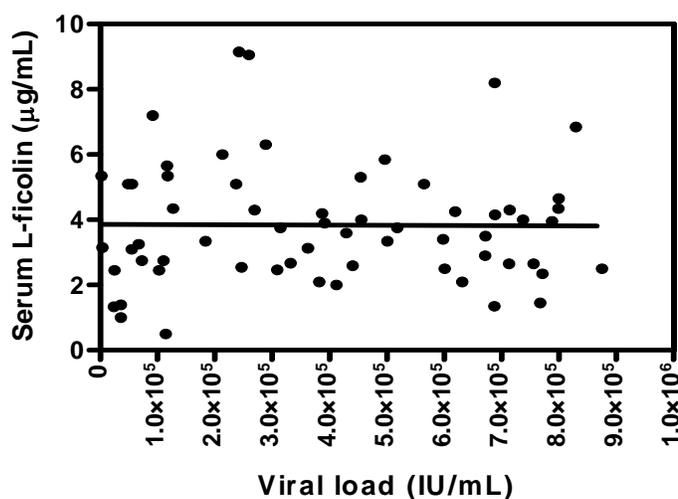


Figure 5.6: *Correlation between serum L-ficolin levels and viral load.*

Correlation between the viral load and serum L-ficolin level showed no statistically significant association ($p = 0.95$).

5.3.5. Correlation with Treatment Outcome

Forty five HCV-infected patients were divided according to their response to treatment, with pegylated interferon plus ribavirin, into two groups; sustained viral responders (SVR), and relapsers and non responders (R/NR). A retrospective assay of the serum L-ficolin level in 22 sustained viral responders and 23 relapsers and non responders was performed, before treatment and after termination of the treatment course (figure 5.7). The ficolin levels in the tested sera did not show a

significant difference before and after treatment in either sustained viral responders ($p = 0.77$) or in relapsers and non responders ($p = 0.32$). In addition, the pre-treatment level of L-ficolin did not show a significant difference between the sustained viral responders (median: 4.93; average: 0.35-7.85 $\mu\text{g/mL}$) and the relapsers and non responders (median: 5.25; average: 2.50-9.01 $\mu\text{g/mL}$).

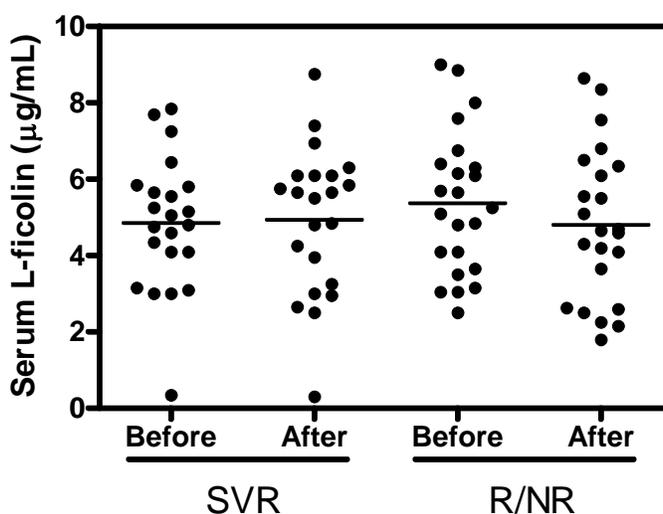


Figure 5.7: Correlation between serum L-ficolin level and response to treatment.

Serum L-ficolin level was evaluated before and after termination of treatment in 22 sustained virologic responders (SVR) or in 23 relapsers and non responders (R/NR). No significant correlation was seen between pre- and post-treatment levels of L-ficolin and response to treatment in sustained virologic responders or in relapsers and non responders. Also, no significant difference was detected between the pre-treatment levels of L-ficolin in both groups of patients.

5.3.6. Correlation with Disease Severity

To investigate the correlation between the serum L-ficolin level and the degree of HCV liver fibrosis, the L-ficolin level in 36 sera from patients with mild HCV

liver fibrosis and 17 patients with severe HCV liver fibrosis was examined (figure 5.8). The serum L-ficolin level was higher in patients with mild liver fibrosis (median: 4.13 $\mu\text{g/mL}$; range: 2.39–12.52 $\mu\text{g/mL}$) than in patients with severe liver fibrosis (median: 3.23 $\mu\text{g/mL}$; range: 1.02–6.33 $\mu\text{g/mL}$) ($p = 0.015$). This result demonstrates an association between serum L-ficolin level and HCV liver disease severity.

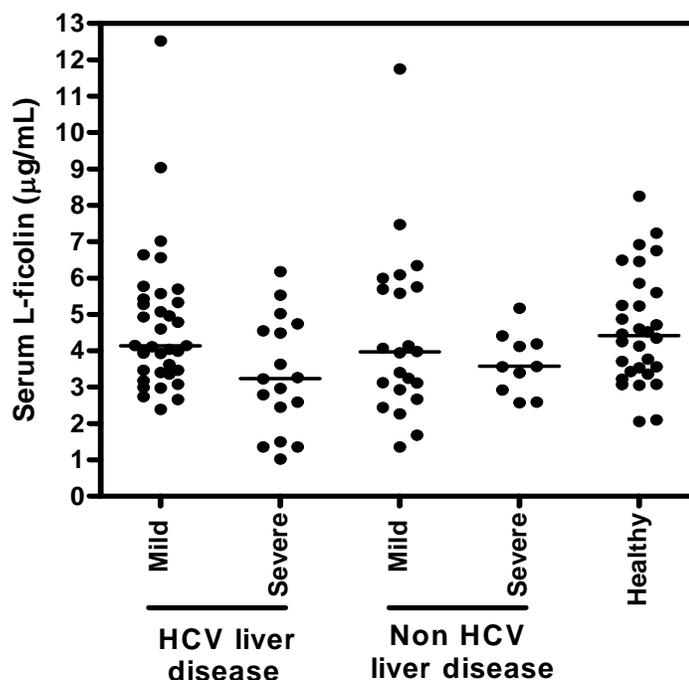


Figure 5.8: Correlation between L-ficolin serum level and degree of liver fibrosis.

Patients with a mild degree of HCV liver fibrosis showed a significantly higher level of L-ficolin compared to patients with a severe degree of HCV liver fibrosis ($p = 0.015$). Although, patients with a mild degree of non-HCV liver fibrosis showed a higher level of L-ficolin compared with patients with a severe degree of non-HCV liver fibrosis, this difference was not significant. The horizontal bars represent the median values.

Patients with non-HCV (NHCV) liver disease, namely alcoholic liver disease and non alcoholic fatty liver disease, were also divided into those with mild fibrosis (n = 22) and those with severe fibrosis (n = 10). They showed lower serum L-ficolin levels in patients with severe liver disease than in patients with mild liver disease. However the difference was not statistically significant (p = 0.32). The median L-ficolin level in the healthy control group (n= 30) was 4.4 µg/mL. The difference in L-ficolin level between the severe HCV liver cirrhosis (n = 17) and the healthy control group was found to be statistically significant with a p value of 0.01. No such statistical significance was achieved between the severe non-HCV liver fibrosis groups and the healthy control group (p = 0.074).

The age, previously found to be negatively correlated with serum L-ficolin level in patients with HCV liver disease (figure 5.4), is one of the host factors that may affect the liver fibrosis progression in HCV liver disease. The age of the examined HCV-infected patients significantly correlated with disease progression (p = 0.001). Thus, it could potentially have a confounding effect on the serum L-ficolin level and liver fibrosis relationship. Binary logistic regression analysis was performed using age as a covariant with L-ficolin concentration. The p value for L-ficolin concentration was different in the adjusted model. However, both serum L-ficolin level (p = 0.03, OR = 0.491, 95% CI = 0.257-0.935) and age (p = 0.021, OR = 1.098, 95% CI = 1.014-1.189) remained significantly associated with liver disease severity.

The L-ficolin level was also determined in the sera of five patients who had biopsy-proven disease progression from mild to severe liver fibrosis (figure 5.9). There was a tendency for L-ficolin level to decrease concurrently with progression of the liver cirrhosis from mild to severe. However, the values were not statistically significant ($p = 0.15$).

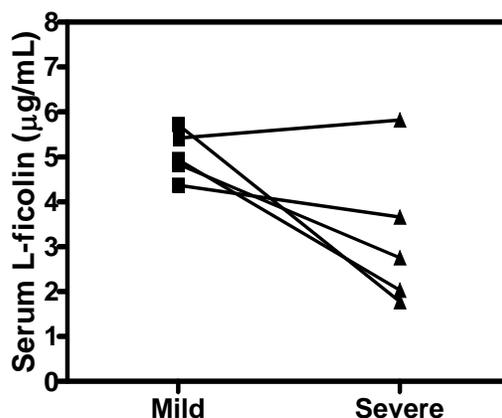


Figure 5.9: Retrospective evaluation of serum L-ficolin level.

Retrospective evaluation of serum L-ficolin level in 5 HCV-infected patients who progressed from mild to severe liver disease. The level of L-ficolin had a propensity to decrease as the fibrosis proceeded from mild to severe. However, the reduction was not statistically significant, $p = 0.15$.

5.3.7. Correlation with Anti-E1E2

To examine the correlation between both serum L-ficolin level and the level of anti-E1E2, the serum L-ficolin concentration in HCV-infected patients was compared to the reactivity against E1E2 proteins (the E1E2 antigen used was homologous to the infecting HCV genotype in the tested sera) (figure 5.10). Linear regression analysis revealed the absence of correlation between the serum

L-ficolin concentration and the level of anti-E1E2 in the examined sera (n = 80), p = 0.69.

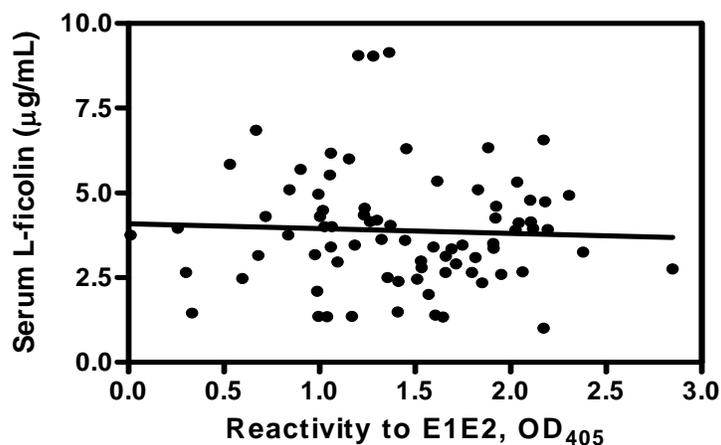


Figure 5.10: Correlation between L-ficolin serum level and seroreactivity to E1E2.

Linear regression analysis revealed absence of a significant correlation between the level of serum L-ficolin and seroreactivity of the tested sera to E1E2 protein, p = 0.69.

5.4. Discussion

Serum L-ficolin level in patients with chronic HCV infection was determined by an ELISA assay in order to explore the clinical impact of L-ficolin binding to HCV and its possible effect on HCV disease outcome. The level of serum L-ficolin was correlated with viral load, infecting HCV genotype, disease severity and response to treatment, to find out the possible associations.

Starting by correlation with demographic characteristics of the examined patients, serum L-ficolin level showed a significant inverse relationship with age with no

correlation with gender. Kipatrick et al., reported the absence of relationship between L-ficolin serum level and either sex or age in healthy blood donors [300]. The significant negative correlation between the age and serum L-ficolin concentration in HCV-infected patients could be a reflection of the association between age and disease severity. The L-ficolin level did not correlate with the infecting HCV genotype or the viral load in the tested sera. As L-ficolin showed a neutralising effect on HCV pseudoparticle infectivity, it was expected that circulating L-ficolin might reduce virus load and diminish the pathogenesis of HCV infection. However, the role of L-ficolin as an immunoprotective agent against viral replication is not evident. It may be possible that a high viral replication can overwhelm the L-ficolin in the serum. There was no apparent relationship between L-ficolin concentration and the response of HCV-infected patients to treatment with ribavarin and pegylated interferon. Similarly, Kilpatrick et al., reported that MBL concentration had no significant association with response to antiviral therapy, in HCV-infected patients [278].

The only positive implication of the serum levels of L-ficolin in the context of HCV disease was the lower serum L-ficolin levels in patients with a severe degree of HCV liver fibrosis (Ishak fibrosis stage: 5–6) compared to patients with mild HCV fibrosis (Ishak fibrosis stage: 0–2) and to the healthy controls. The median L-ficolin level in the healthy control group (4.4 $\mu\text{g}/\text{mL}$) was comparable to the literature. Le et al., reported an average serum L-ficolin level of 4.13 $\mu\text{g}/\text{mL}$ [217]

and Kilpatrick et al. reported a median serum L-ficolin concentration of 3.7 $\mu\text{g/mL}$ and most values were below 6.0 $\mu\text{g/mL}$ [300]. There was an overlap in L-ficolin levels between the two groups of patients, with no clear cut-off value of L-ficolin level to differentiate the two groups. A lower extent of reduction in L-ficolin levels was also observed in patients with severe non-HCV liver fibrosis. The L-ficolin level retained its significant association with liver disease severity even after adjustment using the age, which was associated with liver disease severity in chronic HCV infection, as a covariant.

These data, supported by the ability of L-ficolin to bind HCV glycoproteins (shown in sections 3.2.5 and 3.2.6) and to neutralise HCV pseudoparticle infectivity (shown in section 3.2.7), may implicate a modulating effect of L-ficolin on HCV disease prognosis i.e. a high level of L-ficolin may be associated with protection against HCV liver disease progression. A similar observation of children with lower levels of L-ficolin being more prone to recurrent respiratory infections has been reported [268]. Nevertheless, this finding should be carefully interpreted as L-ficolin is synthesised in the liver [218] and thus the reduction in the serum L-ficolin level in patients with severe liver fibrosis could be simply a result of decreased ficolin production by damaged hepatocytes as a previous study has reported a reduction of L-ficolin levels in liver disease with increasing severity of liver cirrhosis [228]. This cessation in L-ficolin production by the cirrhotic liver explains the relative reduction in serum L-ficolin level found also in non-HCV

patients with severe liver fibrosis and the tendency towards reduction in serum L-ficolin level in patients who progressed from mild to severe liver disease.

From another point of view, it is possible that the binding of L-ficolin to HCV particles plays a role in liver fibrosis. L-ficolin, like MBL, activates MASP-1, which has similar proteolytic activity to thrombin on fibrinogen and factor XIII [280-282]. An association between MBL/MASP-1 enzymatic activity and the severity of fibrosis in HCV patients was previously demonstrated [279]. By such a pathway, L-ficolin may contribute to the pathogenesis of liver fibrosis and in this case serum L-ficolin level could be a marker of HCV-induced fibrogenesis.

Although a higher level of anti-E1E2 antibodies (judged by measuring the reactivity to E1E2) was implicated as a protective modulator in HCV liver disease progression (previously described in section 4.3.5), there was no correlation between serum L-ficolin level and the level of anti-E1E2 in HCV-infected patients. This would suggest an independent effect of both anti-E1E2 and L-ficolin as immune mediators against HCV.

6. General Discussion and Future Work

HCV infection is an important worldwide health problem, affecting 170 million people [3]. It causes acute and chronic hepatitis, and liver cirrhosis [2]. It represents an important risk factor for development of hepatocellular carcinoma [20]. Data suggest that it will continue as a major health burden, at least in the near future [6, 17]. The person-to-person variability in HCV outcome is subject to host and viral factors [27-29]. HCV envelope glycoproteins, E1 and E2, are glycosylated transmembrane proteins essential for host-cell entry [98, 99]. They bind to receptors such as CD81 and SR-B1 and induce fusion with the host-cell membrane [101, 102]. They also provide a target for host immune recognition [56, 106].

The host immune response plays contrasting roles in the pathogenesis of viral hepatitis by contributing either to viral control and clinical recovery, or to the development of liver cirrhosis [23, 173], which is the main complication of chronic liver disease in HCV-infected patients. This project represents an investigation into the role of two immune mediators; L-ficolin and anti-E1E2 in HCV liver disease.

6.1. L-ficolin Binding to HCV Glycoproteins

The main hypothesis before beginning this work was that L-ficolin could interact with HCV glycoproteins, that have multiple N-linked glycosylation sequons in the primary sequence, and thus may affect the course of viral infection. The binding of L-ficolin to target surfaces depends on the interaction of fibrinogen-binding domain with an array of carbohydrates across different N-glycans. L-ficolin has a preference for binding to N-acetyl glucosamine (GlcNAc) residues. Specific interactions between L-ficolin and HCV were demonstrated in terms of binding to HCV glycoproteins sE2 and E1E2 heterodimers by ELISA. This is the first biochemical analysis demonstrating the binding interaction of L-ficolin with HCV envelope glycoproteins. The ability of L-ficolin to bind to authentic HCV particles in patients' sera was also demonstrated by immune precipitation of HCV particles containing viral RNA either with L-ficolin-anti L-ficolin GN5-coated beads or anti L-ficolin GN5-coated beads. This *ex vivo* interaction implies the presence of an accessible conformation of glycans with GlcNAc moieties on HCV particles circulating during infection and suggests that at least a fraction of circulating HCV may form complexes with L-ficolin *in vivo*.

6.2. L-ficolin Functions in HCV Infection

6.2.1. Neutralisation Activity

As an innate immune molecule that binds to HCV glycoproteins, it would be expected that L-ficolin has a beneficial effect in limiting infection in the early

stages before the development of specific adaptive immunity. Thus, to evaluate the potential clinical relevance of L-ficolin binding to HCV glycoproteins, the functional consequence of this binding interaction was addressed by the inhibition of infection in the HCV pseudoparticles system. Recombinant L-ficolin resulted in neutralisation of HCV pseudoparticle infectivity of two strains of HCV (H77c and UKN2B1.1), in a dose-dependent manner, and completely inhibited infection of UKN2B1.1 at a concentration of 6 µg/mL. This concentration is slightly above the average level of L-ficolin in clinical samples of healthy blood donors examined in this study (4.4 µg/mL) or in other studies which reported an average serum L-ficolin level of 3.7 µg/mL [300] and 4.13 µg/mL [217] in healthy populations. This is the first study to demonstrate the *in vitro* neutralisation capacity of L-ficolin in the HCV pseudoparticles system.

6.2.2. Role in Disease Progression

The neutralising effect of circulating L-ficolin might reduce virus load and diminish the pathogenesis of HCV infection. In addition, L-ficolin binding to HCV might activate the complement system, assisting in viral clearance through activation of serine proteases that activate the lectin complement pathway. L-ficolin levels were measured in HCV and non-HCV patient serum samples. Patients with mild HCV liver fibrosis had a significantly higher level of L-ficolin compared to patients with severe HCV liver fibrosis. However, the reduction of L-ficolin level in patients with severe HCV liver fibrosis could be a result of

impaired hepatocyte productivity of L-ficolin, rather than a causal ability to modify HCV disease outcome. Further investigations with increasing the numbers of HCV-infected patients and control groups would provide more clear conclusions.

It may be difficult to draw a conclusion on the role of serum L-ficolin levels in the context of HCV liver disease, as the L-ficolin levels in this study were examined in clinical samples from patients with chronic HCV infection. The median duration of infection at time of sample collection was 18 years, range from 4-37 years. With determination of a neutralisation effect of L-ficolin in the HCV pseudoparticle infectivity assay, the effect of L-ficolin in HCV liver disease could be more relevant in the early phases of infection. Evaluation of the role of L-ficolin in the early stages of HCV infection requires the availability of samples from HCV patients with newly-acquired infection, who subsequently cleared the virus. Simultaneous measurement of serum L-ficolin and viral load in serum samples of acutely-infected HCV patients may indicate a correlation and allow exploration of the implications of serum ficolin levels in the context of HCV disease. However, this will be difficult to achieve, as acute resolving HCV cases represent only 20% of newly infected patients and they are mostly asymptomatic and thus, undetected [18].

6.2.3. Role in Liver Fibrosis

The development of liver cirrhosis is the main complication of chronic liver disease in hepatitis C virus infected patients and is the gateway for the genesis of hepatocellular carcinoma. Despite the neutralising effect of L-ficolin that might result in controlling viral infection, it is possible that the binding of L-ficolin to HCV particles plays a role in liver fibrosis. Ficolins activate MASP-1, which has similar proteolytic activity to thrombin on fibrinogen and factor XIII [280-282]. Thus, it can stimulate the conversion of fibrinogen into fibrin and promote fibrosis in the liver. An association between MBL/MASP-1 enzymatic activity and the severity of fibrosis in HCV patients has been previously demonstrated [279]. Based on the functional similarity between L-ficolin and MBL in activating the lectin pathway, it is possible to assume that L-ficolin may contribute to the pathogenesis of HCV-induced liver fibrosis and progression of disease pathology. However, L-ficolin activity as an independent factor related to liver cirrhosis has not been examined in this study. This functional activity may augment the role of L-ficolin, as an innate immune mediator, in HCV liver disease to be effective not only in early stages (controlling circulating virus and neutralising infectivity), but also in late stages of HCV liver disease. Another potential pathway for development of fibrosis could be initiated by binding of L-ficolin to late apoptotic cells [301], in severe HCV infection where there is destruction of hepatocytes. The ficolin binding to hepatocytes and subsequent complement-coagulation cascade activation may result in the progression of liver fibrosis.

6.3. L-ficolin as a Biotherapeutic

The ability of L-ficolin to neutralise HCV infectivity may be beneficial in terms of therapeutic vaccine development. Administration of L-ficolin as an adjuvant to an HCV vaccine may also increase uptake and antigen presentation for the development of an effective adaptive immune response. However, as L-ficolin may have potentially antagonistic roles in HCV infection, the possible role for L-ficolin in antiviral therapy in the future management of HCV-infected patients must be cautiously considered. The possibility that L-ficolin may interact with MASP-1 and lead to an increase in activity makes L-ficolin therapy seem to be inappropriate during HCV infection.

6.4. Role of anti-E1E2 in HCV Liver Disease

The level of anti-E1E2 detected, using two different E1E2 constructs representing HCV genotypes 1 and 3, in HCV-infected patients was dependent on the genotype from which the E1E2 antigen preparation was derived. This result suggests that at least part of the antibody response is to genotype-specific antigens. Levels of anti-E1E2 against homologous antigens were higher in patients infected with genotype 3 (which is more associated with responsiveness to treatment) than genotype 1, which may impact on the differences between the genotypes in their clinical course and disease association. The level of anti E1E2 was inversely proportional to the viral load and the degree of liver fibrosis, which suggest a potential protective effect of anti-E1E2 against viral replication and disease progression.

6.5. Future Work

The functional activities of L-ficolin in controlling of a range of infectious diseases include opsonisation, neutralisation and complement activation. To date there have been no published studies on the interaction of L-ficolin with HCV. This study characterises the binding of L-ficolin to HCV glycoproteins. Neutralisation assay also showed that such binding is sufficient to prevent HCV pseudoparticles from infecting cells. It is an interesting field to be explored and it is quite clear that further studies are required to provide better understanding of the role of L-ficolin as an innate immune defence against HCV in HCV disease.

As L-ficolin has similar activity to MBL, the role of L-ficolin/MASPs complexes in the pathogenesis of liver cirrhosis through the complement and coagulation pathways could be further investigated. Studies to investigate the functional activation of MASPs on binding of L-ficolin to HCV glycoproteins are required. The L-ficolin associated functional activity could be examined by assays for L-ficolin/MASP-1 and MASP-2 activity upon binding to HCV glycoproteins. MASP-1 activity could be assayed using the Val-Pro-Arg-7-amino-4-methyl coumarin substrate. MASP-2 activity could be evaluated by testing of the ability of L-ficolin/EIE2 to cause C4 deposition.

HCV genotypes differ in their nucleotide sequences by 31–34%, and in their amino acid sequences by ~30% [47, 64]. Some differences between HCV

genotypes in sequence may affect the glycosylation pattern of E1E2, and thus the binding ability to L-ficolin. Therefore, the binding of L-ficolin to E1E2 heterodimers expressed from a representative panel of HCV genotypes could be tested.

As HCV glycoproteins provide a target for immune interaction by anti-E1E2 antibodies, determination of the specific patterns of glycans required for L-ficolin binding would assist in understanding the role of L-ficolin in binding to HCV. E1E2 mutants with different glycosylation patterns could be tested for a potential effect on binding activity. For blocking with antibodies and competition assays, E1E2 could be captured by ALP98, then detection of ficolin binding could be done with biotinylated GN5.

L-ficolin has functional polymorphic sites that regulate both expression as well as function. Single nucleotide polymorphisms (SNPs) in the promoter region of the L-ficolin gene are associated with changes in the serum concentration [235]. Alternatively, SNPs reported in the exon 8, encoding the fibrinogen-like domain, are associated with change in GlcNAc binding ability [267]. Investigation of these polymorphisms in the context of HCV liver disease could be performed to assess the possible relationship with HCV liver disease severity and progression.

A further study to investigate the profile of reactivity of a particular serum to different E1E2 preparations from different genotypes in native and denatured states is required to determine the cross reactivity and epitope specificity of antibody response during chronic HCV infection. This would provide a better understanding of the regions of the HCV envelope glycoproteins recognized by antibodies.

7. Appendix

7.1. Publications

7.1.1. J Viral Hepatitis, 2008. vol 15 (5): p339-45.

Hamed MR, Tarr AW, McClure CP, Ball JK, Hickling TP, Irving WL.

“Association of antibodies to hepatitis C virus glycoproteins 1 and 2 (anti-E1E2) with HCV disease”.

Hepatitis C virus (HCV) causes acute and chronic liver diseases in humans. Its two envelope glycoproteins, E1 and E2, provide a target for host immune recognition. To study the role of anti-HCV E1 and E2 (anti-E1E2) in HCV disease, the correlation between antibody level and viral load, genotype, disease severity and response to treatment was investigated. The levels of antibodies to HCV glycoproteins E1 and E2 antibodies were evaluated in 230 sera of patients with chronic hepatitis C by enzyme-linked immunosorbent assay. The antigens used were recombinant HCV glycoproteins derived from genotype 1 (H77c) and genotype 3 (UKN3A1.28). Seroreactivity was greater when sera were tested against antigen derived from their homologous genotype than against heterologous antigen. Reactivity against UKN3A1.28 in sera from patients infected with genotype 3 was significantly higher than corresponding reactivity between patients infected with genotype 1 and H77c. The seroreactivity was inversely proportional to the viral load and to the degree of liver fibrosis. The pre-treatment level of anti-E1E2 was higher in sustained responders to combination therapy. These results demonstrate that seroreactivity against E1E2 depends upon the genotypic origin of the E1E2 antigens and the infecting genotype, and suggest a possible protective effect of anti-E1E2 against disease progression.

7.1.2. PLoS Pathogens, Submitted July 2009.

Mohamed R. Hamed, Richard J. P. Brown, Anders Krarup, C. Patrick McClure Robert B. Sim, Jonathan K Ball, William L. Irving, Alexander W. Tarr & Timothy P. Hickling

“The Human Soluble Lectin L-Ficolin Neutralises Entry of Hepatitis C Virus by Binding to the E1 and E2 Surface Glycoproteins”

Liver ficolin (L-ficolin) is a soluble pattern recognition molecule of importance in innate immune defence against microorganisms. Upon binding to specific pathogen-associated carbohydrates, L-ficolin activates the lectin complement pathway, resulting in opsonisation or lysis of pathogens. We hypothesised that L-ficolin might play a role in host defence against Hepatitis C Virus (HCV). In this study, both recombinant N-terminal FLAG-tagged L-ficolin and L-ficolin purified from serum were investigated for binding to the envelope glycoproteins E1 and E2 of HCV. Specific, dose-dependent binding of L-ficolin to HCV glycoproteins E1 and E2 was observed. This interaction was inhibited by soluble ligands for L-ficolin. The interaction between L-ficolin and HCV particles in infected sera was also demonstrated. Interaction between L-ficolin and HCV glycoproteins E1 and E2 potentially neutralised entry of retroviral pseudoparticles bearing these glycoproteins. This is the first description of direct neutralisation of HCV entry by a soluble innate immune molecule. These results implicate L-ficolin as a previously undescribed element of the immune defence against HCV infection.

7.2. Presentations and Posters

7.2.1. Presentations

7.2.1.1. 160th Society for General Microbiology Meeting

University of Manchester, UK, 26-29 March 2007.

“Role of Antibodies to HCV Glycoproteins E1E2 in HCV Liver Disease”.

7.2.1.2. 5th UK Meeting on Biology and Pathology of HCV

Rydal Hall, Ambelside, UK, 27-29 April 2007.

“Role of Human Serum L-Ficolin in HCV Disease”

7.2.1.3. 162th Society for General Microbiology Meeting

Edinburgh, Scotland, UK, 31 March - 3 April 2008.

“L-Ficolin/HCV Binding Interaction in HCV Disease”

7.2.1.4. Institute of Clinical Research Annual Meeting

University of Nottingham, UK, 10 June 2008.

“Association of Antibodies to Hepatitis C Virus Glycoproteins 1 and 2 (anti-E1E2) with HCV Disease”.

7.2.2. Posters

7.2.2.1. 14th International Symposium on HCV and Related Viruses

Glasgow, Scotland, UK, 9-13 September 2007.

“Role of Human Serum L-ficolin in HCV Disease”.

7.2.2.2. 15th International Symposium on HCV and Related Viruses

San Antonio, Texas, USA, 5-9 October 2008.

“The Role of Soluble Lectin L-ficolin in HCV Immune Recognition”

7.2.2.3. 44th Annual Meeting of the European Association for the Study of the Liver

Copenhagen, Denmark, 22-26 April 2009.

“Role of Soluble Lectin L-ficolin in HCV Immune Recognition in Hepatitis C Virus Infection”.

7.3. Prizes

7.3.1. 1st Runner Up, Best Poster Prize

Second Postgraduates Research Day, School of Molecular Medical Sciences, University of Nottingham, 25 June 2007.

“Humoral Immune Responses to HCV: Role of Anti-E1E2 and L-ficolin”.

7.3.2. Best Poster Prize (Peer Review)

Graduate School Poster Competition, University of Nottingham, 7 May 2008.

“A Potential Target for Vaccine Production against Hepatitis C Virus”.

7.3.3. European Association for the Study of the Liver Young Investigator Bursary Award.

Copenhagen, Denmark, 22-26 April 2009.

“Role of Soluble Lectin L-ficolin in HCV Immune Recognition in Hepatitis C Virus Infection”.

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