The Effects of the Human Innate Pattern Recognition Receptor L-Ficolin and Its Variants against Hepatitis C Virus Infection

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Student Declaration:

I declare that the data presented is entirely my work, except where otherwise stated.

Abstract

Enveloped virus infections have major impacts to global health and economy. For example, Hepatitis C virus is a leading cause of chronic hepatic disease, and the 2013 Ebola virus outbreak highlighted the need for broadly-active treatments. The immune factors behind successful control of these infections are poorly understood. A potential common target for intervention is the glycosylation patterns that decorate the surface proteins of enveloped virions.

L-ficolin is a liver-expressed lectin that binds several diverse enveloped viruses via their glycosylated surface envelope proteins, and contributes to the innate immune response through blocking of viral entry and complement activation. Two mutations, Thr236Met and Ala258Ser, in L-ficolin are maintained in human populations at high frequencies and influence ligand binding, serum L-ficolin concentration, and response to microbial infection.

In this study, expression of recombinant His_6-tagged L-ficolin was optimised. It was hypothesised that L-ficolin mutants described above have superior ligand binding and antiviral properties. The T236M mutation conferred increased binding of acetylated BSA. In an associated study, L-ficolin was used as an oligomeric scaffold for presenting anti-HCV nanobodies. This novel chimaeric molecule, named ficobody, exhibited superior ligand- and HCV glycoprotein-binding activity when compared to L-ficolin. This is the first instance in which lectins have been used as a scaffold for protein presentation.

Furthermore, correlations between serum L-ficolin concentrations and HCV infection outcome and ethnicity were investigated, finding that chronic HCV patients had significantly lower L-ficolin concentrations. Finally, mutant viruses were used to explore if specific glycosylation sites on the HCV glycoproteins are essential for L-ficolin interaction, producing data that supported previous findings on the roles of specific glycosylation sites in HCV entry.

Understanding the antiviral significance of L-ficolin variants could inform the use of L-ficolin and ficobody in the prognosis, prophylaxis and treatment of a wide variety of enveloped virus infections.

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Abbreviations

AcBSA	Acetylated bovine serum albumin
ALT	Alanine aminotransferase
Amp^r	Ampicillin resistant
AP	Alkaline phosphatase
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
Ca^{2+}	Calcium (II) ions
$CaCl_2$	Calcium chloride
Cat.	Catalogue number
CD81	Cluster of differentiation 81
CIDEB	Cell death-inducing DFFA-like effector B
CLD	Collagen-like domain
CLDN1	Claudin-1
CRP	C-reactive protein
C-terminal	Carboxy-terminal
CysNAc	*N*-acetylcysteine
DAA	Direct-acting Antiviral
DAPI	4',6-Diamidino-2-phenylindole, dilactate
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
E. coli	*Escherichia coli*
EBOV	Ebola virus
EBOVpp	Ebola virus pseudoparticles
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor

ELISA	Enzyme-linked immunosorbent assay
EphA2	Ephrin receptor A2
ER	Endoplasmic reticulum
FBG	Fibrinogen-like
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GAG	Heparin sulphate proteoglycan-associated glycosaminoglycan
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
GNA	*Galanthus nivalis* agglutinin
h	Hours
HBV	Hepatitis B virus
HCV	Hepatitis C Virus
HCVcc	Hepatitis C virus cell culture
HCVpp	Hepatitis C virus pseudoparticles
HEK	Human embryonic kidney
HIV-1	Human Immunodeficiency virus
HRP	Horse radish peroxidase
Huh	Human hepatoblastoma
HVR	Hypervariable region
IAV	Influenza A virus
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulins
LB	Lysogeny broth
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
L-SIGN	Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
MAC	Membrane attack complex
MASP	Mannose-binding lectin-associated serine protease

MBL	Mannose-binding lectin
MHC	Major histocompatibility complex
Midiprep	Midipreparation
min	Minute
Miniprep	Minipreparation
MLD	Mucin-like domain
MW	Molecular weight
MWCO	Molecular weight cut off
nAb	Neutralising antibodies
NaCl	Sodium chloride
NEAA	Non-essential amino acids
NEB	New England Biolabs
nIgG	Natural immunoglobulin G
NPC1L1	Niemann-Pick C1-like 1
N-terminal	Amino-terminal
NXS/T	Asparagine-X-Serine/Threonine
OCLN	Occludin
Opti-MEM	Opti-MEM reduced serum medium
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PBST	PBS containing 0.05% Tween 20
PBSTM	PBS containing 0.05% Tween 20 and 5% non-fat milk
PEI	Polyethylenimine
Pen-Strep	Penicillin-streptomycin
pNPP	*p*-Nitrophenyl phosphate
PRR	Pattern recognition receptor
PTX3	Pentraxin 3
RCL	Recombinant chimaeric lectin
RdRP	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
rpm	Revolutions per minute

RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sGP	Soluble GP
SLS	Scientific Lab Supplies Ltd.
SR-BI	Scavenger receptor class B member 1
ssGP	Small soluble GP
SUMO	Small ubiquitin-like modifier
SVR	Sustained virological response
TAE	Tris acetate-EDTA
TBS	Tris-buffered saline
TBST-Ca	Tris-buffered saline containing 0.05% Tween 20 and 5 mM $CaCl_2$
TfR1	Transferrin receptor 1
UTR	Untranslated region
UV	Ultraviolet
V	Volts
VLDL	Very low-density lipoprotein
VRG	Virus Research Group
VSV	Vesicular stomatitis virus
VWR	VWR International
WGA	Wheat germ agglutinin
Zn^{2+}	Zinc
4PL	Four parameter logistic

Chapter 1 Introduction

Enveloped RNA viruses are responsible for several devastating human diseases worldwide, including human immunodeficiency virus (HIV-1), hepatitis C virus (HCV) and Ebola virus (EBOV), each with a significant impact on health and economy.

Some viruses cause acute infections characterised by rapid onset of disease, a short period of symptoms, followed by resolution of the infection or deterioration of the host. In the case of EBOV, symptoms are generally severe with high case fatality rates. Other viruses, such as HCV, can cause both acute and chronic infections. Acute HCV infection is characterised by a generally asymptomatic elevation of HCV ribonucleic acid (RNA) and alanine amino transferase (ALT) levels and an up-regulation of interferon-stimulated genes, before an HCV-specific antibody response results in viral clearance or the development of a chronic infection (Shin et al., 2016). Chronic HCV infection is characterised by fluctuating HCV RNA and ALT levels, and the establishment of an equilibrium between the down-regulated host immune response and viral persistence with limited cytopathology (Virgin et al., 2009).

While there are several differences between acute and chronic enveloped virus infections, a common factor between HCV and EBOV is the level of glycosylation of the virion glycoproteins. As such, a treatment which could fight infection by both if not several other viruses may be plausible.

1.1 Hepatitis C Virus

In 1989, the hepatitis C virus of the *Hepacivirus* genus and *Flaviviridae* family, was discovered (Choo et al., 1989). HCV is a hepatotropic, spherical virus and is a significant health problem globally, representing a leading cause of hepatitis disease (Catanese et al., 2013; WHO, 2016b). HCV currently infects at least 115 million individuals worldwide, with up to 80 million chronically infected, causing the deaths of 350,000 people a year (Hanafiah et al., 2013; WHO, 2016b). Transmission occurs through several routes, such as medicine- and drug-related injection, sexual transmission and mother-to-child transmission (WHO, 2016b).

While 15 – 45% of those infected will spontaneously clear the infection, in the majority of patients – some 55 – 85% – the virus evades the immune system and establishes a chronic infection after six months (WHO, 2016b). Chronic HCV infection is typically followed by fibrosis, and increases the risk of liver diseases such as cirrhosis and hepatocellular carcinoma (WHO, 2016b). Such disease is not a direct result of the HCV virus, but more likely a result of changes made to the host immune system and metabolism (Grassi et al., 2016). Furthermore, co-infection with HIV-1, hepatitis B virus (HBV) and tuberculosis also occurs (WHO, 2016b). For those who spontaneously clear infection, persistent re-infection is still possible (Burke and Cox, 2010).

1.1.1 Classification of HCV

As a result of the virus' high degree of genetic variability, HCV is categorised into six genotypes with several sub-genotypes, with the prevalence of each varying between geographic locations and ethnicities (WHO, 2016b) (Table 1.1). Genotype classification limits inter-genotypic variation to 31 – 33% of the RNA sequence, with further sub-genotypic categorisation being defined by 20 – 25% nucleotide variation (Simmonds, 2004). Genotype 1 infections are the most common worldwide, with highest prevalence in the Americas, West Africa, Europe, Eurasia, South East Asia and Australia (Figure 1.1). Genotype 3, the second most prevalent genotype worldwide, is most predominant in South Asia and the Middle East, while genotype 4 is particularly widespread in North and Central Africa (WHO, 2016b).

Table 1.1 – Global Prevalence of HCV Genotypes

Percentages of prevalence of each HCV genotype, as determined by Messina et al. (2015).

Hepatitis C Virus Genotype	Estimated Global Prevalence/ % of Cases
Genotype 1	46.2
Genotype 2	9.1
Genotype 3	30.1
Genotype 4	8.3
Genotype 5	0.9
Genotype 6	5.4

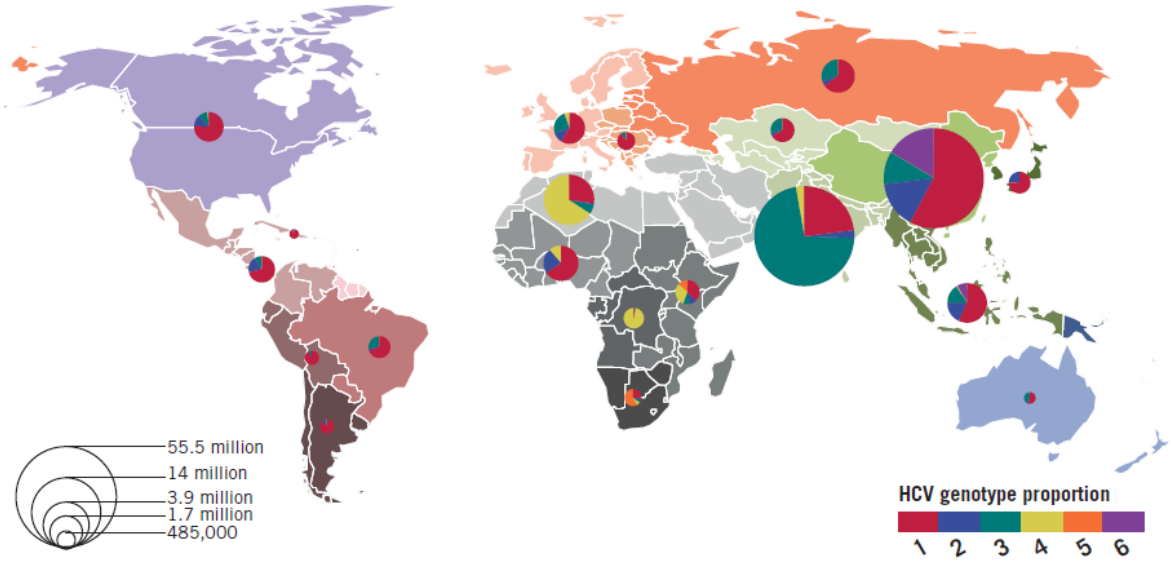

Figure 1.1 – Global Distribution of HCV Genotypes

The prevalence of each HCV genotype varies with geographical location and ethnicity. Reproduced from WHO (2016b).

1.1.2 The HCV Genome

The positive-sense, single-stranded RNA genome of HCV comprises approximately 9600 nucleotides, encoding one continuous open reading frame flanked by a 5'-untranslated region (UTR) – a structured RNA element containing an internal ribosomal entry site which enables cap-independent translation using host ribosomal machinery – and a 3'-UTR (Pavio and Lai, 2003). Secondary structures in the RNA genome, even within protein-coding regions, have several functions, such as genome packaging and regulation of replication and infectivity (Lindenbach et al., 2007; Pirakitikulr et al., 2016; Stewart et al., 2016). The translated product of the HCV genome is approximately 3011 amino acids in length, and is processed by both host and virus proteases to form three structural and six non-structural proteins, as well as p7 (Lindenbach et al., 2007; Pavio and Lai, 2003) (Figure 1.2).

The C gene encodes capsid (Lindenbach et al., 2007). The E1 and E2 proteins are glycoproteins. E1 is believed to have roles in membrane fusion, RNA incorporation into the nucleocapsid, and modulation of E1E2 interaction with certain receptors (Haddad et al., 2017; Li and Modis, 2014). E2 is involved in receptor binding (Miao et al., 2017). The p7 protein is a transmembrane ion channel activated by low pH with an unclear function – hypotheses range from involvement in cytopathy and evasion of the antiviral IFN response to a role in membrane envelopment of nucleocapsid during virion production (Farag et al., 2017; Gentzsch et al., 2013; Qi et al., 2017).

The non-structural protein NS2 is a membrane-bound autoprotease required to cleave the NS2-NS3 junction in the HCV polyprotein, with additional roles in virus assembly (Lindenbach et al., 2007; Popescu et al., 2011). The amino (N)-terminal and carboxy (C)-terminal domains of the NS3 protein have serine protease and RNA helicase/NTPase activities, respectively, both of which are important in RNA replication. While the serine protease has a role in polyprotein cleavage, the purpose of the NTPase-powered RNA helicase is less well-understood (Lindenbach et al., 2007). It may produce negative-strand RNA intermediates and positive-strand RNA genomes (Gu and Rice, 2016). Further NS3 activities in the initiation of fibrosis have also been investigated (Khanizadeh et al., 2016). NS4A acts as a cofactor for zinc (Zn^{2+})-dependent NS3 activity, and anchors the NS3-NS4A complex to cell membranes (Lindenbach et al., 2007). NS4B is a membrane-bound protein with a role in membranous web formation, where replication occurs (Lindenbach et al., 2007). Like p7, the NS5A phosphoprotein has an unknown role that is essential for HCV replication, though roles in the regulation of RNA replication and host cellular signalling, resistance to the host IFN immune response and virion assembly have been suggested (Tran et al., 2015). NS5B is an endoplasmic reticulum (ER) membrane-associated RNA-dependent RNA polymerase (RdRP) which lacks proof-reading ability, with a direct role in replicating the RNA genome in the membranous web (Lindenbach et al., 2007).

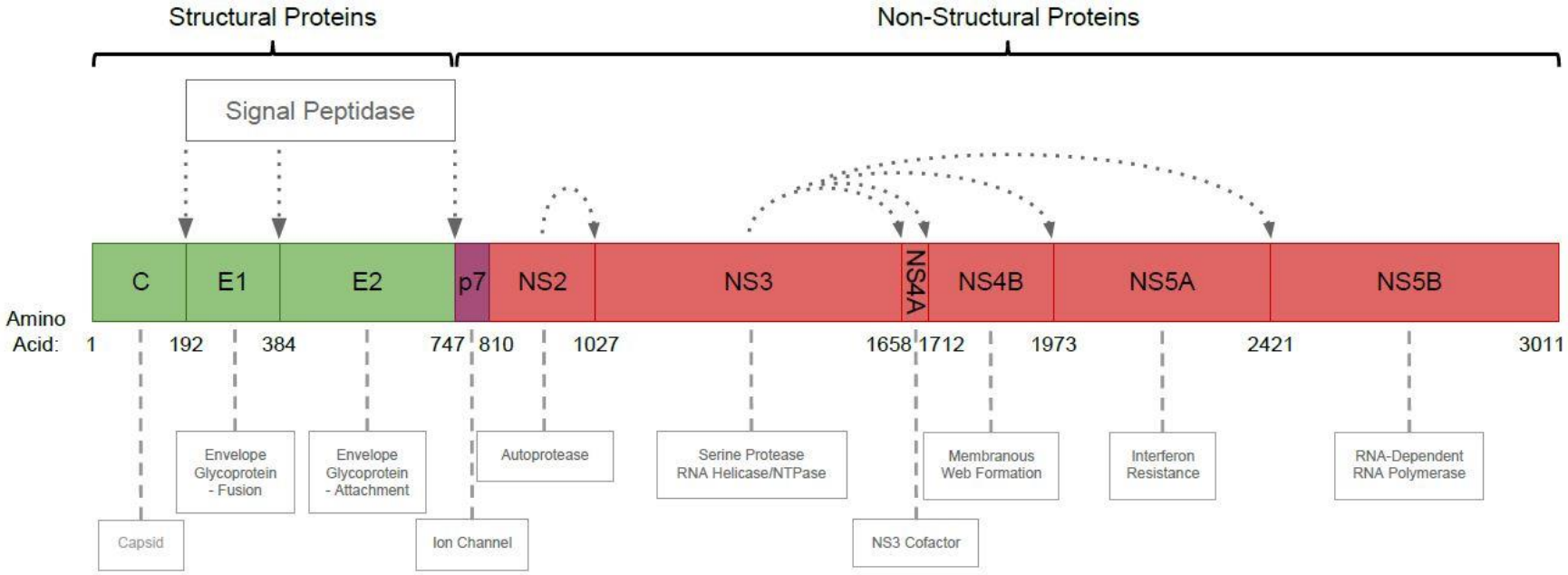

Figure 1.2 – Structure of the HCV Polyprotein and Its Functions

The three structural proteins and seven non-structural proteins encoded by the HCV genome. The starting residue number of each protein is detailed, in addition to their main functions. Dotted arrows indicate cleavage by protease activity.

The lack of RdRP proof-reading is the main cause of HCV hypervariability and the existence of several genotypes, in addition to selection driven by the host immune response (Timm and Roggendorf, 2007). Lastly, ARFP is expressed in an alternate reading frame protein and has an unknown function, possibly immune evasion (Lindenbach et al., 2007).

1.1.3 HCV Interaction with the Human Immune System

Briefly, following detection of HCV pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), innate immune limitation of early HCV infection is mediated by natural killer cells and natural killer T cells through infected cell lysis and interferon (IFN) response. Adaptive immune limitation is achieved by humoral B cell-derived, antibody-mediated and cellular T cell-derived responses via macrophages (Ashfaq et al., 2011a). $CD4^+$ T cells are well known to play a role in limiting the establishment of HCV infection, however such an immune response is not sufficient to prevent chronic infection in many cases, and T cell responses – even to new HCV infections – are dampened over time by chronic infection (Burke and Cox, 2010).

While not fully understood, the ability of HCV to evade an immune response is achieved through several viral mechanisms. For example, mitochondrial quality control is induced in order to attenuate apoptosis and allow persistent, non-cytopathic HCV infection (Kim et al., 2014). Other mechanisms include a high level of virion production at up to 10^{12} virions a day, the low fidelity RdRP allowing rapid mutation – particularly in T cell epitopes – and quasispecies formation, down-regulation of IFN response and cellular signalling, impaired engagement of T cell receptor with antigen-presenting major histocompatibility complex (MHC) and up-regulation of PD-1 on $CD8^+$ T cell (Ashfaq et al., 2011a; Burke and Cox, 2010).

1.1.4 The Life Cycle of HCV

The HCV life cycle (Figure 1.3) begins with virion interaction with host cell receptors via E1E2 to gain entry into the cell via endocytosis (Kim and Chang, 2013). This is followed by fusion and uncoating, then translation and post-translational modification of the RNA genome in the rough ER (Kim and Chang, 2013). The resulting polyprotein is cleaved into the 10 HCV proteins, which perform their functions (Grassi et al., 2016). The sites of RNA replication and capsid formation are separate, at the membranous web and cytosolic lipid droplets, respectively (Grassi et al., 2016). In the ER, replicated RNA genomes associate with the capsid, which multimerises to form the nucleocapsid (Grassi et al., 2016). Correctly-folded transmembrane E1E2 proteins associate and anchor into the ER membrane (Lindenbach et al., 2007). The nucleocapsid migrates to the ER lumen and is enveloped by E1E2-studded ER membrane (Grassi et al., 2016; Lindenbach et al., 2007). Budding occurs followed by release of the virion from the cell via Golgi-mediated exocytosis and likely the VLDL secretory pathway (Gastaminza et al., 2006; Grassi et al., 2016; Kim and Chang, 2013; Lindenbach et al., 2007; Syed et al., 2017).

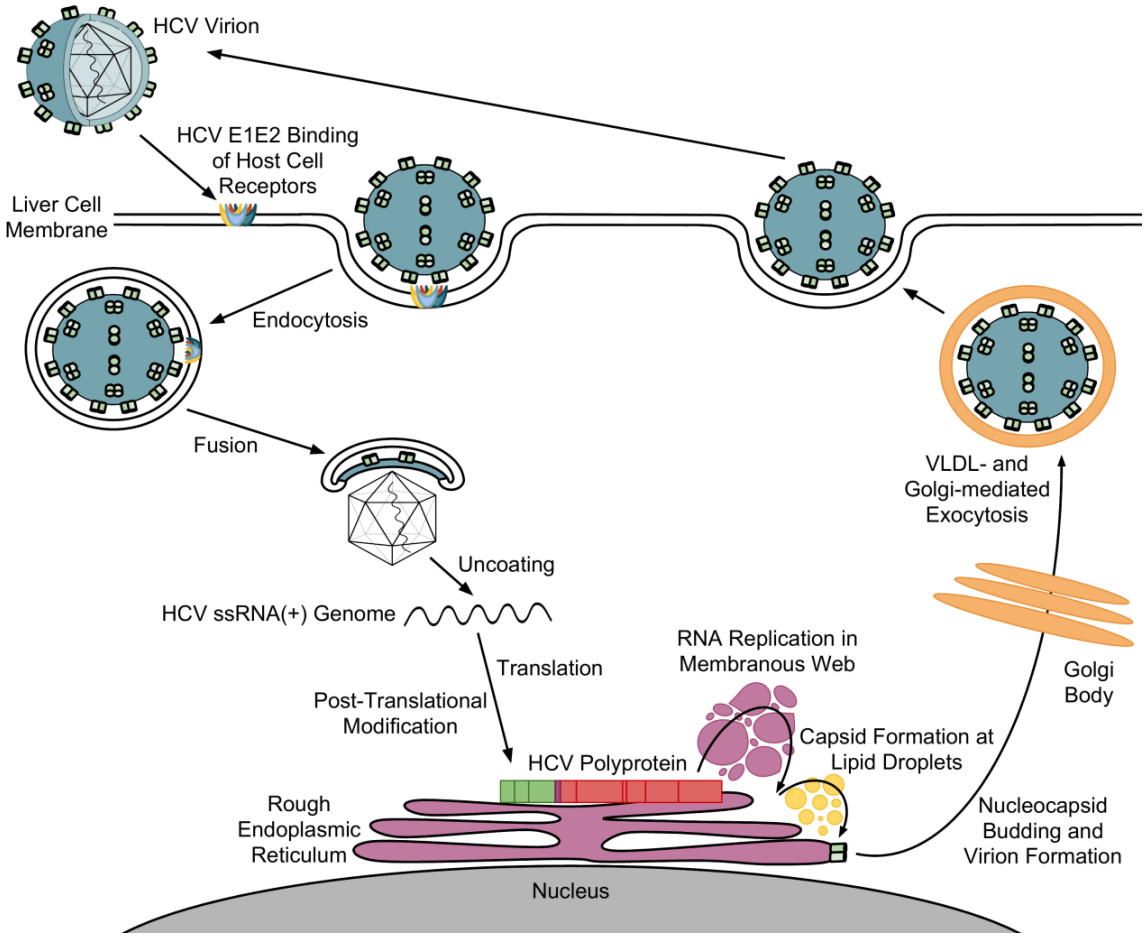

Figure 1.3 – Simplified Schematic of the HCV Life Cycle

Sequential interaction of the HCV E1E2 glycoproteins to several specific liver cell receptors enables virion endocytosis, fusion and uncoating. Next, the single stranded (ss)RNA genome is translated into proteins and matured by host factors in the endoplasmic reticulum. The HCV genome is replicated in the HCV-induced membranous web and packaged into the nucleocapsid in the ER, before budding at E1E2-studded ER membrane. Exocytosis occurs via lipoprotein- and Golgi body-associated pathways.

1.1.4.1 The Significance of Lipoproteins in the Life Cycle of HCV

In the context of human metabolism, lipoproteins are responsible for the transport of lipids via the circulatory system (Lindenbach and Rice, 2013). When associated with an HCV virion, lipoproteins such as low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) bind cell receptors, thus facilitating initial viral interaction with host receptors (Ding et al., 2014;

Grassi et al., 2016). LDL and VLDL bind HCV via lipoprotein components, for example apoproteins apoA-I, apoB-48, apoB-100, apoC-I and apoE. Intracellular low-density lipoprotein receptor (LDLR) and apoA-I were found to be important for optimal replication, while apoB and apoE are believed to be involved in the production of HCV virions (Albecka et al., 2012; Grassi et al., 2016). Lipoproteins may also aid viral immune evasion by masking epitopes (Grassi et al., 2016). It has recently been suggested that lipo-viro-particles form in the ER following translation (Syed et al., 2017).

1.1.5 The E1 and E2 Glycoproteins of Hepatitis C Virus and Their Roles in Viral Entry

The HCV glycoproteins, E1 and E2, non-covalently heterodimerise to form functional E1E2 embedded in the viral envelope (De Beeck et al., 2001; Goffard et al., 2005). They require co-expression for correct folding of E1 and enhanced glycosylation (Dubuisson et al., 2000; Patel et al., 2001). Several neutralising antibody (nAb) epitopes have been found on the E2 glycoprotein (Fafi-Kremer et al., 2012; Tarr et al., 2006).

E1E2 is involved in the interaction of several essential and non-essential host cell receptors in order to gain entry into the cell, as reviewed extensively in Miao et al. (2017). The receptor list includes heparin sulphate proteoglycan-associated glycosaminoglycans (GAGs), occludin (OCLN), claudin-1 (CLDN1), LDLR, scavenger receptor class B member 1 (SR-BI), epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2), Niemann-Pick C1-like 1 (NPC1L1), cell death-inducing DFFA-like effector B (CIDEB), transferrin receptor 1 (TfR1) and cluster of differentiation 81 (CD81), in addition to the possible candidates liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and mothers against decapentaplegic homolog 6 and 7 (SMAD6/7) (Ding et al., 2014; Miao et al., 2017). E2 binding to host CD81 is essential for HCV entry and is a common target of broadly neutralising nAb (Feneant et al., 2014).

Relative spatial distribution of these receptors and the order of interaction with the virus are significant for successful HCV infection. This serves to – at least partially – bias the cell tropism of the HCV virus to hepatocytes, with CD81 and OCLN being the base receptors essential for entry (Ding et al., 2014).

Ding et al. (2014) and Miao et al. (2017) describe the proposed three-step mechanism of entry of lipoprotein-associated HCV virions. Firstly, the GAG, LDLR and SR-BI receptors mediate first contact, causing the lipoprotein to dissociate and inducing E2 glycoprotein conformational changes, which binds CD81. Secondly, EGFR-mediated signalling induces the GTPase HRas to complex HCV-CD81 with CLDN1, resulting in clathrin-mediated endocytosis. TfR1 and OCLN also play unknown roles. Finally, CD81-binding and low pH conditions enable fusion of the virus envelope and endosomal membrane, thus releasing the HCV genome into the cytoplasm. In terms of cell-to-cell transmission of HCV, lipoproteins and the TfR1 receptor are not essential, however several of the aforementioned receptors including EphA2 and NPC1L1 are involved (Ding et al., 2014).

1.1.5.1 The Structure of the E2 Glycoprotein

E2 has a globular fold consisting of an N-terminal ectodomain, a core comprising two near-perpendicular β-sheets with 2 short α-helices, and a C-terminal transmembrane helix which anchors the structure and allows heterodimerisation (De Beeck et al., 2001; Khan et al., 2014). E2 possesses up to three hypervariable (HVR) regions which are prone to high levels of mutation, in particular HVR1, and have roles in infectivity and the evasion of nAb targeting by mutation and epitope masking (Bankwitz et al., 2010; Edwards et al., 2012).

1.1.5.2 N-*Linked Glycosylation of the E1 and E2 Glycoproteins*

Once cleaved from the HCV polyprotein by signal peptidases, the E1E2 proteins are post-translationally modified by *N*-linked glycosylation in the rough ER lumen (Goffard et al., 2005; Kim and Chang, 2013). Here, microheterogeneous and largely high-mannose (Iacob et al., 2008) *N*-linked

oligosaccharides are bound at specific asparagine-X-serine/threonine (NXS/T) consensus sequences in the glycoprotein (Goffard and Dubuisson, 2003) (Figure 1.4). E1 possesses 5 such sites, whereas E2 has 11, however the E2N5 glycosylation site is only present in 75% of HCV sub-genotypes (Goffard and Dubuisson, 2003). The residue in place of X at the NXS/T motif can influence the level of glycosylation and thus glycan function (Kasturi et al., 1995; Shakin-Eshleman et al., 1996). The *N*-linked glycans of E1 and E2 occupy approximately 50% of the heterodimeric molecular weight (MW) (Kong et al., 2013).

Specific *N*-linked glycans have early roles in protein folding through interaction with the calnexin-calreticulin chaperone system, quality control and virion assembly and secretion, however when embedded in the envelope they influence heterodimerisation, virus entry and fusion, and immune modulation (Goffard et al., 2005; Iacob et al., 2008).

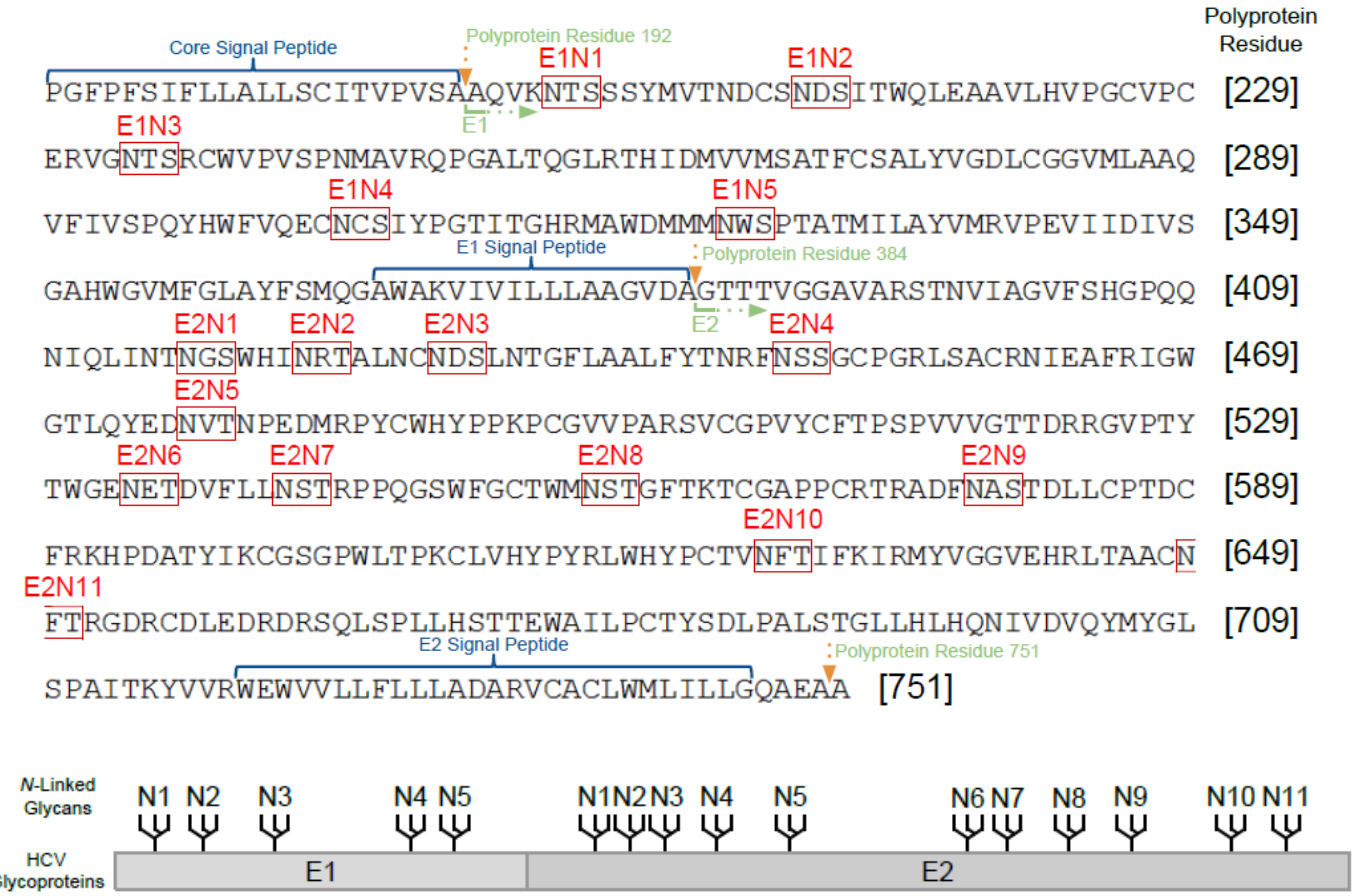

Figure 1.4 – The Locations of the Glycosylation Sites of the HCV E1E2 Glycoproteins

The sequence of the genotype 2a JFH-1 strain of HCV was used as an example in this figure. The N*-linked glycosylation sites – NXS/T – are highlighted in red, and the C-terminal signal peptides of E1 and E2 in blue. These peptides direct the proteins to the ER lumen and anchor the proteins to the ER membrane. Orange arrows indicate the cleavage sites. The first amino acid residues of the glycoproteins are highlighted by green arrows.*

1.1.6 Current Treatment of HCV Infection

For several years, ribavirin and pegylated IFN-α were the standard treatment regime via subcutaneous injection (WHO, 2016b). However, responses to treatment varied with each individual and virus genotype. It is estimated that 40 – 65% of patients who received this regime remained virus-free six months are treatment ended, known as a sustained virological response (SVR) (WHO, 2016b). Furthermore, severe adverse effects were often associated with treatment.

The discovery of second generation direct-acting antivirals (DAAs) began a new era of higher and faster success in HCV treatment via a less invasive, oral means of administration (WHO, 2016b). Examples of DAAs include the NS5A-inhibitors ledipasvir, daclatasvir, ombitasvir and velpatasvir (Foster et al., 2015; Kowdley et al., 2014; Poordad et al., 2014; Sulkowski et al., 2014), the NS5B-inhibitors sofosbuvir and dasabuvir (Kowdley et al., 2014; Lawitz et al., 2013; Zeuzem et al., 2014) and the NS3/NSA4A-inhibitors grazoprevir, asunaprevir and paritaprevir (Chayama et al., 2015; Kanda et al., 2016; Lawitz et al., 2015; Tarao et al., 2017; Wedemeyer et al., 2017).

The majority of these drugs achieved patient SVR rates exceeding 90%, often after 12 weeks, with less severe and less common adverse side effects than with IFN treatment. Even patients with cirrhosis, with whom successful treatment has historically been more difficult, have benefited with high rates of SVR (Bourliere et al., 2015).

To prevent advanced or fatal liver disease, HCV patients often undergo liver transplantation surgery, however the incidence of HCV re-infection of the liver is high, at 90% of cases (Chan and Rosen, 2003). The use of DAAs has provided an improved scenario for those awaiting or who have received a liver transplant, however more time and research is needed to determine their effect on long-term liver disease such as fibrosis and HCC (Baumert et al., 2017; Ponziani et al., 2017; Righi et al., 2015).

While some DAAs display genotype specificity, some such as sofosbuvir and velpatasvir have shown pan-genotypic success. DAA success rates can be enhanced when used in tandem with ribavirin (Afdhal et al., 2014; Curry et al., 2015), which in turn improves in safety (Feld et al., 2017), and even further enhanced when used in combination with other DAAs (Foster et al., 2015).

1.1.6.1 Current Issues in the Treatment of HCV Infection

Although several successful HCV treatments exist, spread of the virus and disease continues due to several factors, particularly in areas of the world with poorer socio-economic climates. These include insufficient education and healthcare systems, unsafe medical procedures, high drug costs and the lack of access to treatment in developing countries (Gupta et al., 2017; Thomas, 2013; WHO, 2016b). Moreover, many individuals are unaware of their infected status due to the asymptomatic nature of early infections (WHO, 2016b). These factors and HCV re-infection mean that DAAs are by no means a 'cure'. Furthermore, HCV variants resistant to DAA activity have been observed – particularly for NS5A inhibitors – at a proposed baseline of 15% of the HCV variant population within a patient (Jimenez-Perez et al., 2016; Pawlotsky, 2016; Raj et al., 2017). Some HCV mutations responsible for resistance have been proposed.

A prophylactic vaccine able to induce pan-genotypic HCV-specific T cell responses as well as cross-reactive nAb production to prevent initial HCV infection would control transmission more effectively, but at present has remained elusive (Torresi et al., 2011; WHO, 2016b). This is due to the high genetic variability of HCV, which complicates the discovery of a target conserved across HCV genotypes and serotypes, and the virus' ability to evade immune activities (Naderi et al., 2014; Torresi et al., 2011).

While the E2 glycoprotein is the most well-established natural target of both nAbs and lectins, it is also the antigen which displays the most genetic and post-translational variation (Kong et al., 2013). Besides traditional approaches, other vaccines have been explored with limited success, such as vaccines using a peptide able to trigger MHC-mediated T cell responses,

recombinant proteins, virus-like particles and DNA to compensate for HCV-mediated immune dampening (Abdelwahab and Ahmed Said, 2016; Naderi et al., 2014). Treatments and vaccines investigating the blocking of viral entry have also been investigated (Torresi et al., 2011).

1.1.7 Current Research Models of HCV Infection

No efficient natural animal models exist to facilitate HCV research (Vercauteren et al., 2015). Chimpanzees have been used previously due to their similarity to humans in response to infection, however their use poses financial and ethical problems (Edwards et al., 2012). Chimaeric immunodeficient uPA/SCID mouse models with humanised livers can be infected with HCV, and have proven useful for study of the virus life cycle and drug efficacy (Mercer et al., 2001; Tesfaye et al., 2013). This rodent animal model has many disadvantages, such as rejection of transgenes and the development of kidney disorders, however genetic improvements are being made to combat these issues (Tateno et al., 2015).

The non-structural and p7 genes of the HCV genome are critical for HCV replication (Lindenbach et al., 2007). The advent of replicons – selectable HCV genomes consisting of non-structural genes transfected into human hepatoma Huh7 cells – allowed the investigation of such proteins and RNA replication intracellularly, however infectious particles were not formed and the full life cycle could not be studied using this system (Lohmann et al., 1999). For the *in vitro* study of E2, soluble, recombinant, truncated and full-length E2 constructs have been produced in eukaryotic cells (Edwards et al., 2012; Flint et al., 2000; Pileri et al., 1998).

To better understand their role in viral entry and attachment, infectious HCV pseudoparticle (HCVpp) models – which can infect but not replicate – have been optimised in which the envelope glycoproteins are expressed on retroviral or lentiviral core particles (Bartosch et al., 2003) (Figure 1.5). While not all HCV glycoproteins are able to form infectious pseudoparticles, a chimaeric JFH-1 based cassette – to which E1E2 glycoproteins can be cloned – has been developed to enhance entry (McClure et al., 2016).

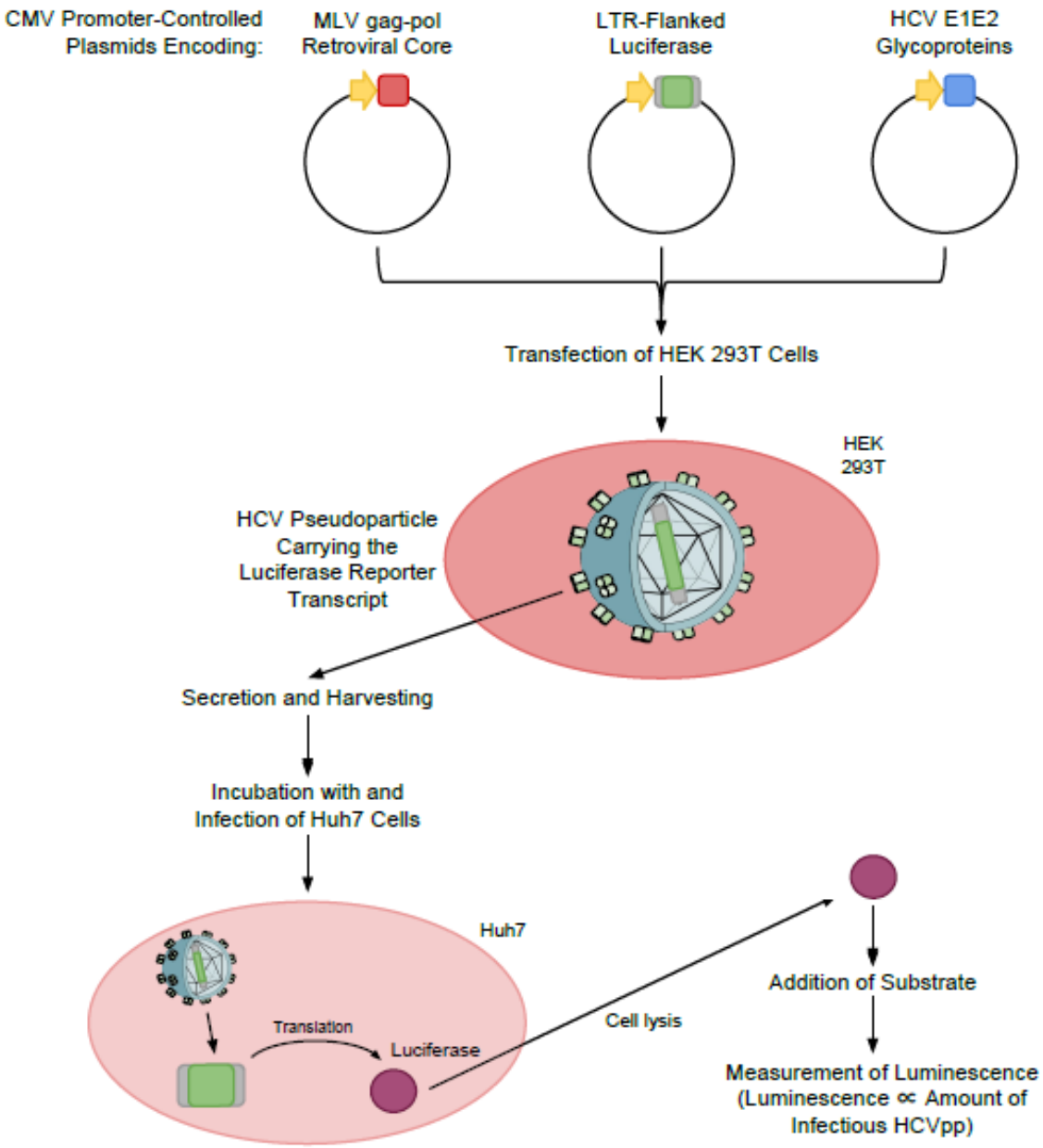

Figure 1.5 – The Process of HCV Pseudoparticle Production

While alternative backbone and reporter plasmids can be used, the method described here was used in the present study. Plasmids encoding retroviral core, a reporter gene and HCV glycoproteins are co-expressed in HEK 293T cells to produce infectious pseudoparticles. Infection of Huh7 cells can be measured using the luciferase reporter system.

Furthermore, infectious HCV cell culture (HCVcc) virions have been produced by transient expression, and assemble and function in a manner similar to authentic infectious HCV, and are therefore are considered a more accurate representation of the virus and its full life cycle (Helle et al., 2010; Wakita et al., 2005). Huh7.5 cells – Huh7 cells with a single point mutation to dampen an antiviral response – are used for HCVcc infection as they are more permissive of HCV RNA replication (Bartenschlager and Pietschmann, 2005; Wilson and Stamataki, 2012). Recently, replication of HCVcc was achieved in Monkey kidney epithelial Vero cells modified to produce microRNA 122, SR-BI and ApoE – factors which are essential for the replication, entry and production of infectious HCV particles, respectively (Murayama et al., 2016). Nevertheless, only the genotype 2a JFH-1 strain of HCV has produced fully infectious HCVcc particles, and a chimaeric related strain produced even more infectious particles (Lindenbach et al., 2007). This limits research using HCVcc, due to the extent of differences in immune interaction and virus characteristics between genotypes and sub-genotypes.

1.2 Ebola Virus

Ebolaviruses are filamentous viruses of the *Filoviridae* family with a negative-sense, single stranded RNA genome (Bharat et al., 2012). Five EBOV species have been identified: Reston, Taï Forest, Sudan, Bundibugyo and Zaire (Goeijenbier et al., 2014). While all species but Reston are pathogenic to humans, it is believed that potential human pathogenicity of Reston EBOV is separated by only a few amino acids in the VP24 protein (Pappalardo et al., 2016).

The Zaire EBOV outbreak in West Africa beginning in December 2013 and ending in 2016 was the largest, most widespread and most devastating epidemic yet, with more than 28,000 cases and 11,000 deaths across three countries (Coltart et al., 2017; WHO, 2016a). Previous to this outbreak, 29 epidemics had been recorded since 1976 (Coltart et al., 2017).

Human-to-human transmission occurs through mucosal surfaces, breaches of the skin or parental transmission (Goeijenbier et al., 2014). EBOV causes severe, rapid-onset viral haemorrhagic fever, characterised by symptoms including diarrhoea, fever and even haemorrhage and multiple organ failure, resulting in death (Goeijenbier et al., 2014). Case fatality rates vary from 50 – 90%, typically over the course of 14 – 21 days (Goeijenbier et al., 2014; Sullivan et al., 2003).

1.2.1 The EBOV Genome

All EBOV species possess a genome of approximately 19,000 bp, consisting of seven genes flanked by 5’ leader and 3’ trailer RNA sequences necessary for replication and transcription (Feldmann and Klenk, 2004). Non-coding RNA between genes regulates gene expression (Brauburger et al., 2015; Feldmann and Klenk, 2004). From the seven genes, 10 proteins are encoded (Feldmann and Klenk, 2004) (Figure 1.6).

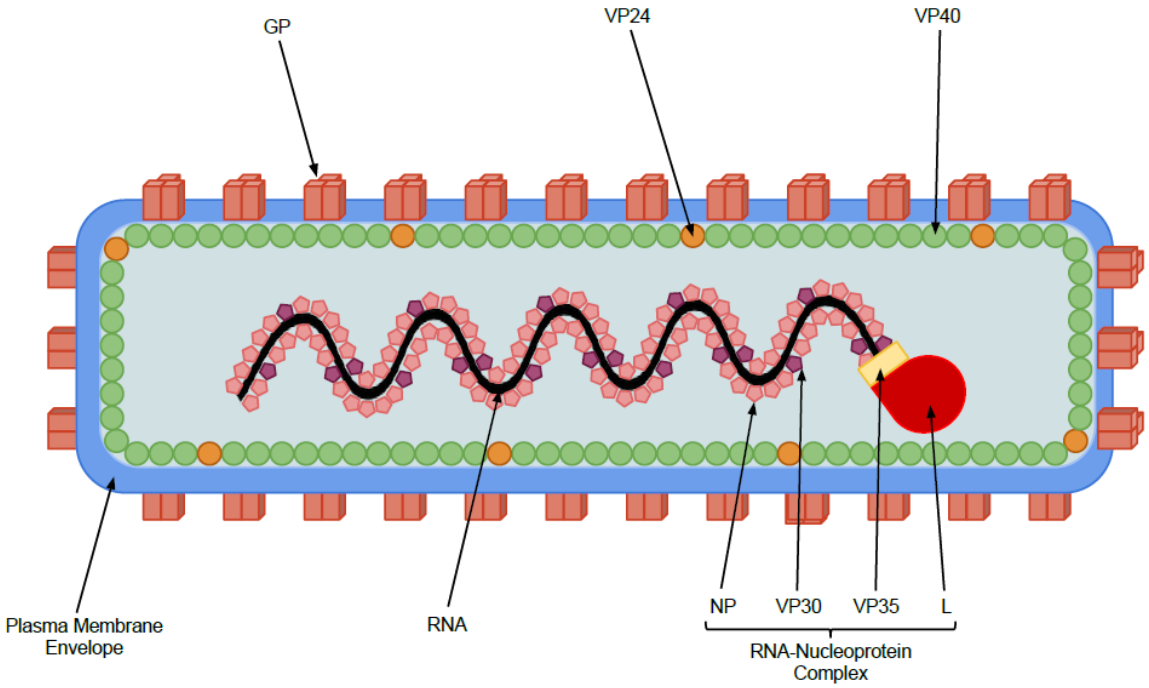

Figure 1.6 – Simplified Schematic of EBOV Structure and Proteins

The Ebola virion is enveloped and studded with GP glycoproteins responsible for viral entry and fusion. VP40 and VP24 are matrix proteins with roles in structural integrity. The NP, VP30, the L RNA-dependent RNA polymerase and the VP35 cofactor proteins complex with the EBOV RNA genome and have roles in transcription and replication. Figure constructed using information from Feldmann and Klenk (2004) and Yu et al. (2017).

NP encapsulates the viral genome in conjunction with VP35, VP30 and L protein (Feldmann and Klenk, 2004). VP35 is a cofactor to the polymerase with further roles in NP-RNA dissociation for replication and regulation of host immune responses (Yu et al., 2017). VP40 is a matrix protein with roles in budding and providing structural stability to the virus.

GP encodes the EBOV glycoprotein which consists of two subunits – GP_1 and GP_2 – formed by post-translational cleavage (Yu et al., 2017). The two subunits have individual roles in receptor binding and membrane fusion, respectively. Three other alternate transcription variants of GP exist: soluble GP (sGP), small soluble GP (ssGP) and the Δ-peptide (Yu et al., 2017). sGP acts as a decoy to subvert immune activity and enhance EBOV infectivity, whereas the Δ-peptide has a proposed role in entry (Basler, 2013; Yu et al., 2017). The purpose of ssGP is unconfirmed.

VP30 activates transcription and nucleocapsid formation and interference of the IFN response (Feldmann and Klenk, 2004; Yu et al., 2017). VP24 is another matrix protein involved in nucleocapsid assembly and inhibition of the host IFN response (Baseler et al., 2017). The L gene encodes the RdRP with a main purpose in allowing replication and transcription (Feldmann and Klenk, 2004).

1.2.2 EBOV Interaction with the Human Immune System

EBOV infection of several cell types results in up-regulated pro-inflammatory cytokine and chemokine release, allowing infection of more immune cells, impairment of the T cell and IFN response, direct virion-associated tissue damage and cell death through TNF-α activity (Falasca et al., 2015; Wong et al., 2014). Such an attack overwhelms an anti-EBOV immune response, which in fact contributes to its pathogenesis (Caballero et al., 2016). Alongside systemic dissemination, the action of inflammatory cytokines leads to vascular leakage and cell death. To overcome infection, the initial immune response is important, however a full perspective of all of the adaptive and innate immune factors involved has not yet been attained (Sullivan et al., 2003).

1.2.3 The Life Cycle of EBOV

Endocytosis via several possible pathways, such as clathrin-dependent or caveolin-mediated uptake or macropinocytosis, follows EBOV GP-mediated receptor binding (Yu et al., 2017). Upon GP cleavage by cathepsin under low pH conditions, host enzyme-mediated fusion and uncoating occur (Yu et al., 2017). Nucleoprotein complex-associated RNA is replicated and transcribed. New nucleocapsids form, while the GP glycoprotein is translated in the ER, post-translationally modified and cleaved by furin in the Golgi body (Yu et al., 2017). All components migrate to the cell membrane for budding (Yu et al., 2017). EBOV GP prevents host cell tetherin from inhibiting viral release, through an unknown mechanism (Cook and Lee, 2013; Yu et al., 2017).

1.2.4 The GP Glycoprotein of EBOV and Its Role in Viral Entry

The GP_1 surface and GP_2 transmembrane glycoprotein subunits form heterodimers, which further heterotrimerise to form mature surface GP (Lee and Saphire, 2009; Ueda et al., 2017). Receptor candidates for attachment and endocytosis include human macrophage galactose and acetylgalactosamine-specific C-type lectin, asialoglycoprotein receptor, lymph node sinusoidal endothelial cell C-type lectin, DC-SIGN, L-SIGN, Tyro3 protein kinases and T-cell immunoglobulin mucin domain (Yu et al., 2017).

The cell tropism of EBOV reaches far wider than that of HCV. EBOV initially infects immune cells such as immature dendritic cells, monocytes and macrophages, and can later disseminate to the liver, lung, kidney, skin and spleen via the lymphatic and vascular systems (Falasca et al., 2015; Martines et al., 2015; Wong et al., 2014). EBOV is zoonotic, able to infect several other mammals including non-human primates, rodents, fruit antelope and fruit bats – the unconfirmed natural reservoir of EBOV (Baseler et al., 2017; Rewar and Mirdha, 2014; Urbanowicz et al., 2016b; Wong et al., 2014).

1.2.4.1 Glycosylation of the EBOV Glycoproteins

The GP_1 glycoprotein consists of a receptor-binding domain, a glycan cap and a mucin-like domain (MLD) (Yu et al., 2017). In addition to roles in glycoprotein function, glycosylation in the glycan cap and MLD serve to protect the glycoprotein from antibody and immune cell recognition (Wong et al., 2014; Yu et al., 2017).

GP expresses both *N*-linked and sialylated *O*-linked glycans, with 15 and 2 *N*-linked glycosylation sites on the GP_1 and GP_2 glycoproteins, respectively (Cook and Lee, 2013; Wang et al., 2017). In fact, glycans contribute to more than a third of the EBOV GP molecular weight (Wang et al., 2017). The *N*-linked glycans are of heterogeneous high-mannose, hybrid and complex type, with approximately 60 variations (Cook and Lee, 2013; Wang et al., 2017). While these modifications are not essential for entry, and may even hinder entry to an extent (Lennemann et al., 2014), two glycosylation sites are critical for GP incorporation, maturation and function (Dowling et al., 2007; Wang et al., 2017).

Anti-EBOV nAbs are known to only target GP_1, particularly the MLD, making this a significant target for antibodies, immune cells and therapeutics (Cook and Lee, 2013; Hofmann-Winkler et al., 2015). Furthermore, EBOV GP expressed on immune cells via proteins such as MHC class I proteins blocks antiviral immune interactions by glycan shielding (Cook and Lee, 2013).

1.2.5 Current Treatment of EBOV Infection

Several therapeutics have been developed to tackle EBOV infection, targeting essential life cycle stages such as GP-mediated entry or RdRP-mediated transcription, however none have yet been approved and licensed (Bixler et al., 2017). ZMapp – a pool of three GP-specific monoclonal chimaeric antibodies – was effective during the 2013 West Africa outbreak, however its use was limited and statistics are lacking to prove efficacy (Bixler et al., 2017).

Unlicensed vaccines exist, in which GP and NP have been used to prime the host immune system, however they have proven less effective in humans and non-human primates than rodents (Sullivan et al., 2003). NP in particular is a major target in early robust T cell and B cell responses against EBOV infection, making this viral protein a potential candidate for vaccines (McElroy et al., 2015). Several vaccines are under development, using different approaches such as DNA vaccines, recombinant viral vectors, replicating vesicular stomatitis virus (VSV) vectors and virus-like particles (Sridhar, 2015). Additionally, efforts should be placed into preventative measures, improved identification of infection and improved healthcare systems (Coltart et al., 2017).

1.2.5.1 Current Issues in the Treatment of Ebola Virus Infection

Due to the fast, sporadic and unpredictable nature of EBOV outbreaks, research into treatment has historically not been at the forefront of research (Bixler et al., 2017). Furthermore, clinical trials have often used North American populations rather than the genetically and immunologically distinct African populations to which EBOV infection is most relevant (Bixler et al., 2017).

1.2.6 Current Research Models of EBOV Infection

EBOV pseudoparticles (EBOVpp) have been developed (Wool-Lewis and Bates, 1998) and optimised (Urbanowicz et al., 2016a) for the study of viral entry and neutralisation (Chin et al., 2015; Hofmann-Winkler et al., 2015). Recently, a replicon consisting of NP, VP35, VP30 and L proteins was developed for research in Huh7 cells under biosafety level -2 conditions (Tao et al., 2017). The typical models used for research are Guinea pigs, mice and non-human primates (Baseler et al., 2017; Sullivan et al., 2003). The full life cycle of the virus can be studied using Vero cells, however this requires biosafety level 4 conditions (Schuit et al., 2016).

1.3 The Complement System of Innate Immunity

The complement system of immunity plays a major role in the initial defence against viral infections of the human body (Mason and Tarr, 2015; Sarma and Ward, 2011). Through the action of over 40 soluble and membrane-bound proteins (Liu et al., 2013), the complement system contributes towards the eradication of pathogens through a sequential activation cascade of proteins, thus enabling effector processes such as opsonisation, phagocytosis and apoptosis (Sarma and Ward, 2011).

1.3.1 The Complement Cascade

There are three known pathways of the complement system, which differ in their manner of activation. However, each pathway converges at the formation of C3 convertase (Figure 1.7), which cleaves complement factor C3 to form C3a and C3b (Sarma and Ward, 2011). C3b associates with C3 convertase to form C5 convertase (Sarma and Ward, 2011). In a similar fashion, C5 convertase cleaves C5 to produce C5a and C5b, the latter of which complexes with complement factors C6, C7, C8 and several C9 proteins to constitute the membrane attack complex (MAC) (Sarma and Ward, 2011). The MAC subsequently forms pores in the lipid membranes of enveloped viruses and infected cells, causing osmotic lysis (Blue et al., 2004; Sarma and Ward, 2011).

Parallel to the cascade, complement factors such as C3b and C4b act as opsonins, which are deposited onto pathogen surfaces in order to attract phagocytes, encourage aggregation and prevent viral entry (Blue et al., 2004). Furthermore, the anaphylatoxin peptides C3a, C4a and C5a have roles in the chemoattraction of immune cells and the mediation of inflammation by binding to their respective receptors (Blue et al., 2004; Sarma and Ward, 2011).

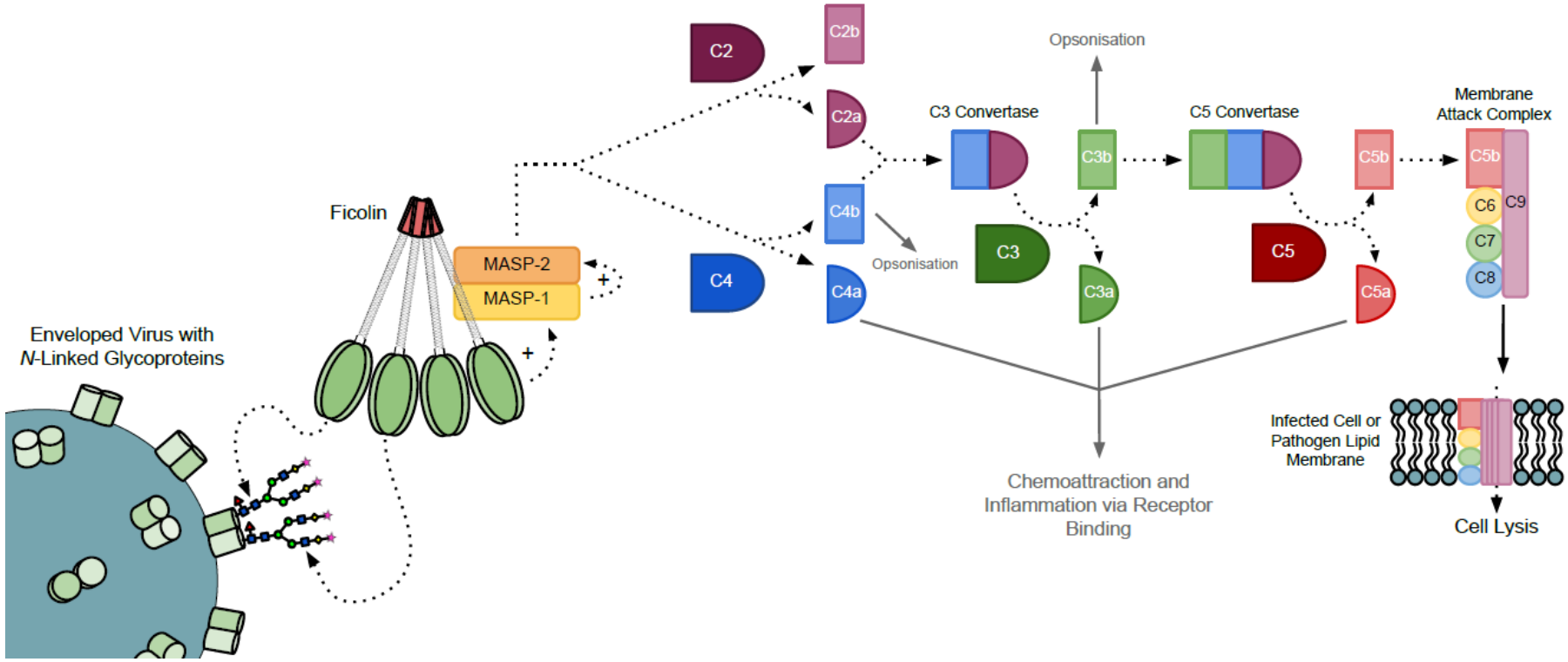

Figure 1.7 – The Lectin Pathway of Complement Activation

Upon the binding of lectins, such as L-ficolin, to a pathogen-associated carbohydrate moiety, lectin-associated MASP-2 proteins are activated and cleave the C2 and C4 complement factors. C3 convertase is subsequently formed, which cleaves C3. The product C3b associates with C3 convertase to form C5 convertase, which acts similarly. C5b associates with complement factors C6 – C9 to form the membrane attack complex which lyses infected host or pathogen cells.

To avoid unnecessary and potentially damaging complement system activity, stringent regulation mechanisms at several points in the complement cascade are imposed, including the cleavage of complement factors, the direct and irreversible inhibition of enzymes, or the endocytotic shedding of MACs (Blue et al., 2004).

1.3.2 The Pathways of Complement Activation

One pathway of complement activation is the alternative pathway, which is triggered by foreign surface carbohydrates, proteins and lipids, and relies on the association and cleavage of the protein Factor B with target-bound C3b to form C3 convertase C3bBb (Sarma and Ward, 2011). The classical pathway is triggered by antigen:antibody complexes, which interact with the C1 complex to induce the formation of C3 convertase C4bC2a (Sarma and Ward, 2011).

Thirdly, the lectin pathway involves the binding of microbial surface carbohydrate moieties to serum lectins (Sarma and Ward, 2011) as shown in Figure 1.7, followed by conformational autoactivation of a lectin-bound MBL-associated serine protease (MASP)-2 enzyme dimer (Mason and Tarr, 2015; Matsushita, 2010). The classical and lectin pathway are similar in that both antibodies and lectins require direct interaction with antigens to directly induce a response.

1.3.2.1 The MBL-Associated Serine Proteases

MASP-1, MASP-3 and MAP-1 are alternative splice products of the *MASP1* gene (Dahl et al., 2001; Skjoedt et al., 2010), while MASP-2 and sMAP are encoded by the *MASP2* gene (Stover et al., 1999). MASP-1 and MASP-2 cleave C3 and C4, respectively, while C2 is cleaved by both (Matsushita et al., 2000). MASP-2 can produce the C3 convertase C4bC2a and thus activate complement (Sarma and Ward, 2011).

Upon ligand binding, lectins undergo a conformational change that brings MASP serine protease domains within close proximity of each other, thus allowing proteolytic autoactivation (Dong et al., 2007; Feinberg et al., 2003; Gál et al., 2005; Gingras et al., 2011) via the cleavage of an arginine-isoleucine bond in the serine protease domain (Ambrus et al., 2003).

MASP-2 activation appears to be primarily dependent on MASP-1, which may autoactivate then *trans*-activate the MASP-2 proenzyme (Megyeri et al., 2013). This may be mediated either by heterodimeric MASP complexes, MBL-MASP-1-MASP-2 co-complexes, or separate MBL-MASP complexes (Degn et al., 2013; Parej et al., 2014). If these complexes are separate, the binding sites of MASP-2 and MASP-1 and -3 on the lectin are likely to be within close proximity and overlapping, but not identical (Wallis et al., 2004).

sMAP and MAP-1 are truncated MASP proteins lacking serine protease domains with putative roles in the modulation of MASP-associated complement activation (Iwaki et al., 2006; Skjoedt et al., 2010; Stover et al., 1999). MAP-1 may inhibit MASP-2 activation by the disruption of inter-MASP and lectin-MASP co-complexes (Degn et al., 2013; Rosbjerg et al., 2014).

The MASPs may also associate the lectin pathway of complement with the coagulation system (Gulla et al., 2010; Kozarcanin et al., 2016; Takahashi et al., 2011). MASP-3 has a putative role in regulation of MASP-2 activity (Dahl et al., 2001), and embryonic development (Sirmaci et al., 2010).

1.3.3 The Lectins of the Complement System

Lectins are a diverse group of proteins broadly defined as non-immunoglobulin proteins that exhibit high avidity for glycoprotein- and/or glycolipid-associated carbohydrates, but display no enzymatic activity (Loris, 2002; Mason and Tarr, 2015). Phylogenetic studies indicate that the primitive immune system depended on lectin protease-mediated opsonophagocytosis (Sekine et al., 2001). Lectins differ in tissue expression, ligand affinities, structure and function, and are classified by the phylogeny and primary and tertiary amino acid structures of their carbohydrate-recognition domains (CRD). However there are several inconsistencies in this classification system (reviewed in Varki et al. (2009)).

Lectin-glycan interactions are generally achieved by hydrogen bonding and Van der Waals forces, and often depend on cations and multivalent interactions between multiple CRDs and multiple, clustered target glycans in order to achieve sufficient affinities for lectin activity (Varki et al., 2009).

The roles of human lectins include protein modulation, cell growth and homeostasis (Ghazarian et al., 2011). As glycoproteins are found on the surfaces of several pathogens to a diverse and widespread degree, some lectins act as PRRs. They are able to recognise PAMPs, including glycans and nucleic acid, related to invading microorganisms and malignant, apoptotic or dead host cells. This can lead to the induction of an immune response against the invading pathogen. However, the relationship between lectins and viruses is complex. In addition to immune evasion, glycosylation is often essential for protein expression, assembly and entry steps in virus replication cycles (Vigerust and Shepherd, 2007), and many viruses have evolved mechanisms to exploit lectins to enhance infection.

Examples of lectins include mannose-binding lectin (MBL), of the collectin family, and ficolins (Sarma and Ward, 2011). The former has been well studied, whereas the role of the latter in the protection against and in the clearance of pathogens has not yet been well-defined (Gupta and Surolia, 2007).

1.4 Ficolins

The ficolin family is similar in function to the collectin family of calcium ion (Ca^{2+})-dependent lectins (Mason and Tarr, 2015; Matsushita and Fujita, 2001), however it is defined by its subunit structure, which contains a fibrinogen-like (FBG) domain rather than a C-type lectin domain (Kilpatrick and Chalmers, 2012).

1.4.1 The Genetic and Structural Characteristics of the Ficolins

Three human ficolins have been described: L-ficolin (Endo et al., 1996), M-ficolin (Ichijo et al., 1991; Lu et al., 1996) and H-ficolin (Sugimoto et al., 1998). The ficolins differ in several ways, for example in their localisation in the human body and their capacity to trigger an immune response.

1.4.1.1 The Genetics of Ficolins

The M-, L- and H-ficolin proteins are encoded by the *FCN1*, *FCN2* and *FCN3* genes, respectively, encoding polypeptides of 326, 313 and 299 amino acids (Garred et al., 2010) - including the signal peptide. The *FCN1* and *FCN2* genes are both situated on chromosome 9q34 whereas FCN3 is found on chromosome 1p36.11 (Garred et al., 2010). The *FCN2* and *FCN3* genes consist of 8 exons (Figure 1.8), whereas *FCN1* comprises 9 exons (Garred et al., 2010).

1.4.1.2 Ficolin Orthologues

Several orthologues have been identified in genetically diverse lineages of animals, an indication of the ancient ancestral origins of these lectins. These include the invertebrate ascidians (Kenjo et al., 2001), and vertebrates such chickens (Lynch et al., 2005), non-human primates (Hummelshøj et al., 2011) and pigs (Ichijo et al., 1993; Ichijo et al., 1991).

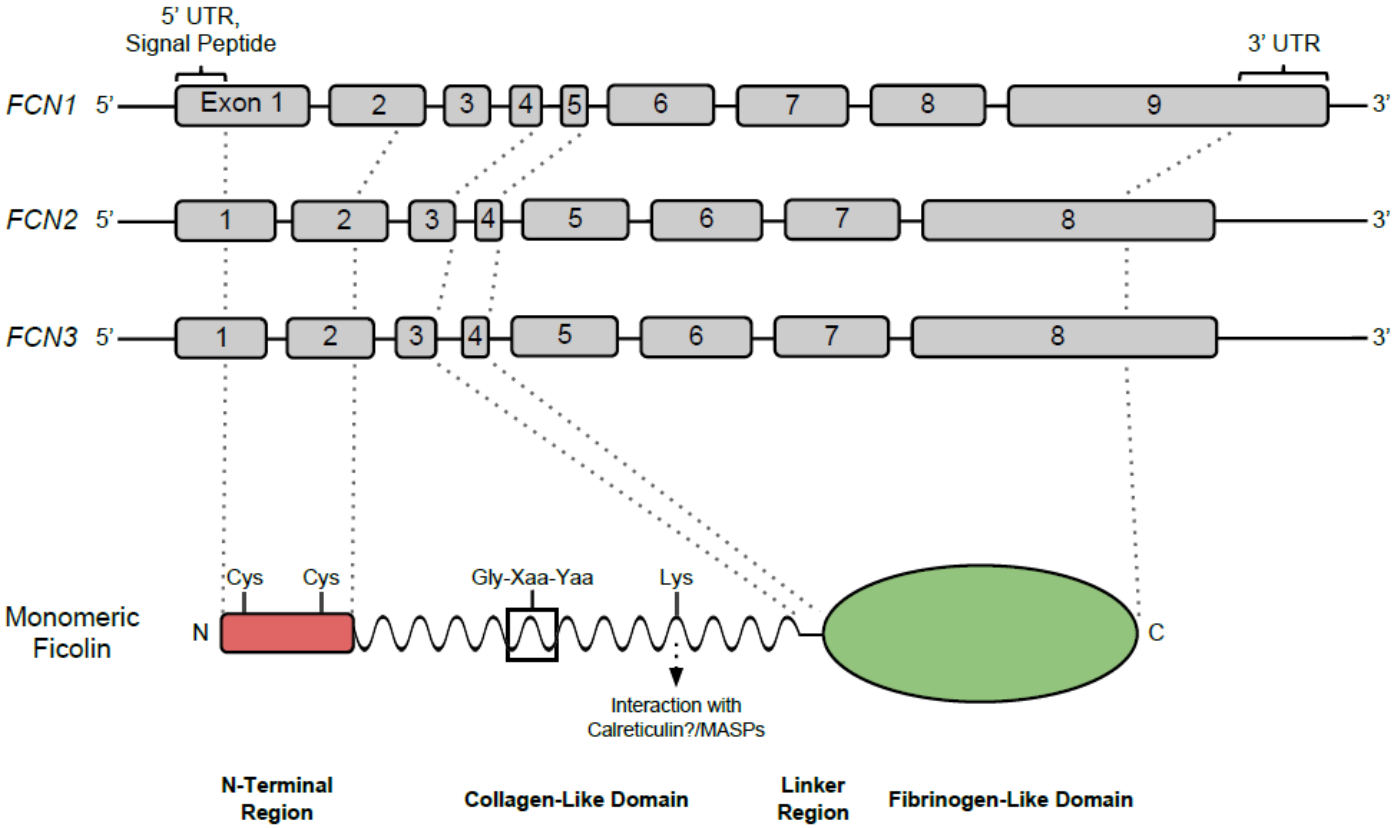

Figure 1.8 – The Exon Arrangement of the *FCN* Genes and the Structure of the L-Ficolin Monomer

The FCN2 *gene consists of 8 exons. Exon 1 encodes the 5' untranslated region (UTR) and the signal peptide. Exons 1 and 2 encode the N-terminal region of the L-ficolin monomer, which contains two cysteine residues involved in oligomerization. Exon 3 encodes the collagen-like domain (CLD) involved in MASP interaction and phagocytosis. Exon 4 encodes the linker region, and exons 5 – 8 encode the C-terminal fibrinogen-like (FBG) domain involved in pathogen recognition. Exon 8 also encodes the 3' UTR. Diagram created by author for Mason and Tarr (2015).*

1.4.1.3 The Structure of the Ficolins

Each glycosylated ficolin monomer comprises an N-terminal region with two functionally important cysteine residues, a collagen-like domain (CLD) containing Gly-Xaa-Yaa repeats, a linker region and, characteristically, a C-terminal globular FBG domain (Hummelshøj et al., 2007; Kilpatrick and Chalmers, 2012; Matsushita et al., 1996) (Figure 1.8). The FBG domain recognises specific pathogen-associated carbohydrates, and the CLD is responsible for signalling to induce an immune response via MASP proteins (Hummelshøj et al., 2007; Matsushita et al., 1996). Active, oligomeric L-ficolin and M-ficolin are dodecamers comprised of 4 homotrimeric subunits to form what has been labelled as a "bouquet" structure, whereas H-ficolin is octadecameric (Hummelshøj et al., 2007; Lacroix et al., 2009; Matsushita et al., 1996) (Figure 1.9). Ficolin homotrimers are stabilised by interactions between hydrophobic residues in the CLDs (Sheriff et al., 1994; Weis and Drickamer, 1994), and oligomerise by inter-monomer and -trimer disulphide bridges between the N-terminal cysteine residues (Hummelshøj et al., 2007; Ohashi and Erickson, 2004).

Slight stabilisation occurs via hydrogen bonding, salt bridging and van der Waals forces between several residues in the FBG domains of L-ficolin monomers (Garlatti et al., 2007). These interactions confer great flexibility, with inter-monomeric angles varying between 117° and 122° (Garlatti et al., 2007).

L-ficolin possesses two potential *N*-linked glycosylation sites at residues Asn240 and Asn300, however only glycosylated Asn215 has been observed (Garlatti et al., 2007).

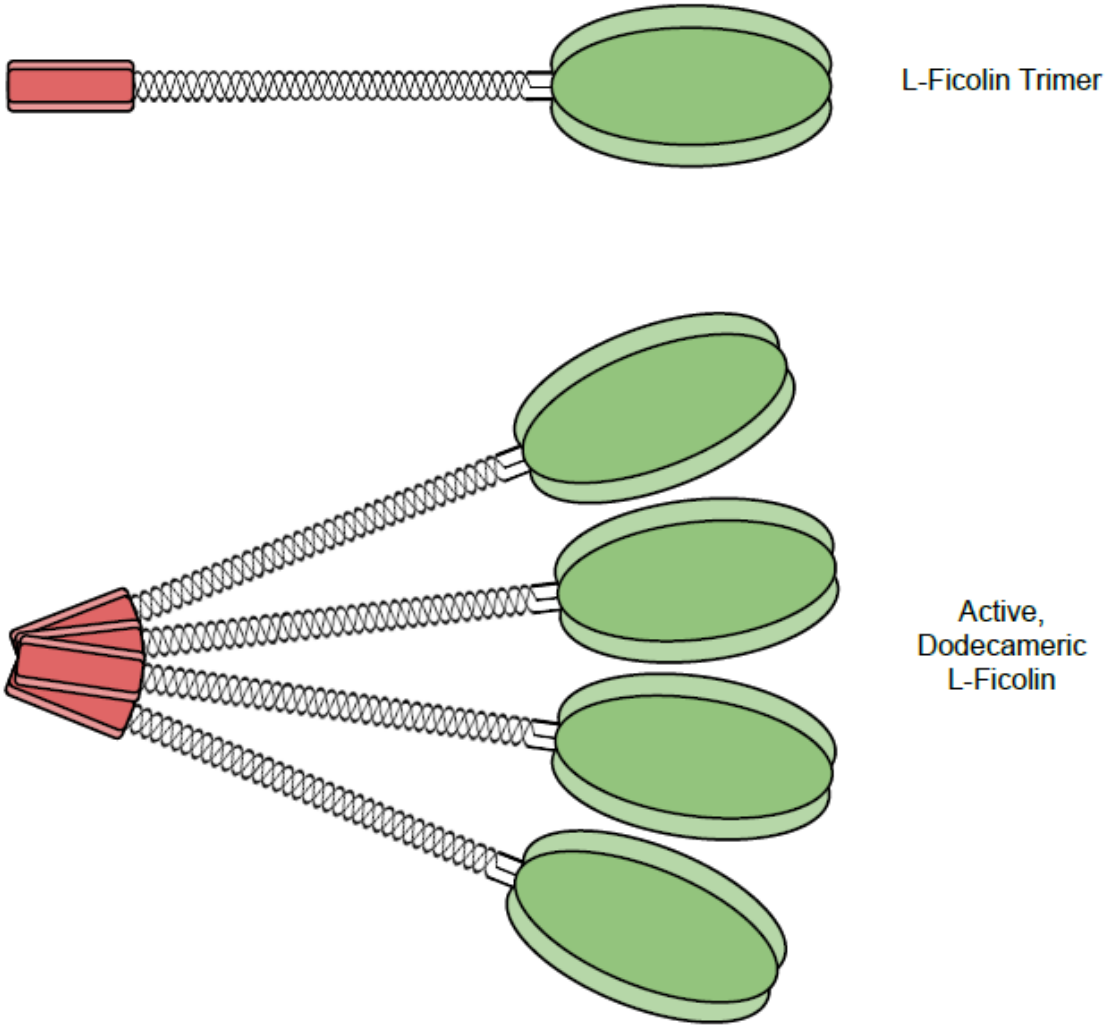

Figure 1.9 – The Trimeric and Dodecameric Forms of L-Ficolin
L-ficolin monomers bind via two functional cysteine residues in their N-terminal domains (in red) to form trimers with triple α-helical coiled-coil collagen-like domains (CLDs; in black). Active L-ficolin consists of 4 homotrimers and is able to recognise pathogens via its C-terminal fibrinogen-like (FBG) domains (in green).

1.4.1.4 The Expression of the Ficolins

Hepatocytes are the main site of expression and secretion of both L- and H-ficolin (Akaiwa et al., 1999; Matsushita et al., 1996), though H-ficolin is also highly expressed in type II alveolar and bronchial epithelial cells (Akaiwa et al., 1999). Despite minor lung and blood expression, most M-ficolin is associated with the surface of peripheral blood leukocytes (Liu et al., 2005; Lu et al., 1996). L-ficolin has also been found in the lung (Bidula et al., 2015).

H-ficolin is the most abundant serum ficolin (median concentration of ~26 µg/mL; range 6 – 83 µg/mL) (Schlapbach et al., 2009) followed by L-ficolin (median of 3.7 – 5.4 µg/mL; range ~1 – 13 µg/mL) (Kilpatrick et al., 1999; Munthe-Fog et al., 2007) and M-ficolin (median of 1.07 µg/mL; range 0.28 – 4.05 µg/mL) (Wittenborn et al., 2010).

1.4.1.5 The Binding Specificities of the Ficolins

Each ficolin possesses its own specific binding activities – typically through hydrogen bonding – for different carbohydrate moieties, albeit most ficolin ligands are acetylated compounds (Garlatti et al., 2007; Matsushita, 2010). All ficolins bind *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) (Garlatti et al., 2007; Liu et al., 2005; Sugimoto et al., 1998). H-ficolin binds GalNAc and D-fucose, but not mannose and lactose (Garlatti et al., 2007; Sugimoto et al., 1998). M-ficolin binds sialic acid (Liu et al., 2005).

These differing ligand specificities are conferred by sequence differences in the binding site – S1 – near the Ca^{2+}-binding site of the FBG (Garlatti et al., 2007). In addition to S1, L-ficolin has 3 additional binding sites, S2 – S4, thus allowing the sites to accommodate a wide variety of ligands in both Ca^{2+}-dependent and -independent ways (Figure 1.10). Ligands include phosphocholine moieties of bacterial teichoic acids, in addition to acetylated compounds (Garlatti et al., 2007; Vassal-Stermann et al., 2014b). For example, S2 is the major binding site for galactose and *N*-acetylcysteine (CysNAc), whereas S3 and S4 cooperate to bind (1,3)-β-D-glucan, among others (Garlatti et al., 2007).

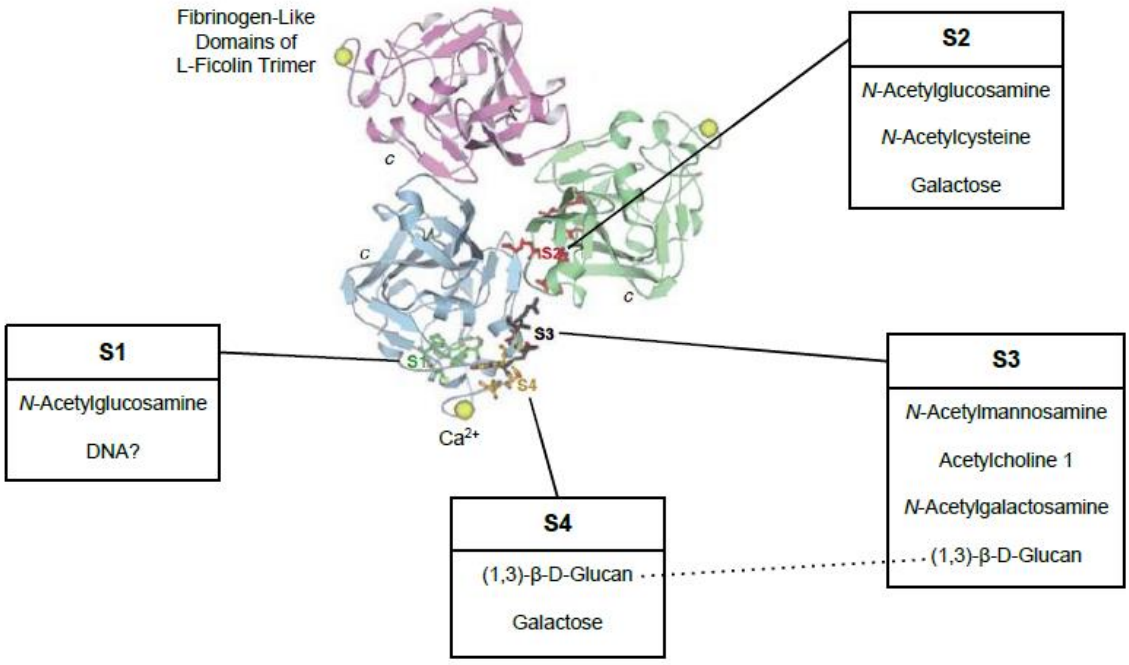

Figure 1.10 – The Binding Sites and Ligands of L-Ficolin

A 3D structure of the FBG domains of an L-ficolin trimer as seen head-on, from the C-termini. Yellow circles represent the calcium-binding sites. Binding sites S1 – S4 and their confirmed ligands are indicated. Binding of (1,3)-β-D-glucan spans the S3 and S4 sites. Adapted from Garlatti et al. (2007) and Garlatti et al. (2010).

1.4.1.6 The Binding Sites of L-Ficolin

Amino acids constituting the binding sites of L-ficolin have been identified (Garlatti et al., 2007) (Figure 1.11). The FBG domain of each L-ficolin monomer consists of nine α-helices and 12 β-sheets. Residue 1 represents the methionine start codon of the L-ficolin signal sequence.

The hydrophobic Ca^{2+}-binding site of L-ficolin was determined to involve Asp249, Asp251, Asn253 and Gly255. The S1 site lies near the Ca^{2+}-binding site and comprises a mix of non-polar, polar and charged amino acids: Phe261, Cys270, His271, Gly285, Phe287, Tyr299 and Asn300. Interestingly, Asn300 is a putative *N*-linked glycosylation site, however no oligosaccharides have been detected here (Garlatti et al., 2007). The S2 binding site exists at the inter-monomer interfaces of the trimer and consists of Ser116, Asp134, Asp136, Arg147, Glu307 and Lys309, in addition to Ser168 from an adjacent monomer, which form a ring-like arrangement. S3 lies between the S2 and Ca^{2+}-binding sites, consisting of the hydrophilic Arg157, Asp158, Thr161 and Lys246 residues. Lastly, S4 is located very close to S3, between the S3 and Ca^{2+}-binding sites, consisting of both hydrophobic and hydrophilic residues: Trp160, Ala161, Asp247 and Gln248.

L-ficolin displays extensive plasticity of recognition, with the atomic mechanism of interaction varying with each ligand for each binding site (Garlatti et al., 2007).

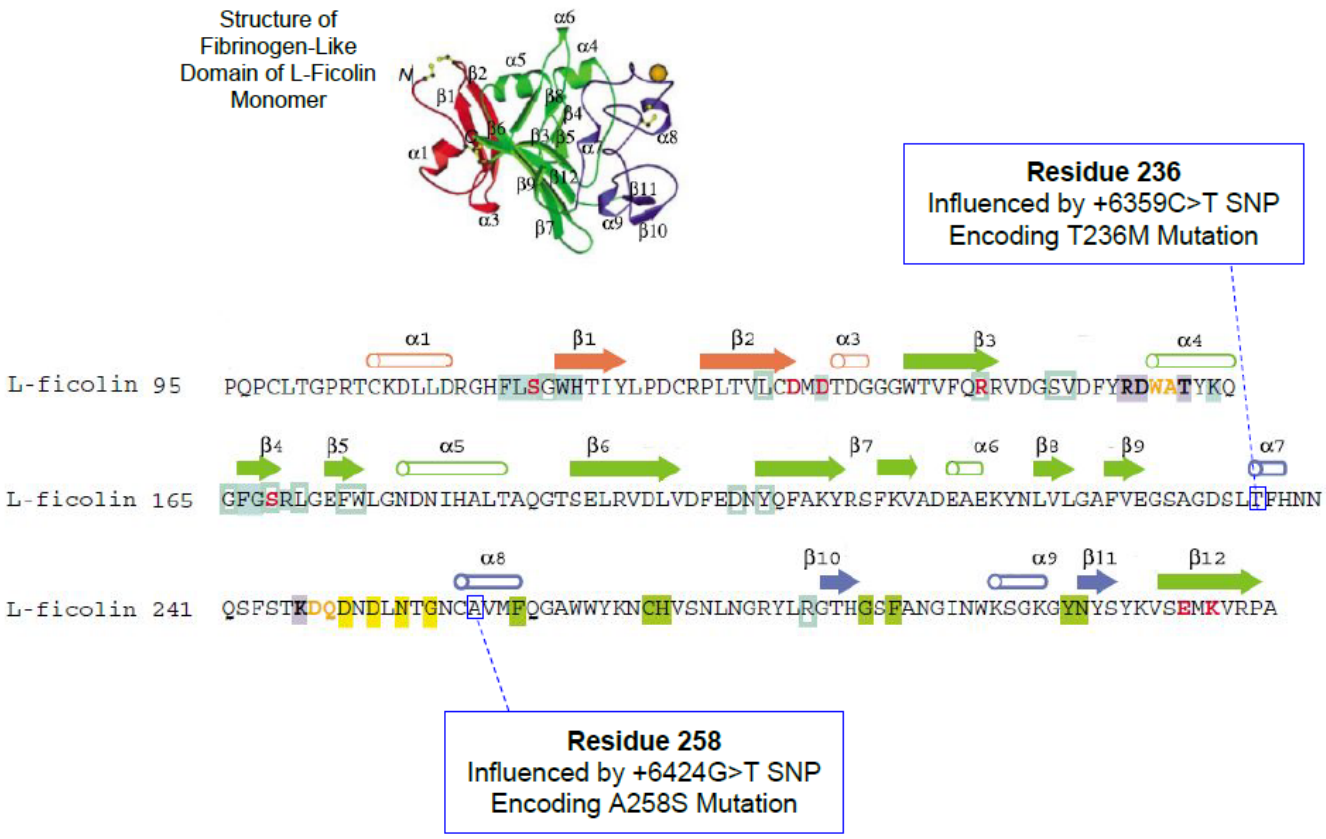

Figure 1.11 – The Amino Acid Residues Constituting the L-Ficolin Binding Sites

A 3D structure of the fibrinogen-like (FBG) domain of an L-ficolin monomer, and its corresponding amino acid sequence. Amino acid numbering commenced from the signal peptide. Residues constituting the S1, S2, S3 and S4 binding sites are highlighted in green, red, black (purple box) and orange, respectively. Coloured and empty cyan boxes represent residues consistently and variably involved in inter-monomer interaction, respectively. The residues of interest in this study are highlighted in blue. Adapted from Garlatti et al. (2007).

1.4.2 The Immune Activities of the Ficolins

Ficolins indirectly activate the lytic complement pathway via MASP activation, induce phagocytosis by opsonisation, and stimulate the production and secretion of inflammatory cytokines and nitric oxide by macrophages (Luo et al., 2013) (Figure 1.12). Interaction with phagocytes is mediated by a lysine in the CLD, at residues 57 and 47 for L- and H-ficolin respectively, which binds calreticulin on phagocyte cell surfaces (Lacroix et al., 2009). The same residue is responsible for interaction with MASPs, therefore it is possible that the phagocytic and complement effects of L-ficolin are competitive (Lacroix et al., 2009; Wallis et al., 2004). L-ficolin also clears apoptotic and necrotic host cells through the binding of apoptosis-associated ligands, and directly prevents viral entry into host cells (Hamed et al., 2014; Jensen et al., 2007).

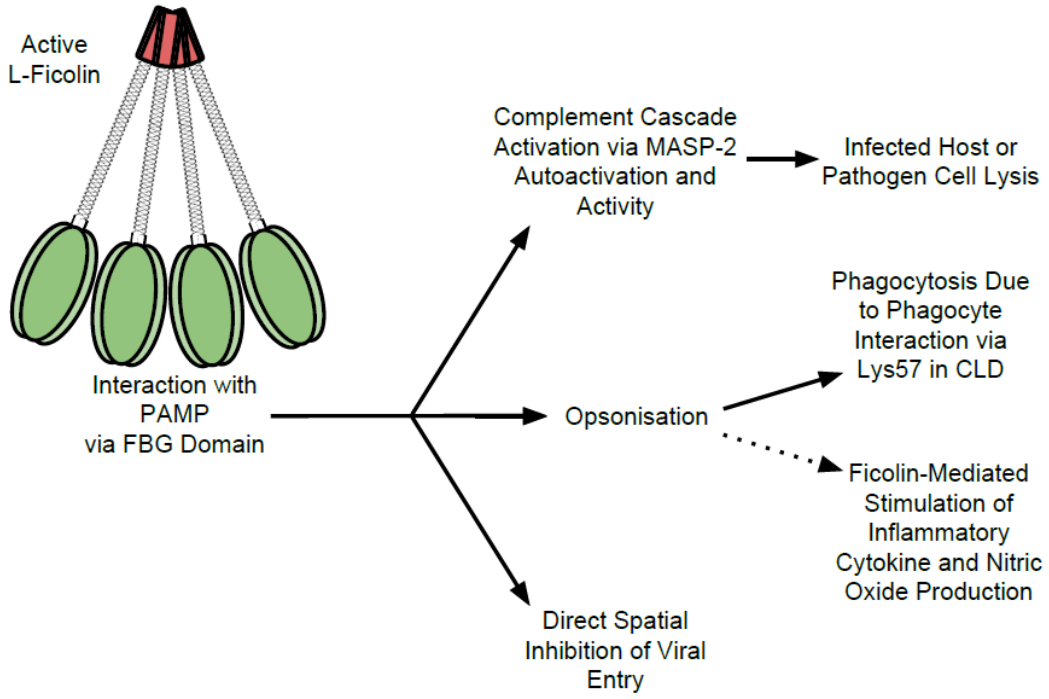

Figure 1.12 – The Immune Activities of L-Ficolin

Upon interaction with pathogen-associated carbohydrate moieties, active L-ficolin can produce an immune effect through three known pathways. Complement activation leads to pathogen or infected host cell lysis. Opsonisation can lead to phagocytosis or inflammation. Spatial blocking can prevent viral entry and infection.

1.4.2.1 Cooperative Relationships between Lectins and Other Immune Proteins

A consequence of complement activation is the subsequent activation of the humoral immune system against a pathogen, thus enhancing the adaptive immune response and memory (Sarma and Ward, 2011). L-ficolin can also directly interact with immune components and enhance the antimicrobial response (Figure 1.13).

Natural antibodies are produced without prior exposure to infection or immunisation, and are important in the protection of individuals exposed to pathogens for first time, such as neonates (Panda et al., 2013). They are able to initiate complement alone, however under mild acidosis and reduced calcium levels – conditions found at infection-inflammation sites – an additional binding site is exposed on the ficolin FBG to allow complex formation with natural immunoglobulin G (nIgG) (Panda et al., 2013; Panda et al., 2014). This allows indirect nIgG-based phagocytosis via L-ficolin opsonisation, leading to a stronger immune response (Panda et al., 2013).

Similar to nIgG:ficolin complexes, infection-inflammation conditions significantly increase interaction between the acute phase protein C-reactive protein (CRP) and L-ficolin, leading to a stronger classical- and lectin-mediated complement response against *Pseudomonas aeruginosa* (Zhang et al., 2009). A pH- and calcium-sensitive binding site on the ficolin FBG domain enables binding to CRP (Zhang et al., 2011). Pentraxin 3 (PTX3) also interacts with L-ficolin to enhance its binding of *Aspergillus fumigatus* and its induction of C4 deposition (Ma et al., 2009).

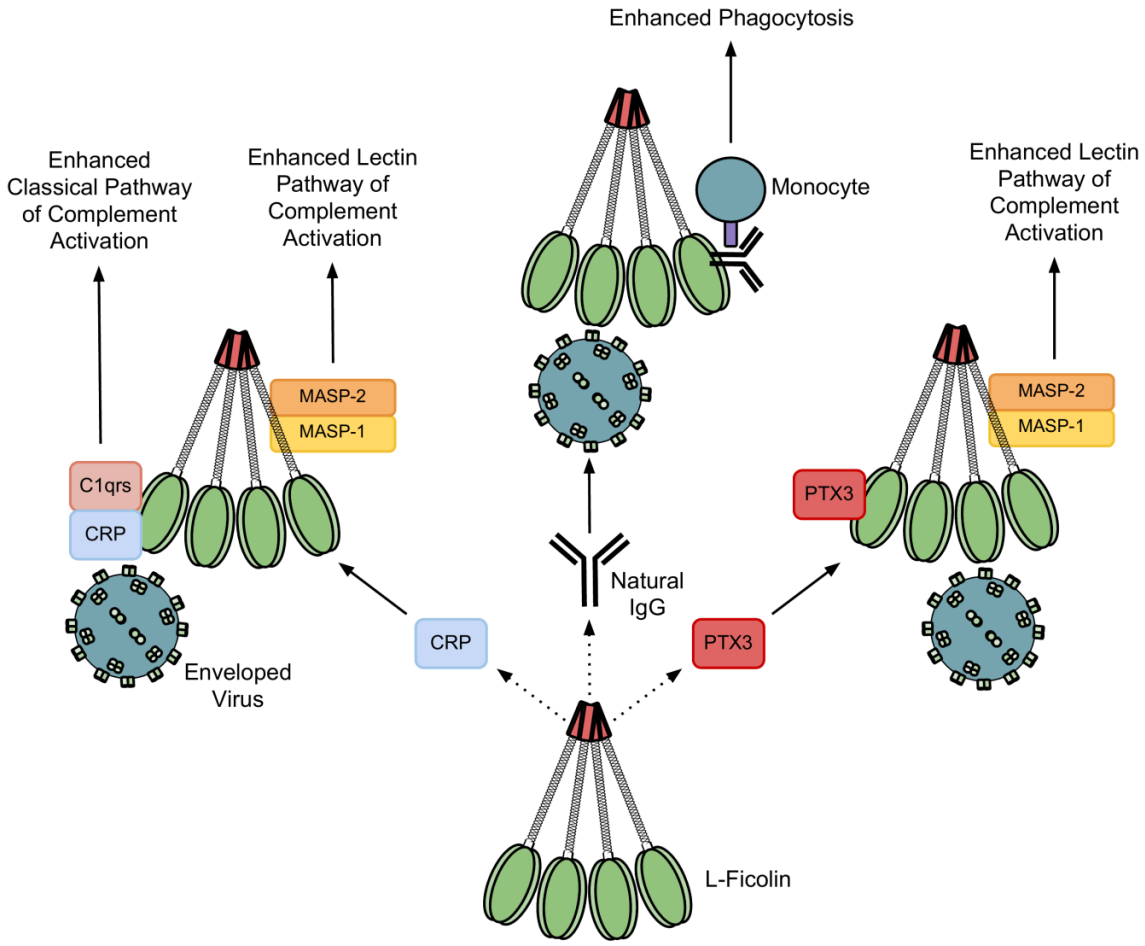

Figure 1.13 – Cooperative Relationships between L-Ficolin and Other Immune Components

L-ficolin is able to interact with C-reactive protein (CRP) in order to amplify both the classical and lectin pathways of complement activation. Interaction with natural immunoglobulin G (IgG) allows enhanced phagocytosis after L-ficolin-based opsonisation. L-ficolin interaction with pentraxin 3 (PTX3) has been shown to enhance complement activation.

1.5 The Activity of L-Ficolin in Pathogenic Infections

Since their discovery, the role of ficolins in the engineering of an immune response against – and the clearance of – several pathogens has become increasingly evident; however their mechanism of action against viruses requires greater investigation. L-ficolin interacts with viruses via *N*-linked glycans on viral envelope glycoproteins (Keirstead et al., 2008; Liu et al., 2009; Pan et al., 2012).

1.5.1 Interactions between L-Ficolin and HCV

L-ficolin binding of HCV via HCV glycoproteins triggers infected-cell lysis via C4 deposition (Liu et al., 2009). Biologically relevant levels of recombinant oligomeric L-ficolin, which displayed similar binding activity and structure to serum L-ficolin, neutralised HCV entry in a dose-dependent manner by preventing E2 interaction with cell surface receptors (Hamed et al., 2014; Zhao et al., 2014). Monomeric L-ficolin can activate complement (Liu et al., 2009) but not inhibit HCV entry (Hamed et al., 2014).

1.5.2 Interactions between L-Ficolin and Other Pathogenic Viruses

L-ficolin neutralises replication and infection of influenza A virus (IAV) *in vivo* by directly inhibiting IAV entry and promoting complement-mediated lysis of IAV and infected cells (Pan et al., 2012). L-ficolin also mediates complement activation following interaction with HIV-1 gp120 and may spatially block entry (Luo et al., 2016; Ren et al., 2014). L-ficolin interaction with EBOV has not yet been reported, however M-ficolin and MBL have both been implicated in enhancement of EBOV infection by binding the glycans in the MLD of EBOV GP (Brudner et al., 2013; Favier et al., 2016).

1.5.3 Interactions between L-Ficolin and Other Pathogens

Due to the widespread presence of acetylated compounds on the surfaces of microorganisms, ficolins interact with a wide variety of other pathogens. For example, ficolins bind and combat infection by *Mycobacterium tuberculosis* (Luo et al., 2013), and Gram-positive bacteria such as *Staphylococcus aureus* and several streptococci (Ali et al., 2013; Lynch et al., 2004) and Gram-negative bacteria such as *Escherichia* species (Sugimoto et al., 1998) and *Pseudomonas aeruginosa* (Panda et al., 2013).

Ficolin interaction with protozoans such as *Trypanosoma cruzi* (Cestari Idos et al., 2009) and *Giardia intestinalis* (Evans-Osses et al., 2010) via *N*-linked carbohydrate moieties on the cell surface leads to complement activation. L-ficolin binds (1,3)-*β*-D-glucan (Ma et al., 2004), a major component of fungal cell walls, and enhances opsonophagocytosis of the fungus *Aspergillus fumigatus* in the lung (Bidula et al., 2015; Ma et al., 2009).

1.6 The Effects of L-Ficolin Polymorphisms and Serum Levels in Clinical Cases

There have been several clinical studies monitoring the part that ficolins play in disease outcome, typically focusing on one or both of two factors: ficolin gene polymorphisms and ficolin concentrations in the serum.

1.6.1 The Polymorphisms of the FCN2 *Gene*

Hummelshøj et al. have extensively described the SNPs of the highly polymorphic *FCN* genes (Table 1.2) (Hummelshøj et al., 2005; Hummelshøj et al., 2008). In general, polymorphisms in the promoter regions of the ficolin genes are expected to affect gene regulation and protein concentration whereas coding region polymorphisms likely affect protein stability, modification, folding and activity, thus altering protein function. Non-synonymous substitutions alter protein activity, however non-synonymous mutations may influence mRNA processing and protein expression.

While polymorphisms have been identified in the *FCN1* and *FCN3* genes, several more significant SNPs have been identified in the *FCN2* gene. The frequencies of *FCN* gene polymorphisms often differ between ethnicities, with some existing solely in a particular geographical population, more so in African populations (Herpers et al., 2006; Hummelshøj et al., 2008). This likely arose from distinct geographical selective pressures, such as genetically-determined and infectious diseases. The *FCN2* gene has three as yet undetected splicing variants (Endo et al., 1996; Garred et al., 2009).

Table 1.2 – Identified Polymorphisms in the *FCN2* Gene with Phenotypic Influences

A list of FCN2 *polymorphisms and their associated effects on L-ficolin serum concentrations, activities and virus interactions. Polymorphisms of interest in this study are in bold.*

***FCN2* dbSNP**	**Nucleotide Position**	**Major Allele**	**Minor Allele**	**Region**	**Amino Acid Mutation**	**Phenotype and Relevance to Specific Virus Infections**
rs3124952	-986	G	A	Promoter	-	Reduced serum concentration (Hummelshøj et al., 2008); HBV (Hoang et al., 2011)
rs3124953	-602	G	A	Promoter	-	Increased serum concentration (Hummelshøj et al., 2008); HBV (Hoang et al., 2011)
rs17514136	-4	A	G	Promoter	-	Increased serum concentration (Hummelshøj et al., 2008); HBV (Hoang et al., 2011)
ss76901565	+4423	C	T	Exon 5	Arg103Cys	Likely affects chemical and structural properties (Hummelshøj et al., 2008)
ss76901566	+4526	C	T	Exon 5	Thr137Met	Likely affects chemical and structural properties (Hummelshøj et al., 2008)
ss76901570	+4957	G	A	Exon 6	Arg147Gln	Likely affects ligand binding (Hummelshøj et al., 2008)
ss76901571	+4987	G	A	Exon 6	Arg157Gln	Likely affects ligand binding (Hummelshøj et al., 2008)
rs17549193	**+6359**	**C**	**T**	**Exon 8**	**Thr236Met**	**Reduced binding to GlcNAc (Hummelshøj et al., 2008) and PTX3 (Ma et al., 2009);**
rs7851696	**+6424**	**G**	**T**	**Exon 8**	**Ala258Ser**	**Increased binding to GlcNAc (Hummelshøj et al., 2008); CMV (de Rooij et al., 2011), HBV (Hoang et al., 2011)**
rs28357091	+6443_44	CT	A	Exon 8	Ala264fs	Truncated protein (Hummelshøj et al., 2008)

1.6.1.1 Significant FCN2 *Single Nucleotide Polymorphisms*

Of the 36 SNPs in the *FCN2* gene, five significant polymorphisms have been identified. Promoter polymorphisms -986A>G, -602G>A and -4A>G affect serum levels of L-ficolin and exon 8 polymorphisms +6359C>T and +6424G>T – conferring Thr236Met and Ala258Ser mutations respectively – in the FBG alter L-ficolin affinity to GlcNAc. Several of these and other polymorphisms were in strong linkage disequilibrium, namely the SNPs at -4 and +6359, and the SNPs at -64 and +6424 (Szala et al., 2013). Additional linkages observed in Japanese and African populations were between the -986, intronic +2545 and +6424 SNPs with +6359, and -557 and intronic +5121 with the +6424 SNP (Hummelshøj et al., 2008).

Additional *FCN2* SNPs were detected and their effects hypothesised, however no associated phenotype has yet been described. The Arg147Gln and Arg157Gln mutations are found in the S2 and S3 binding sites respectively, and are therefore expected to affect ligand binding. Furthermore, the Arg103Cys and Thr137Met mutations are expected to affect the chemical and structural properties of L-ficolin. A rare frame shift mutation encoding Ala264fs has also been described, however a homozygote for this polymorphism has not been found, hence the physiological implications are unknown (Hummelshøj et al., 2005).

1.6.1.2 The Significance of FCN2 *Gene Single Nucleotide Polymorphisms in Viral Infections*

One *FCN2* haplotype – involving the +6424G SNP – is associated with protection against HBV infection (Hoang et al., 2011). L-ficolin levels were higher in patients with acute rather than chronic HBV infection, suggesting that the protein is directly involved in immediate clearance of the virus, and influences subsequent liver disease (Hoang et al., 2011). The L-ficolin Ala258Ser mutation appears to confer a protective effect against CMV re-infection in liver transplantation when compared to wild-type L-ficolin (de Rooij et al., 2011).

The Thr236Met mutation reduced affinity towards PTX3, a serum protein which enhances L-ficolin-mediated complement response to *Aspergillus fumigatus*, suggesting that *FCN2* polymorphisms also alter affinity towards cooperative proteins and thus affect the immune response (Ma et al., 2009). The +6424T SNP is associated with low serum levels of L-ficolin (Munthe-Fog et al., 2007). On the hypothesis that lectins involved in the lectin pathway of complement activation compensate for each other, Bjarnadottir et al. (2016) found that the +6424T SNP is rare in healthy individuals with MBL deficiency.

1.6.1.3 The Significance of FCN2 *Gene Single Nucleotide Polymorphisms in Other Pathogenic Infections*

Although there are few investigations of the role of L-ficolin in viral infections, *FCN2* polymorphisms were found to be significant in susceptibility to and disease severity of several bacterial diseases, including cutaneous and visceral leishmaniasis (Assaf et al., 2012; Mishra et al., 2015), *Mycobacterium leprae* (de Messias-Reason et al., 2009), *Pseudomonas aeruginosa*-associated bronchiectasis (Chalmers et al., 2011), and *Streptococcus pygones*-associated rheumatic fever and chronic rheumatic heart disease (Messias-Reason et al., 2009). However, the SNPs were not associated with invasive pneumococcal disease (Chapman et al., 2007) and other respiratory tract infections (Ruskamp et al., 2009). Contrarily, the +6424T SNP was implicated in predisposition to *Coxiella burnetii* pneumonia (van Kempen et al., 2017). These studies have been relatively small-scale and geographically limited, and often did not measure the serum concentrations of L-ficolin to confirm the relationship of the polymorphisms and haplotypes with L-ficolin levels, therefore more rigorous larger scale studies would yield more reliable results.

1.6.2 The Significance of L-Ficolin Serum Concentrations in Viral Infections

Serum ficolin levels are dependent on the expressed alleles; homozygotes for particular SNPs exhibit the highest or lowest levels whereas heterozygotes display intermediate levels of ficolin (Hummelshøj et al., 2005). Specific *FCN2* SNPs that are associated with low levels of L-ficolin tend to cause higher susceptibility to infection (Assaf et al., 2012). L-ficolin levels are significantly increased in the serum of HCV-infected individuals, and concentrations correlate with the severity of fibrosis (Liu et al., 2009). Chronic HCV-associated liver damage also did not reduce the levels of L-ficolin expressed (Hamed et al., 2014). In chronic HCV-infected patients with abnormal levels of the HCV marker ALT, serum concentrations of L-ficolin correlated with ALT levels (Hu et al., 2013a). After successful therapy, ALT and HCV RNA levels of these patients all decreased to normal values, followed by a decrease in L-ficolin levels, suggesting a correlation of alanine aminotransferase (ALT) and RNA levels with disease outcome, as a result of L-ficolin activity (Hu et al., 2013a; Hummelshøj et al., 2005). HIV-1 infection has also been implicated in increased L-ficolin expression (Luo et al., 2016).

In the context of non-viral pathogenic infections, L-ficolin serum concentrations have been shown to play a role. For example, higher L-ficolin serum concentrations also appear to confer protective effects against microorganism-induced inflammation in allergic respiratory disease (Cedzynski et al., 2009). L-ficolin levels were higher in acute severe cases of *Plasmodium falciparum*-based malaria, rather than mild cases (Faik et al., 2011).

1.7 Research Aims

Human lectins such as MBL, M-ficolin, DC-SIGN and L-SIGN have been shown to interact with enveloped viruses such as HCV and EBOV (Alvarez et al., 2002; Brown et al., 2010; Brudner et al., 2013; Favier et al., 2016). Due to the wide ligand specificity and biological significance of the L-ficolin protein, it was hypothesised that L-ficolin can neutralise a wide variety of virus infections. HCV and EBOV are acute and chronic enveloped viruses, respectively, with largely differing life cycles and mechanisms of pathogenesis. Due to their highly glycosylated nature, they are strong candidates for interaction with L-ficolin.

Using these viruses as models, several hypotheses were tested. To assess whether the T236M and A258S mutations in the L-ficolin FBG domain, encoded by the +6359C>T and +6424G>T SNPs, confer an enhanced effect on L-ficolin interaction with ligands and other viruses, binding assays and virus pseudoparticle entry neutralisation assays were carried out. Furthermore, a chimaeric lectin exploiting the unique structure and ligand affinities of L-ficolin was created to explore its potential for enhanced binding and antiviral activity.

In a separate project, correlations between serum L-ficolin concentrations and HCV infection outcome and patient ethnicity were investigated. Lastly, to identify the HCV E2 *N*-linked glycans involved in L-ficolin interaction, a clone library of E2 glycosylation site mutants was created and assessed in HCV pseudoparticle infectivity and neutralisation assays.

Chapter 2 Characterisation of Binding by L-Ficolin Variants

2.1 Aims

The common L-ficolin variants containing Thr236Met and Ala258Ser mutations in their FBG domains have been observed to correlate with varying levels of binding to GlcNAc, L-ficolin serum concentrations and predisposition to certain microbial infections. However, the physical effect of these mutations on L-ficolin interaction with viruses has not been characterised. It was hypothesised that specific combinations of these FBG mutations confer an enhanced effect on L-ficolin interaction with several viruses, and thus an enhanced ability to neutralise virus entry.

Here, novel recombinant His_6-tagged L-ficolin variants were produced by adapting a protocol from Hamed et al. (2014). Comparison and characterisation of their binding activity to acetylated BSA was performed, and their ability to neutralise enveloped virus pseudoparticle entry was investigated.

2.2 Materials and Methods

All solutions used were either purchased in sterile condition, or sterilised by autoclaving at 121°C for 20 minutes (min) or filtration through a 0.45 µm or 0.22 µm Minisart® Polyethersulfone High Flow Syringe Filter (Catalogue number [Cat.] 16537----------K or 16532----------K; Sartorius). All human cell work was performed in MSC-Advantage™ Class II Biological Safety Cabinet (Thermo Fisher Scientific [Thermo]) sterilised using Trigene disinfectant and 70% ethanol. An MCO-18AC CO_2 incubator (Panasonic) was used for all human cell lines. An MJ Research PTC-200 gradient thermal cycle (Bio-Rad Laboratories, Inc. [Bio-Rad]) was used for all molecular biology manipulations. Sterile molecular biology work was performed under a Bunsen burner. Room temperature (RT) was considered to be 22 – 25°C. Sterile filtered water (Cat. W3500; Sigma) was used for small volumes of samples and reagents, whereas distilled water was used for reagents exceeding 100 mL; both will be referred to as dH_2O.

2.2.1 Growth Conditions and Storage

2.2.1.1 Bacteria

Bacterial strains were grown aerobically at 37°C overnight (16 hours [h]). Suspension cultures were grown in Lennox lysogeny broth (LB) media (Cat. L3022; Sigma-Aldrich [Sigma]) – containing 10 g of tryptone, 5 g of yeast extract and 5 g of sodium chloride (NaCl) per litre of dH_2O – with shaking at 200 revolutions per minute (rpm) with the appropriate antibiotics. Alternatively, sterile 10 cm diameter petri dishes containing Lennox LB agar (Cat. L2897; Sigma) composed of LB broth with 15 g of agar per litre of dH_2O were used, also with the appropriate antibiotics.

NEB® 10-beta Competent *Escherichia coli* (*E. coli*) (ampicillin resistant [Amp^r]; Cat. C3019; New England BioLabs Inc. [NEB]) was used for high efficiency transformation.

2.2.1.2 Human Cell Lines

Human cell lines (Table 2.1) were passaged in sterile, 25 cm^2 (T25), 75 cm^2 (T75) or 225 cm^2 (T225) Corning® tissue culture-treated flasks with flat, orange, vented caps (Cat. CLS430639, CLS430641 or CLS431082; Sigma). Sterile Gibco® 1X Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L D-glucose, L-glutamine and a phenol red pH indicator (Cat. 41965-039; Thermo) – with 10% Gibco™ heat-inactivated foetal bovine serum (FBS; qualified, EU-approved, South America origin; Cat. 10500-064; Thermo) and Gibco® 1X minimum essential medium non-essential amino acids (NEAA; Cat. 11140-050; Thermo) added – at 37°C with 5% CO_2. Unless stated otherwise, this preparation of DMEM was used in all situations. Cell centrifugation was performed at 300 x g for 7 min.

Table 2.1 – Human Cell Lines Used in This Study

Cell Line	Use	Cat. (If Available); Source
Human Embryonic Kidney Cells (HEK) 293T ATCC® CRL-3216™	Adherent cell line stably transfected with the SV40 large T-antigen, which binds to the SV40 origin of replication that is often engineered into expression vectors, thus increasing protein expression.	MRC-University of Glasgow Centre for Virus Research.
Human Hepatoblastoma 7 (Huh7) Cells	Adherent cell line highly permissive for HCV and EBOV infection and replication, thus allowing use as a host for a measure of HCVpp entry and infectivity.	Nakabayashi et al. (1982). Medical Research Council – University of Glasgow Centre for Virus Research.
Human Hepatoblastoma 7.5 (Huh7.5) Cells	Adherent cell line with further enhanced permissiveness for HCV replication, conferred by a single mutation in the RIG-I open reading frame (Sumpter et al., 2005), thus allow use for HCVcc propagation.	Blight et al. (2002). The Rockefeller University, USA.

2.2.1.2.1 Passage of Human Cell Lines

Growing cell stocks were passaged at a density of 6 x 10^6 in 50 mL of DMEM using a T225 flask every 72 – 96 h until 90% confluence was reached. Briefly, this process involved aspiration of media following by a wash using 8 mL of Dulbecco's phosphate buffered saline (PBS; modified, without calcium chloride or magnesium chloride, sterile-filtered; Cat. D8537; Sigma) before aspiration. Adherent cells were dissociated from the flask by incubating cells with 8 mL of Gibco® trypsin-EDTA (0.05%, phenol red; Cat. 25300-062; Thermo) at 37°C (5% CO_2) for 5 min. DMEM was added up to 50 mL to inactivate trypsin. Three cell counts were averaged using a haemocytometer and extrapolated to determine total cell count. Cells were centrifuged and resuspended in 10 mL of DMEM, before the appropriate concentration of cells/mL was used.

2.2.1.2.2 Freezing and Thawing of Human Cells

Cell storage in liquid nitrogen prevents mutation and contamination therefore all cells lines had frozen aliquots. Cells were suspended at a density of 5 x 10^6 – 2 x 10^7 in 1 mL of freezing media (DMEM containing 10% FBS, 10% dimethyl sulfoxide [Hybri-Max™ sterile-filtered; Cat. D2650; Sigma]) in cryovials, to prevent cell damage by freezing (Lovelock and Bishop, 1959). Using a Mr. Frosty™ Nalgene Freezing Container (Cat. 1562, Sigma) containing 100% isopropanol, vials were stored at -80°C overnight for cell freezing at a controlled rate of -1°C/min. Vials were then rapidly transferred to storage at -196°C in liquid nitrogen.

Frozen cryovials were thawed in a water bath set to 37°C for 2 min until defrosted, before the drop-wise addition of 10 mL of DMEM, in order to prevent osmotic damage. Cells were transferred to a T25 flask, incubated at 37°C (5% CO_2) for 24 h and passaged before seeding at a density of 2 x 10^6 cells/mL in 20 mL of DMEM in a T75 flask. After incubation for 48 – 72 h, cells were passaged into 50 mL of media into a T225 flask at the standard cell density.

2.2.1.3 Antibiotics

Antibiotics were stored at -20°C. Ampicillin (Cat. A5354; Sigma) was dissolved in dH_2O and used at 100 µg/mL. Penicillin-Streptomycin (Pen-Strep; Cat. 15140-122; Thermo) was used at 100 µg/mL.

2.2.2 DNA Cloning

2.2.2.1 Plasmids

Plasmids were stored at -20°C. Glycerol stocks for long-term storage at -80°C were made by growing transformed *E. coli* in selective LB broth overnight before freezing in 25% [v/v] glycerol (≥99.5%; Cat. G9012; Sigma).

Gateway™ pcDNA™-DEST40 (Thermo; Amp^r) plasmids encoding C-terminal His_6-tagged L-ficolin-WT, L-ficolin-T236M, L-ficolin-A258S and L-ficolin-T236M/A258S were previously developed by the Virus Research Group (VRG) at the University of Nottingham. pcDNA™3.1D/V5-His-TOPO® plasmid (Cat. K490040; Thermo; Amp^r) was used in the cloning of N-terminal His_6-tagged L-ficolin variant constructs, for CMV promoter-enhanced protein expression. Plasmids used for production of virus pseudoparticles are summarised in Table 2.2.

2.2.2.2 Primers

Primers (Table 2.3) were synthesised by Eurofins Genetic Services Ltd and stored at -20°C.

Table 2.2 – List of Plasmids Used for Pseudoparticle Production in This Study

Plasmid	Properties	Use	Source
Retroviral Core and Reporter Proteins			
phCMV5349	Encodes the Murine leukaemia virus (MLV) Gag-Pol structural and enzymatic genes. Amp^r.	CMV promoter-enhanced expression of MLV proteins encoded by gag-pol genes, which act as pseudoparticle backbone.	Centre International de Recherche en Infectiologie, École Normale Supérieure de Lyon, France.
pTG126	Encodes firefly luciferase reported protein. Amp^r.	When co-expressed with phCMV5349, the luciferase reporter gene is encapsulated in the pseudoparticle for detection via luminescence.	
pNL4-3.Luc.R^-E^-	pUC19-derived plasmid containing genes of NL4-3 strain of HIV-1, with ΔEnv and ΔVpr genotype, and firefly luciferase gene inserted into the *nef* gene; ~16500 bp, Amp^r.	Encodes both backbone and reporter plasmids for use in pseudoparticle expression.	He et al. (1995), NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

Virus Envelope Glycoproteins			
H77.20	E1E2 glycoprotein of H77c genotype 1 strain of HCV cloned into pcDNA™3.1D/V5-His-TOPO®.	Encodes glycoprotein plasmids which, when co-expressed with retroviral backbone and reporter plasmids, produce virus pseudoparticles to study entry and infectivity.	Tarr et al. (2011).
VSV	VSV G glycoprotein of Vesicular Stomatitis Virus.		Didier Trono (École Polytechnique Fédérale de Lausanne, Switzerland)
pCAGGS:SEBOV	GP glycoprotein of Sudan strain of EBOV.		Public Health Agency of Canada's National Microbiology Laboratory.
pCAGGS:ZEBOV	GP glycoprotein of Mayinga Zaire strain of EBOV.		
pCAGGS:REBOV	GP glycoprotein of Reston strain of EBOV.		

Table 2.3 – List of Primers Used in This Chapter

Forward represents 5' to 3'. Reverse represents 3' to 5'.

Primer Name	Orientation	Primer Sequence (5'→3')	Size/ bp
General Primers			
T7F	Forward	TAATACGACTCACTATAGGG	20
BGHR	Reverse	TAGAAGGCACAGTCGAGG	18
Cloning of N-Terminal His_6-Tagged L-Ficolin Variants			
FCN2 Signal Peptide	Forward	CACCCAGCGAGTCAACACCATGGAGCTGGACAGAG	35
	Reverse	ATGGTGATGGTGATGATGAGCCCAGGCCATGC	32
FCN2 Mature Peptide	Forward	CATCATCACCATCACCATCTCCAGGCGGCAGACACCTGTCCAG	43
	Reverse	GCCGCCTGTCTATCCTCACTCTCTAGGCAGGTCGCACCTTCATC	44
Custom *FCN2* Tag	Forward	CACCCAGCGAGTCAACACC	19
	Reverse	GCCGCCTGTCTATCCTCACTCT	22

2.2.2.3 Polymerase Chain Reaction

For DNA amplification, a typical PCR reaction in 0.2 mL thin-walled PCR tubes consisted of 1 µL of plasmid template diluted 1:100, 1.25 µL each of forward and reverse primers (8 µM), 0.5 µL of 10 mM dNTP and 0.25 of Q5® Hot Start High-Fidelity DNA Polymerase (2000 Units mL^{-1}; Cat. M0493; NEB) in Q5 reaction buffer, topped up to a total volume of 25 µL with nuclease-free distilled dH_2O. The reaction involved initial denaturation at 98°C for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at the appropriate temperature for 20 sec and extension at 72°C for 60 sec. A final extension was performed at 72°C for 120 sec.

For colony PCR, a typical 12.5 µL PCR reaction consisted of an *E. coli* colony as template, 0.5 µL each of T7F forward and BGHR reverse primers (10 µM), 0.5 µL of 10 mM dNTP and 0.06 of JumpStart™ *Taq* DNA Polymerase (2.5 Units μL^{-1}; Cat. D9307; Sigma) in 10X buffer, topped up with nuclease-free distilled dH_2O. Colony PCR reaction involved initial denaturation at 94°C for 60 sec, followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 90 sec. A final extension was performed at 72°C for 120 sec.

2.2.2.4 Agarose Gel Electrophoresis

A 2% [w/v] agarose gel was prepared by heating and dissolving agarose in 1X Tris acetate-EDTA (TAE) buffer (10X, pH 8.3, non-sterile, filtered; Cat. T9650; Sigma). Once cooled to approximately 50°C, ethidium bromide (Electran®, 10 mg/mL; Cat. 443922U; VWR International [VWR]) was added at 0.5 µg/mL, before solidifying in a sealed casting tray. The gel was immersed in 1X TAE buffer in a gel tank. A maximum of 10 µL of sample was mixed with DNA gel loading dye (6X; Cat. SM0333; Thermo), and 3.5 µL of GeneRuler DNA ladder mix (0.1 µg/mL; Cat. SM0333; Thermo) was used. The gel was run at 90 volts (V) for 36 – 42 min. DNA bands were visualised using an ultraviolet (UV) light transilluminator and captured using the HeroLab video capture printer.

2.2.2.5 Restriction Enzyme Digestion

The DpnI restriction enzyme (20,000 units/mL; Cat. R0176; NEB) was used to cleave methylated DNA according to manufacturer's instructions, in which 0.5 – 1 µL of enzyme was added to up to 50 µL of DNA sample and incubated at 37°C for 1 hr, before inactivation at 80°C for 20 min.

2.2.2.6 DNA Purification

Purification of linear PCR products and circular plasmids were carried out using the GenElute™ PCR clean-up kit (Cat. NA1020; Sigma), and the GenElute™ HP plasmid minipreparation (miniprep) or midipreperation (midiprep) kits (Cat. NA0150 or NA0200; Sigma), respectively. Manufacturer's instructions were used.

For miniprep, 5 mL bacterial cultures were grown overnight in LB broth (containing relevant antibiotic) before centrifugation at 12,000 x g for 5 min using a microcentrifuge. For midiprep, 70 mL bacterial cultures were used, with centrifugation at 18,000 x g for 10 min using a SLA-1500 rotor (Thermo). All pellets were pooled together in one volume of resuspension solution.

For both PCR and miniprep purification methods, elution volumes varied from 25 – 100 µL. For midiprep, elution volumes ranged from 700 – 1000 µL. DNA concentrations ($OD_{260/280\ nm}$) were measured using a NanoDrop® spectrophotometer (Thermo) and ND-1000 v3.7.1 software (Thermo).

2.2.2.7 Ligation

Ligation into the pcDNA™3.1D/V5-His-TOPO® plasmid was performed at 22°C for 20 min before being placed on ice for use in transformation. Using the manufacturer's protocol, the 3 µL reaction used 0.5 µL plasmid, 0.5 µL salt solution, 0.5 µL dH_2O and 1.5 µL of purified PCR product.

2.2.2.8 Transformation of Competent Bacterial Cells

Into 17.5 µL, 25 µL or 50 µL of chemically competent *E. coli* cells, 0.5 µL, 1.0 µL or 1.5 µL of plasmid was transformed. Briefly, plasmid was added to cells and incubated on ice for 30 min. Heat shock was performed at 42°C for 30 sec before immediate incubation on ice. To the cells, 250 µL of SOC medium was added, followed by incubation at 37°C for 1 hr with shaking at 200 rpm. Next, 100 µL of the transformed cell mixture was plate on an LB agar plate overnight at 37°C. Resulting colonies were analysed for the correct insert by colony PCR.

2.2.2.9 DNA Sequencing and Analysis

DNA sequencing was performed through the Source BioScience service using the PeakTrace™ basecaller. Sequencing primers were used at an amount of 3.2 pmol. Analysis was performed using Chromas 2.4.3 (Technelysium), Lasergene 11.2.1 SeqMan (DNASTAR) and MEGA 7.0.26 software.

2.2.3 Transient Transfection of HEK 293T Cells

2.2.3.1 Virus Pseudoparticle Production

Virus pseudoparticles were produced as previously described (Bartosch et al., 2003) and shown in Figure 1.5. Typically, HEK 293T cells were seeded at a density of 1.2 x 10^6 in DMEM (10% FBS, 1X NEAA) in a sterile 10 cm diameter Corning® Primaria™ standard dish (Cat. 734-0072; VWR) and incubated overnight to achieve 50% confluency. Cells were co-transfected with 2000 ng of backbone/reporter plasmid and virus envelope glycoprotein plasmid. Plasmids encoding HCV and VSV glycoproteins were transfected at 2000 ng, whereas EBOV glycoproteins were transfected at 200 ng.

A total volume of 300 µL of room temperature Gibco™ Opti-MEM™ I Reduced Serum Medium (Opti-MEM; Cat. 31985-054; Thermo) was spiked with the plasmids. To the plasmid mix, 300 µL of Opti-MEM containing the linear cationic transfection reagent polyethylenimine (PEI; 40 µg/mL; Cat.

23966-1; Polysciences, Inc.) was added and mixed thoroughly. The plasmid-PEI mixture was incubated at RT for 1 h. Media on the incubated cells was replaced with 7 mL of Opti-MEM and the plasmid-PEI mixture was added in a drop-by-drop manner, before gentle rocking side-to-side three times. The cells were incubated at 37°C (5% CO_2) for 6 h before replacement of media with 10 mL of DMEM. After 72 – 96 h of further incubation, the resulting media containing the secreted pseudoparticles was syringe-filtered using a 0.22 µm filter and stored at 4°C for up to a month. A ΔE negative control was produced with every batch, in which the glycoprotein plasmid was omitted from the expression process.

2.2.3.2 Protein Expression in Mammalian Cells

This was achieved in a similar manner to section 2.2.3.1, using only a desired protein expression plasmid. Protein was expressed either in 10 cm diameter dishes or T225 flasks. For the latter, HEK 293T cells were seeded at a density of 10 x 10^6 in 28 mL of DMEM (10% FBS, 1X NEAA) overnight, before transfection of 8000 ng plasmid in a total of 2400 µL of Opti-MEM. The plasmid-PEI mixture was added to 28 mL of Opti-MEM media for the 6 h incubation stage, and replaced with 40 mL of media prior to the 72 h incubation stage.

2.2.4 Characterisation of Virus Pseudoparticles

2.2.4.1 Pseudoparticle Infectivity Assay

A Costar® flat-bottomed, tissue culture-treated, 96-well white plate (Cat. 3917; Corning Inc.) was seeded with 1.5 x 10^4 Huh7 cells/well in 100 µL of DMEM. After overnight incubation, media was replaced with 100 µL of pseudoparticles per well in triplicate. After 6 h of incubation, 150 µL of DMEM containing Pen-Strep was added, followed by a further 72 h of incubation.

2.2.4.2 Virus Pseudoparticle Neutralisation Assay

This was similar to section 2.2.4.1, however the protein of interest was mixed with a pseudoparticle preparation in a total of 300 µL, in a V-bottomed, gamma-irradiated, 96-well Sterilin™ clear microtitre™ plate (Cat. 612V96; Thermo). Further serial dilutions were made if required. The protein-pseudoparticle mixtures were incubated at RT for 1 h before 95 µL of preparation was added to the Huh7 cells (with aspirated media) in triplicate, to continue at the 6 h incubation stage. A positive control of the pseudoparticle of interest in the presence of a 1:10 dilution of 1X PBS and a non-infectious ΔE negative control lacking glycoprotein were consistently used.

2.2.4.3 Measurement of Luminescence

Following an assay, the luciferase reporter gene system of pseudoparticles was exploited to measure pseudoparticle entry. Media was aspirated from wells and dried by tapping. To each well, 50 µL of 1X luciferase cell culture lysis reagent (5X; Cat. E153A; Promega) was added and incubated at RT for 15 min with rocking at 15 rpm. Next, 50 µL of luciferase assay substrate (Cat. E151; Promega) was added to each well for 5 min. Using a FLUOstar Omega microplate reader (BMG Labtech), luminescence was measured at a wavelength of 420 nm with a photomultiplier optical gain of 3600. When VSVpp was produced using the pNL4-3.Luc.R^-E^- backbone, luminescence was measured at a gain of 1800 to avoid optic bleaching.

2.2.5 Protein Detection

Proteins were stored at 4°C for short-term storage or -20°C for long-term storage.

2.2.5.1 Antibodies Used

Antibodies (Table 2.4) were stored at 4°C or -20°C, according to manufacturer's instruction.

2.2.5.1.1 Biotinylation of Antibodies

Antibody biotinylation was performed using EZ-Link® sulfo-NHS-LC-biotin (100 mg, Cat. 21335; Thermo), according to manufacturer's protocol. Briefly, 0.2 µL of 10 mM biotin was added to 150 µL of 100 µg/mL anti-L-ficolin monoclonal antibody GN5 and incubated at RT for 1 h. The sample was centrifuged at 12,000 x g for 10 min using a Millipore Amicon® Ultra 0.5 mL 3 kDa molecular weight cut off (MWCO) centrifugal filter unit (Sigma) to remove biotin, and resuspended in a final volume of 150 µL of 1X PBS.

2.2.5.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Precast 15 µL 15-well 10% Mini-PROTEAN® TGX™ sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Cat. 456-1036; Bio-Rad) were used, running 13 µL protein sample at 160 V for 50 min. Samples were denatured by heating in 2X non-reducing SDS-PAGE loading dye (50 mM Tris-HCl [pH 7], 5% [w/v] SDS (Cat. L4390; Sigma), 20% [v/v] glycerol, bromophenol blue) at 100°C for 10 min. Protein ladders used were ColorPlus™ prestained protein ladder (broad range, 10 – 230 kDa; Cat. P7711; NEB) and Thermo Scientific™ Spectra™ multicolor broad range protein ladder (10 – 260 kDa; Cat. 26634; Thermo). 1X running buffer containing 192 mM glycine (Cat. BP381-1; Fisher Bioreagents), 25 mM Tris base (Cat. T1503; Sigma) and 0.1% [w/v] SDS was used. Gel staining was performed using Dodeca™ Silver Stain Kit (Cat. 1610480; Bio-Rad).

Table 2.4 – List of Antibodies Used in This Chapter

Working concentrations may vary. WB and IF stand for western blot and immunofluorescence imaging, respectively.

Antibody	Specificity	Clonality	Developed in	Stock Concentration/ µg/mL	Working Concentration/ µg/mL (or Dilution)			Cat. (If Available); Supplier
					WB	ELISA	IF	
Primary Antibody	Anti-Human L-Ficolin GN4	Monoclonal	Mouse	100	0.5	1		HM2090: Hycult® Biotech.
	Anti-Human L-Ficolin GN5	Monoclonal	Mouse	100	0.5	1	1	HM2091; Hycult® Biotech.
	Anti-Polyhistidine Tag	Polyclonal	Rabbit		1:200			ab5000; abcam.
	Anti-HCV E1E2 AP33	Monoclonal	Mouse	150		1		Centre for Virus Research, University of Glasgow, UK
	Anti-fd Bacteriophage	Polyclonal	Rabbit			1:1000		B7786; Sigma.

Antibody	Specificity	Clonality	Developed in	Stock Concentration/ µg/mL	Working Concentration/ µg/mL (or Dilution)			Cat. (If Available); Supplier
					WB	ELISA	IF	
Secondary Antibody	Anti-Mouse Immunoglobulins (Ig)-Horse Radish Peroxidase (HRP) Conjugate	Polyclonal	Rabbit	1300	1.3			P0161; Dako.
	Anti-Rabbit Immunoglobulins-HRP Conjugate	Polyclonal	Pig	340	0.34			P0399; Dako.
	Anti-Mouse IgG (Fc Specific)-Alkaline Phosphatase (AP) Conjugate	Polyclonal	Goat			1:1000		A1418; Sigma.
	Anti-Rabbit IgG (Whole Molecule)-Alkaline Phosphate	Polyclonal	Goat			1:2000		A2556; Sigma.
	Streptavidin-AP Conjugate					1:1000		AR001; R&D Systems.
	Anti-Mouse IgG (H+L) Alexa Fluor 488	Polyclonal	Goat				1:1000	A11001; Invitrogen.

2.2.5.3 Western Blot

An SDS-PAGE gel was assembled next to a GE Healthcare Hybond® enhanced chemiluminescent (ECL)™ nitrocellulose membrane (Cat. GERPN303D; Sigma), encased either side by three pieces of filter paper, and placed on a Transblot® SD semi-dry transfer cell (Bio-Rad). All components were soaked in 1X transfer buffer (1.92M glycine, 25 mM Tris base, 0.5% [w/v] SDS, 20% [v/v] methanol [Cat. 32213; Sigma]). The membrane was placed closest to the positive electrode. Transfer was run at 100 mA per gel for 1 h.

The membrane was blocked using 50 mL of 1X PBS (Cat. BR0014; Oxoid) with 0.05% [v/v] TWEEN® 20 (Tween 20; Cat. P9416; Sigma) (PBST) and 5% [w/v] non-fat milk (Marvel) (PBSTM) at RT for 1 h at 10 rpm. Tween 20 prevents background staining and non-specific protein interactions. The membrane was immersed in 50 mL of PBSTM containing primary antibody, and incubated at 4°C overnight. The membrane was washed three times in 20 mL of PBST for 5 min with rocking at 30 rpm. Next, the membrane was immersed in 50 mL of PBST containing secondary antibody-HRP conjugate at RT for 1 h with rocking, followed by three washes.

The membrane was immersed in GE Healthcare Amersham™ ECL western blotting detection reagents (Cat. 10340125; Fisher) for 5 – 8 min. The membrane was placed between two acetate sheets, with bubbles removed, and compressed against Amersham Hyperfilm ECL (8 × 10"; Cat. 28906838; Scientific Laboratory Supplies Ltd. [SLS]) in dark conditions. The film was developed using GE Healthcare Amersham™ Hyperfilm™ ECL high performance chemiluminescence film (Cat. 10479214; Fisher), Carestream® Kodak® autoradiography GBX developer/replenisher and fixer/replenisher (Cat. P7042 and P7167; Sigma).

2.2.6 Protein Purification and Concentration

Protein samples were stored in low-binding Eppendorf tubes.

2.2.6.1 Nickel Affinity Chromatography

An ÄKTAPrime system (GE Healthcare) was used according to manufacturer's instructions. Absorbance (OD_{280nm}) of UV light and conductivity were monitored using PrimeView software. Injection was achieved using a Superloop (GE Healthcare). Flow-through and elution fractions were collected in 50 mL and 1 mL fractions, respectively.

Briefly, His_6-tagged protein sample was filtered, buffered to 300 mM of NaCl and 20 mM of imidazole (Cat. I2399; Sigma), and injected into a GE Healthcare HisTRAP™ HP nickel sepharose high performance 5 mL column (Cat. 17524802; SLS) previously primed with buffer A (300 mM NaCl, 20 mM imidazole, 20 mM NaH_2PO_4, 20 mM Na_2HPO_4; pH 7.4). The column was washed, followed by elution using buffer B (300 mM NaCl, 500 mM imidazole, 20 mM NaH_2PO_4, 20 mM Na_2HPO_4; pH 7.4) and a final wash. All buffer volumes used were five-column volumes.

2.2.6.2 Protein Concentration by Centrifuge Filtration

Protein was concentrated in EMD Millipore Amicon® Ultra 15 mL 10 kDa or 50 kDa MWCO centrifugal filter units (Cat. 10781543 or 10550443; Fisher) according to manufacturer's instructions, at 5000 x g for 10 min at a time in a swinging bucket rotor. Sample was retrieved by aspiration.

2.2.6.3 Buffer Exchange by Centrifuge Filtration

Protein was applied to a Millipore Amicon® Ultra 0.5 mL 3 kDa MWCO centrifugal filter units (Cat. Z677094; Sigma) according to manufacturer's instructions. This method of dialysis was used in all cases unless stated otherwise.

2.2.7 Protein Characterisation

2.2.7.1 PNGase F Digestion

Flavobacterium meningosepticum PNGase F (Cat. P0705; NEB), with specificity for *N*-linked glycans, was used according to manufacturer's instruction. Using 1 – 20 µg of protein sample, the sample was denatured by heating at 100°C for 10 min in the presence of 10X glycoprotein denaturation buffer. Sample was digested with PNGase F at 37°C for 1 h in the presence of 10% NP-40 and 10X G7 reaction buffer. The enzyme was inactivated by heating at >75°C for 10 min.

2.2.7.2 Enzyme-Linked Immunosorbent Assays

Indirect enzyme-linked immunosorbent assays (ELISAs) were performed in non-sterile Nunc-immuno™ Maxisorp™ flat-bottomed, clear 96-well polystyrene plates (Cat. 442404; Thermo). Incubation steps were performed using a moist chamber to prevent evaporation. Samples were added in duplicate. Samples and antibodies were diluted in TBST- Ca, prepared using Tris-buffered saline (TBS) tablets (pH 7.6; Cat. 94158; Sigma) with 0.05% Tween 20 [v/v] and 5 mM calcium chloride ($CaCl_2$; AnalaR; Cat. 22317.460; VWR). Controls used wash buffer to replace certain components of the ELISA, such as sample or antibody.

Wells were coated with the relevant coating reagent or antibody at 4°C overnight. The plate was blocked using 200 µL of PBSTM at RT for 2 h. Wells were washed three times using 200 µL of wash buffer. Wash buffer consisted of TBST-Ca. Next, 50 µL of samples was added and incubated at 4°C overnight. After three washes, 50 µL of primary antibody was added at RT for 2 h, followed by three more washes and incubation with 50 µL of secondary antibody at RT for 1 h. Finally, wells were washed three times and 100 µL of 1 mg/mL SIGMAFAST™ *p*-nitrophenyl phosphate (pNPP; Cat. N2770; Sigma) substrate was added for 15 – 60 min.

2.2.7.2.1 Protein Quantification using Anti-L-Ficolin mAb GN4

By adapting a protocol used by Hummelshøj et al. (2005), 1 µg/mL of anti-L-ficolin mAb GN4 was used to capture and quantify L-ficolin. Biotinylated anti-L-ficolin mAb GN5 and streptavidin-AP conjugate were used as primary and secondary antibodies, respectively (Figure 2.1.1).

For the purposes of interpolation of L-ficolin concentration, a serum sample of a healthy donor known to contain 4 µg/mL of L-ficolin was used in an eight-point serial dilution diluted two-fold, typically starting with a 1:3.125 or 1:6.25 dilution. This sample was kindly provided by Dr Anders Krarup (MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford). Standard curves using serum L-ficolin were fitted with a sigmoidal, four parameter logistic (4PL) regression for interpolation of L-ficolin concentration.

2.2.7.2.2 Acetylated BSA ELISA

To coat, 50 µg/mL of Ambion™ acetylated bovine serum albumin (AcBSA; Cat. AM2614; Invitrogen) was diluted in 1X PBS. For the detection of L-ficolin, anti-L-ficolin mAb GN5 and anti-mouse Ig-HRP conjugate were used as primary and secondary antibodies (Figure 2.1.2). The IC_{50} of inhibitors of AcBSA binding was determined by robustly fitting data with a log(inhibitor) vs. response (three parameters) non-linear regression.

2.2.7.3 Protein Quantification using Bicinchoninic Acid Assay

The Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Cat. 23227; Thermo) was used according to manufacturer's instructions, using nine provided bovine serum albumin (BSA) size standards between 2000 and 0 µg/mL in duplicate. Following incubation at 37°C for 30 min in a moist chamber, $OD_{562\ nm}$ was measured using the FLUOStar Omega plate reader. Standard curves were fitted with a linear regression for interpolation of total protein concentration.

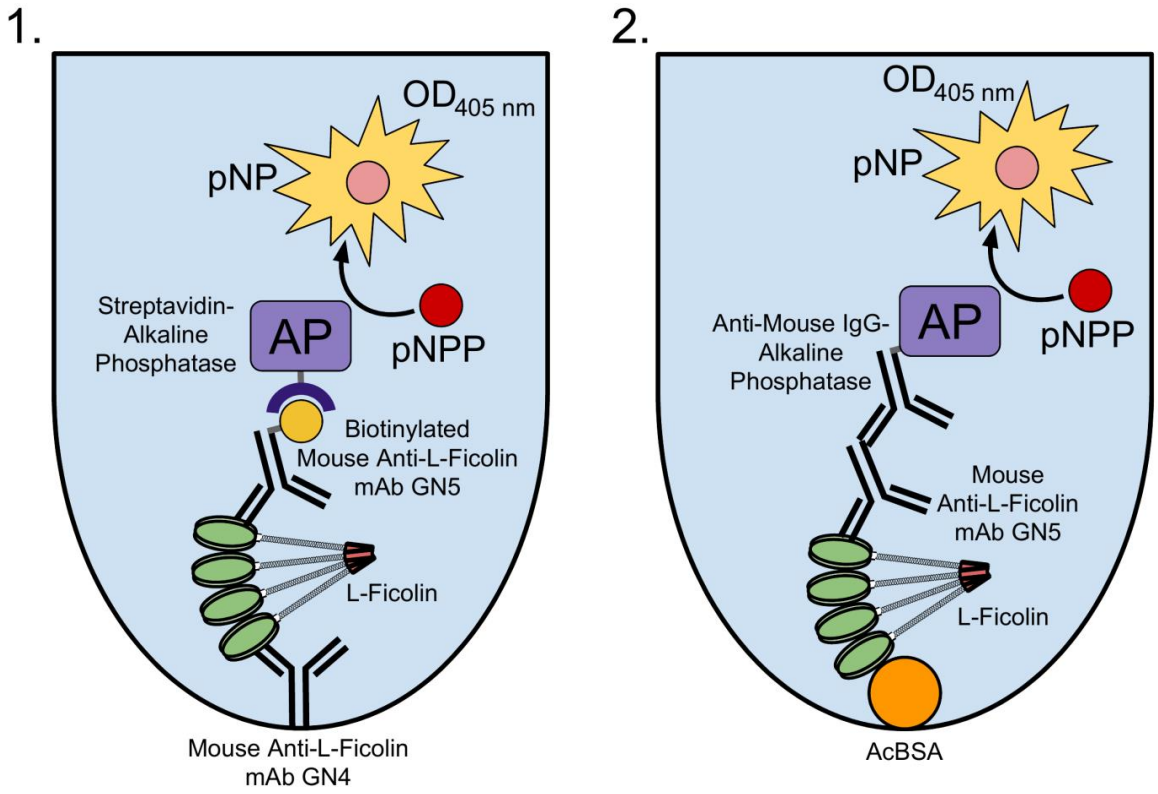

Figure 2.1 – Schematic of Enzyme-Linked Immunosorbent Assays Used in This Chapter

The schematics above represent one of several identical ELISA interactions in a plate well. ***1.*** *The L-ficolin-specific monoclonal antibody (mAb) GN4 is used to capture L-ficolin, which is in turn detected by biotinylated anti-L-ficolin mAb GN5. The biotin is bound by streptavidin conjugated to alkaline phosphatase (AP), which converts* p*-nitrophenyl phosphate to* p*-nitrophenol and omits light which can be measured at* $OD_{405\ nm}$ *to correlate to the amount of bound L-ficolin.* ***2.*** *AcBSA is bound by L-ficolin, which is detected by anti-L-ficolin mAb GN5. This is in turn detected by anti-mouse immunoglobulin-AP conjugate.*

2.2.7.4 Immunofluorescence Imaging of L-Ficolin

In order to visualise the protein production in cells, immunofluorescence (IF) imaging was used. Following L-ficolin expression in HEK 293T cells, cells were passaged and resuspended in 1X PBS with 2% BSA (pH 7; Cat. A7906; Sigma). Next, 3 µL of cells at a density of 15 x 10^6 were seeded onto a 24-spot PTFE/Teflon®-coated microscope slide (TEKDON, Inc.). Slides were air-dried and fixed with either cold 100% acetone or a 1:1 ratio [v/v] of 100% acetone and 100% methanol at RT for 10 min. The dried slides were stored at -80°C.

Frozen slides were brought to RT and air-dried. Incubations were performed in a moist chamber. Slides were blocked with 10 µL of 1X PBS (2% BSA) at RT for 1 h. Following aspiration, 25 µL of 1 µg/mL of anti-L-ficolin mAb GN5 in 1X PBS was used as primary antibody and incubated at RT for 1 h, before aspiration. After washing with 1X PBS in a glass Coplin jar on a stirrer at RT for 5 min, excess 1X PBS was aspirated and the plate was air-dried. Secondary antibody incubation was performed using 25 µL of goat anti-mouse IgG (H+L) Alexa Fluor Plus 488 (Cat. A32723; Thermo). Following washing, counter-staining was performed by immersing the slides in 0.04 µg/mL of Molecular Probes™ 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI; Cat. 11530306; Fisher) for 2 min in a Coplin jar. After a final wash, a spot of VECTASHIELD Antifade Mounting Medium (Cat. H-1000; Vector Labs) was applied to the well and covered with a glass slip, which was immobilised using nail varnish. Slides were stored in a dark box at RT.

Cells were visualised using a Leica DMRB upright fluorescence microscope, using DAPI and fluorescein isothiocyanate (FITC) filters, and images were processed using ImageJ software (NIH). As images were captured using identical settings such as aperture, 10X zoom and others, the brightness/contrast of images in each dataset were equalised for each filter for comparison.

2.2.7.5 Biopanning

Biopanning was kindly performed by Dr Abdulrahim Hakami of the VRG, University of Nottingham. The Ph.D™-12 Phage Display Peptide Library Kit (Cat. E8110S; NEB) and *E. coli* strain K12 ER2738 (Cat. E4104; NEB). The phage library consisting of ~1 x 10^9 12-amino acid sequences fused to bacteriophage surface proteins. Briefly, the library was added to plates coated with 10 µg/mL of the anti-L-ficolin mAbs GN4 or GN5. Following washing, the bacteriophage in wells with strong binding to the antibodies were eluted, amplified in *E. coli* and titrated in a plaque assay. This cycle was repeated three times to validate phage clones. DNA was then sequenced and the peptide sequences were aligned with L-ficolin sequence for homology.

2.2.7.5.1 Phage-Detection ELISA

To detect phage bound to a specific antibody, the antibody was used at 2 µg/mL to coat the plate. Phage sample was diluted 1:10 in PBSTM and added to a single well. Anti-fd Bacteriophage and anti-rabbit IgG-AP were used as primary and secondary antibodies, respectively.

2.2.8 Analysis of Protein Characterisation

Neutralisation assay and ELISA data was analysed using GraphPad Prism 6 software (GraphPad Software, Inc.). The p value threshold for significance is 0.05. Molecular weights were predicted using the ExPASy Compute pI/Mw tool (Swiss Institute of Bioinformatics [SIB]). For nucleotide and protein sequence alignments, the Basic Local Alignment Search Tool (BLAST) service (National Centre for Biotechnology Information [NCBI]) was used. L-ficolin sequences were aligned with *Homo sapiens* ficolin-2 (*FCN2*), transcript variant SV0 (GI Number 61744444, Accession Number NM_004108.2). UCSF Chimera 1.12 (Resource for Biocomputing, Visualization and Informatics; University of California, San Francisco) was used for the interactive analysis of protein structures. The SAROTUP database of target-unrelated phages was used during biopanning (Huang et al., 2010).

2.3 Results

2.3.1 Expression of L-Ficolin Variants

To compare the effect of the Thr236Met and Ala258Ser mutations on L-ficolin binding activity, four L-ficolin constructs were produced for comparison in this study (Table 2.5). Due to its small size, ease of purification and potential for up-scaling, an N-terminal His_6-tag was used (Kimple et al., 2013; Zhao et al., 2013). Expression of these constructs was performed using a pre-established HEK 293T cell line designed for enhanced protein expression.

Table 2.5 – The DNA and Amino Acid Configurations of the L-Ficolin Variants Used in This Study

Four recombinant L-ficolin samples with N-terminal His_6-tags were produced, with different combinations of the +6359C>T polymorphism encoding a Thr236Met mutation and the +6424G>T polymorphism encoding an Ala258Ser mutation.

L-Ficolin Variant	SNP at +6359 in *FCN2*		SNP at +6424 in *FCN2*	
	Nucleotide at +6359	Amino Acid at Residue 236	Nucleotide at +6424	Amino Acid at Residue 258
L-Ficolin-WT	C	Thr	G	Ala
L-Ficolin-T236M	T	Met	G	Ala
L-Ficolin-A258S	C	Thr	T	Ser
L-Ficolin-T236M/A258S	T	Met	T	Ser

The four L-ficolin samples were produced by transient transfection of expression plasmid into HEK 293T cells in five T225 flasks using the PEI transfection reagent. Secreted L-ficolin was harvested in 200 mL of serum-free Opti-MEM media supernatant and filtered. A negative control sample was produced using the same process, but omitting an expression plasmid.

L-ficolin samples were prepared using two different paths of production: purified L-ficolin as described in section 2.3.1.1 and non-purified L-ficolin as described in section 2.3.1.2.

2.3.1.1 Production of Purified L-Ficolin

The supernatant was concentrated to 50 mL using a centrifugal filter unit (50 kDa cut-off) and purified by nickel affinity chromatography. L-ficolin that bound to the nickel column by its His_6-tag was eluted using 500 mM imidazole, and was collected in 1 mL fractions. During purification, all samples showed two OD_{280nm} peaks – corresponding to protein concentration – at the 9^{th} and 11^{th} elution fractions (Figure 2.2). This indicates that protein is present even in the negative control. L-ficolin in the corresponding fractions was detected by western blot using the L-ficolin-specific monoclonal antibody GN5. Western blot profiles were similar between variants (Figure 2.3). L-ficolin bands of ~40 kDa, ~80 kDa, ~160 kDa and >260 kDa were detected in the concentrated purification load samples, and from elution fractions 9 upwards. These represent L-ficolin monomers, oligomers and higher order multimers, and were similar to blots in literature of serum L-ficolin and other recombinant L-ficolin. Protein was not lost in the flow-through. No L-ficolin was detected in the negative control. These western blots were representative of all expressions of N-terminal His_6-tagged L-ficolin variants throughout the study.

Fractions containing L-ficolin were pooled in 15 mL and dialysed against 1.0 – 1.5 mL of PBS using centrifuge filtration.

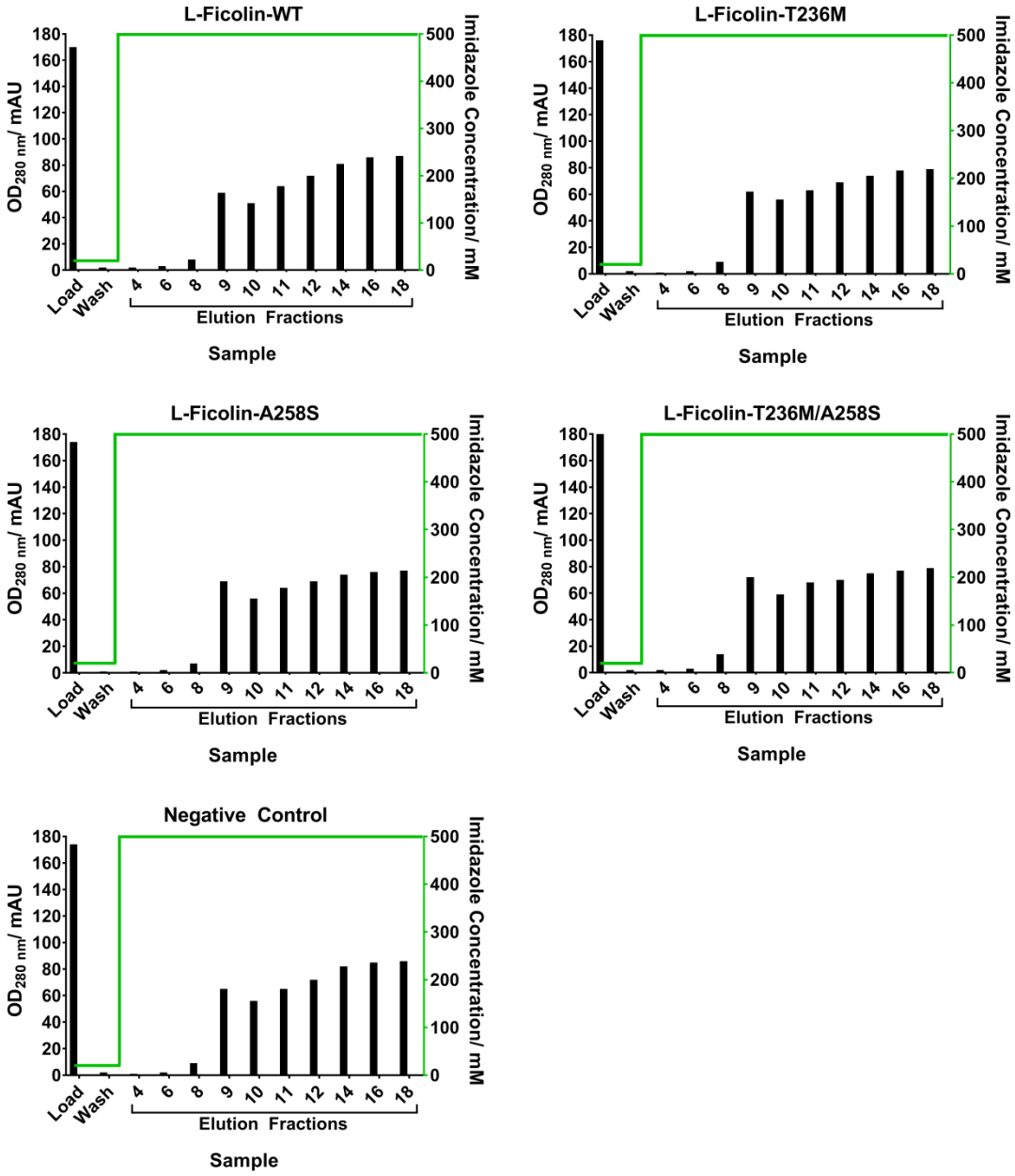

Figure 2.2 – Detection of L-Ficolin Variants during Nickel Affinity Chromatography

L-ficolin variants expressed by HEK 293T and negative control (no expression plasmid) were harvested, concentrated and purified by nickel affinity chromatography using 500 mM imidazole (in green). Protein content was monitored during purification by absorbance (OD_{280nm}). In all samples, protein was detected in the load sample and in elution fractions 9 onwards, with two absorbance peaks of ~65 and ~80 mAU. The graphs are representative of all purifications in this project.

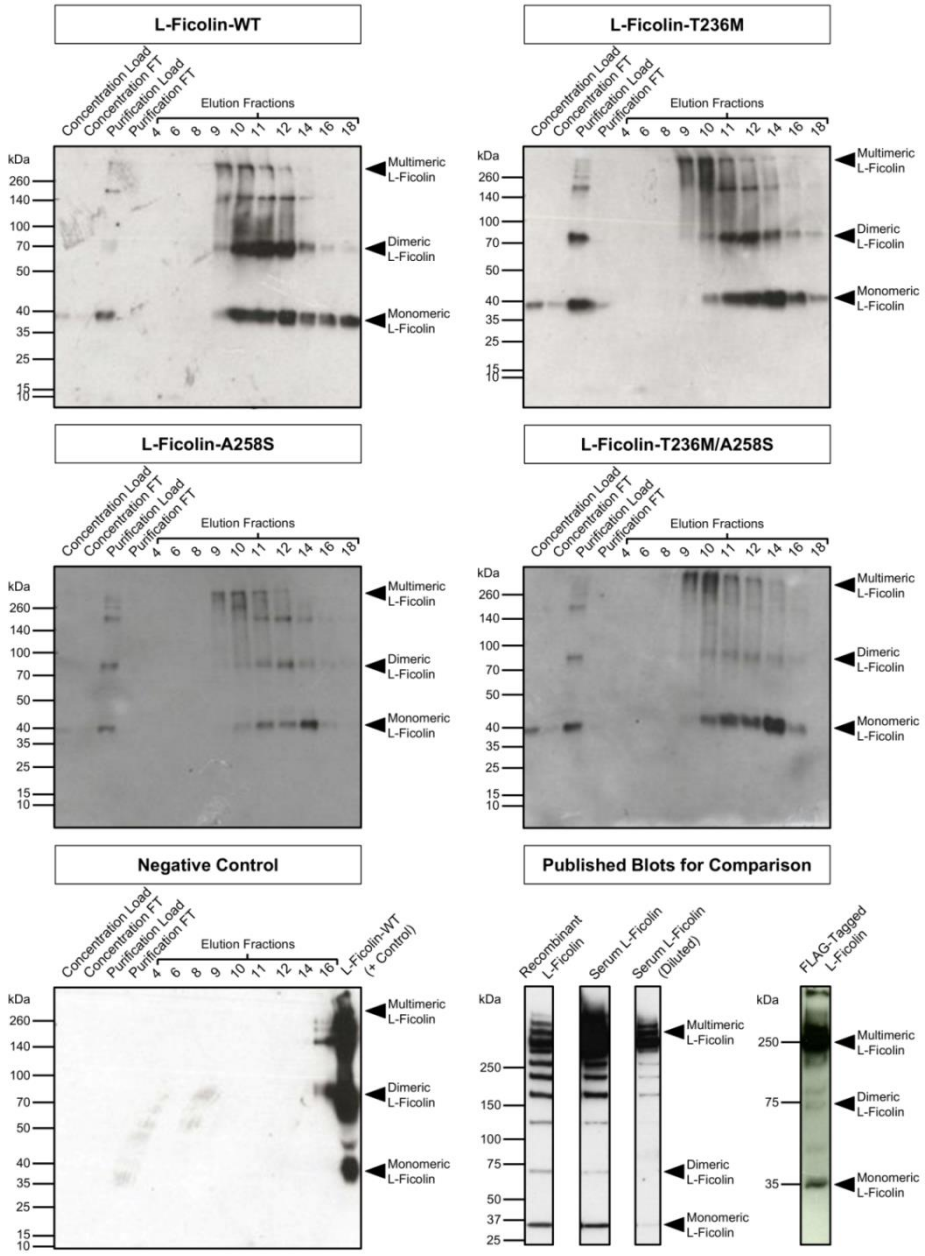

Figure 2.3 – Detection of Purified L-Ficolin Variants

Secreted L-ficolin variants expressed in HEK 293T cells were concentrated and purified by nickel affinity chromatography using 500 mM imidazole. The load material, flow-through (FT) and elution fractions were detected on 10% SDS-PAGE gels using anti-L-ficolin monoclonal antibody (GN5). Elution fractions 9 onwards contain bands of ~40 kDa, ~80 kDa, 160 kDa and > 260 kDa, representing L-ficolin monomer, dimer and higher order multimers. The negative control (transfection without expression plasmid) contained no L-ficolin. Blot profiles were comparable to serum L-ficolin from Hummelshøj et al. (2007) and recombinant FLAG-tagged L-ficolin from Hamed et al. (2014).

2.3.1.2 Production of Non-Purified L-Ficolin

Due to low L-ficolin yields, an alternative approach involved concentrating the 200 mL supernatant to 1.5 mL using a centrifugal filter unit to reduce loss of L-ficolin during purification. Western blots using the anti-L-ficolin mAb GN5 gave similar profiles to the purified western blots, as seen in Figure 2.7.

To confirm that all L-ficolin variants were expressed, HEK 293T cells used to produce the variants were prepared for IF imaging using 100% acetone or acetone:methanol to fix and permeabilise the cells. A mock-transfection HEK 293T negative control was used.

Cells showed consistent DAPI staining of nuclear DNA across all samples (Figure 2.4). L-ficolin-specific FITC signals were detected in all L-ficolin samples, albeit weaker in the L-ficolin-T236M/A258S sample. The negative control sample produced no FITC signal. These results show that L-ficolin was indeed being produced during the protein production process.

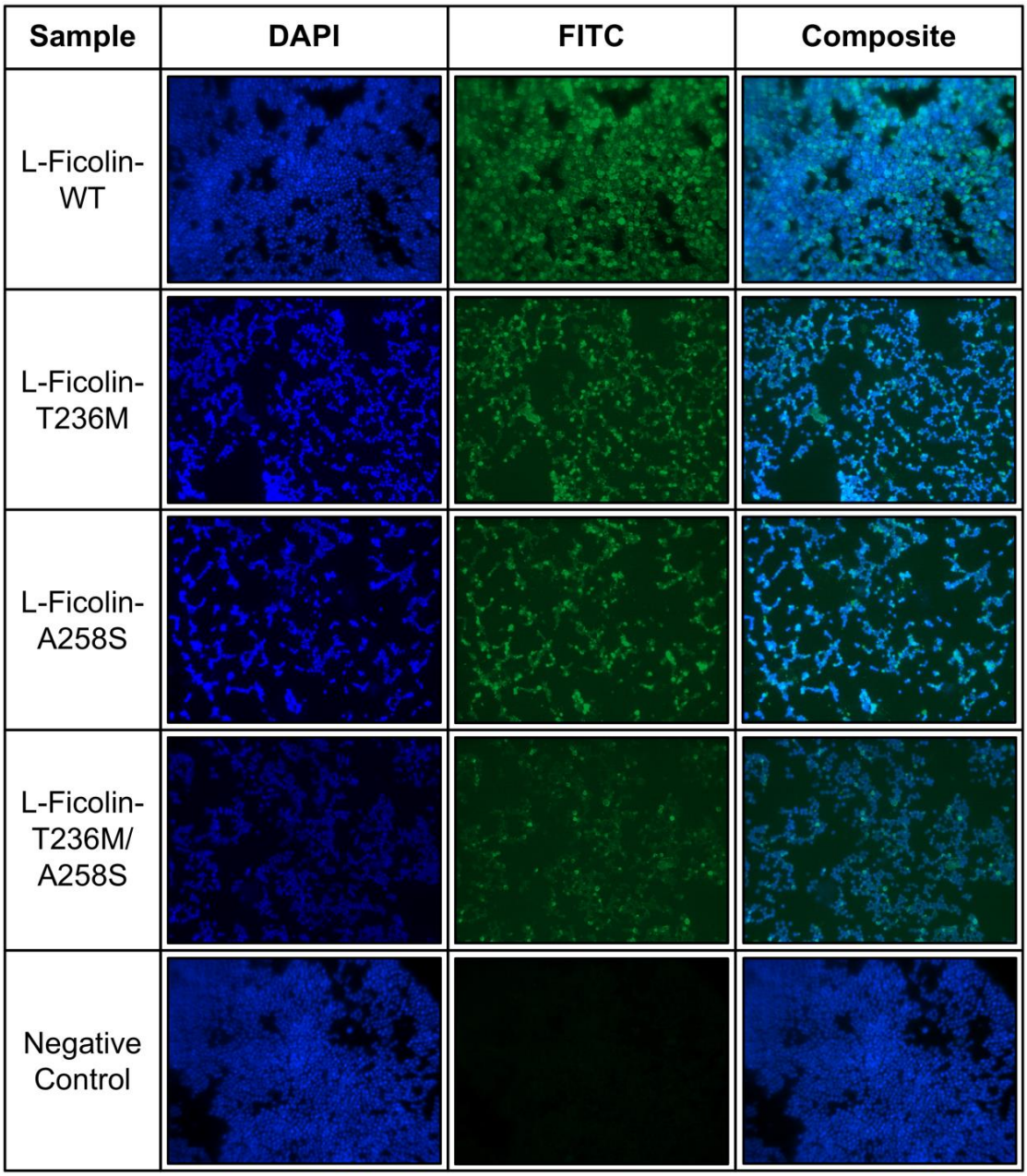

Figure 2.4 – Immunofluorescence Staining of Fixed HEK 293T Cells Producing L-Ficolin

HEK 293T cells used to produce L-ficolin were fixed with acetone (L-ficolin-WT and negative control) or acetone:methanol. A mock-transfection negative control was used. Images were captured using identical aperture, 10X zoom and other settings, and processed using ImageJ software with the brightness/contrast equalised for each filter. DNA-specific DAPI stains were detected in all samples. A FITC signal, mediated by anti-L-ficolin mAb GN5 detection, was present in all samples producing L-ficolin, indicating that L-ficolin production by transient transfection was successful for all variants.

2.3.1.3 Quantification of L-Ficolin Samples

Over the project, L-ficolin samples were quantified using different techniques (Figure 2.5). Total protein concentrations were quantified using a BCA assay, which interpolates protein concentrations using the manufacturer's BSA standard dilution scheme. This assay does not differentiate between L-ficolin and other proteins in the sample. L-ficolin-specific quantification was performed using an established L-ficolin-specific mAb GN4-capture ELISA. A two-fold serial dilution of a serum sample known to contain 4 µg/mL of L-ficolin (1/3.125 to 1/200 dilutions) was used to create a standard curve.

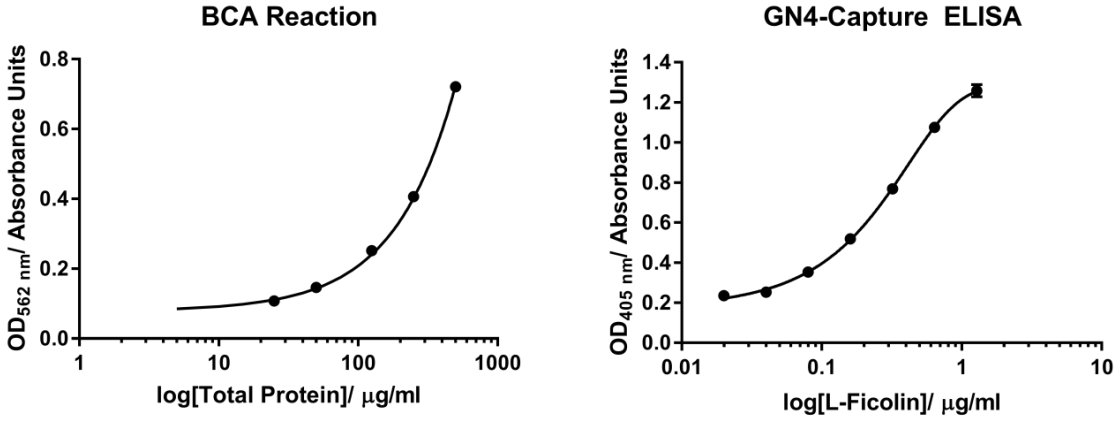

Figure 2.5 – Standard Curves Used to Quantify L-Ficolin Concentration
Commercial BCA assays were used to determine total protein concentration in samples from curves fitted with a linear regression. An in-house ELISA using anti-L-ficolin mAb GN4 was used to determine L-ficolin concentration using a serum standard containing 4 µg/mL of L-ficolin, fitting the curve with sigmoidal, 4PL regression. This figure is an example of standard curves used to interpolate concentrations using the absorbance results of samples. Error bars represent standard error of mean.

2.3.1.4 The L-Ficolin Variants Do Not Affect Oligomerisation

Previous studies found that reducing conditions, which disrupt disulphide bonds, cause L-ficolin to monomerise (Hamed et al., 2014). To ensure that the mutations of the L-ficolin variants do not alter this observation by impacting oligomerisation, non-purified samples were run in a western blot using the anti-L-ficolin mAb GN5. Reducing conditions were achieved using 20 mM DTT as in Hamed et al. (2014). All L-ficolin variants were reduced to monomers of ~40 kDa under reducing conditions, indicating that the mutations do not affect oligomerisation (Figure 2.6).

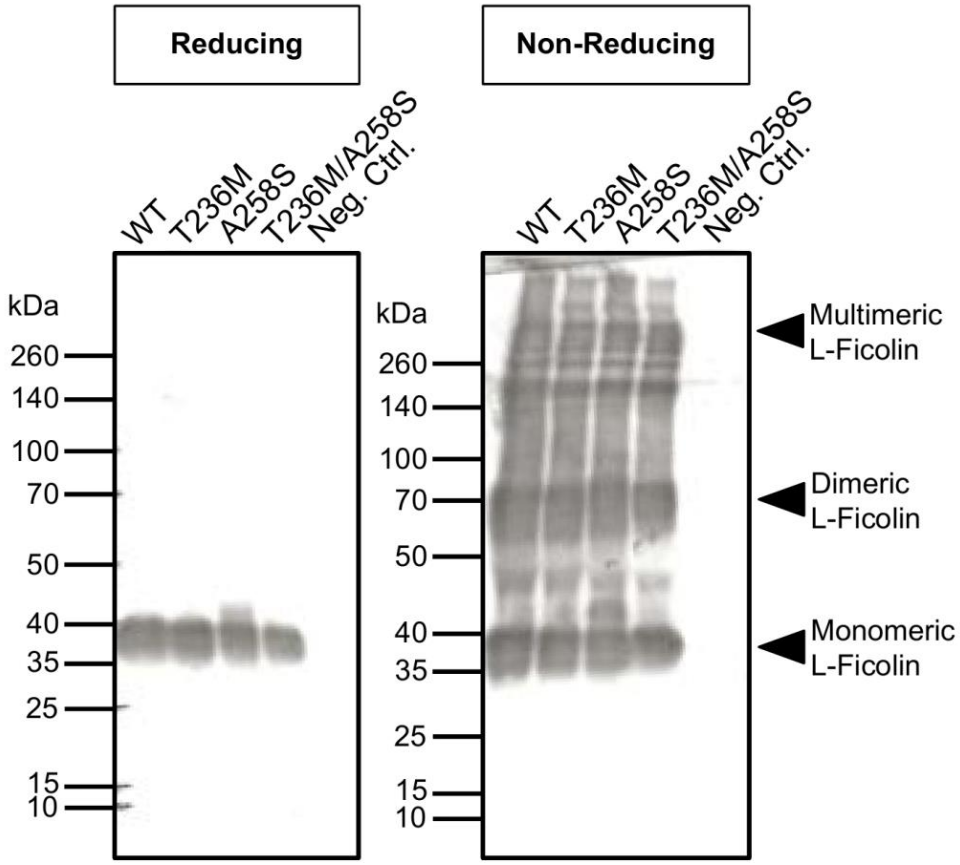

Figure 2.6 – Oligomerisation of L-Ficolin Variants under Reducing Conditions

Western blots of non-purified L-ficolin variants using L-ficolin-specific monoclonal antibody GN5 under reducing (20 mM DTT) and non-reducing (0 mM DTT) conditions. All variants monomerised in a similar way under reducing conditions, suggesting no major alterations in oligomerisation between variants.

The theoretical size of an L-ficolin monomer with a cleaved signal sequence is 36 kDa, however western blots consistently produced ~40 kDa monomers. This was hypothesised to be due to post-translational modifications such as glycosylation. PNGase F – a glycoamidase with specificity towards the bond between asparagine residues and the first GlcNAc residue of *N*-linked glycans (Freeze and Kranz, 2010; Trimble and Tarentino, 1991) – was used to remove any possible glycans.

A 4 µg (total protein concentration as determined by BCA assay) purified L-ficolin-WT sample was denatured in a reducing buffer (0.5% SDS, 40 mM DTT), to cause monomerisation, and digested. A control lacking PNGase F was prepared. Western blot detection using the anti-L-ficolin mAb GN5 revealed a ~40 kDa band in the control and a ~36 kDa band in the digested sample (Figure 2.7). This indicates that L-ficolin is indeed glycosylated, and explains the higher MW consistently seen across SDS-PAGE and western blot analysis.

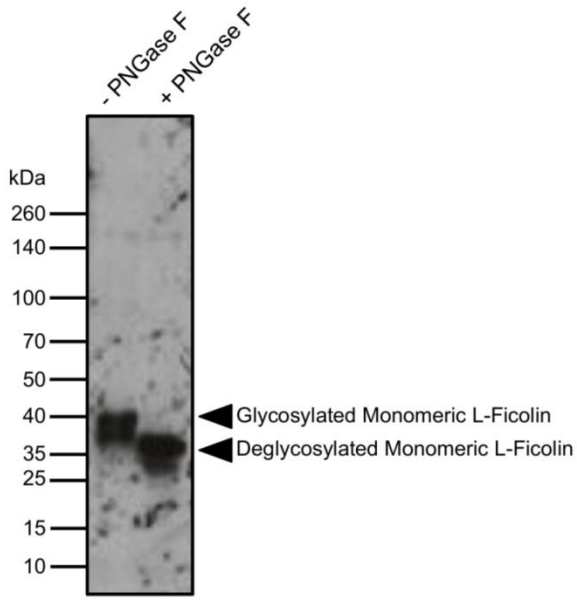

Figure 2.7 – De-Glycosylation of L-Ficolin

Under reducing conditions, PNGase F was used to de-glycosylate monomerised L-ficolin. In a western blot using anti-L-ficolin monoclonal antibody GN5, de-glycosylated L-ficolin had a ~36 kDa band, closer to the theoretical size of an L-ficolin monomer. This explains the consistently higher molecular weights found in SDS-PAGE and western blot detection of L-ficolin.

2.3.1.5 Optimisation of L-Ficolin Production

The original method used HEK 293T cells seeded at a density of 1.5 x 10^6 in 10 cm^2 diameter plates and secreted L-ficolin was harvested in 50 mL of DMEM (10% FBS). L-ficolin production was extensively optimised over the project in order to enhance yields and activity for characterisation, and avoid FBS contamination, as summarised in Table A in the Appendix. In brief, several steps of the production process were investigated.

Transfection was investigated by altering the amount of plasmid used, and exploring an alternative transfection reagent. A stably-transfected HEK 293T cell line was also designed to produce C-terminal His_6-tagged L-ficolin-WT.

Expression was optimised, such as the seeding density of HEK 293T cells, supernatant volume and the amount of PBS used following dialysis by centrifugation. A ~50 kDa FBS-derived contaminant was a persistent issue in L-ficolin samples as detected in L-ficolin-specific western blots. This was overcome by altering FBS content in media and the MW cut off of the centrifugal filter, before settling on the use of serum-free Opti-MEM media.

Alternative purification methods were explored, including FLAG-tag and GlcNAc affinity chromatography, however the best up-scaling and yields were produced using nickel affinity chromatography. The methods described in sections 2.3.1.1 and 2.3.1.2 represent the techniques with the optimal results.

2.3.1.6 L-Ficolin Variants Require an N-Terminal His_6-Tag

When expressing the L-ficolin variants, it was consistently observed that C-terminal His_6-tagged L-ficolin-A258S and L-ficolin-T236M/A258S could not be detected in western blots using the anti-L-ficolin monoclonal GN5 and anti-His tag polyclonal antibodies (Figure 2.8). On an SDS-PAGE gel, all L-ficolin samples had similar band patterns, whereas the negative control (produced using no expression plasmid) had no bands. This implies that protein was indeed expressed and purified in the L-ficolin-A258S and L-ficolin-T236M/A258S samples, and that the issue was in detection.

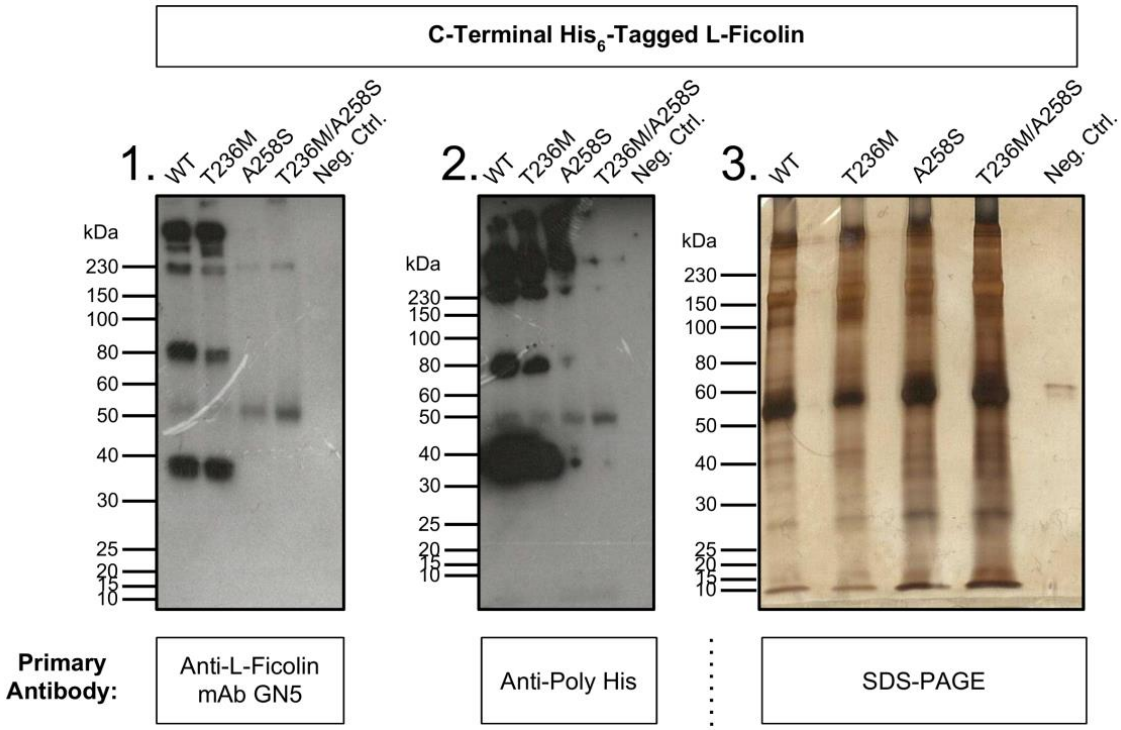

Figure 2.8 – Detection of C-Terminal His_6-Tagged L-Ficolin Variants

Nickel affinity chromatography-purified and dialysed C-terminal His_6-tagged L-ficolin variant samples were detected in a western blot using: ***1.*** *L-ficolin-specific monoclonal antibody GN5 and* ***2.*** *a polyhistidine-specific polyclonal antibody, to detect the His_6-tag. In both blots, only the L-ficolin-WT and L-ficolin-T236M samples contained L-ficolin bands of ~40 kDa, 80kDa and >230 kDa. A 50 kDa band represents a contaminant which was later removed through optimisation.* ***3.*** *Silver staining of an SDS-PAGE gel using the same samples showed protein in all samples except the negative control (produced identically, with no expression plasmid). Therefore, the L-ficolin-A258S and L-ficolin-T236M/A258S samples contained protein which wasn't detected in the blot.*

Due to the versatility and established use of the anti-L-ficolin mAb GN5 in both western blot and ELISA assays, its use for detection of all variants was essential for successful characterisation of these proteins. Therefore, constructs were designed in which the C-terminal His_6-tag was moved to the N-terminus.

To allow secretion, the N-terminal His_6-tagged L-ficolin gene sequence would comprise the N-terminal *FCN2* signal sequence, followed by six codons encoding a His_6-tag, and succeeded by the *FCN2* mature peptide sequence. After two failed methods, successful cloning of N-terminal His_6-tagged L-ficolin variants was performed using custom primer sequence tags which were not found in the *FCN2* gene to prevent amplification of template DNA. The custom tags were designed to encompass the *FCN2* gene while maintaining the Kozak sequence.

For each L-ficolin variant, two PCR reactions were performed (Figure 2.9). The pcDNA-DEST40 plasmid encoding the corresponding C-terminal His_6-tagged L-ficolin variant was used as template, with the *FCN2* signal peptide and *FCN2* mature peptide primer pairs (Figure 2.10). The resulting custom-tagged *FCN2* fragments 1 and 2 were used as templates in a PCR using the custom *FCN2* tag forward and reverse primers, allowing homologous recombination at the respective 3' and 5' His_6-tag DNA sequences of the fragments. The resulting *FCN2* fragment was DpnI-digested to remove methylated plasmid DNA and was purified. Ligation into the pcDNA3.1D/V5-His-TOPO® plasmid was performed, and transformed *E.coli* NEB colonies grown with selection were screened using the T7F and BGHR primers. Plasmid was purified and sequenced using the T7F and BGHR primers, thus confirming the successful cloning and orientation of each pcDNA3.1:N-His-FCN2 plasmid.

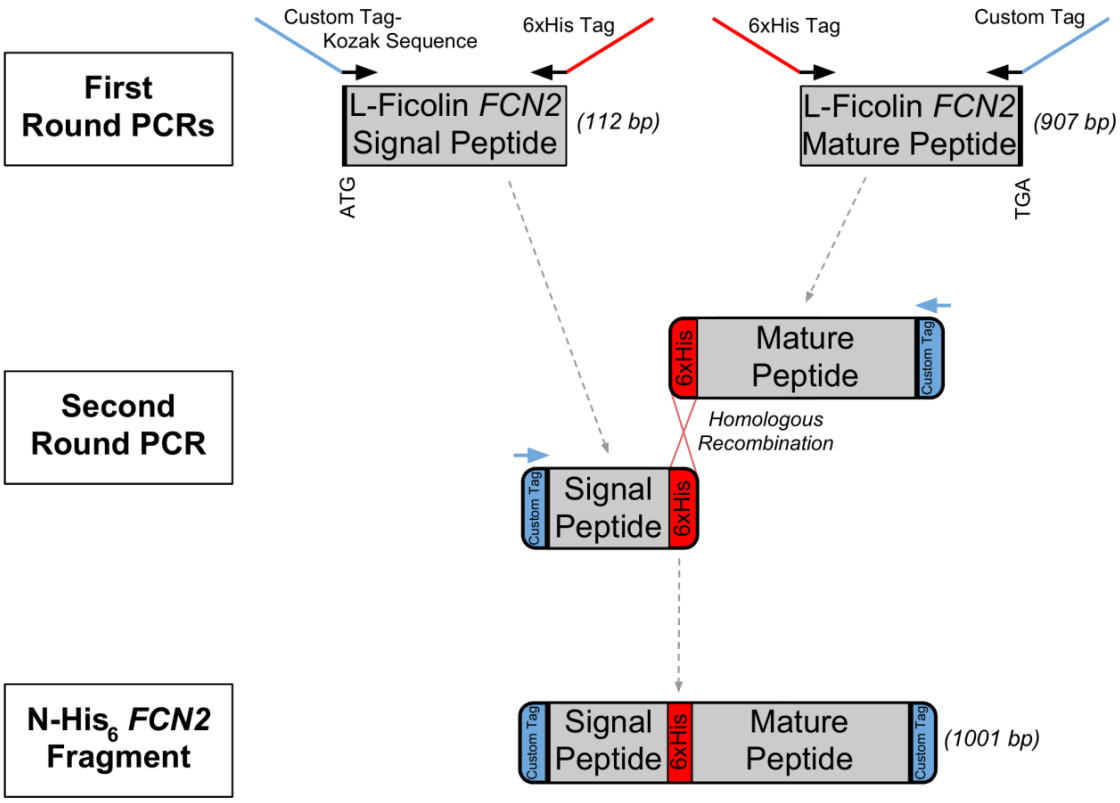

Figure 2.9 – Cloning Method to Produce N-Terminal His_6-Tagged L-Ficolin

Using pcDNA-DEST40 plasmid encoding L-ficolin as a DNA template, the FCN2 *signal peptide and mature peptide DNA sequences would be amplified separately, using primers to introduce custom primer and His_6-tag sequences. Using custom* FCN2 *tag primers (→ and ←), homologous recombination would occur at the His_6-tag sequence (red).*

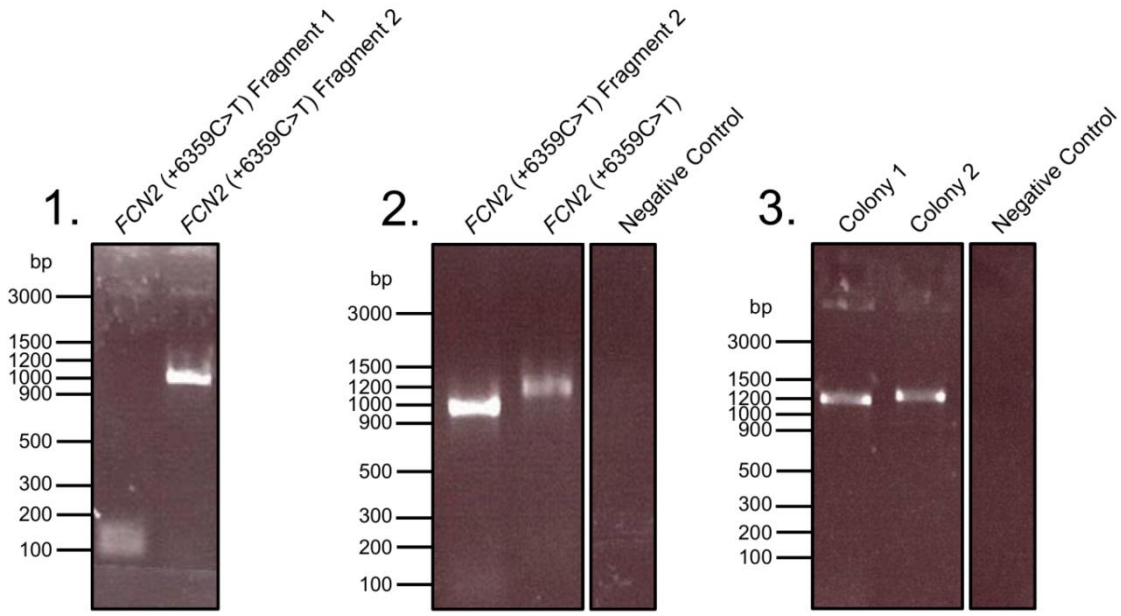

Figure 2.10 – Cloning of *FCN2* Sequence Encoding N-Terminal His_6-Tagged L-Ficolin

Agarose gel electrophoresis and ethidium bromide staining of PCR reactions used to create FCN2 *encoding L-ficolin with an N-terminal His_6-tag. Here, the* FCN2 *gene containing the +6359C>T SNP, encoding the T236M mutation, was used as an example. DNA size markers are shown in base pairs (bp). Negative controls represent PCR reactions containing no DNA template.* ***1.*** *The pcDNA-DEST40 plasmid encoding C-terminal His_6-tagged L-ficolin-T236M was used as template with the* FCN2 *signal peptide and* FCN2 *mature peptide primer pairs. The two reactions produced the expected 112 bp and 907 bp fragments.* ***2.*** *Using these fragments and the custom* FCN2 *tag primers, homologous recombination took place at the His_6-tag sequence, producing the expected 1001 bp band.* ***3.*** *Following ligation into the pcDNA3.1D/V5-His-TOPO® plasmid and transformation into* E. coli*, the T7F and BGH-R primers were used to screen colonies for the expected 1266 bp plasmid. Sequencing of these colonies confirmed successful cloning.*

Both the C-terminal His_6-tagged L-ficolin variants and, using the new plasmids, the N-terminal His_6-tagged L-ficolin variants were produced using the non-purified method. A negative control was produced identically with a mock-transfection of HEK 293T cells. Western blots of all samples were performed using the anti-L-ficolin mAbs GN5 and GN4, along with an SDS-PAGE of the N-terminal His_6-tagged L-ficolin samples (Figure 2.11).

The C-terminal His_6-tagged samples had identical profiles to previous observations, with the anti-L-ficolin mAb GN4 also lacking detection of L-ficolin containing the A258S mutation. The N-terminal His_6-tagged L-ficolin variants, however, produced the expected bands of multiples of ~40 kDa for all samples except the negative control. A ~50 kDa contaminant was detected by GN4 in all samples.

Banding in the SDS-PAGE gels was similar between all samples. It should be noted that the C-terminal His_6-tagged samples on the SDS-PAGE were purified whereas the N-terminal His_6-tagged samples were not, due to time constraints. This explains why the negative control of the C-terminal His_6-tagged samples contains no protein.

As they were detectable, the N-terminal His_6-tagged variants were opted for use. This detection issue had not previously been reported.

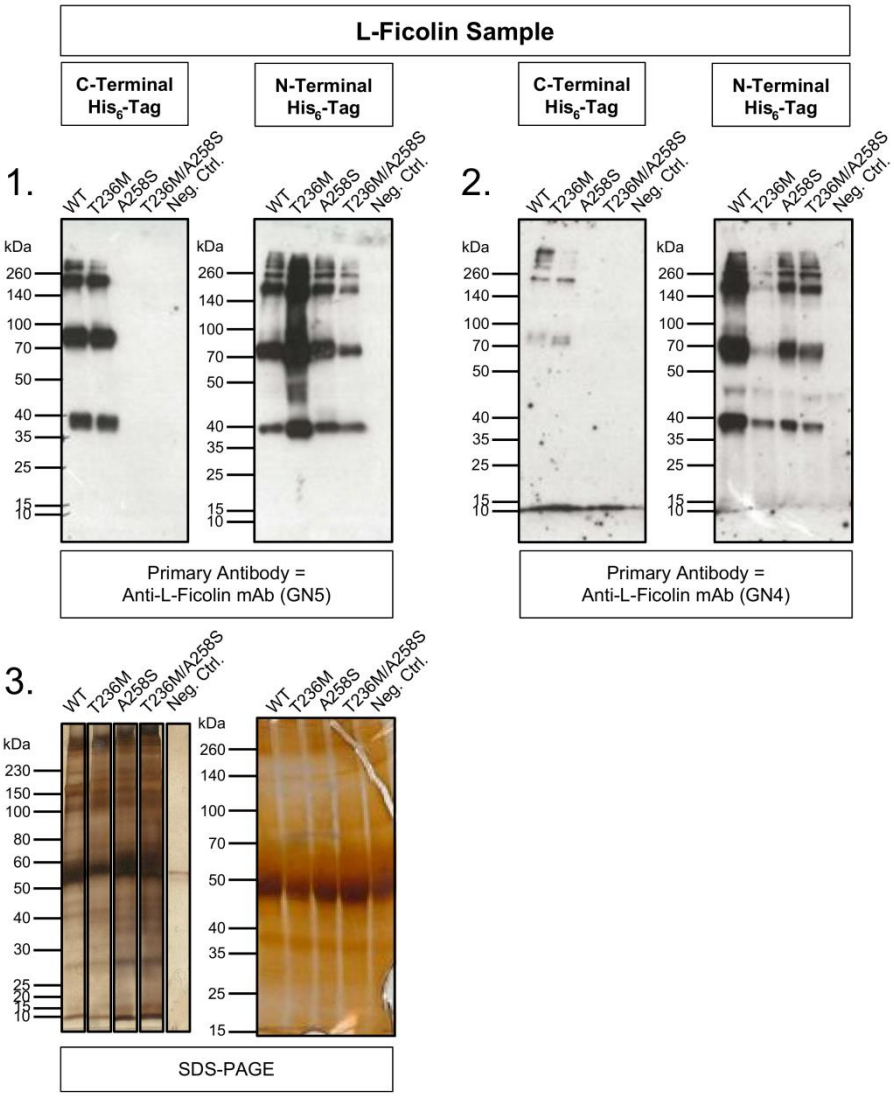

Figure 2.11 – Detection of L-Ficolin Variants with C-Terminal and N-Terminal His_6-Tags

*Non-purified L-ficolin variants were produced with C-terminal or N-terminal His_6-tags. However, the C-terminal His_6-tagged L-ficolin samples used in the SDS-PAGE were purified. L-ficolin was detected in western blots using anti-L-ficolin monoclonal antibodies **1.** GN5 and **2.** GN4. In both instances, L-ficolin containing an A258S mutation were not detected when C-terminal His_6-tagged, whereas all N-terminal His_6-tagged L-ficolin variants were detected and were thus appropriate for characterisation. The expected ~40 kDa, ~80 kDa and higher order multimer bands were seen. The negative controls (no expression plasmid) contained no L-ficolin. **3.** Silver staining of an SDS-PAGE gel of the samples showed similar protein band patterns in all samples except the purified negative control of the C-terminal His_6-tagged samples.*

2.3.1.6.1 Epitope Mapping of Anti-L-Ficolin mAb GN5

The epitopes of the anti-L-ficolin monoclonal antibodies GN4 and GN5 have not yet been described. By establishing these epitopes, it could help explain why the C-terminal His_6-tagged L-ficolin-A258S and L-ficolin-T236M/A258S samples could not be detected.

Biopanning was performed using a random phage library against the anti-L-ficolin mAbs GN4 and GN5, with three cycles of phage clone validation. DNA of 20 selected phage clones – three for mAb GN4 and 17 for mAb GN5 – was sequenced and the peptide sequences were aligned with L-ficolin sequence for homology.

An ELISA was performed using anti-L-ficolin mAbs GN4, GN5 and anti-HCV E1E2 mAb AP33 to capture the phage samples and confirm binding. A known AP33 phage was used as a positive control for phage binding, while a standard phage clone unrecognisable to the antibodies was used as a negative control. Binding was only detected for six of the anti-L-ficolin mAb GN5 phage clones, with mAb GN5 (Figure 2.12). A search in the SAROTUP database showed that these phage sequences were unique and not recognised as target-unrelated phages. The AP33 phage positive control bound to anti-HCV E1E2 mAb AP33, whereas the negative control phage bound no antibodies.

Only two of the clones which bound had homologous sequence in the L-ficolin peptide: GN5-11 and GN5-17 (Table 2.6). GN5-11 had the highest binding signal, indicating that the anti-L-ficolin mAb GN5 epitope involves YKQGFGSRLGEF at amino acid residues 162 – 173 in the L-ficolin FBG domain. This is near residue 258 in the FBG structure, and may account for the lack of detection in combination with the C-terminal His_6-tag.

In a 3D structure, this sequence is found within proximity of residue 258, though distant from the C-terminus (Figure 2.13). An alternative epitope candidate, with weaker binding to mAb GN5, was VSNLNGRYLRGT. This is situated further from residue 258.

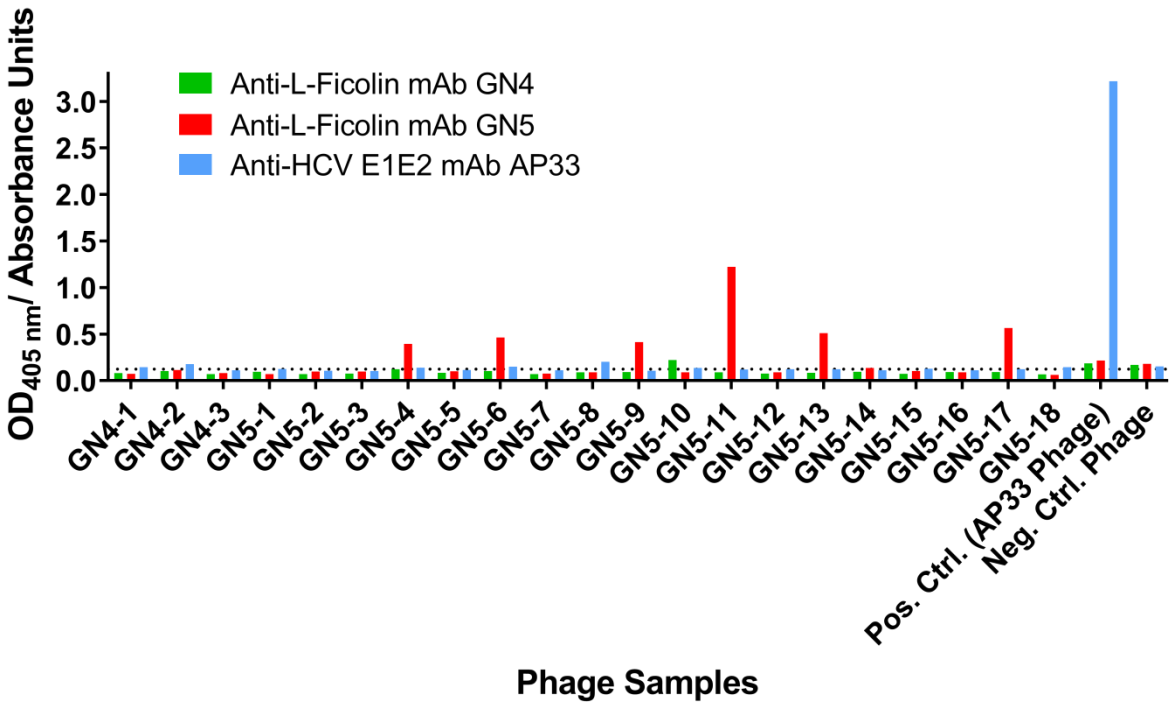

Figure 2.12 – Selected Phage Bound by Anti-L-Ficolin Antibodies

The anti-L-ficolin monoclonal antibodies GN4 and GN5 were used to coat ELISA plates and biopanning-deduced phage clones representing putative antibody epitopes were added (n = 1). Six mAb GN5 phage clones showed binding. GN5-11 exhibited the strongest binding, and had a homologous sequence in the L-ficolin FBG domain. The AP33 phage positive control bound the anti-HCV mAb AP33, and the negative control phage bound no antibodies. The dotted line indicates the lower limit of detection of the assay (mean of negative sample + (2 x S.D.)).

Table 2.6 – Amino Acid Sequences of Putative L-Ficolin-Specific Monoclonal Antibody GN5 Epitope

In an ELISA, phage clone GN5-11 bound the anti-L-ficolin mAb (GN5) with the strongest signal, and is homologous to a sequence of the L-ficolin FBG domain. ***** *= fully conserved residue:* **:** *= strongly similar residue;* **.** *= weakly similar residue.*

Phage	Amino Acid Sequence	Putative Homologous *FCN2* Sequence
GN5-4	ISAPASHYKKPL	
GN5-6	ISAPASHYKKPL	
GN5-9	ISAPASHYKKPL	
GN5-11	HANVVGIRAGEW	(FCN2) YKQGFGSRLGEF : : .* * **: (Phage) HANVVGIRAGEW
GN5-13	HLALRSPPHYQK	
GN5-17	WSPHSKLYVRAS	(FCN2) VSNLNGRYLRGT * . *:*.: (Phage) WSPHSKLYVRAS

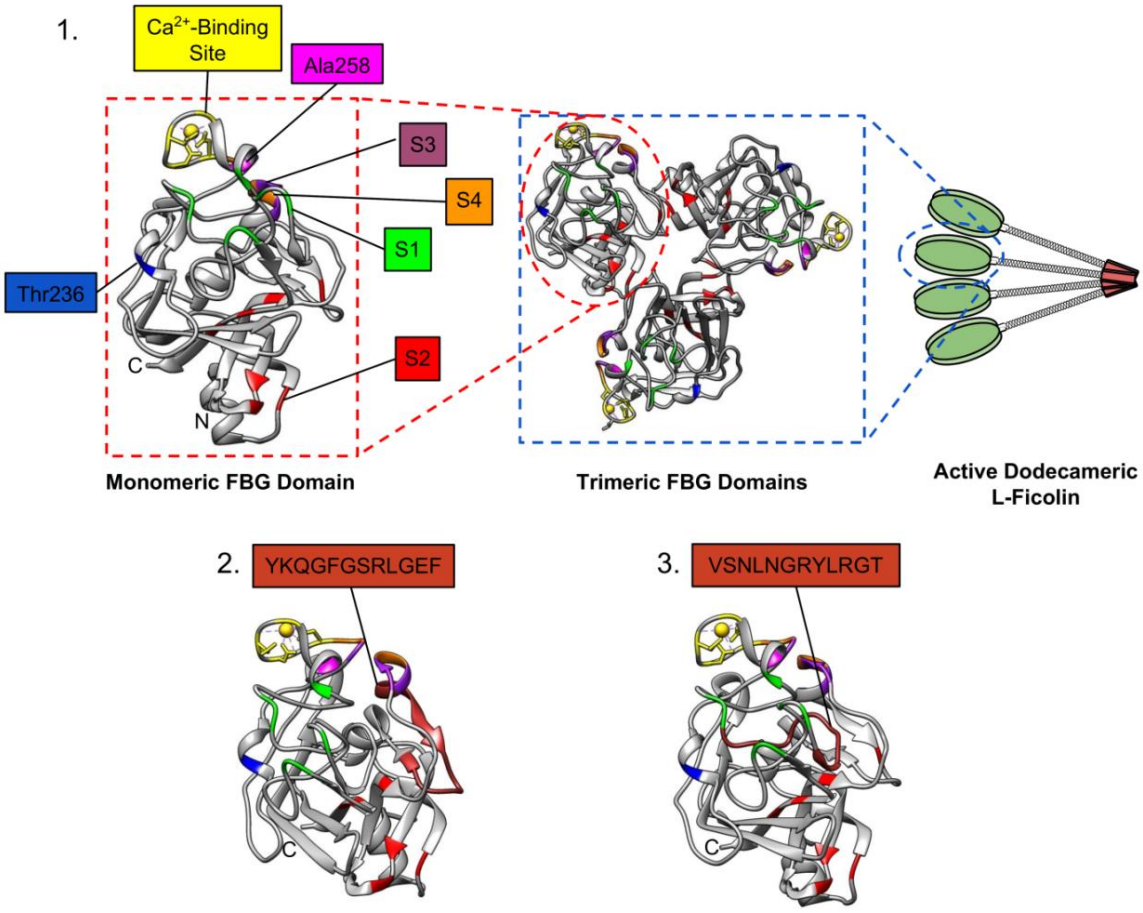

Figure 2.13 – Potential L-Ficolin Epitopes of Anti-L-Ficolin Monoclonal Antibody GN5

The epitope of the L-ficolin-specific monoclonal antibody GN5 was determined through phage biopanning and ELISA. The 3D structure was adapted from Garlatti et al. (2007) (Protein Data Bank [PDB] code 2j3g) using UCSF Chimera 1.12 software. ***1.*** *Structural detail of a monomeric FBG domain from a wild-type L-ficolin trimer. The amino acids of the Ca^{2+} (yellow), S1 (green), S2 (red), S3 (purple) and S4 (orange) binding sites are highlighted. The Thr236 and Ala258 residues of interested are highlighted in blue and pink, respectively.* ***2.*** *The putative GN5 mAb epitope YKQGFGSRLGEF (in brown) and is in the vicinity of residue 258. A serine at 258 in combination with a C-terminal His_6-tag may interfere with GN5 interaction.* ***3.*** *The possible epitope VSNLNGRYLRGT was also detected, but was deemed less likely from the results.*

2.3.2 L-Ficolin-T236M Exhibits Enhanced Binding to Acetylated BSA

Previously, L-ficolin-A258S was found to have superior binding to GlcNAc than the wild-type, whereas serum L-ficolin-T236M had lower binding levels (Hummelshøj et al., 2005).

It was hypothesised that the recombinant L-ficolin variants produced here would also have differing binding levels towards another known ligand, acetylated BSA (AcBSA). Two batches of L-ficolin variants were quantified by anti-L-ficolin mAb GN4-capture ELISA and normalised to 1.1 µg/mL and 0.4 µg/mL. These were tested in three independent AcBSA assays. A negative control produced by mock-transfection of HEK 293T cells was used.

There was a significant difference in binding to AcBSA between all variants and L-ficolin-WT ($p = 0.0001$; one-way ANOVA with Dunnett statistical test). L-ficolin-T236M displayed the highest mean binding level, followed by L-ficolin-WT, L-ficolin-A258S and finally L-ficolin-T236M/A258S had the lowest binding, close to that of the negative control.

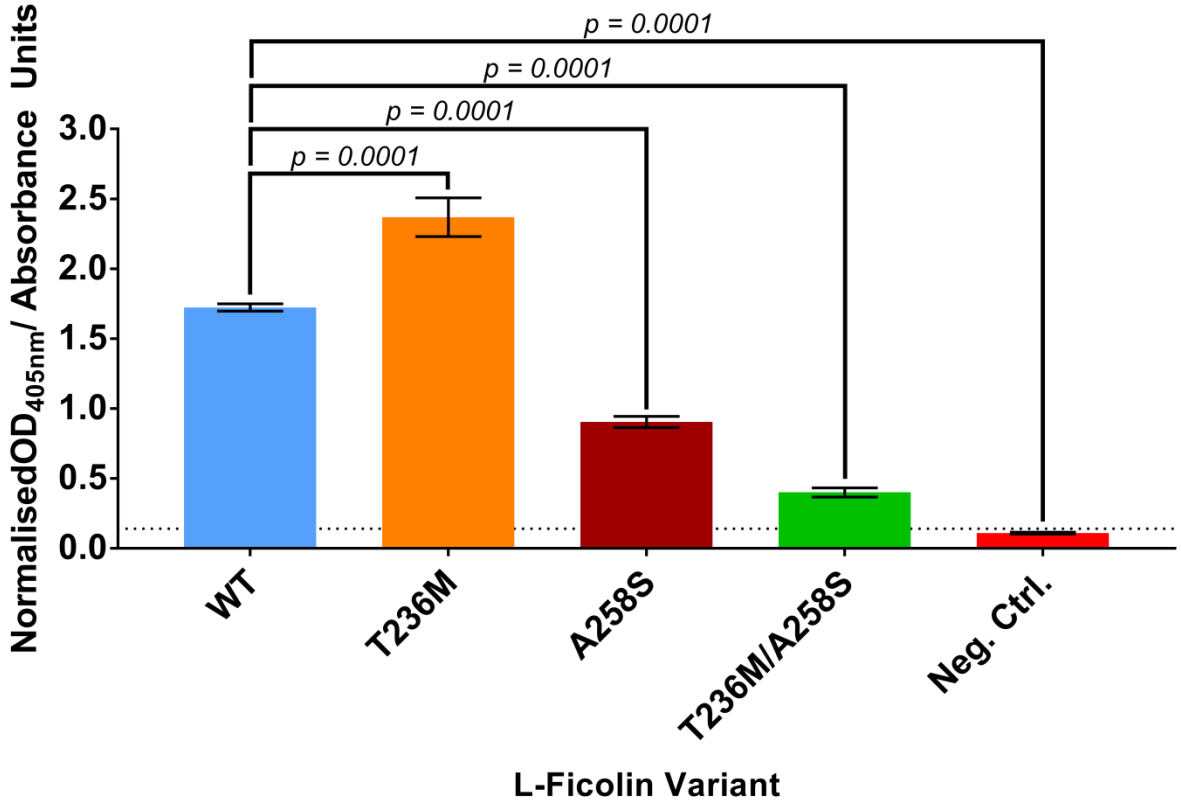

Figure 2.14 – Differences in Binding to Acetylated BSA between L-Ficolin Variants

An ELISA was performed to assess binding to AcBSA by normalised L-ficolin variants added in duplicate (n = 3). Absorbance was normalised between assays to the absorbance of L-ficolin-WT. One-way ANOVA analysis and Dunnett's test for multiple comparison was performed (p value threshold of significance = 0.05). L-ficolin-T236M had significantly higher binding to AcBSA than L-ficolin-WT (p = 0.0001), whereas L-ficolin-A258S had inferior binding, and L-ficolin-T236M/A258S had even lower binding. The negative control, produced by omitting expression plasmid, had no activity. Error bars represent standard error of mean. The dotted line indicates the lower limit of detection of the assay (mean of negative sample + (2 x S.D.)).

2.3.2.1 L-Ficolin Variants Bind Acetylated BSA in a Calcium-Dependent Manner

Levels of free calcium ions within serum have previously been reported to be 1.41 mM (Sava et al., 2005). Depending on the binding site used, L-ficolin binding can be calcium-dependent (Hamed et al., 2014; Krarup et al., 2004). To confirm calcium ions affect binding activity rather than oligomerisation (Hamed et al., 2014; Hummelshøj et al., 2007), western blot analysis using anti-L-ficolin mAb GN5 was performed in which non-purified variants were treated with 5 mM $CaCl_2$. The multimeric L-ficolin band profiles, with multiples of ~40 kDa, did not differ with or without the presence of $CaCl_2$. The mock-transfection negative control contained no L-ficolin. This supports the observation that Ca^{2+} ions affect L-ficolin binding rather than oligomerisation.

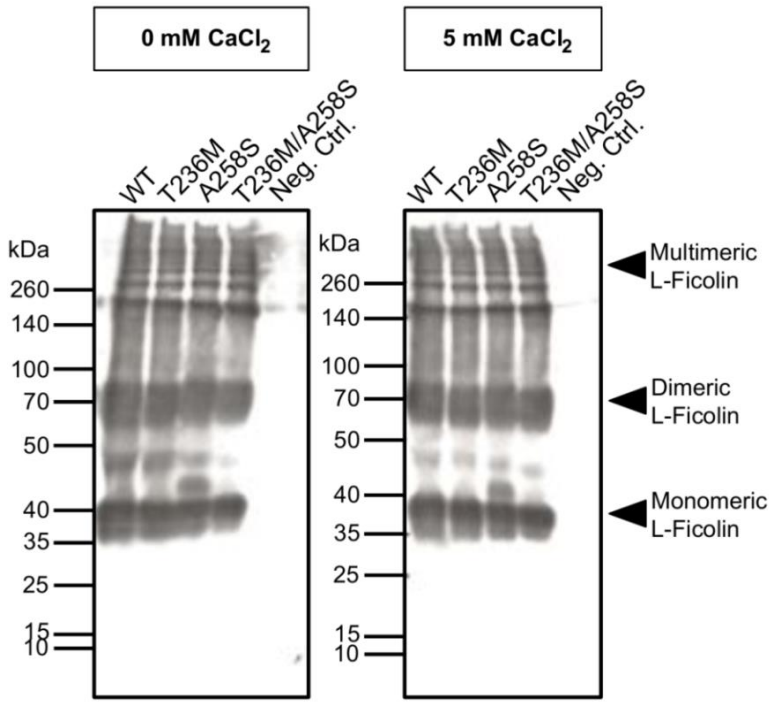

Figure 2.15 – The Effect of $CaCl_2$ on L-Ficolin Oligomerisation

Non-purified L-ficolin variants were treated with 0 mM and 5 mM $CaCl_2$ and detected by western blot using anti-L-ficolin monoclonal antibody GN5. No difference in the ~40 kDa multimer band pattern was seen in the presence or absence of $CaCl_2$ for any L-ficolin variant, thus disulphide-mediated oligomerisation is unaffected. The negative control, produced without expression plasmid, contained no L-ficolin.

To identify if AcBSA binding by L-ficolin is Ca^{2+}-dependent, and to assess whether the mutations of the L-ficolin variants affect the putative Ca^{2+}-dependence, ELISAs were performed using varying levels of $CaCl_2$ and ethylenediaminetetraacetic acid (EDTA) – a calcium inhibitor.

Normalised L-ficolin variant samples (at 1.1 µg/mL and 0.4 µg/mL as quantified by anti-L-ficolin mAb GN4-capture ELISA) were added to the plate with three 10-fold dilutions of $CaCl_2$ and EDTA at concentrations of 0 mM, 0.5 mM, 5 mM and 50 mM in TBST. The L-ficolin samples used in the EDTA ELISA were diluted in TBST containing 5 mM $CaCl_2$. Due to constraints in time and amounts of sample with sufficient yields, this assay was performed twice. Representative data from one assay is shown in Figure 2.16.

The observations of AcBSA binding by the L-ficolin variants in Figure 2.14 were supported by this data. Despite a slight increase of L-ficolin-WT binding in the presence of 0.5 and 5 mM $CaCl_2$, the presence of $CaCl_2$ did not alter binding levels for all variants. However, non-purified samples which were not dialysed from Opti-MEM into PBS were used in this assay. As Opti-MEM media contains $CaCl_2$, the binding levels seen at 0 mM $CaCl_2$ in the ELISA likely still contain Ca^{2+} ions. Nevertheless, EDTA would have chelated calcium ions from L-ficolin.

EDTA inhibited AcBSA binding by all L-ficolin variants. The mean EDTA concentrations of each variant that resulted in 50% inhibition of AcBSA binding (IC_{50}) were similar between all variants, at 1.8 mM for L-ficolin-WT, 1.7 mM for L-ficolin-T236M, 1.6 mM for L-ficolin-A258S and 1.6 mM for L-ficolin-T236M/A258S. These results imply that AcBSA binding is Ca^{2+}-dependent, the manner of which is unaffected by the variants.

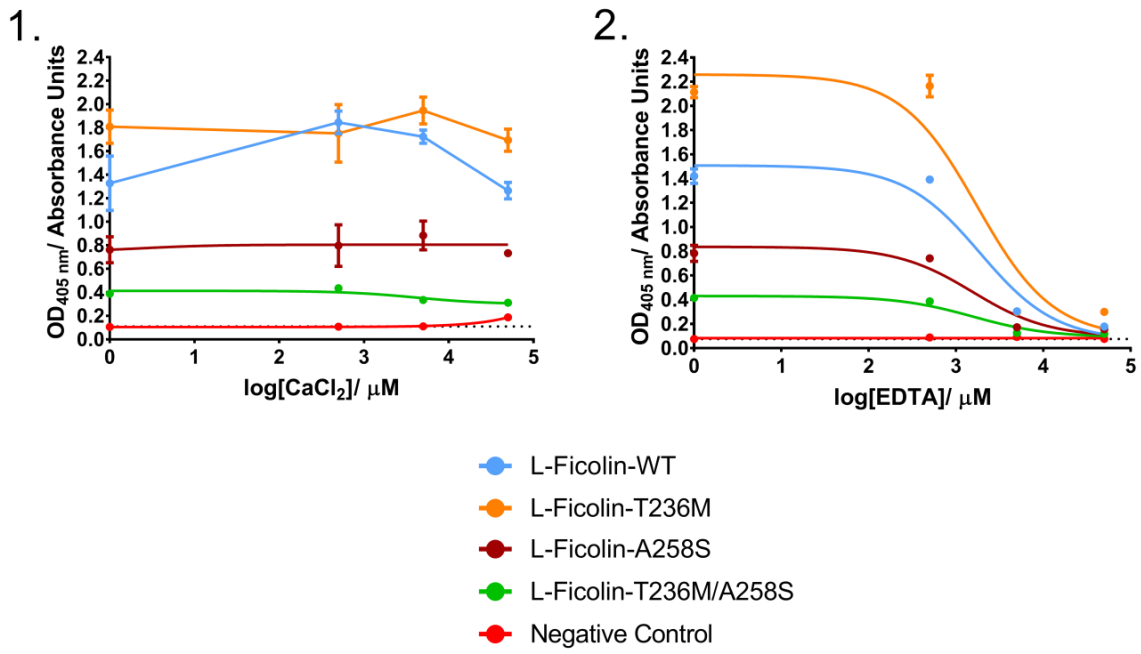

Figure 2.16 – The Effects of $CaCl_2$ and EDTA on L-Ficolin Binding to AcBSA

An AcBSA-binding ELISA was performed using non-purified L-ficolin variants in duplicate, exposed to 0 mM, 0.5 mM, 50 mM and 500 mM of $CaCl_2$ or EDTA (n = 2). ***1.*** *$CaCl_2$ had little effect on L-ficolin binding, perhaps because the sample media likely contained $CaCl_2$.* ***2.*** *EDTA, a calcium inhibitor, inhibited all L-ficolin binding in a similar manner between variants. By fitting the data with a non-linear regression, IC_{50} concentrations of 1.8 mM, 1.7 mM, 1.6 mM and 1.6 mM were deduced for L-ficolin-WT, -T236M, -A258S and –T236M/A258S, respectively. Error bars represent standard error of mean. Dotted lines indicate the lower limit of detection of the assay (mean of negative sample + (2 x S.D.)).*

2.3.2.2 Alternative Ligands Inhibit Acetylated BSA Binding by L-Ficolin *Variants*

L-ficolin has four binding sites with their own specificities (Garlatti et al., 2007). Due to the proximity of the L-ficolin variant mutations to the binding sites, it was hypothesised that the non-synonymous T236M and A258S mutations would alter the affinity of L-ficolin towards other ligands.

To investigate this, two known acetylated L-ficolin ligands – *N*-acetylcysteine (CysNAc) and *N*-acetylglucosamine (GlcNAc) – were added as competitors against L-ficolin binding of AcBSA. Due to time and L-ficolin yield constraints only one assay was performed, therefore these results require verification.

In an ELISA coated with 50 µg/mL of AcBSA, non-purified L-ficolin variants were normalised (to 1.1 µg/mL as quantified by anti-L-ficolin mAb GN4-capture ELISA) and were incubated with 0 mM, 0.05 mM, 5 mM and 500 mM of either CysNAc or GlcNAc. Both competitive ligands inhibited binding to AcBSA to some extent for all variants (Figure 2.17). In this set, the L-ficolin-WT had higher AcBSA binding levels than L-ficolin-T236M, inconsistent with the results of Figure 2.14 and Figure 2.16.

Data was fitted with a non-linear regression to establish IC_{50} concentrations of each ligand for each sample. For L-ficolin-WT, L-ficolin-T236M, L-ficolin-A258S and L-ficolin-T236M/A258S, the IC_{50} concentrations of CysNAc were 2.0 mM, 1.2 mM, 0.9 mM and 1.1 mM, respectively. The lower IC_{50} values may indicate that both mutations alter L-ficolin structure to allow competitive CysNAc binding more easily. With GlcNAc, the non-linear regression curves were incomplete, implying that concentrations of GlcNAc exceeding the 500 mM range of this assay are required for full inhibition of AcBSA binding by L-ficolin. As such, IC_{50} concentrations could not reliably be interpolated.

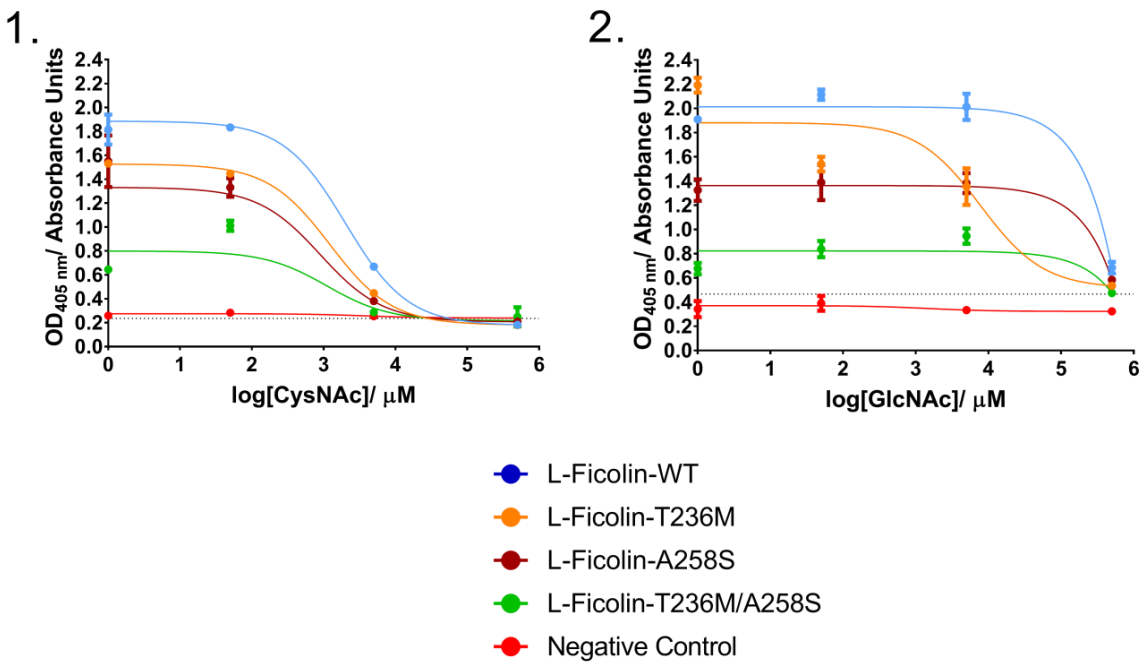

Figure 2.17 – The Effects of Competitive Ligands on the Binding of L-Ficolin to AcBSA

An ELISA coated with 50 µg/mL of AcBSA was performed using non-purified L-ficolin variants in duplicate exposed to 0 mM, 0.05 mM, 5 mM and 500 mM of L-ficolin ligands CysNAc and GlcNAc (n = 1). Both ligands inhibited L-ficolin binding to AcBSA. ***1.*** *By fitting the data with a non-linear regression, CysNAc IC_{50} concentrations of 2.0 mM, 1.2 mM, 0.9 mM and 1.1 mM were determined for L-ficolin-WT, -T236M, -A258S and –T236M/A258S, respectively.* ***2.*** *Despite inhibition of AcBSA binding by GlcNAc the inhibition curve was incomplete with the range of concentrations used in this assay, therefore reliable IC_{50} values could not be deduced. Error bars represent standard error of mean. Dotted lines indicate the lower limit of detection of the assay (mean of negative sample + (2 x S.D.)).*

2.3.3 Interaction of L-Ficolin Variants with Viruses

HCV and EBOV are enveloped viruses with largely differing life cycles and mechanisms of pathogenesis. L-ficolin is known to interact with HCV by its E1E2 glycans however at the time of testing EBOV interaction had not been documented. Due to the highly glycosylated nature of EBOV, it is a strong candidate for binding by L-ficolin. As the variants have displayed differing levels of binding towards AcBSA and GlcNAc, it was hypothesised that the L-ficolin variants would also bind virus glycoproteins at differing levels.

L-ficolin neutralises HCVpp entry into Huh7 cells through interaction with the E1E2 envelope glycoproteins (Hamed et al., 2014). An assay was carried out to investigate the neutralising effect of L-ficolin variants on virus pseudoparticle entry into Huh7 cells.

Purified C-terminal His_6-tagged L-ficolin variants were normalised to a total protein concentration of 1.2 mg/mL as determined by BCA assay, and were used in three neutralisation assays. A five-fold serial dilution of L-ficolin from 117 µg/mL to 0.2 µg/mL was incubated with the pseudoparticles before addition to the cells in triplicate. Pseudoparticle entry was normalised to the luminescence of cells infected with a negative control pseudoparticle expressed with no glycoprotein (0%) and the pseudoparticle of interest in the absence of L-ficolin (100%). The anti-HCV E1E2 monoclonal antibody AP33 (10 µg/mL) was also serially diluted and used as positive control, as it neutralises a broad range of HCV strains (Owsianka et al., 2005). A negative control sample produced through a mock-transfection of HEK 293T cells was used.

HCV genotype 1 (H77.20 strain) glycoproteins were used to produce retroviral pseudoparticles. Vesicular stomatitis virus (VSV) glycoproteins were also used as a control. VSV is an enveloped virus able to infect Huh7 cells, and has been reported susceptible to L-ficolin neutralisation (Hamed et al., 2014).

There was minor, insignificant neutralisation by all samples against HCVpp and VSVpp, including the mock-transfection negative control (Figure 2.18). One-way ANOVA (Dunnett's multiple comparison) of infectivities at the highest concentration of each L-ficolin sample showed no significant differences in HCVpp entry when compared with the negative control, while the AP33 mAb positive control showed significant HCV-specific neutralisation ($p = 0.0001$). There was no signficant neutralisation of VSVpp by any sample ($p = 0.1727$).

A similar lack of neutralisation was observed when tested against lentiviral pseudoparticles expressing glycoproteins of EBOV and other HCV genotypes (data not shown). These results imply that insufficient L-ficolin was present in all samples for an effect in the neutralisation assay, a theme consistently observed throughout the project, as will be discussed.

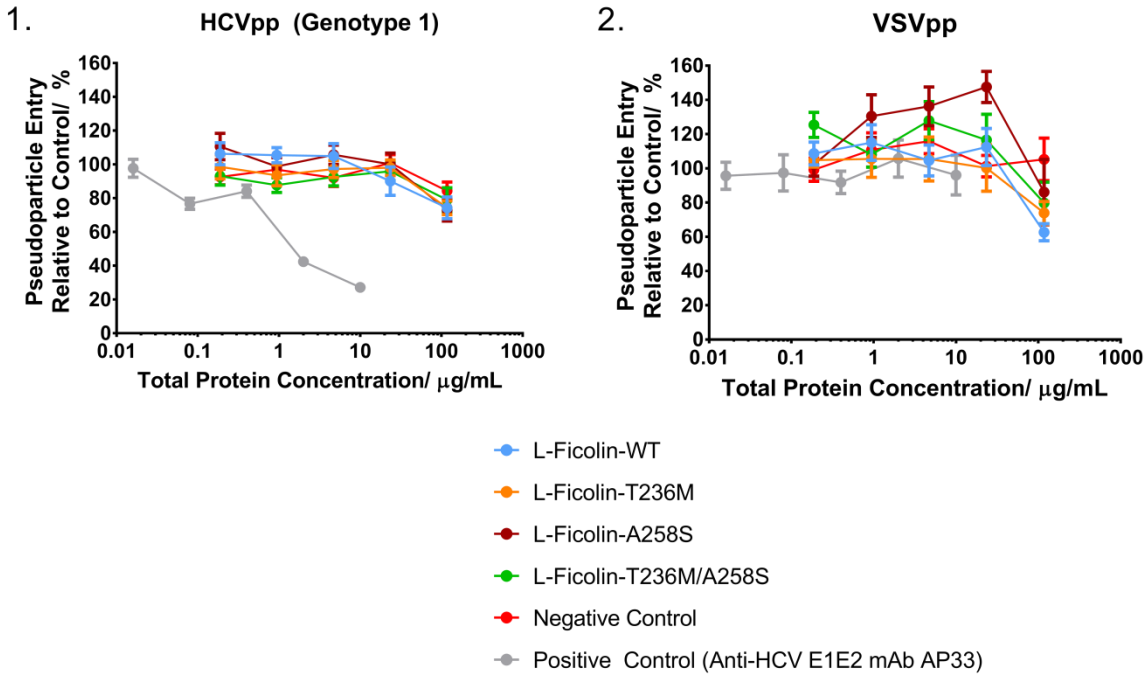

Figure 2.18 – Neutralisation of HCV and VSV Pseudoparticle Entry into Huh7 Cells by L-Ficolin Variants

L-ficolin variants normalised by total protein concentration were diluted five-fold from 117 µg/mL to 0.2 µg/mL and pre-incubated in triplicate with retroviral pseudoparticles, before being added to Huh7 cells (n = 3). Infectivity was determined by pseudoparticle-based luciferase activity in the Huh7 cells and normalised to glycoprotein-deficient (0%) and uninhibited (100%) pseudoparticle controls. ***1.*** *HCVpp were not neutralised by any L-ficolin sample, however the HCV-specific monoclonal antibody AP33 control neutralised entry.* ***2.*** *VSVpp, a virus control able to infect Huh7 cells, was not significantly neutralised by the L-ficolin variants or the AP33 mAb control. These results suggest insufficient L-ficolin protein for use in this assay. Error bars represent standard error of mean.*

2.4 Discussion

Of the three human ficolins – H-ficolin, L-ficolin and M-ficolin – L-ficolin was selected for study for several reasons. For example it is a significant immune component as no humans have been reported with L-ficolin deficiency (Hummelshøj et al., 2005). It has a wide ligand specificity and established interplay with several viruses, allowing research and possible exploitation against various infections. Furthermore, it has a more extensive catalogue of scientific research.

Prior studies have used purified serum L-ficolin to study the protein (Ma et al., 2009) however for the purpose of studying the effect of three mutant constructs, recombinant L-ficolin was chosen. Active recombinant L-ficolin has previously been produced and purified in many ways, for example FLAG-tagged L-ficolin (Hamed et al., 2014), GlcNAc affinity-purified L-ficolin (Geno et al., 2015), CysNAc affinity-purified L-ficolin (Vassal-Stermann et al., 2014a) and His_6-tagged L-ficolin (Hummelshøj et al., 2007). Of these, the common His_6-tag was chosen for expression of the L-ficolin variants, due to its small size, ease of purifying several constructs, and its potential for up-scaling (Kimple et al., 2013; Zhao et al., 2013).

L-ficolin variants with exon 8 polymorphisms had previously been identified and correlated through DNA sequencing with virus infections such as CMV and HBV. However, other than GlcNAc binding, the variants had not been characterised for physical interactions with other ligands and viruses. If one of these mutants was found to have an increased affinity towards ligands and virus glycoproteins, and therefore potentially an enhanced neutralisation effect against virus entry, this could make a particular L-ficolin construct an optimal and viable candidate for therapeutic use.

2.4.1 Expression of L-Ficolin

In this study, HEK 293T cells were used. It has since been shown that the HEK 293T and Huh7 cell lines used in this study secrete very low, insignificant levels of L-ficolin (Jalal et al., 2019a). C-terminal His_6-tagged L-ficolin was originally produced, as this would avoid interference of the tag with crucial N-terminal oligomerisation. Although a C-terminal His_6-tag could risk interference with ligand binding activity and FBG trimer formation, Hummelshøj et al. (2007) had published use of a C-terminal His_6-tagged L-ficolin with GlcNAc-binding activity.

However, upon production of the variants it became clear that variants containing the A258S mutation could not be detected in western blots. SDS-PAGE detection of protein in all of the purified L-ficolin samples indicated that it was an issue with antibody detection rather than expression. Biopanning of the anti-L-ficolin mAb GN5 indicated that the epitope amino acid sequence in L-ficolin is YKQGFGSRLGEF, found within the vicinity of the 258 residue. Alanine scanning mutagenesis of the putative epitopes could identify essential amino acids for antibody binding.

The original C-terminal His_6-tagged L-ficolin constructs made use of the supplied vector's C-terminal His_6-tag, by omitting the naturally-occurring stop codon of the *FCN2* gene and allowing a 39-amino acid spacer sequence followed directly by a His_6-tag and a stop codon. This spacer sequence may have allowed interaction between the serine at residue 258 in the A258S samples and the His_6-tag, thus affecting detection by the GN5 mAb. X-ray crystallography of this construct could elucidate the placement of the C-terminal His_6-tag.

As L-ficolin-specific antibody interaction was essential for characterisation through western blotting and ELISAs, N-terminal His_6-tagged constructs modelled on the N-terminal FLAG-tagged L-ficolin-WT construct from Hamed et al. (2014) was explored. This removed the presence of a large spacer sequence, and the His_6-tag was placed immediately downstream of the signal sequence and upstream of the *FCN2* mature peptide sequence.

The consensus Kozak sequence is 5'-GCCRCCATGG-3' (Xia, 2007), where R represents a purine base (A or G) and ATG represents the start codon. While the native L-ficolin Kozak DNA sequence (5'-GAAGAGATGG-3') was not used, the vector-suggested Kozak sequence of 5'-AACACCATGG-3' was used. This sequence may have caused the lower expression levels observed in this study. Indeed, SNPs at -986, -602 and -4 have been shown to influence L-ficolin levels (Hummelshøj et al., 2008). The use of more native or alternative Kozak sequences could be explored.

Geno et al. (2015) argued that a C-terminal His_6-tag is necessary due to the N-terminal signal sequence cleavage of L-ficolin, however they reported that C-terminal His_6-tagged L-ficolin lacked activity. The results of this project contradict this statement, as both forms of His_6-tagged L-ficolin showed secretion, oligomerisation and activity. Nevertheless, despite successful secretion of the protein, it is possible that the signal sequence was not cleaved as a result of the downstream addition of the tag. This could be confirmed through mass spectrometry. Immunofluorescence staining confirmed L-ficolin production, however it should be repeated for clearer images, with a later optimised protocol using 4% formaldehyde for fixation, 0.1% Triton X-100 for permeabilisation and a 40 – 60X magnification.

Western blotting showed that L-ficolin presented as a ~40 kDa glycosylated monomer with several oligomeric bands, agreeing with previous blots of serum and recombinant L-ficolin reported by several laboratories (Hamed et al., 2014; Hummelshøj et al., 2007; Krarup et al., 2004; Ma et al., 2009). A higher MW protein ladder such as HiMark™ Prestained Protein Standard (30 - 460 kDa; Cat. LC5699; Thermo) could be used to identify the sizes of the higher order oligomers. Oligomerisation ratios could be further defined using gel filtration and sucrose density gradient centrifugation (Hummelshøj et al., 2007).

As dodecameric L-ficolin is the active form, less monomeric L-ficolin would be preferred. The stability and oligomerisation of L-ficolin in several conditions, such as pH and buffer type, and additive components, such as amino acids and cofactors, could be investigated using a Thermofluor screen (Boivin et al.,

2013). Briefly, fluorescent protein-binding dyes are used to determine the temperature at which a protein denatures under several conditions. Nevertheless, the presence of active, dodecameric L-ficolin is supported by activity in the ELISA assays.

An early persistent issue was the presence of a contaminant of L-ficolin samples produced in DMEM (10% FBS) carried through during nickel affinity purification. Geno et al. (2015) detailed the persistent contamination and inhibitory effects of FBS and albumin during L-ficolin production. The contaminant was often identified as a ~50 kDa band in western blots, indicating detection by anti-L-ficolin mAb GN5 (data not shown). A potential candidate contaminant in FBS that could interact with L-ficolin is fetuin, a 48.4 kDa serum glycoprotein that possesses sugars such as glucosamine and *N*-acetyl derivatives of oligosaccharides (Spiro, 1960). To identify the contaminating protein, proteolytic digestion of the sample followed by matrix-assisted laser desorption/ionisation-time of flight tandem mass spectrometry (MALDI-TOF-MS/MS) could be carried out (Gundry et al., 2009). This obstacle was overcome in this assay through the use of Opti-MEM, at the expense of a minor impact to cell growth. An additional purification step such as ion exchange chromatography was not used to conserve the yields of L-ficolin.

2.4.2 L-Ficolin Variant Patterns of Binding Differs Between Ligands

Previous studies showed that the T236M and A258S mutations conferred decreased and increased binding to GlcNAc, respectively (Hummelshøj et al., 2005). In the present study, the opposite was observed for binding to AcBSA. The L-ficolin-T236M/A258S double mutant had even less binding still. These results suggest that there is no one superior L-ficolin variant, but that binding to ligands and possibly virus glycoproteins varies between variants on a case-to-case basis, due to the several binding sites of L-ficolin. In the future, more ligands could be used to perhaps identify if the mutants consistently affect the affinities of each binding site. A GlcNAc-coated ELISA should be used to confirm previous findings with the current constructs.

The differences in binding between the variants are perhaps a result of alterations in FBG domain structure. X-ray crystallography of all variants when bound to AcBSA, and when not, could be performed to study structural differences and responsible binding sites. The amino acids involved in the mutations have widely varying propensities for α-helix formation (Pace and Scholtz, 1998). Study of the 3D structure of L-ficolin shows that residue 236 is in the relative vicinity of the Ca^{2+}-dependent S1 binding site, while residue 258 is the external centre of the S1, S3 and S4 binding sites (Figure 2.19). While no assumptions can be made, it is possible that mutations at these residues could alter their structural relationship with the binding sites.

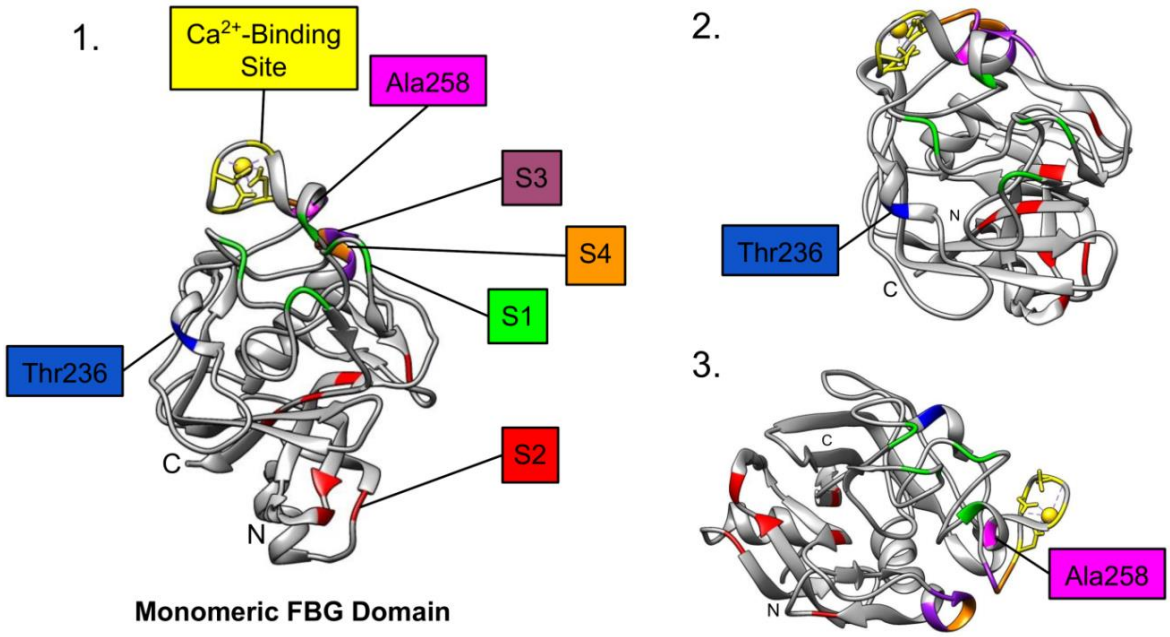

Figure 2.19 – 3D Structure of Monomeric Wild-Type L-Ficolin Fibrinogen Domain

The 3D structure was adapted from Garlatti et al. (2007) (Protein Data Bank code 2j3g) using UCSF Chimera 1.12 software. ***1.*** *Positions of binding sites on a monomeric L-ficolin FBG domain: The occupied Ca^{2+}-binding site is highlighted in yellow. The S1, S2, S3 and S4 binding sites are highlighted in green, red, purple and orange, respectively. The external Thr236 and Ala258 residues are highlighted in blue and pink, respectively.* ***2.*** *Thr236 is situated close to the S1 site.* ***3.*** *Ala258 is located between the S1, S3, S4 and Ca^{2+}-binding sites.*

AcBSA binding was found to be at least partially Ca^{2+}-dependent due to the inhibitory effect of EDTA. Lacroix et al. (2009) also reported a strongly inhibited AcBSA-binding activity of L-ficolin in the presence of EDTA during SPR spectroscopy, theorising the involvement of the Ca^{2+}-dependent S1 binding site, therefore these results support this theory. There was no difference in the EDTA IC_{50} concentration between variants, indicating that the mutations likely do not affect calcium dependence. A wider range of EDTA concentrations with more data points should be used in the future, to improve the resolution of each IC_{50} concentration.

There was a slight difference in the IC_{50} concentrations of CysNAc between variants, with lower CysNAc concentrations needed to inhibit AcBSA binding by variants containing the A258S mutation. Putative structural changes in the FBG caused by the A258S mutation could alter L-ficolin affinity towards CysNAc. The IC_{50} concentrations of GlcNAc could not reliably be interpolated. In the future, more concentrations of these and other competitive ligands across wider ranges should be used, to improve the resolution of each IC_{50} concentration. A non-L-ficolin ligand control such as D-mannose should be used to confirm specificity.

Such competition may give some insight into the L-ficolin binding site required for AcBSA interaction. Garlatti et al. (2007) demonstrated that binding sites S1 and S2 were implicated in GlcNAc interaction, whereas the S2 and possibly S3 were necessary for CysNAc. S3 was also implicated in the binding of acetylated compounds. This could suggest a role for S2 or S3 in AcBSA interaction. However, speculations can only be confirmed by X-ray crystallography, and it is equally feasible that the competing ligands displace AcBSA interaction regardless of binding site.

Understanding the interaction of L-ficolin variants with these ligands and virus glycoproteins would help to predict binding and solidify or dispel the concept of a superior variant.

2.4.3 L-Ficolin Yields Require Optimisation to Detect Virus Glycoprotein Interaction

For the purpose of ELISAs the use of non-purified L-ficolin was suitable, however this was not true for virus pseudoparticle neutralisation assays. Persistently, any neutralisation by the L-ficolin samples was accompanied by identical neutralisation by the mock-transfection negative control. This implies that there was insufficient L-ficolin in the sample to confer a neutralising effect, and that another factor in the media caused neutralisation. Such an effect was not observed in the ELISA.

Several other experiments were performed to characterise L-ficolin variant interaction with HCV and EBOV, however issues in L-ficolin production hindered progress. Dr Barnabas King of the VRG performed neutralisation of HCVcc (cell culture) in Huh7.5 cells to allow interaction in a more authentic environment, however no effect was observed. Several ELISAs were optimised to study L-ficolin interaction with virus glycoproteins extracted by lysis from HEK 293T cells used in pseudoparticle production. The *Triticum vulgaris* lectin wheat germ agglutinin binds EBOV GP (Maruyama et al., 1999) and was used to capture glycoprotein, but no interaction was observed. Snowdrop *Galanthus nivalis* agglutinin (GNA) lectin binds HCV E1E2 but no interaction was observed (Ashfaq et al., 2011b). The same result was seen when HCV E1E2-specific antibodies such as ALP98 and AP33 were used. Only when neat L-ficolin sample was used was a signal produced, again indicating that there was insufficient active L-ficolin in the sample for the assay.

In the future, the use of purified L-ficolin is necessary for more reliable results, however higher concentrations are needed. Despite optimisation, it is apparent that there was an issue in the system used. Yields of L-ficolin never exceeded 1.2 µg/mL. Other systems have reported the production of 2 – 6 µg/mL (Ma et al., 2009) to even 1 mg/mL (Geno et al., 2015). Later work by the VRG successfully expressed L-ficolin in Huh7.5 cell lines containing a lentiviral transduction cassette, producing concentrations up to 7 µg/mL (Jalal et al., 2019b). These cells were more resistant to cell-to-cell HCV

transmission and HCVpp entry, and to a lesser extent EBOVpp and VSVpp entry, whereas rabies virus pseudoparticle entry was enhanced. This nevertheless confirmed L-ficolin activity against EBOV. A HEK 293T cell line stably-transfected to produce His_6-tagged L-ficolin-WT was explored in the present study (data not shown). While good amounts of L-ficolin were produced, the cell line's dependence on FBS for growth led to contamination during purification. This could also be an issue with the modified Huh7.5 cell line. A cell line that can grow in serum-free media such as that described in Geno et al. (2015) would be ideal.

In the future, all assays should be repeated using higher concentrations of L-ficolin. Often, the limited yields of L-ficolin alongside the ongoing optimisation of L-ficolin production meant that an assay could only be repeated once, therefore having higher concentrations would strengthen findings.

Chapter 3 L-Ficolin in Synthetic Biology – Scaffolds For Antiviral Proteins

3.1 Aims

L-ficolin has the desirable qualities of a flexible dodecahedral structure and multiple binding sites with affinities towards several ligands, though its antiviral activity could be improved. Nanobodies are highly stable and widely interactive partial antibodies found in animals such as sharks and camelids (Rajan et al., 2015; Tarr et al., 2013; Vanlandschoot et al., 2011). They are heavy chain antibodies consisting of a single variable domain and two constant domains. The *Camelidae* D03 nanobody has the desirable quality of strong affinities towards and neutralisation of several strains of the HCV virus by entry inhibition (Tarr et al., 2013), but has the disadvantage of a short *in vivo* half-life due to its small size (Bannas et al., 2017; Van Audenhove and Gettemans, 2016).

Merit has been proven in combining the superior biological activities of two separate immune proteins in a hybrid molecule (Mason and Tarr, 2015). For example recombinant chimaeric lectins (RCLs) – consisting of the structurally similar lectins MBL and L-ficolin – have been studied (Chang et al., 2011; Michelow et al., 2010; Takahashi et al., 2013). Certain RCL constructs showed stronger antiviral immune activities than their original counterparts.

In this study, production of an alternative recombinant chimaeric molecule named ficobody was investigated by combining L-ficolin and the nanobody D03. It was theorised that the combination of flexible L-ficolin and small D03 would result in a humanised molecule with high affinities towards HCV and a high immunological capacity to neutralise infection. Assays similar to Chapter 2 were performed to allow comparison to L-ficolin-WT.

3.2 Materials and Methods

3.2.1 Growth Conditions and Storage

One Shot™ TOP10 Chemically Competent *E. coli* (Amp^r; Cat. C4040-04; Thermo) were used for rapid high efficiency transformation. Bacteria and the human HEK 293T and Huh7 cell lines were treated as described in Chapter 2.2.1. An additional antibiotic – Kanamycin sulphate (Kan; Cat. 15160-054; Thermo) – was used at 10 µg/mL.

3.2.2 DNA Cloning

Plasmids and primers (Table 3.1) were treated as in Chapter 2.2.2. PCR reactions, agarose gel electrophoresis, restriction enzyme digestion, DNA purification, ligation, transformation and DNA sequencing were performed as detailed in Chapter 2.2.2.

Table 3.1 – List of Primers Used in This Chapter

Forward represents 5' to 3'. Reverse represents 3' to 5'.

Primer Name	Orientation	Primer Sequence (5'→3')	Size/ bp
General Primers			
T7F	Forward	TAATACGACTCACTATAGGG	20
BGHR	Reverse	TAGAAGGCACAGTCGAGG	18
Cloning of Ficobody			
FCN2	Forward	CTTGACCAGGACTACACCATGGAGCTGGACAGAG	34
	Reverse	CGATCCGCCTGACCCACCGGCAGGTCGCACCTTCATC	37
D03	Forward	GGTGGGTCAGGCGGATCGATGGCGGAAGTCCAAC	34
	Reverse	CTGTGGATTACACTGTCCTCACTCGAGTGCGGCC	34
Custom *FCN2-D03* Tag	Forward	CTTGACCAGGACTACACC	18
	Reverse	CTGTGGATTACACTGTCC	18

3.2.2.1 Plasmids

In addition to the virus pseudoparticle plasmids described in Table 2.2, the pcDNA™3.1D/V5-His-TOPO® plasmid (Cat. K490040; Thermo; Amp^r) was again used in the cloning of a ficobody plasmid – pcDNA3.1:FCN2-D03 – for CMV promoter-enhanced protein expression. The pT783:D03 (Kan^r) plasmid for expression of the alpaca nanobody D03 was kindly provided by the Institute of Virology, Medizinische Hochschule Hannover, Germany.

3.2.3 Transient Transfection of HEK 293T Cells

Virus pseudoparticle production and protein expression in HEK 239T cells were carried out as in Chapter 2.2.3.1.

3.2.3.1 Lysis of HEK 293T Cells

To make lysates, 1 mL of lysis buffer (50 mM Tris, 140 mM NaCl, 1 mM EDTA (E6635; Sigma], 1% Igepal® CA-630, pH 8; Cat. I8896; Sigma) containing 100 mM of iodoacetamide (Cat. I1149; Sigma) was added to cells in a 10 cm diameter dish, respectively. Iodoacetamide prevents protein aggregation via non-specific disulphide bridges between cysteines. After rocking at RT for 15 min at 15 rpm, lysates were pooled and centrifuged at 10000 x g for 5 min using a microcentrifuge. The protein-containing supernatant was harvested and stored at -20°C.

3.2.4 Characterisation of Virus Pseudoparticles

Pseudoparticle infectivity assays and virus pseudoparticle neutralisation assays were performed as in Chapter 2.2.4.

3.2.5 Protein Detection

Antibodies (Table 3.2), SDS-PAGE and western blots were treated as in Chapter 2.2.5.

Table 3.2 – List of Antibodies Used in This Chapter

Working concentrations may vary. WB = western blot.

Antibody	Specificity	Clonality	Developed in	Stock Concentration/ µg/mL	Working Concentration/ µg/mL (or Dilution)		Cat. (If Available); Supplier
					WB	ELISA	
Primary Antibody	Anti-Human L-Ficolin Clone GN4	Monoclonal	Mouse	100	0.5	1	HM2090: Hycult® Biotech.
	Anti-Human L-Ficolin Clone GN5	Monoclonal	Mouse	100	0.5	1	HM2091; Hycult® Biotech.
	Anti-HCV E1E2 ALP98	Monoclonal	Mouse		1:400	1:200	Centre for Virus Research, University of Glasgow, UK.
	Anti-HCV E1E2 AP33	Monoclonal	Mouse	150	2	1	
Secondary Antibody	Anti-Mouse Ig-HRP Conjugate	Polyclonal	Rabbit	1300	1.3		P0161; Dako.
	Anti-Mouse IgG (Fc Specific)-AP Conjugate	Polyclonal	Goat			1:1000	A1418; Sigma.
	Streptavidin-AP Conjugate					1:1000	AR001; R&D Systems.

3.2.6 Protein Sample Manipulation.

Nickel affinity chromatography, and protein concentration and buffer exchange by centrifuge filtration, were performed as in Chapter 2.2.6.

3.2.7 Protein Characterisation and Analysis

Several ELISAs were performed as in Chapter 2.2.7, including anti-L-ficolin mAb GN4-capture ELISA and AcBSA ELISA. The same tools for analysis described in Chapter 2.2.8 were also used. The p value threshold for significance is 0.05.

3.2.7.1 Glycoprotein-Capture ELISA

Antibodies or lectins were used to capture glycoprotein (Figure 3.1). Antibodies specific for HCV envelope glycoprotein were used at 1 µg/mL. Lectins were used at 50 µg/mL. Snowdrop *Galanthus nivalis* agglutinin (GNA; Cat. L8275; Sigma) is a lectin that binds several enveloped virus glycoproteins including HCV E1E2 (Ashfaq et al., 2011b). The *Triticum vulgaris* lectin wheat germ agglutinin (WGA) binds EBOV GP (Maruyama et al., 1999). The protocol for the use of lectins was adapted from Flint et al. (2000).

Coating reagents were diluted in 50 mM of carbonate-bicarbonate buffer (pH 9.6; Cat. C3041; Sigma). Following washing and blocking, 200 µL of lysates diluted 1:3 in 1X PBS were added at RT for 2 h. After three washes, sample was diluted 1:3 and added to the wells. Detection was performed as detailed in Chapter 2.2.7.2.

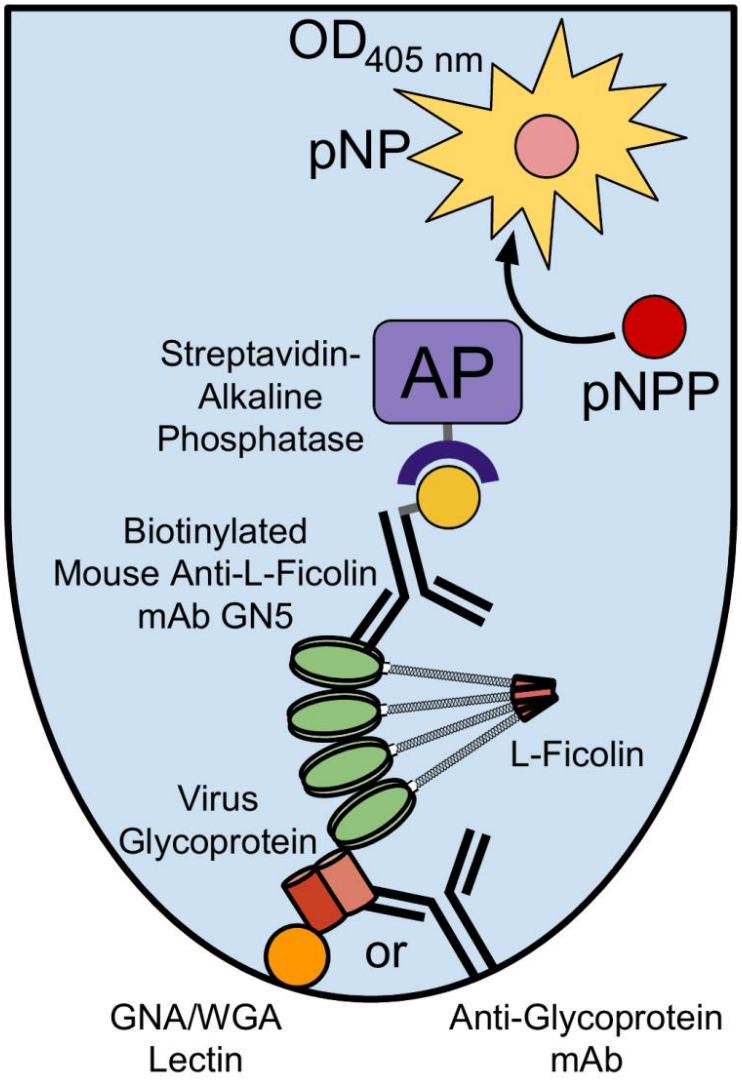

Figure 3.1 – Schematic of Glycoprotein-Capture Enzyme-Linked Immunosorbent Assay

The schematic above represents one of several identical ELISA interactions in a plate well. Lectins – such as Galanthus nivalis *agglutinin (GNA) or wheat germ agglutinin (WGA) – or glycoprotein-specific antibodies are used to capture glycoproteins, which are bound by L-ficolin, which is in turn detected by biotinylated anti-L-ficolin mAb GN5. The biotin is bound by streptavidin conjugated to alkaline-phosphatase (AP), which converts* p*-nitrophenyl phosphate to* p*-nitrophenol and omits light which can be measured at* $OD_{405\ nm}$ *to correlate to the amount of bound L-ficolin.*

3.3 Results

3.3.1 Cloning and Expression of Ficobody

To create the proposed ficobody molecule, *D03* nanobody DNA was combined to the 3' end of the N-terminal His_6-tagged wild-type *FCN2* fragment using a sequence encoding a Gly-Gly-Ser-Gly-Gly-Ser linker (Figure 3.2).

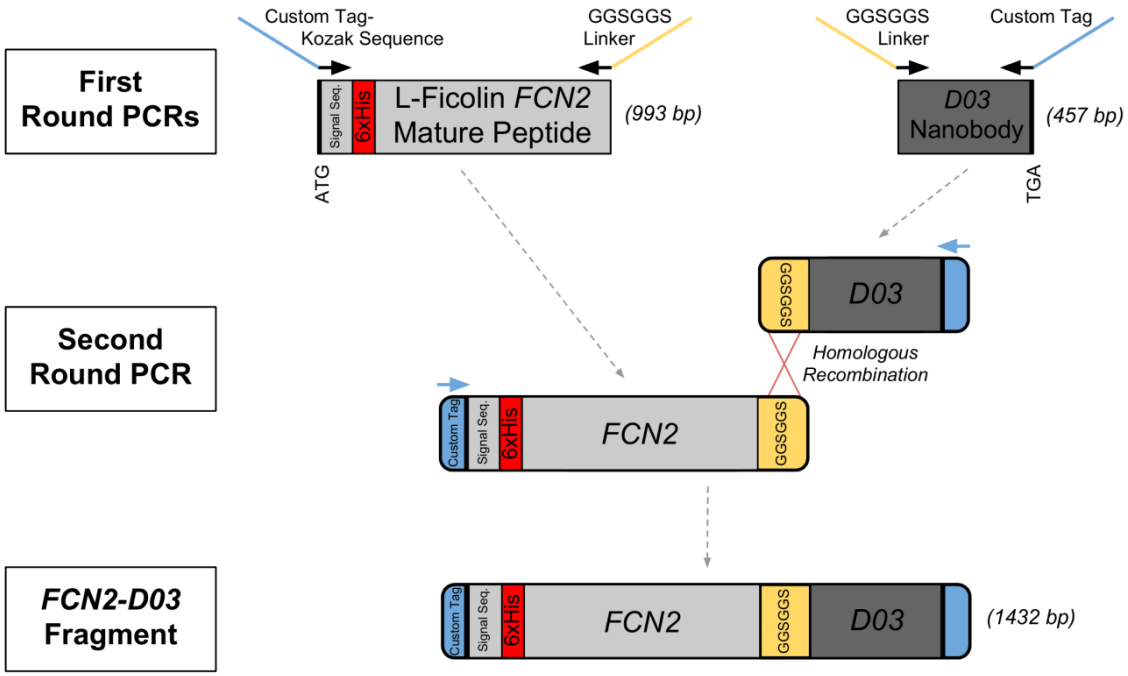

Figure 3.2 – Cloning Method to Produce Ficobody

First round PCRs would be used to amplify two custom-tagged fragments; Firstly, an FCN2 *(L-ficolin) fragment containing a Kozak sequence, the* FCN2 *sequence with a His_6-tag downstream of the signal sequence, followed by a 3' GGSGGS linker DNA sequence. Secondly, a* D03 *(nanobody) fragment with a 5' GGSGGS linker sequence. A second round PCR reaction using custom FCN2-D03 tag primers (→ and ←) would allow homologous recombination at the GGSGGS linker to produce the* FCN2-D03 *fragment encoding the N-terminal His_6-tagged ficobody protein.*

The two first round PCR reactions were performed using the *FCN2* primer pair with the pcDNA3.1:N-His-FCN2-WT plasmid as template, and the *D03* primer pair with the pT783:D03 plasmid (Figure 3.3.1).

The resulting custom-tagged *FCN2* and *D03* fragments were DpnI-digested, purified and used in a PCR with the custom *FCN2-D03* tag primer pair to allow homologous recombination at the GGSGGS linker DNA sequence (Figure 3.3.2). Sequencing revealed the successful amplification of the *FCN2-D03* fragment, which was ligated into the pcDNA™3.1D/ V5-His-TOPO® plasmid and transformed into *E. coli*. After ampicillin selection on LB agar, colonies were screened using the vector-specific T7F and BGHR primers, and the T7F and fragment-specific custom *FCN2-D03* tag reverse primer, to confirm insert orientation (Figure 3.3.3).

In several colonies, the expected 1522 bp and 1697 bp fragments containing the *FCN2-D03* fragment were amplified using the fragment-specific and generic primer sets, respectively. The successful pcDNA3.1:FCN2-D03 plasmid was isolated by miniprep. Sequencing using the T7F and BGHR primers confirmed successful ligation.

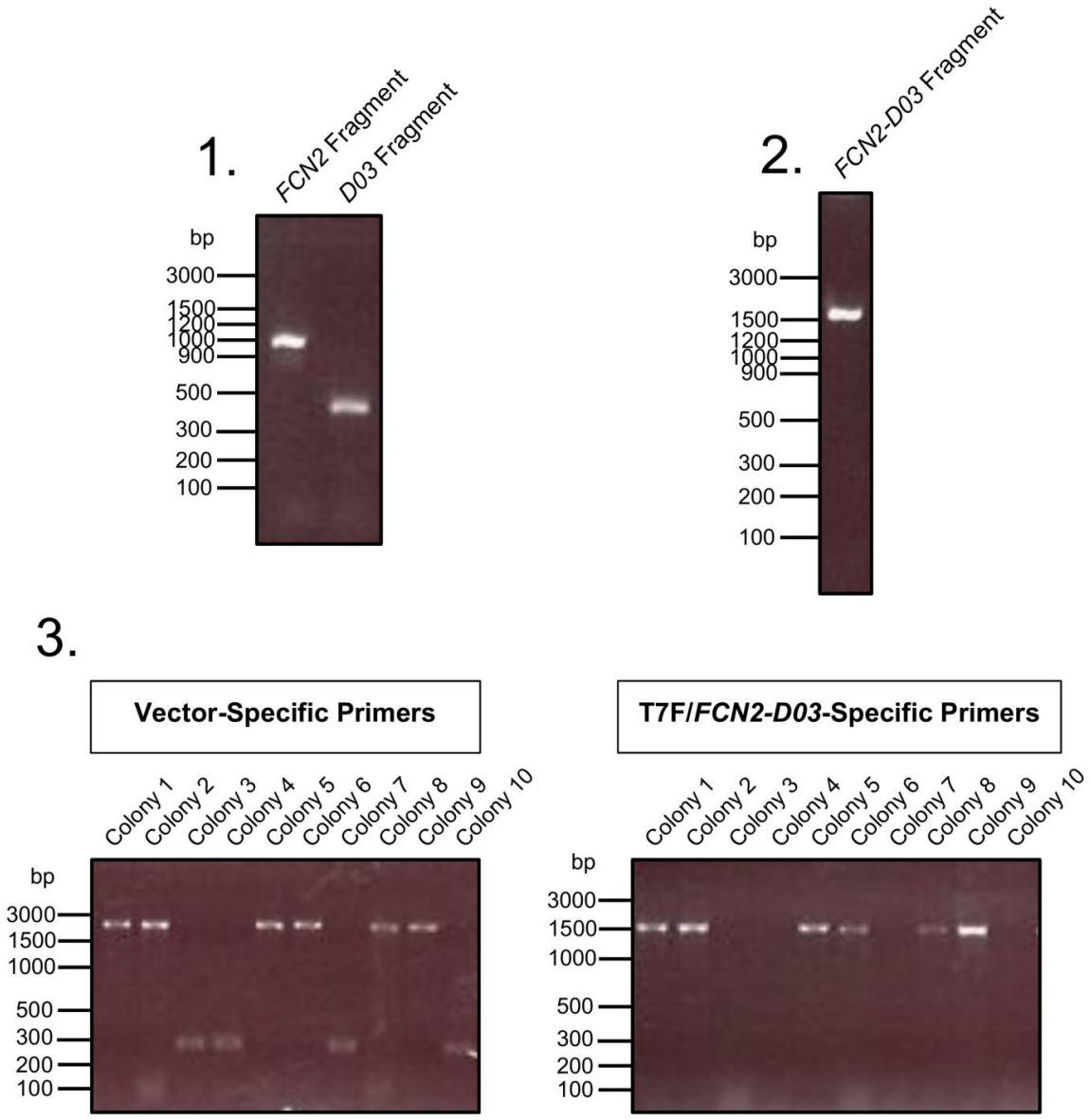

Figure 3.3 – Cloning of the *FCN2-D03* Sequence Encoding Ficobody

Gel electrophoresis and ethidium bromide staining of PCR products with DNA size markers shown in base pairs (bp). ***1.*** *The N-terminal* His_6*-tagged* FCN2 *fragment (993 bp) and* D03 *fragment (457 bp) were amplified.* ***2.*** *Homologous recombination of the fragments produced the 1432 bp* FCN2-D03 *fragment.* ***3.*** *Using colonies of transformed* E. coli *TOP10 cells under selection, PCR was performed with the T7F forward primer and the vector-specific BGHR reverse primer (1697 bp band) or the* FCN2-D03*-specific reverse primer (1522 bp band). This, and sequencing, confirmed successful ligation of the pcDNA3.1:FCN2-D03 plasmid in the correct orientation.*

Using the pcDNA3.1:FCN2-D03 plasmid, recombinant ficobody protein was produced in 200 mL of serum-free Opti-MEM using HEK 239T cells. A negative control using no expression plasmid was also produced. As in Chapter 2.3.1, samples would be either non-purified or purified.

Samples were purified using nickel affinity chromatography, as described in Chapter 2.3.1.1. During purification, both samples showed two OD_{280nm} peaks – corresponding to protein concentration – at the 10^{th} and 12^{th} elution fractions (Figure 3.4.1). In a western blot using the anti-L-ficolin mAb GN5, bands of ~50 kDa representing monomeric ficobody and multimers were detected in the elution fractions corresponding to the OD_{280nm} peaks. No L-ficolin or ficobody was detected in the negative control (Figure 3.4.2). The 8^{th} to 25^{th} elution fractions were pooled for ficobody and the negative control, then filtered and concentrated before dialysis into 1.4 mL of PBS.

Concentrated, non-purified and non-dialysed ficobody produced similar bands of multiples of ~50 kDa in a western blot using anti-L-ficolin mAb GN5 (Figure 3.5). Ficobody bands had a higher MW than the L-ficolin-WT control which produced the expected ~40 and higher multimer kDa band pattern. The mock-transfection negative control contained no L-ficolin. Silver staining of the same samples in an SDS-PAGE gel showed a heavy 50 kDa band, likely representing an Opti-MEM media component. The ~80 kDa and ~200 kDa bands of L-ficolin as seen in the western blot are perhaps seen in the SDS-PAGE, while the ~100 kDa and ~250 kDa bands of ficobody may also be visible. These results were consistent throughout expression, and suggest that the production, secretion and purification of ficobody were successful. It also shows that the anti-L-ficolin mAb GN5 epitope is still accessible to the antibody.

Samples were quantified using the L-ficolin-specific mAb GN4-capture ELISA as detailed in Figure 2.5.

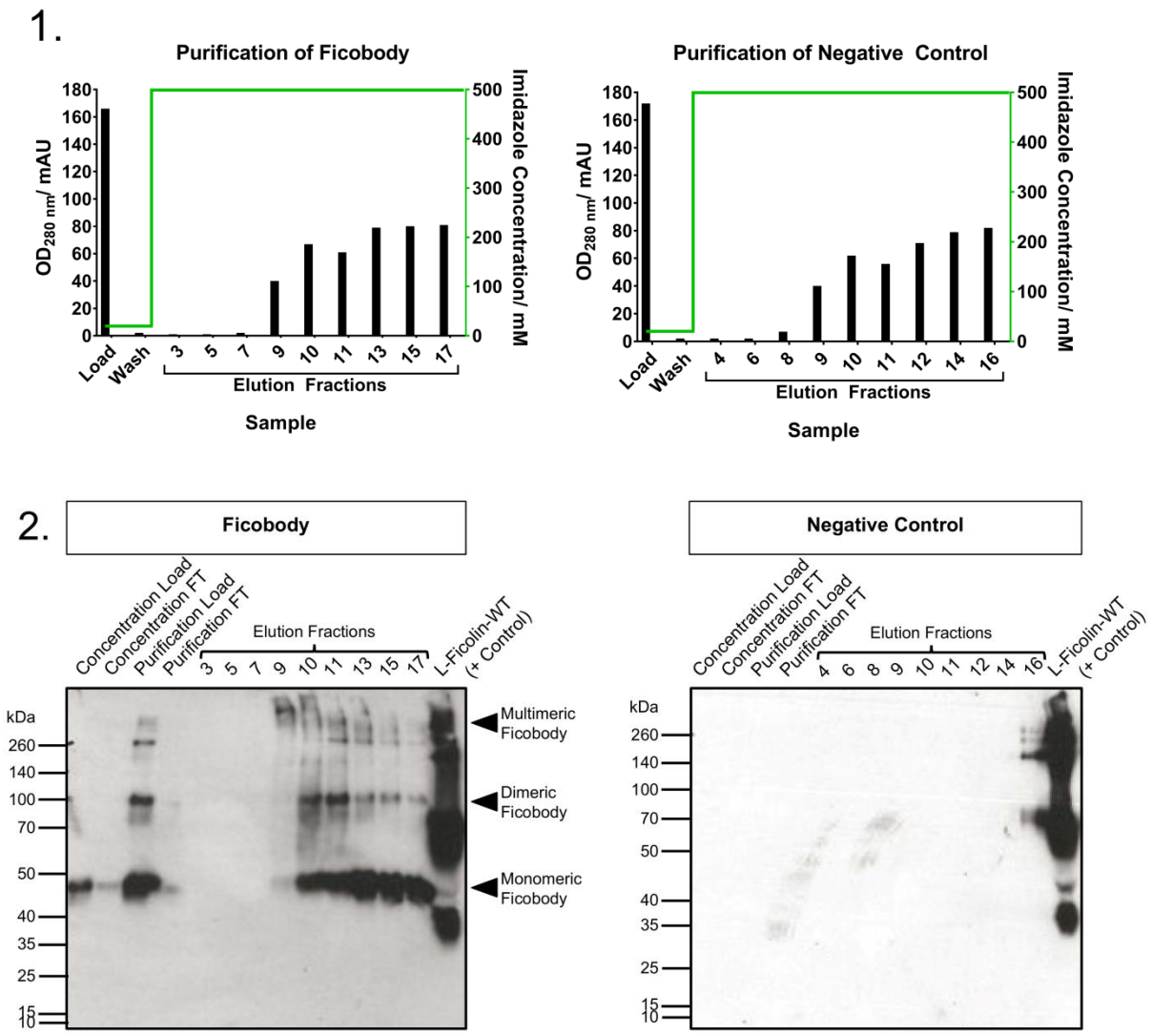

Figure 3.4 – Detection of Ficobody Purified by Nickel Affinity Chromatography

Ficobody expressed by HEK 293T and negative control (no expression plasmid) were harvested, concentrated and purified by nickel affinity chromatography using 500 mM imidazole. ***1.*** *Protein content was monitored during purification by absorbance (OD_{280nm}). In both samples, protein was detected in the load sample and in elution fractions 9 onwards, with two absorbance peaks of ~65 and ~80 mAU.* ***2.*** *Western blot analysis using the L-ficolin-specific antibody GN5 detected ficobody monomers (50 kDa) and multimers in elution fractions 9 onwards, whereas no protein was detected in the negative control. The L-ficolin control was detected as expected in both western blots. This shows that ficobody can be expressed and purified.*

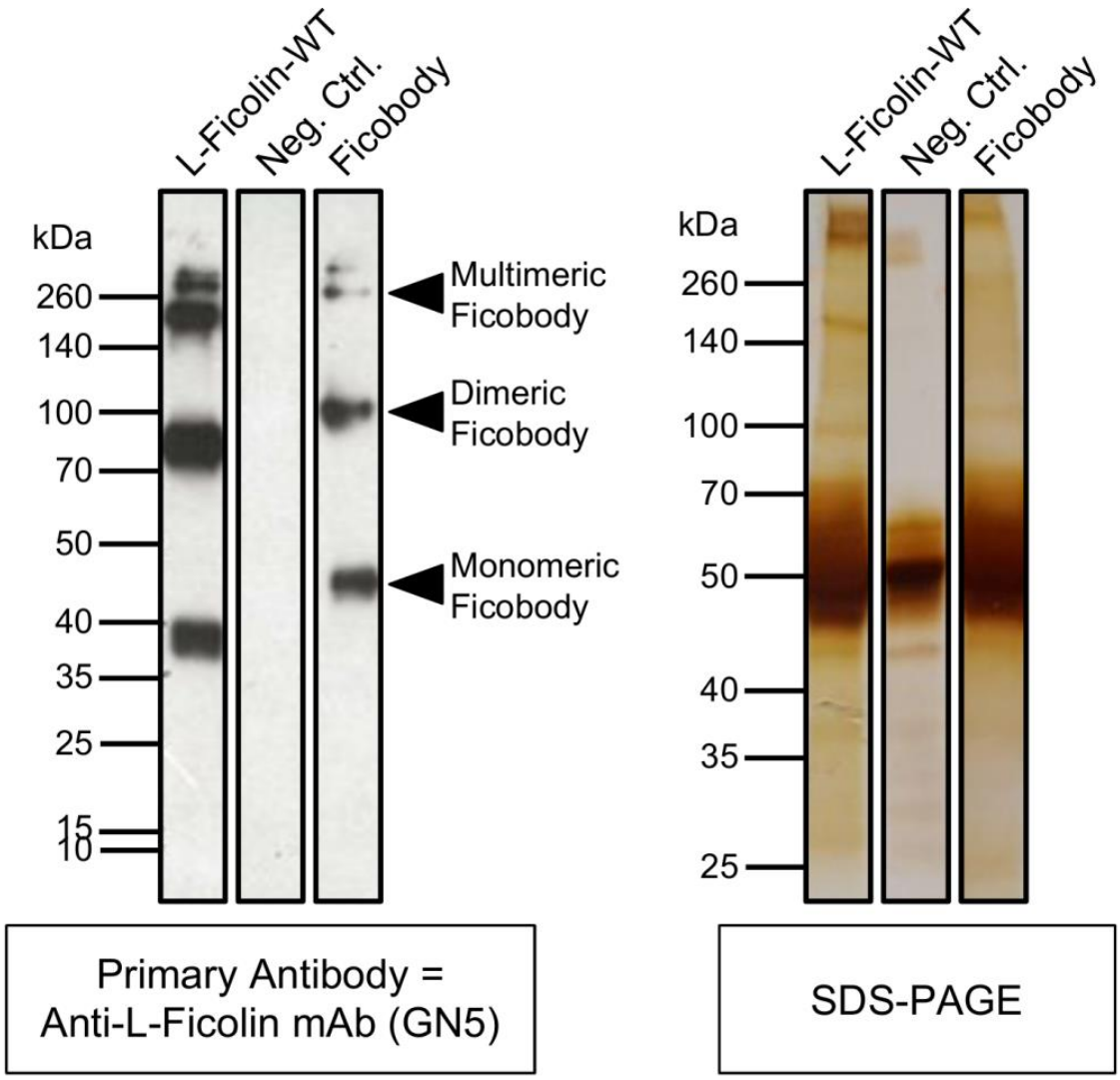

Figure 3.5 – Detection of Non-Purified Ficobody

Non-purified ficobody, an N-terminal His_6-tagged L-ficolin-WT positive control and a mock transfection negative control were run on an SDS-PAGE gel. ***1.*** *Western blot detection using the L-ficolin-specific monoclonal antibody GN5 detected ~50 kDa ficobody monomer, ~100 kDa dimer, and >~260 kDa multimers. The expected band pattern of multiples of ~40 kDa was detected for the L-ficolin control, while the negative control had no bands.* ***2.*** *Silver staining of the SDS-PAGE showed prevalent ~50 kDa proteins in all samples, with faint detection of the bands detected in the western blot. This indicates successful production of ficobody.*

3.3.2 Ficobody Exhibits Stronger Binding to Acetylated BSA Compared to L-Ficolin

Several of the assays in Chapter 2.3 were repeated to compare the capacity of ficobody to bind AcBSA with the L-ficolin-WT construct on which ficobody was based. It was hypothesised that the D03 modification in ficobody would enhance L-ficolin binding of AcBSA. To investigate this, a batch of non-purified ficobody was produced with a concentration of 0.3 µg/mL. In three independent AcBSA ELISAs, ficobody and a mock-transfection negative control were tested. An L-ficolin-WT sample normalised to 0.3 µg/mL was also used in two of these assays, due to limited sample.

ANOVA and Dunnett's multiple comparison analysis of the results found the ficobody had significantly higher binding levels ($p = 0.0001$) than the L-ficolin sample (Figure 3.6). The negative control had no activity, with a signal below the detection threshold of the assay. This indicates that the addition of D03 enhances binding to AcBSA.

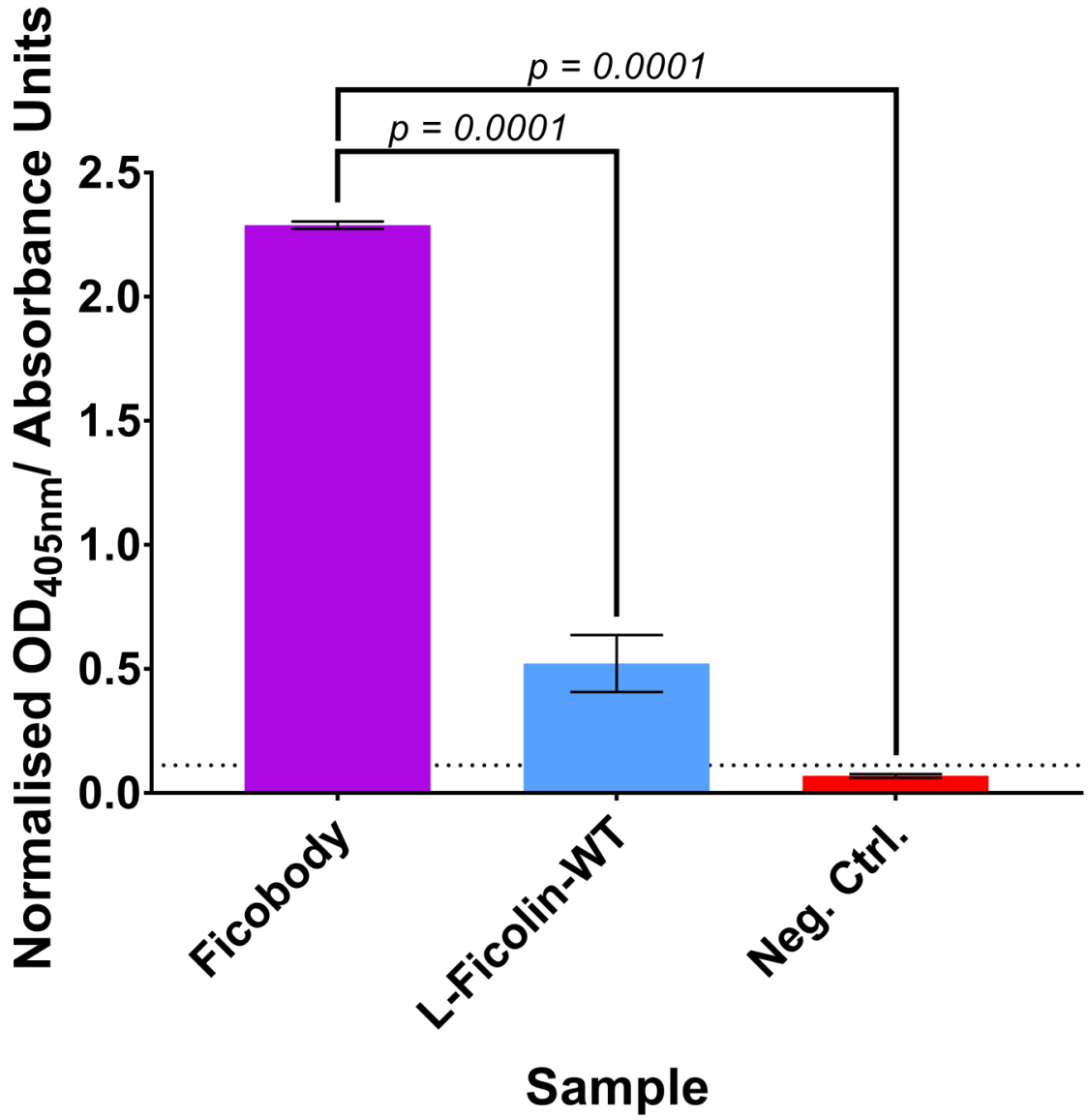

Figure 3.6 – Differences in Binding to Acetylated BSA between Ficobody and L-Ficolin

Binding of ficobody to AcBSA was tested in duplicate in an ELISA (n = 3). Wild-type L-ficolin normalised to the ficobody concentration (0.3 µg/mL) was also used (n = 2). Ficobody had an almost five-fold higher binding level than L-ficolin (p = 0.0001). A mock-transfection negative control had no binding activity. One-way ANOVA and Dunnett's multiple comparison analysis were used (p value threshold of significance = 0.05). Error bars represent standard error of mean. The dotted lines indicates the lower limit of detection of the assay (mean of negative sample + (2 x S.D.)).

To assess if ficobody binding is also Ca^{2+}-dependent, an AcBSA ELISA was performed in the presence of varying concentrations of $CaCl_2$ or EDTA, a calcium chelator. If binding was Ca^{2+}-independent, EDTA would not affect binding.

Ficobody and L-ficolin-WT (at 0.3 µg/mL) were pre-incubated with 0 mM, 0.5 mM and 5 mM and 50 mM of $CaCl_2$ or EDTA. Samples were diluted in TBST containing 5 mM $CaCl_2$ in the EDTA ELISA. A negative control produced without expression plasmid was also used. Due to expression issues, time constraints and limited sample, only one assay was performed, therefore data was not conclusive.

Binding by both samples was unaffected by varying $CaCl_2$ concentrations (Figure 3.7.1). As with the assay performed using the L-ficolin variants, the Opti-MEM media of the non-purified sample likely still contained Ca^{2+} ions, and therefore the 0 mM $CaCl_2$ value likely do not truly represent 0 mM $CaCl_2$. Nevertheless, high concentrations of $CaCl_2$ had no effect.

As seen with L-ficolin, EDTA inhibited ficobody binding to AcBSA (Figure 3.7.2) with 50% inhibition of binding (IC_{50}) of 2.2 mM for ficobody and 1.7 mM for L-ficolin-WT. In both assays, the negative control had no activity. These results imply that ficobody binding of AcBSA is Ca^{2+}-dependent, however higher concentrations of EDTA were required to inhibit binding. This supports the increased strength of ficobody binding to AcBSA.

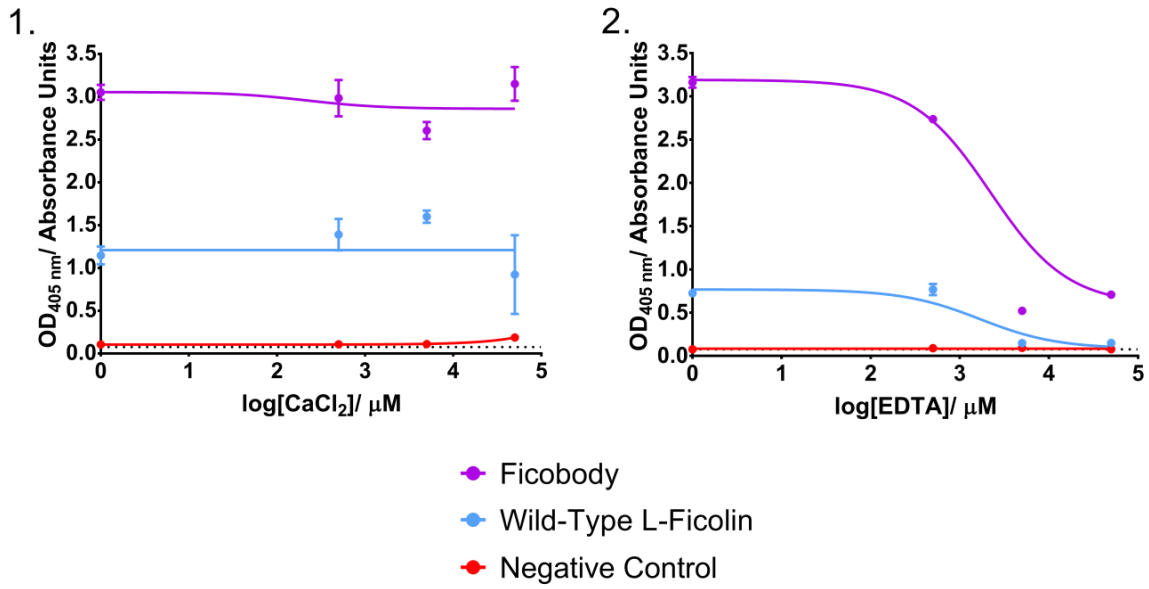

Figure 3.7 – The Effects of $CaCl_2$ and EDTA on Ficobody Binding to AcBSA

An AcBSA-binding ELISA was performed using non-purified ficobody and wild-type L-ficolin in duplicate exposed to 0 mM, 0.5 mM, 50 mM and 500 mM of $CaCl_2$ or EDTA (n = 1). ***1.*** *High concentrations of $CaCl_2$ did not affect ficobody binding. The sample media likely contained $CaCl_2$, therefore 0 mM $CaCl_2$ is not representative.* ***2.*** *EDTA, a calcium inhibitor, inhibited ficobody (IC_{50} = 2.2 mM) and L-ficolin-WT (IC_{50} = 1.7 mM) binding of AcBSA, as determined by fitting the data with non-linear regression. This supports the observation that ficobody binding to AcBSA is strengthened. Error bars represent standard error of mean. Dotted lines indicate the lower limit of detection of the assay (mean of negative sample + (2 x S.D.)).*

3.3.3 Interaction of Ficobody with Viruses

As nanobodies have shown interaction with HCV, and due to the superior binding activity of ficobody to AcBSA it was proposed that ficobody would also have an enhanced virus glycoprotein-binding and pseudoparticle-neutralising activity than L-ficolin.

The effect of ficobody against virus entry was investigated in a virus pseudoparticle neutralisation assay. Virus pseudoparticles were produced using an HIV-1 backbone and HCV, VSV and EBOV glycoproteins. The purified samples were non-normalised, to increase potential for activity. Ficobody was used at 1.0 µg/mL and L-ficolin-WT being used at 0.9 µg/mL. A negative control produced without expression plasmid was also used.

The data shown in Figure 3.8 is representative of several assays using several preparations of ficobody, as well as all other recombinant L-ficolin samples prepared in this project. With all pseudoparticles, no difference in neutralisation was observed between samples and the negative control. Despite purification, a contaminating factor present in all of the samples including the negative control, allowing partial neutralisation of HCVpp and EBOVpp entry. The seemingly enhancing effect of Sudan EBOVpp entry by samples was consistently seen throughout the project, including by the L-ficolin variants (data not shown).

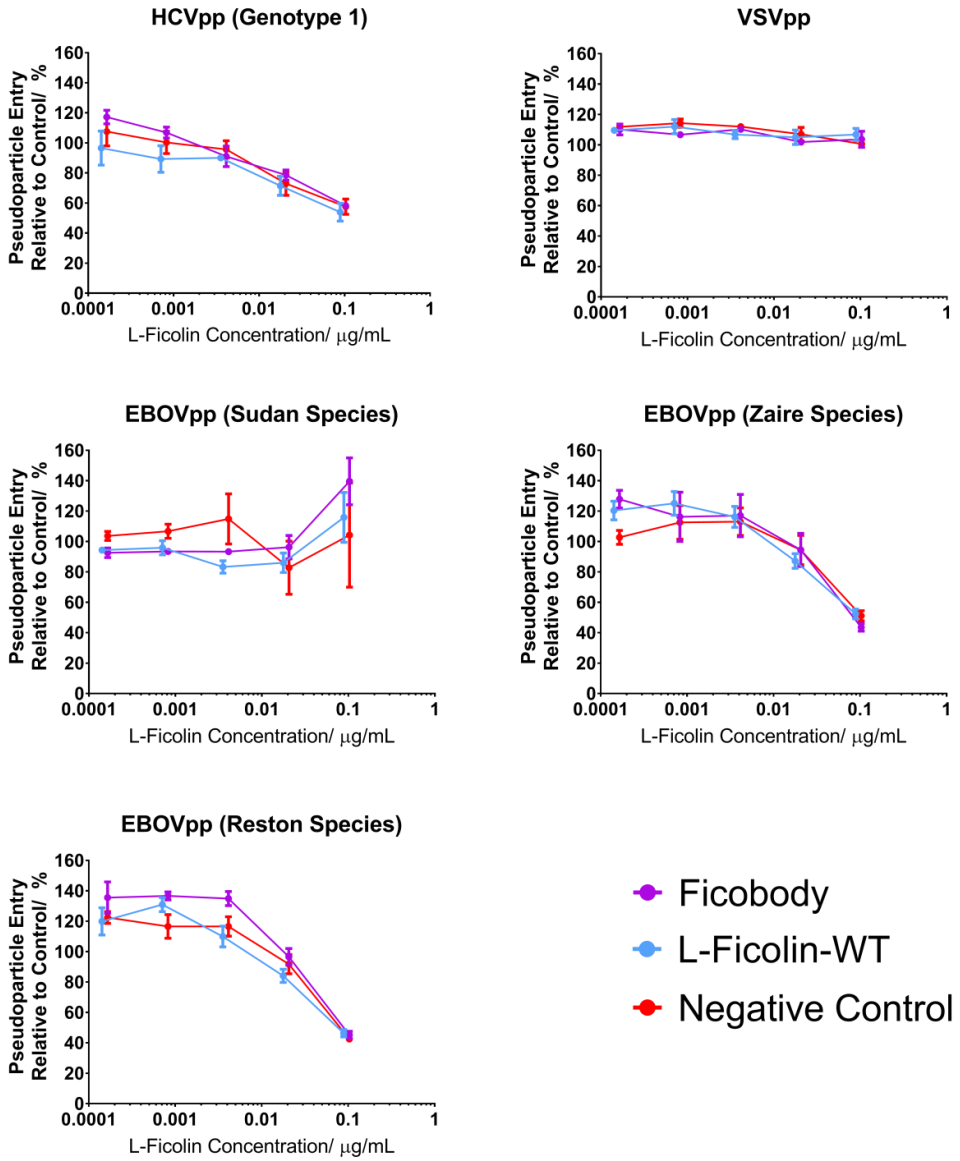

Figure 3.8 – Neutralisation of Virus Pseudoparticle Entry by Ficobody

Nickel affinity-purified ficobody (1.0 µg/mL) and wild-type L-ficolin (0.9 µg/mL) were pre-incubated with retroviral pseudoparticles in triplicate and added to Huh7 cells. Pseudoparticles expressing HCV, VSV and EBOV envelope glycoproteins were used (n = 1). Infectivity was determined by pseudoparticle-based luciferase activity in the Huh7 cells and normalised to glycoprotein-deficient (0%) and uninhibited (100%) pseudoparticle controls. In all cases, protein sample did not differ from the mock-transfection negative control, suggesting insufficient concentrations were used. Dose-dependent neutralisation was seen for several pseudoparticles, indicating that a contaminating protein was able to inhibit virus pseudoparticle entry.

Due to low protein yields, ELISA was explored as an alternative method with higher sensitivity for the detection of glycoprotein interaction with ficobody. Lectins known to interact with the viruses of interest were used to capture virus glycoproteins retrieved from the lysates of HEK 293T used for pseudoparticle expression. L-ficolin interaction with these glycoproteins was measured using AP-derived absorbance. Due to extensive optimisation efforts and restricted amounts of sample, each of the following assays were only performed once using non-normalised samples, therefore the data can be considered as preliminary.

The lectin GNA was used to capture HCV and VSV glycoproteins, and WGA was used to capture EBOV glycoproteins. A 0.3 µg/mL ficobody sample was used while a 0.4 µg/mL L-ficolin-WT was used, as determined by anti-L-ficolin mAb GN4-capture ELISA. A two-way ANOVA with Tukey's multiple comparison test found a significant difference between all samples when binding to all glycoproteins. However there was a significant difference even in binding to the non-glycoprotein lysate sample, indicating that the ficobody and L-ficolin samples were able to bind another possibly acetylated factor within the GNA/WGA-captured lysate.

Thus, a two-way ANOVA with Tukey's multiple comparison test was instead used to compare the binding of different glycoproteins to binding of the no glycoprotein lysate, for each sample. For all HCV, VSV and EBOV glycoproteins, no binding was detected from the L-ficolin and negative control samples. However, binding of ficobody to both the genotype 1 (H77.20) and genotype 2 (J6) strains of HCV was highly significant ($p = 0.0001$), but not to VSV or EBOV glycoproteins.

This shows that ficobody is able to bind specifically to HCV, as previously reported for L-ficolin and D03 nanobody. As L-ficolin is known to bind HCV, protein yields must be enhanced. Background binding demonstrates that the ELISA protocol also requires optimisation.

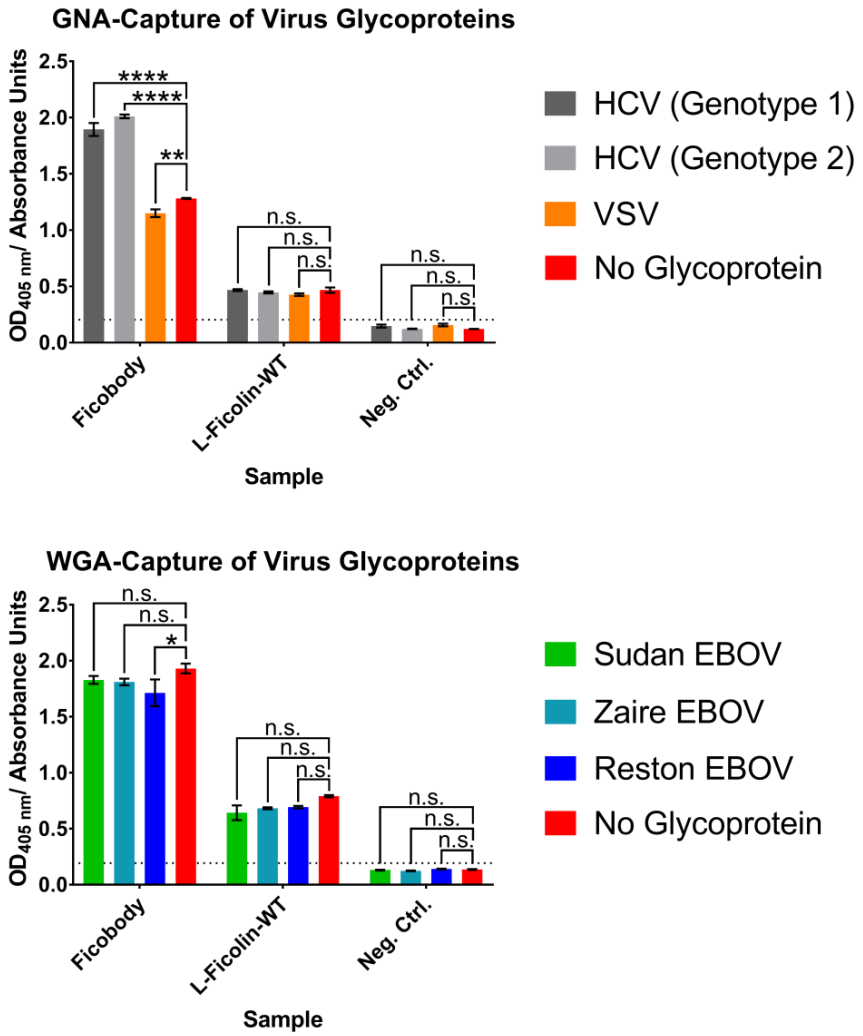

Figure 3.9 – Binding of Ficobody to Virus Glycoproteins

*An ELISA was performed using the lectins GNA and WGA to capture virus glycoproteins in lysates (n = 1). Ficobody (0.3 µg/mL), L-ficolin (0.4 µg/mL) and a mock-transfection negative control were added in duplicate. Two-way ANOVA (Tukey's multiple comparison) analysis compared binding to a no glycoprotein control. The p value threshold of significance is 0.05 (No significance (n.s.) ≥ 0.1234; * = 0.0110; ** = 0.0033; **** = 0.0001). Ficobody significantly bound HCV genotype 1 and genotype 2 strains of E1E2 glycoprotein (p = 0.0001). L-ficolin and negative control showed no significant binding, though L-ficolin bound another GNA/WGA-captured factor. This data shows the strength of binding of ficobody, and the need for higher yields of L-ficolin. Error bars represent standard error of mean. Dotted lines indicate the lower limit of detection of the assay (mean of negative sample + (2 x S.D.)).*

3.4 Discussion

3.4.1 Design of the Ficobody Chimaeric Molecule

Research into recombinant chimaeric lectins of L-ficolin and MBL provided evidence for the advantages of such hybrid molecules. For example, recombinant active MBL is complicated and expensive to produce, due to its complex structure (Michelow et al., 2010), whereas RCLs can exhibit superior protective potency, cost-effectiveness and safety (Hartshorn et al., 2000; Jensenius et al., 2003; Petersen et al., 2006; White et al., 2000). RCL2 used the dodecameric structure and flexibility of L-ficolin to enhance MBL-mediated ligand binding (Michelow et al., 2010). RCL2 also displayed strong complement activation and opsonophagocytic properties due to combination of MBL and L-ficolin MASP- and calreticulin-binding sites (Michelow et al., 2010). Physiological levels of RCL2 neutralised influenza A, Hendra, Nipah and EBOV virus infection (Michelow et al., 2010), when MBL was previously found to enhance EBOV infection (Brudner et al., 2013; Mason and Tarr, 2015). These antiviral activities were mediated by enhanced complement activation, viral aggregation and inhibition of virus envelope glycoprotein activities (Chang et al., 2011). RCL3 also diminished coagulation system activities and maintained a cytokine and inflammatory balance *in vivo*, which is more advantageous for the host (Chang et al., 2011; Takahashi et al., 2013). These studies revealed the favourable qualities and activities of L-ficolin for use as a scaffold in a chimaeric molecule, such as a flexible structure and ability to interact with several pathogens.

The nanobody D03 is a strong HCV inhibitor (Tarr et al., 2013). However, nanobodies are known to have a short half-life *in vivo*, which can be extended by fusing the protein to another nanobody with specificity for serum albumin to increase size and reduce loss via renal filtration (Bannas et al., 2017; Van Audenhove and Gettemans, 2016). This led to the proposal that a chimaera of L-ficolin and D03 would enhance the activity and stability of both molecules. A wild-type L-ficolin scaffold was chosen rather than a variant to allow a wider comparison in literature. Unlike Michelow et al. (2010), it was decided to retain

the full structures of both molecules in the ficobody due to the small size of the nanobody. Theoretically, the increased size gifted to D03 by L-ficolin should humanise the protein and prevent its size-based filtration in the kidney. This could be confirmed through half-life investigations *in vivo* in mice.

A GGSGGS amino acid linker was chosen to combine the two proteins, due to its previous success (Evers et al., 2006; Lindenburg and Merkx, 2014; Nordlund et al., 2004). This linker provides flexibility and solubility, as seen with nanobody fusion (Bannas et al., 2017). Coiled loop structures formed by glycine-rich linkers are favoured in nature and recombinant design, and can influence protein stability and function (Reddy Chichili et al., 2013). Loop length may inversely correlate with stability (Nagi and Regan, 1997), therefore a smaller linker was used. The length of this linker could be optimised, though current results suggest that a GGSGGS linker still allows enhanced activity.

Use of the ficobody expression plasmid did not appear to alter expression levels. The consensus Kozak sequence is 5'-GCCRCCATGG-3' (Xia, 2007), while the native L-ficolin Kozak DNA sequence is 5'-GAAGAGATGG-3'. L-ficolin used in this study had a Kozak sequence of 5'-AACACCATGG-3', whereas the ficobody Kozak sequence was 5'-TACACCATGG-3'. The T base at -6 may influence protein yields and could be further optimised.

3.4.2 Binding Activity of Ficobody

Binding of ficobody to AcBSA was found to be superior to L-ficolin, even when a lower protein concentration was used. This enhanced binding could be attributed to the D03 domain, as this was the only difference between the ficobody and L-ficolin constructs, although D03 binding to acetylated compounds has not previously been described. The D03 region of ficobody may induce a conformational change which could inhibit L-ficolin activity. In which case, the binding observed would solely be a result of the D03 domain. A D03 molecule comparison would solve this. Nanobody stability has been enhanced by specific mutagenesis and the addition of a disulphide bond (Liu et al., 2017). These modifications could be also applied to the nanobody region of ficobody to improve stability.

Similar to L-ficolin, AcBSA binding by ficobody appeared to be Ca^{2+}-dependent. L-ficolin possesses a Ca^{2+}-binding site and a Ca^{2+}-dependent S1 binding site (Garlatti et al., 2007), however Ca^{2+}-dependence of nanobodies has not yet been described. These results could suggest that nanobody binding of AcBSA is Ca^{2+}-dependent, and possibly that L-ficolin activity contributes towards ficobody activity. The EDTA IC_{50} against AcBSA binding was marginally higher for ficobody (2.2 mM) than for L-ficolin (1.7 mM). This is likely a result of stronger binding by the ficobody protein. The assay should be repeated using samples of higher concentrations and a wider range of EDTA concentrations, to provide more accurate IC_{50} results. Furthermore, the D03 nanobody should also be used to confirm Ca^{2+}-dependence. Though it would likely have been removed from the ELISA plate before addition of the secondary antibody, it should be noted that high EDTA concentrations can interfere with alkaline phosphatase activity (Tate and Ward, 2004).

Both L-ficolin and D03 nanobody are known to interact with and neutralise entry of the H77.20 strain of HCV (Hamed et al., 2014; Tarr et al., 2013). While a neutralisation assay was not sufficiently sensitive to detect virus interaction with the concentrations used, ficobody bound both the H77.20 genotype 1a and J6 genotype 2a strains of HCV in an ELISA. This suggests that the wide HCV genotype specificity of D03 and possibly L-ficolin is retained in the ficobody protein. Tarr et al. (2013) used a D03 sample at a concentration of 10 µg/mL in a pseudoparticle neutralisation assay; a concentration 150 times higher than that of the working concentration of ficobody used in this assay. Ficobody yields must be increased to better establish glycoprotein interactions. This would also allow more efficient purification, such as nickel affinity, size exclusion or ion exchange chromatography. Such purification could also remove the neutralising activity of the negative mock-transfection control in the neutralisation assay.

No interaction of L-ficolin with EBOV was detected, however the highly glycosylated nature of EBOV GP makes it a likely candidate for L-ficolin and possibly D03 interaction. Indeed, MBL and M-ficolin have been shown to bind EBOV GP (Brudner et al., 2013; Favier et al., 2016) and further work by the

VRG has since shown L-ficolin neutralisation of EBOV activity ((Jalal et al., 2019b)). This contradicted Favier et al. (2016) who reported no interaction of L-ficolin with the Mayinga strain of Zaire EBOV glycoprotein.

The ELISAs used for glycoprotein interaction require optimisation. Instead of WGA, a glycoprotein-specific monoclonal antibody would be superior to capture lysates in an ELISA. Weak interaction was detected using the anti-HCV E1E2 mAb AP33 to capture HCV glycoproteins, however sample yields again prevented reliable data from being obtained. At the time of testing, an anti-EBOV glycoprotein antibody was not easy to obtain. Efforts were made to produce the anti-EBOV glycoprotein monoclonal antibody KZ52. Efforts were also made to bio-label the glycoproteins of a variety of strains of Zaire EBOVpp lysates (data not shown). The bio-labels of these glycoproteins could be exploited to detect ficobody and L-ficolin interaction.

Lysates were used to obtain glycoproteins from pseudoparticles, to maintain a more authentic structure. Modified and purified soluble HCV glycoproteins produced in HEK 293T cells have been successfully used in a GNA-capture ELISA (Ruwona et al., 2014). Lectins such as jacalin or WGA could be used to purify glycoproteins from lysates for use in the ELISA (Lee et al., 2012).

Once the data presented here is optimised, further experiments could be performed. For example, a wider array of ligands and viruses could be investigated for ficobody interaction both *in vitro* and *in vivo*, such as in mice models infected with IAV. How the structure of L-ficolin varies from its original counterparts could be determined through X-ray crystallography. It cannot be confirmed from the current dataset if binding is purely D03-based, or a combination of L-ficolin and D03. In the future, various combinations of mutations of the ligand-binding sites of L-ficolin and D03 should be made to determine the binding sites involved in ficobody binding and specificities. Furthermore, more ficobody constructs could be produced using other nanobodies that are biologically active against a range of pathogens, for example the nanobodies specifically raised against EBOV by Liu et al. (2017).

The potential therapeutic uses of ficobody will be discussed in Chapter 6.

Chapter 4 Characterisation of L-Ficolin Serum Concentrations in HCV-Infected Patients

4.1 Aims

The significance of serum L-ficolin concentrations has been investigated for several viral and other microbial infections. For example, higher L-ficolin concentrations – influenced by specific *FCN2* allele haplotypes – were observed during acute rather than chronic HBV infection, suggesting a role of L-ficolin protection against persistent HBV infection (Hoang et al., 2011). Furthermore, ethnicity has been observed to influence HCV infection outcome (Sugimoto et al., 2003) and frequencies of *FCN2* haplotypes (Hummelshøj et al., 2008).

Here, two HCV patient cohorts were used to investigate the effects of serum L-ficolin concentrations against HCV infection and ligand binding activity. Correlations with ethnicity and specific *FCN2* SNPs were also investigated. By identifying such correlations, immune responses to HCV could be predicted and an optimal form of the protein could be exploited for anti-HCV treatment.

4.2 Materials and Methods

4.2.1 Serum Samples

The Trinity College Dublin (TCD) cohort consisted of serum samples collected in 2010 from a unique HCV infection cohort (Barrett et al., 2001), provided by Trinity College Dublin. Virus was inactivated by detergent by the TCD.

The ethnicity cohort of chronic HCV patients was selected from the Trent HCV Cohort with the permission of the VRG of the School of Life Sciences and NIHR Biomedical Research Unit in Gastrointestinal and Liver Diseases, Faculty of Medicine and Health Sciences, University of Nottingham, UK. Samples had live virus and were stored at -80°C, often for several years.

4.2.2 Indirect Enzyme-Linked Immunosorbent Assays

Two ELISAs were used to capture L-ficolin, using the basic protocol described in Chapter 2.2.7.2. Interpolation of L-ficolin concentrations was performed using the serum sample containing 4 µg/mL of L-ficolin, as detailed in Chapter 2.2.7.2.1. The anti-human L-ficolin mAbs GN4 and GN5 used in this chapter are detailed in Table 2.4.

4.2.2.1 AcBSA-Capture ELISA

AcBSA-binding ELISAs were performed as in Chapter 2.2.7.2.2 however samples were diluted 1:50. To interpolate the L-ficolin concentration of serum samples using absorbance values, the standard curve was fitted with a linear regression.

4.2.2.2 Anti-L-Ficolin Monoclonal Antibody GN4-Capture ELISA

This ELISA was performed for L-ficolin quantification as described in Chapter 2.2.7.2.1. Samples were diluted 1:10. Standard curves using serum L-ficolin were fitted with a sigmoidal, 4PL regression for the interpolation of L-ficolin concentration.

4.2.3 Virus Pseudoparticle Production and Characterisation

Virus pseudoparticle production, infectivity assay, neutralisation assay and the measurement of luminescence was performed as described in Chapters 2.2.3.1 and 2.2.4. Plasmids encoding the genotype 1 H77.20 strain of HCV E1E2 and VSV glycoprotein were produced using the phCMV5349/pTG126 backbone/reporter plasmids in this chapter.

A positive control of the pseudoparticle of interest in the presence of a 1:10 dilution of 1X PBS and a non-infectious ΔE negative control lacking glycoprotein were consistently used to allow the normalisation of luminescence values, representing 100% and 0% infectivity, respectively.

4.2.4 Polymerase Chain Reaction

PCR amplification of DNA using serum samples was achieved using the Thermo Scientific™ Phusion™ blood direct PCR kit (Cat. F547S; Thermo), according to manufacturer's instructions.

A typical PCR reaction in 0.2 mL thin-walled PCR tubes consisted of 1 µL of serum sample as template, 0.5 µL each of forward and reverse primers (5 pmol/µL) and 0.2 µL of Phusion Blood II DNA polymerase in 2X Phusion Blood PCR buffer containing 1.5 mM $MgCl_2$ and 2.5 mM EDTA, topped up to a total volume of 10 µL with nuclease-free distilled dH_2O. Amplification was carried out by an initial denaturation at 98°C for 5 min, followed by 40 cycles of denaturation at 98°C for 5 sec, annealing at 56°C for 15 sec and extension at 72°C for 30 sec. A final extension was performed at 72°C for 120 sec.

The primers used in this chapter are listed in Table 4.1. Amplified DNA was visualised using agarose gel electrophoresis as detailed in Chapter 2.2.2.4, and sequenced as described in Chapter 2.2.2.9.

Table 4.1 – List of Primers Used in This Chapter

Forward represents 5' to 3'. Reverse represents 3' to 5'.

Primer Name	Orientation	Primer Sequence (5'→3')	Size/ bp
FCN2 Exon 8	Forward	CTGTCTGTAATGATGTTACTGC	22
	Reverse	TACAAACCGTAGGGCCAAGC	20

4.2.5 Analysis

Absorbance and luminescence were analysed using GraphPad Prism 6 software (GraphPad Software, Inc.). The p value threshold for significance is 0.05. For nucleotide sequence alignments, the NCBI BLAST service was used.

4.3 Results

4.3.1 The TCD Cohort

In 1977, a cohort of Irish women was exposed to the same genotype 1b strain of HCV from a single donor of anti-D immunoglobulin (Barrett et al., 2001). The cohort is homogenous in several factors, including sex, ethnic origin, mode of transmission, and duration of disease (Barrett et al., 2001). A wide range of responses to this infection event were reported, in which some patients became infected but responded to treatment, some developed a chronic HCV infection and some naturally cleared infection during the acute phase. The reason for such differences in response has not been discovered. Here it is hypothesised that higher serum L-ficolin concentrations may result in innate resistance to HCV infection, whereas lower levels increase the patient's risk of infection.

4.3.1.1 Chronic HCV Patients Have Significantly Lower L-Ficolin Concentrations

Serum L-ficolin levels of each patient were quantified using an AcBSA-capture assay through interpolation of a standard curve, performed three times. L-ficolin concentrations were interpolated using a standard curve fitted with a linear regression (Figure 4.1).

The infection responses of each patient were grouped into five categories:

- Patients who developed **chronic HCV** infection (n = 5)
- **Spontaneous resolvers** who eliminated the infection without the need for treatment (n = 5)
- Patients who achieved **sustained virological response (SVR)** and were cured following treatment (n = 8)
- Patients who were **innately resistant** to infection (n = 19)
- **Healthy control** patients (n = 10)

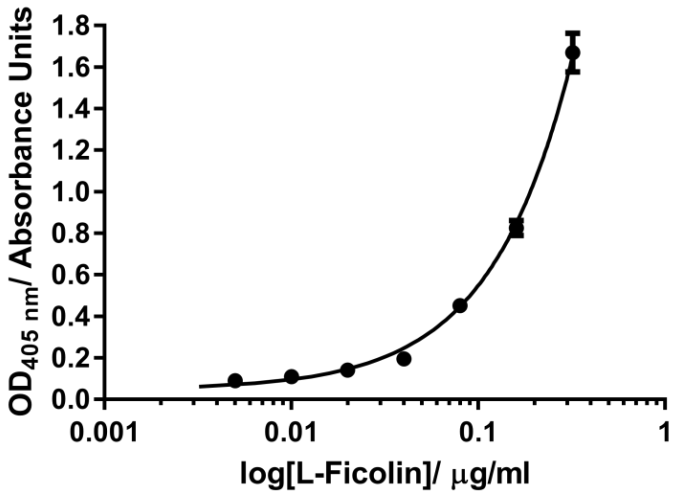

Figure 4.1 – Standard Curve Used to Quantify L-Ficolin Concentration by Acetylated BSA-Capture ELISA

An in-house ELISA was performed in which AcBSA was used to capture L-ficolin. L-ficolin concentration was determined against a standard curve produced by diluting a serum sample containing 4 µg/mL of L-ficolin two-fold, fitted with a linear regression. This graph is an example of standard curves used to interpolate concentrations using the absorbance results of samples. Error bars represent standard error of mean.

Response to infection was correlated to serum L-ficolin concentration (Figure 4.2). The chronic HCV group had the lowest average L-ficolin concentration (median = 1.4 µg/mL; range = 1.1 – 3.2 µg/mL). The spontaneous resolvers had the next highest concentration (median = 1.4 µg/mL; range = 1.2 – 10.1 µg/mL) followed by the treated SVR patients (median = 5.5 µg/mL; range = 2.3 – 16.3 µg/mL). The innate resistant patients had the highest serum L-ficolin concentration (median = 11.1 µg/mL; range = 2.5 – 20.8 µg/mL). Despite the wide range of concentrations, Kruskal-Wallis (Dunn's test of multiple comparisons) analysis of the concentrations revealed that only the chronic patients had a slightly significantly different L-ficolin concentration ($p = 0.0119$) than the healthy controls (median = 9.3 µg/mL; range = 3.3 – 18.5 µg/mL). This may suggest that serum L-ficolin concentration plays an important role in preventing the establishment of chronic HCV infection.

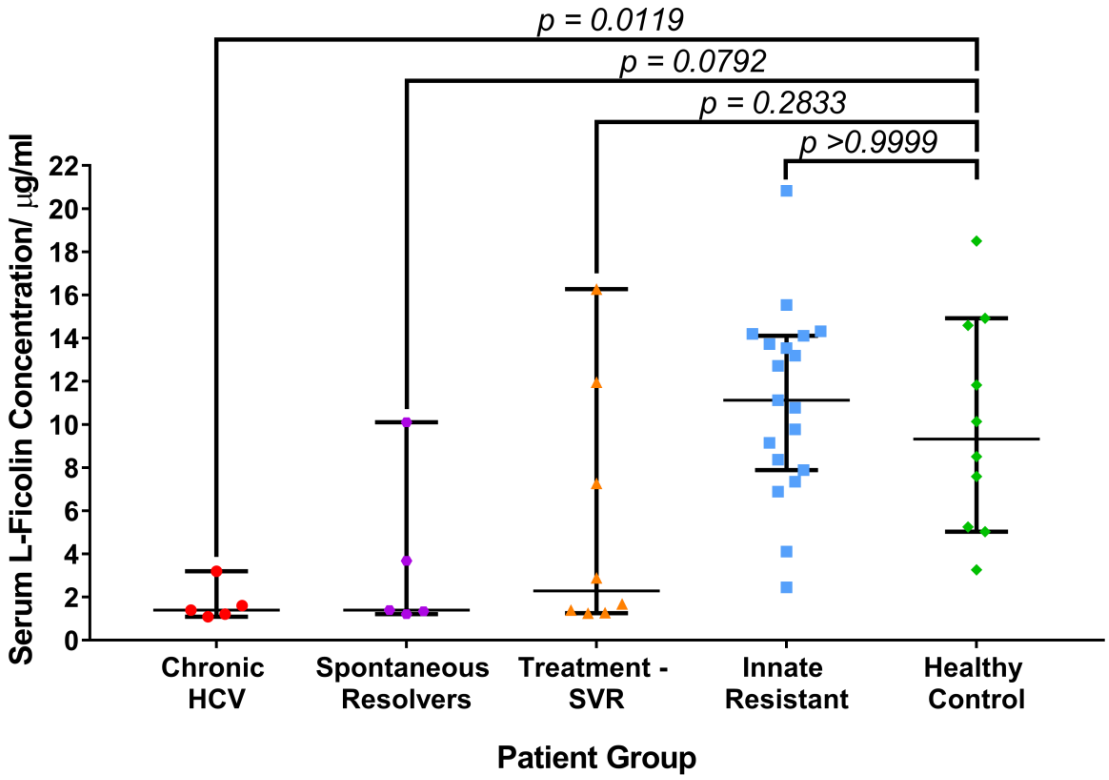

Figure 4.2 – Correlation of Serum L-Ficolin Concentrations with HCV Infection Response

Patients exposed to a single HCV source had varying responses to infection. The mean average serum L-ficolin concentrations of each patient were interpolated using an AcBSA-binding ELISA in duplicate (n = 3). The Kruskal-Wallis and Dunn's multiple comparison tests were used for analysis (p value significance threshold = 0.05). Patients who developed chronic HCV infection had significantly lower serum L-ficolin concentrations than the healthy controls (p = 0.0119). There was no significance for all other samples compared to the healthy control. Bars represent median serum L-ficolin concentrations with 95% confidence intervals.

4.3.1.2 Serum Neutralisation of HCV Entry in Chronic Infections

As chronically-infected HCV patients had significantly lower circulating L-ficolin concentrations, it was hypothesised that the L-ficolin of the chronic HCV patients would be less effective than the other patient groups at neutralising HCV infection. A neutralisation assay was carried out using 20 samples from the cohort against pseudoparticles expressing glycoproteins of the H77.20 genotype 1 strain of HCV. VSVpp were used as a control, as response to HCV would not be expected to influence neutralisation of this virus. A non-infected plasma sample determined to have a very low L-ficolin concentration served as a control to which infectivity was normalised. Data was compared to chronic HCV neutralisation levels using one-way ANOVA analysis with the Tukey test.

All samples neutralised HCVpp entry below 30% relative to the plasma control (Figure 4.3.1). The chronic HCV samples exhibited highly significant neutralisation ($p = 0.0001$; mean = 6% infectivity; sample size [n] = 5) when compared to the spontaneous resolvers (mean = 21% infectivity; n = 4) and innate resistant samples (mean = 21% infectivity; n = 6). The chronically-infected HCV serum samples also neutralised entry stronger than the SVR patient serum samples, although to a lesser extent (mean = 12% infectivity; $p = 0.0652$; n = 5). These results could suggest that the L-ficolin of chronic HCV patients is more readily effective at neutralising HCVpp entry than other samples.

The chronic HCV serum samples with low serum L-ficolin concentrations neutralised HCVpp entry the strongest. Infectivity values were plotted against the L-ficolin concentration of each sample (Figure 4.3.2). Linear regression analysis showed no significant correlation between the neutralisation and L-ficolin concentration ($p = 0.0775$; $r^2 = 0.163$), therefore the neutralisation observed was likely the result of another factor, for example the L-ficolin variant of the patients.

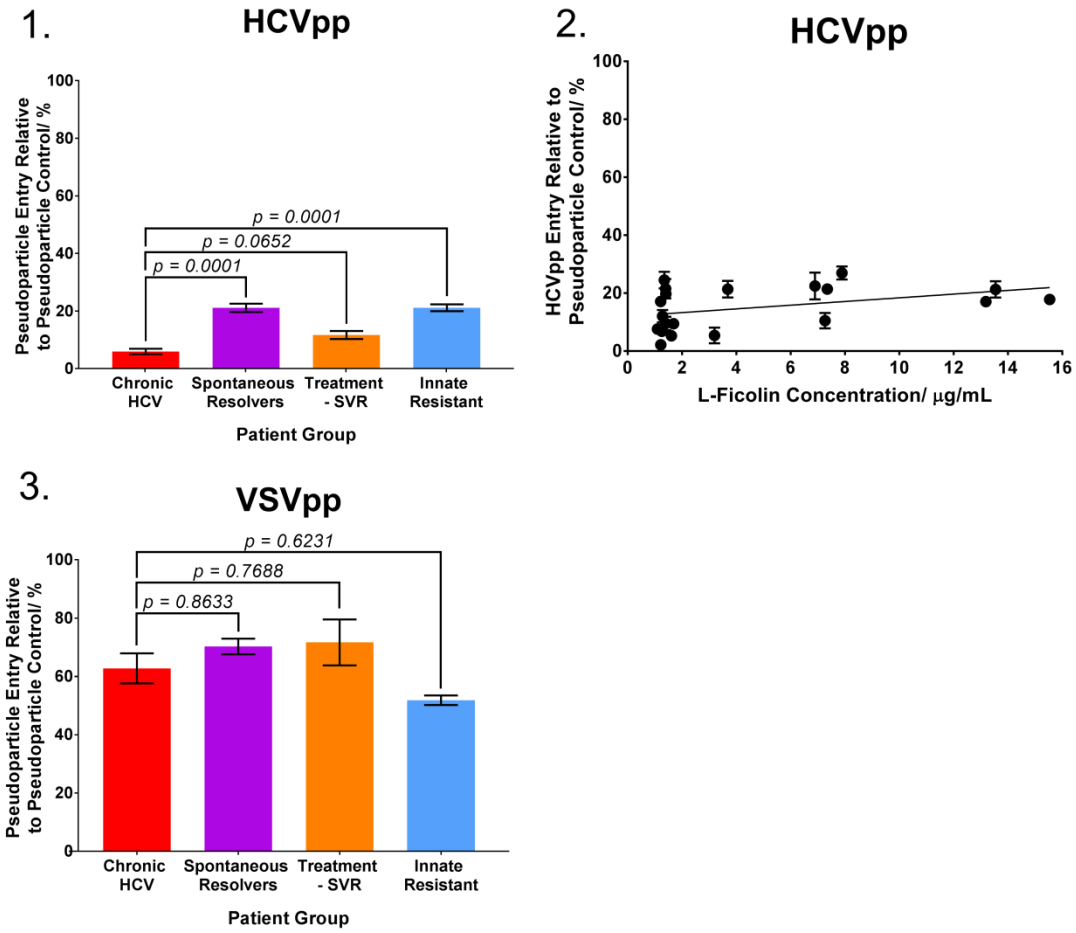

Figure 4.3 – Correlation between Response to HCV Infection and L-Ficolin Neutralisation Activity

The serum of patients with varying responses to HCV infection from a single source were used to neutralise virus pseudoparticle entry into Huh7 cells. Entry was normalised to infectivity in the presence of a plasma control with a very low L-ficolin concentration. ***1.*** *Serum of chronic HCV patients neutralised HCVpp entry more significantly than other patient groups, as determined by one-way ANOVA with Tukey test (p value threshold of significance = 0.05).* ***2.*** *There was no correlation between L-ficolin concentration and HCVpp neutralisation ($p = 0.0775$; $r^2 = 0.163$).* ***3.*** *Despite neutralisation of the VSVpp control entry there was no significant difference between HCV patient groups, indicating that response to HCV does not impact neutralisation of VSVpp entry. Other immune components such as lectins and antibodies were not accounted for in this assay, therefore the differences in neutralisation cannot solely be attributed to L-ficolin.*

While all serum samples neutralised VSVpp infectivity, with the strongest neutralisation by innate resistant samples (mean = 52% infectivity), there was no significant difference between any samples when compared to the chronic HCV samples (mean = 63% infectivity) (Figure 4.3.3). This implies no correlation between HCV patient group and neutralisation of VSVpp entry.

While there are significant differences between neutralisation by the HCV patient groups, the neutralisation observed is likely a combined effect of L-ficolin and other immune components, such as MBL and antibodies (Brown et al., 2010), which were not accounted for in this experiment.

4.3.2 The Ethnicity Cohort of Chronic HCV Patients

A separate investigation was undertaken using chronic HCV patients of European and Middle Eastern and Asian backgrounds. HCV infection is unevenly distributed across the world. As ethnicity influences both *FCN2* haplotype and response to HCV infection, it was hypothesised that serum L-ficolin concentrations and +6359C>T and +6424G>T SNPs differ between these ethnic groups and influence ligand-binding and response to HCV infection. The cohort consisted of European (n = 50) and Asian (Middle Eastern, South Asian and North African; n = 72) patients (Table 4.2).

Table 4.2 – The Range of HCV Genotype in the Ethnicity Cohort
Chronic HCV patients were separated by European and Asian (Middle Eastern, South Asian and North African) ethnicity.

HCV Genotype	Number of HCV Patients	
	European	Asian
1	14	11
2	14	1
3	15	52
4	6	6
5	1	1
Total	**50**	**72**

4.3.2.1 Quantification of Serum L-Ficolin

ELISAs were performed using anti-L-ficolin mAb GN4 to capture and quantify serum L-ficolin using a standard curve fitted with a non-linear regression (Figure 4.4). Samples were diluted 1:5 and added in duplicate, though the ELISA was performed once due to limited amounts of serum sample.

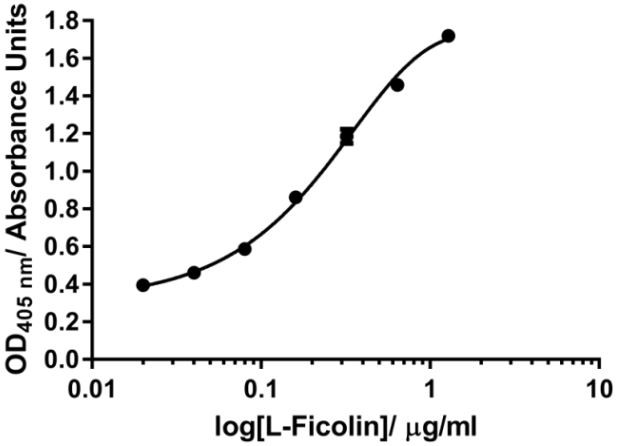

Figure 4.4 – Standard Curve Used to Quantify L-Ficolin Concentration by Anti-L-Ficolin Monoclonal Antibody GN4-Capture ELISA
An in-house ELISA was performed using anti-L-ficolin mAb GN4 to capture L-ficolin in serum in duplicate. A standard curve was made using a serum standard containing 4 µg/mL of L-ficolin. This graph represents standard curves used for interpolation of L-ficolin concentration by absorbance, fitted with sigmoidal, 4PL regression. Error bars represent standard error of mean.

The European serum L-ficolin concentrations ranged from 0.1 – 4.6 µg/mL, whereas the concentrations of the Asian cohort ranged from 0.1 – 4.8 µg/mL (Figure 4.5). Analysis was performed using Kruskal-Wallis and Dunn's test, in which genotypes 2 and 5 were removed due to their singular sample sizes. The median concentrations of the chronic HCV patients showed no significant difference between each ethnicity across all HCV genotypes, with 0.4 µg/mL (range = 0.1 – 4.6 µg/mL) for European patients and 0.4 µg/mL (0.1 – 4.8 µg/mL) for Asian patients ($p = 0.5646$).

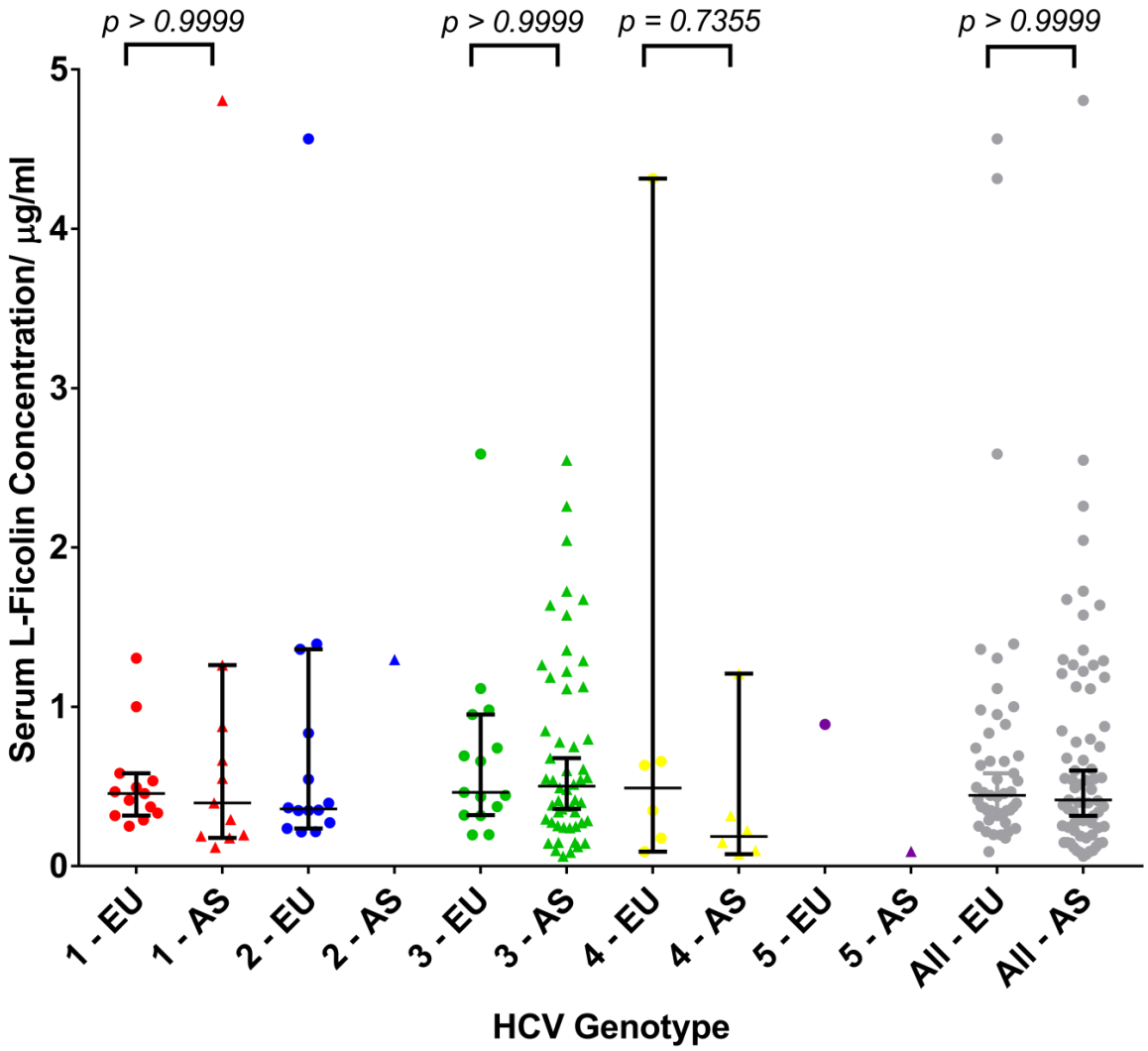

Figure 4.5 – Correlation between Serum L-Ficolin Concentration and Ethnicity of HCV Patients

Serum L-ficolin concentrations of European (EU) and Asian (AS) chronic HCV patients were determined in duplicate using anti-L-ficolin monoclonal antibody GN4-capture ELISA (n = 1). The L-ficolin concentrations of each ethnicity were compared using the Kruskal-Wallis test (Dunn's multiple comparison) within each HCV genotype (p value threshold of significance = 0.05). No significant difference in L-ficolin concentration was identified between the ethnicities in each genotype, both with median average concentrations of 0.4 µg/mL. Due to the singular sample sizes for the Asian genotype 2 and the genotype 5 data sets, analysis could not be performed. Bars represent median serum L-ficolin concentrations with 95% confidence intervals.

4.3.2.2 Correlation between Ethnicity and L-Ficolin Binding of Acetylated BSA

It was hypothesised that, as there were no significant differences in average serum L-ficolin concentration between the ethnicities, that there would be no difference in L-ficolin binding activity of ligands such as AcBSA.

An AcBSA-capture ELISA was carried out in which the L-ficolin concentrations of all samples were normalised to the lowest concentration of the cohort (0.1 µg/mL) which was diluted 1:10 for the assay and used in duplicate. A serum control was used at 0.3 µg/mL L-ficolin to allow normalisation of binding and the combination of data from several plates. Due to limited amounts of serum samples, assays were performed once. Once again, analysis was performed using Kruskal-Wallis and Dunn's tests, with data for genotypes 2 and 5 removed due to their singular sample sizes. No significant difference in the mean levels of binding was detected between the ethnicities for any genotype ($p = 0.4435$) (Figure 4.6).

The low levels of statistical difference could well be explained by the significantly low L-ficolin normalised concentration, at which perhaps no activity could be detected. Therefore it was investigated if samples with higher L-ficolin concentrations had higher binding activities of AcBSA, by performing an identical ELISA in which the samples were not normalised (Figure 4.7). No linear or non-linear correlation was identified for either cohort, thus higher L-ficolin concentrations did not necessarily correlate with AcBSA binding.

The boxed samples in Figure 4.7 represent samples with low L-ficolin concentrations yet high AcBSA-binding activity and samples with high L-ficolin concentrations yet low binding activity. To identify possible correlating L-ficolin characteristics of these samples, efforts were turned towards sequencing.

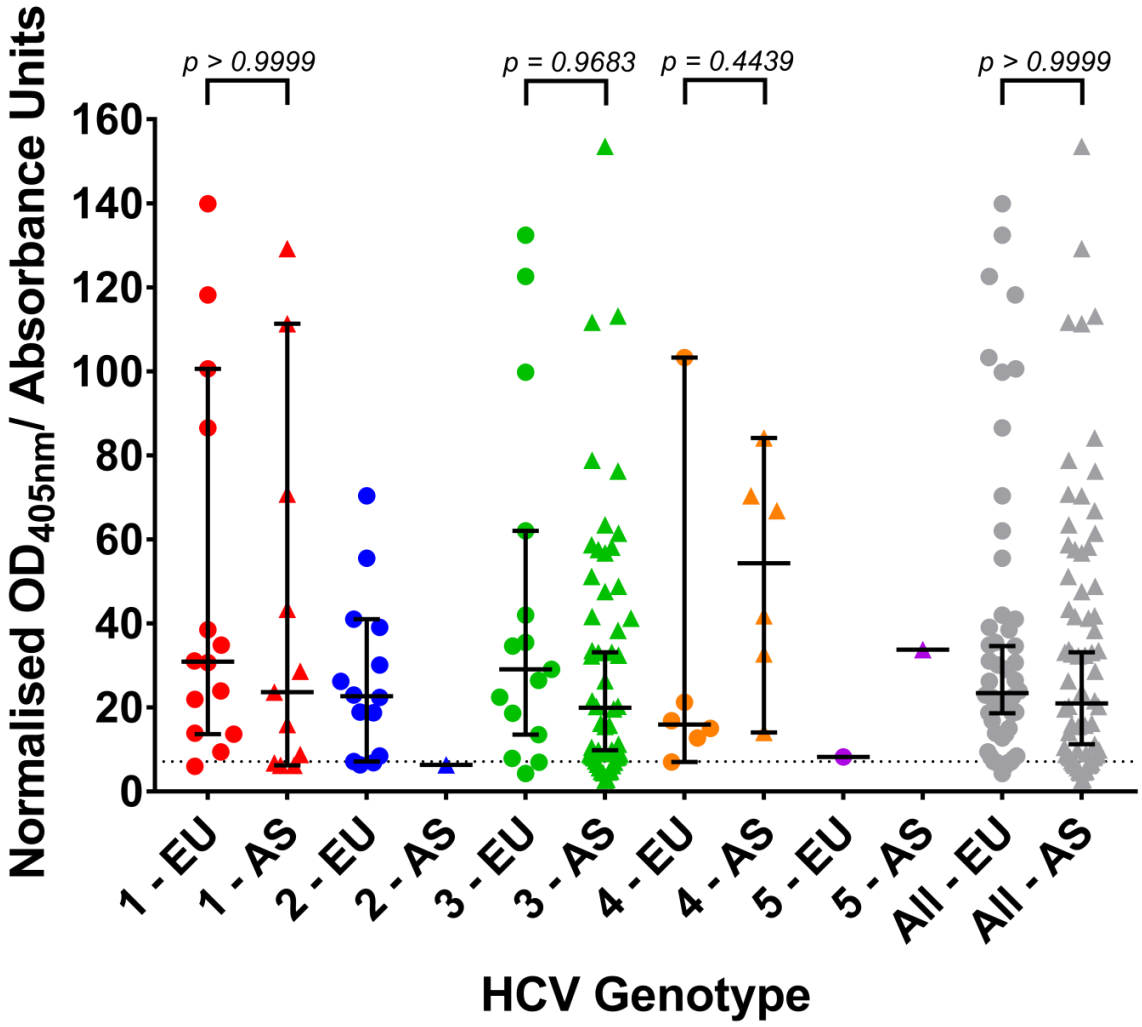

Figure 4.6 – Correlation between Ethnicity and L-Ficolin Binding to AcBSA

Levels of binding to AcBSA by L-ficolin in European (EU) and Asian (AS) chronic HCV patients normalised to 0.01 µg/mL and used in duplicate was investigated in an ELISA (n = 1). Binding was normalised to the activity of a 0.3 µg/mL serum L-ficolin control (100%) and 0 absorbance units (0%). No significant difference in AcBSA binding was identified between the ethnicities within all genotypes. Statistical analysis was achieved using the Kruskal-Wallis and Dunn's tests, omitting genotype 2 and 5 data due to small sample sizes. The p value threshold of significance is 0.05. Bars represent median serum L-ficolin concentrations with 95% confidence intervals. The dotted line indicates the lower limit of detection of the assay (mean of negative sample + (2 x S.D.)).

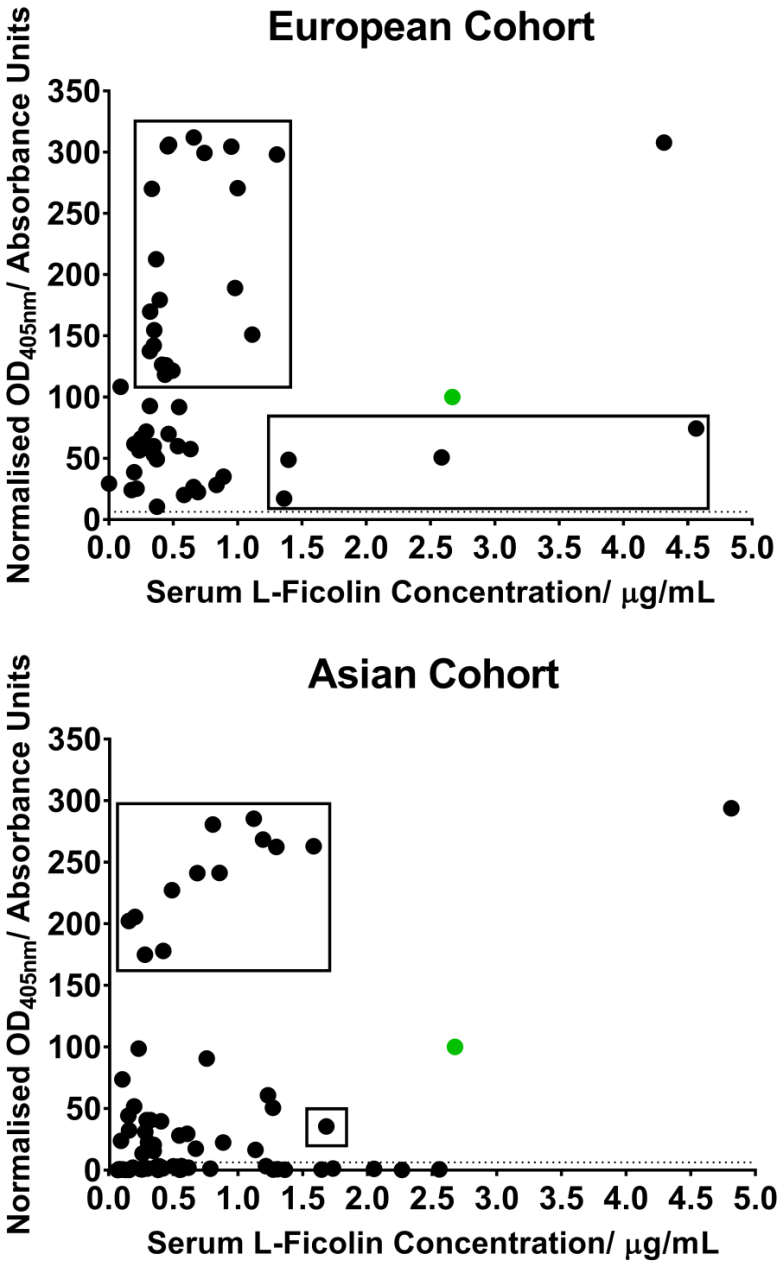

Figure 4.7 – Correlation between Serum L-Ficolin Concentration and AcBSA Binding Activity

An AcBSA-capture ELISA was performed using European and Asian chronic HCV patients (n = 1). Binding levels were normalised to the activity of a 0.3 µg/mL serum L-ficolin control (100%, shown in green) and 0 absorbance units (0%). No correlation was identified between L-ficolin concentration and binding to AcBSA for either ethnicity cohort. The boxed samples had higher concentrations but low AcBSA-binding activity, and vice versa. The L-ficolin of these samples may have certain characteristics that allow these activities.

4.3.2.3 Sequencing of FCN2 *Exon 8 in Ethnicity Cohort*

It was hypothesised that the samples with low concentrations yet high AcBSA-binding levels, and vice versa, were a result of the +6359 and +6424 SNPs encoding non-synonymous T236M and A258S mutations in the L-ficolin FBG domain, which affect ligand binding as shown in Chapter 2. To confirm a relationship, the *FCN2* exon 8 sequences of each patient were essential.

PCR to amplify FCN2 exon 8, to be used for sequencing, was performed using the *FCN2* Exon 8 forward and reverse primers. These primers were previously used to successfully amplify the exon. The expected 384 bp band was detected in multiple European and Asian serum samples, however additional bands of approximately 1000 bp and 2000 bp were also identified (Figure 4.8). These were likely the result of primer binding to other sequences in the serum DNA.

Due to the previous success of these primers in another project, optimisation was attempted. For example, PCRs were performed by altering the volumes used of primers, $MgCl_2$, EDTA and the total reaction. A gradient PCR to optimise annealing temperature was also attempted (data not shown). In all cases, the 384 bp band did not appear alone, if at all, though a total volume of 20 µL or a lower volume of EDTA appeared necessary for the presence of the expected band.

Sequencing of a small set of serum samples was attempted using the *FCN2* Exon 8 forward primer, despite multiple banding. No sequence similar to the *FCN2* exon 8 sequence was produced. However a common sequence was identified in all samples with 100% homology to a region of human chromosome 7. The *FCN2* gene is located in the q34.3 region of human chromosome 9 therefore the primer must have shown affinity towards more than one region within the human genome.

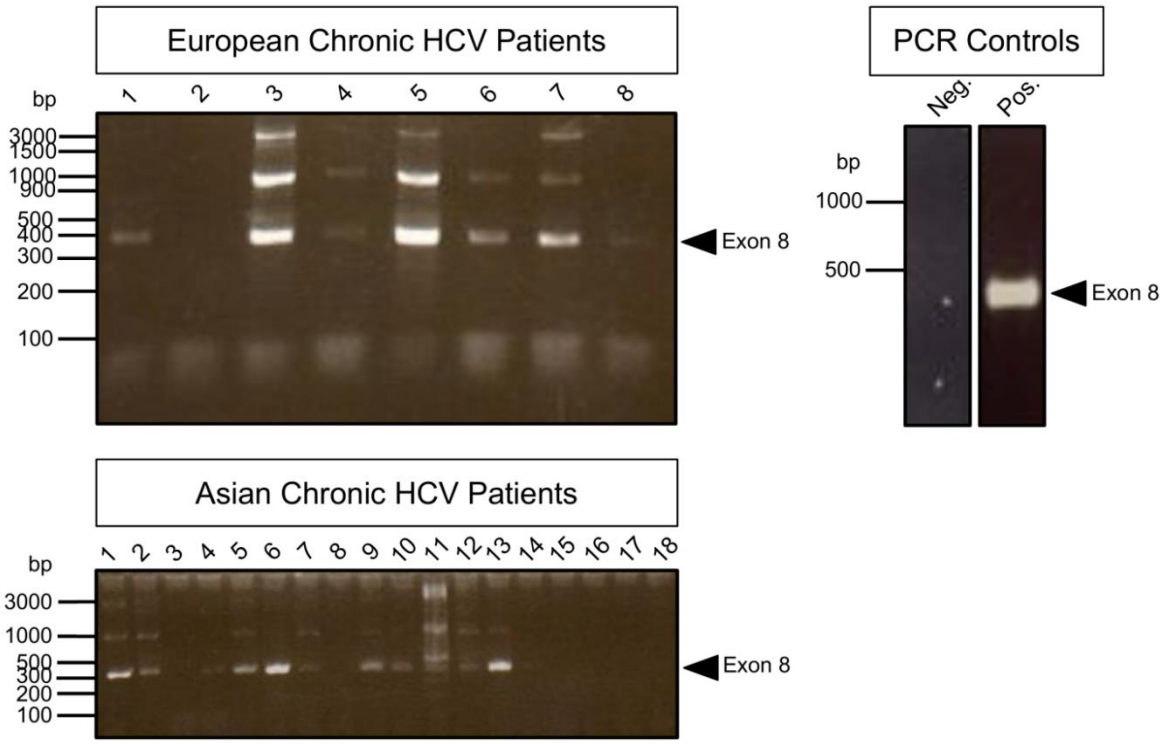

Figure 4.8 – Amplification of *FCN2* Exon 8

Agarose gel electrophoresis and ethidium bromide staining of PCR reactions used to amplify exon 8 of the FCN2 *gene encoding L-ficolin in the serum samples of chronic HCV patients. These would be used to determine exon 8 polymorphisms through sequencing. However the expected 384 bp band was not isolated, being detected alongside bands of ~1000 bp and ~2000 bp. The negative control contained no template, whereas the positive control used the serum of an HCV patient from another cohort. Images of controls were provided by Paywast Jalal.*

4.4 Discussion

4.4.1 The Serum L-Ficolin Concentrations of the Cohorts

Previous studies have shown median serum L-ficolin concentrations between 3.0 and 5.4 µg/mL, with a range of 0.72 – 12.8 µg/mL (Hummelshøj et al., 2005; Kilpatrick et al., 1999; Munthe-Fog et al., 2007). Although Munthe-Fog et al. (2007) used an alternative means of quantification, Kilpatrick et al. (1999) and Hummelshøj et al. (2005) used a similar anti-L-ficolin mAb GN4-capture ELISA to the ethnicity cohort, in which biotinylated anti-L-ficolin mAb GN5 was also used as a primary antibody, thus the results are comparable.

However previous studies did not use AcBSA-binding assays to determine L-ficolin concentration as was performed with the TCD cohort. This assay does not differentiate between L-ficolin variants with mutations such as T236M and A258S which have varying AcBSA-binding activities, as shown in Chapter 2. This may account for the wide range of median concentrations ranging from 1.4 to 11.1 µg/mL, with values up to 20.8 µg/mL. Use of an anti-L-ficolin mAb GN4-capture ELISA would resolve this issue.

In the ethnicity chronic HCV cohort, the average serum L-ficolin concentrations were low for both ethnicities (median = 0.4 µg/mL), similar to the chronic HCV patients of the TCD cohort (median = 1.4 µg/mL). Several samples had concentrations below 1.0 µg/mL, lower than the medians described above. This may have been an effect of HCV infection on serum L-ficolin concentrations, as the present study used HCV-infected blood whereas the previous studies used the blood of healthy Scottish and Danish donors. Previous studies have found links between L-ficolin serum concentrations and HCV infection and disease (Mason and Tarr, 2015). In chronic HCV-infected patients, L-ficolin serum concentrations were increased in conjunction with elevated ALT levels – a marker of fibrosis and inflammation (Hu et al., 2013a). L-ficolin levels have been seen to positively correlate with the severity and progression of HCV disease, such as fibrosis (Liu et al., 2009) with supporting data that liver damage as a result of chronic

HCV infection does not reduce L-ficolin levels (Hamed et al., 2014). After successful therapy of HCV disease, ALT and HCV RNA levels have been seen to decrease to normal levels, followed by a decrease in L-ficolin levels, suggesting a correlation of ALT and RNA levels with disease outcome, as a result of L-ficolin activity (Hu et al., 2013a; Hummelshøj et al., 2005; Mason and Tarr, 2015). Furthermore, data later published by the VRG showed that age and gender also affect L-ficolin serum concentration, and found that L-ficolin levels slightly increased with HCV infection, with a marked increase upon shortly prior to the development of HCV-based hepatocellular carcinoma (Jalal et al., 2019a). The severity and stage of HCV infection of patients within the ethnicity cohort and their history of HCV infection was not recorded, and therefore many factors may influence their L-ficolin concentrations.

The results of both cohorts, in particular the TCD cohort, contradict the previous observations described above, as there was instead a decreased average serum L-ficolin concentration in chronic HCV patients, and higher levels in healthier patients. This may suggest that higher serum L-ficolin levels play an important role in preventing the establishment of HCV infection. HCV in the samples was not heat-inactivated, with only detergent being used for the TCD cohort, therefore L-ficolin would likely have remained undegraded.

An alternative explanation for this contradiction is the effect of HCV infection on levels of L-ficolin. For example those with chronic HCV may have low levels of L-ficolin either due to increased activity and usage of serum L-ficolin to combat infection, or due to immune dampening, thus reducing L-ficolin production. Innate resistant patients may have levels similar to the healthy controls due to their lack of infection. Furthermore, it is unlikely that all healthy controls would be innate resistant if infected with HCV. Kilpatrick et al. (2015) suggest that the disease status of a serum sample can affect the quantification of L-ficolin. Hypothetical reasons for the low L-ficolin serum concentrations are immune suppression, increased binding to HCV or HCV-related PAMPs followed by ‘exhaustion’ of the L-ficolin supply in serum upon L-ficolin-mediated phagocytosis, or migration of the protein from the serum to the liver for targeted immune activity.

Another possible explanation for low concentrations is the storage of serum samples for several years at -80°C. Taking into account unknown numbers of freeze-thaw cycles of the serum samples, it is possible that the stability of proteins within the serum samples had decreased over time before use in this investigation. Indeed, research by Geno et al. (2016) highlighted the importance of using fresh serum samples when investigating L-ficolin, due to the apparent increase in prevalence of L-ficolin inhibitors in serum samples stored long-term at -80°C. These inhibitors interfere with L-ficolin activity and quantification using anti-L-ficolin antibodies. Proposed explanations included chemical alternations in or degradation of the L-ficolin protein during storage, or the presence of *N*-linked glycosyl chains which arise from glycoproteins in serum, thus posing as potential ligands for L-ficolin. Brady et al. (2014) have described the sensitivity of serum L-ficolin to different blood collection tubes, which affected protein concentration and activity. Silica clot activator was the causative agent however glass vials produced no such effect. Silica clot activator did not affect other lectins including MBL and H-ficolin.

4.4.2 L-Ficolin Concentrations in the TCD Cohort

The genetically similar TCD cohort of patients exposed to a single source of HCV infection offers a rare opportunity to discover immune factors that lead to differing responses to infection. L-ficolin concentration appeared to negatively correlate with the severity of disease outcome, opposing previous findings for the possible reasons described above.

The serum samples of patients with chronic HCV were the most effective at neutralising HCVpp entry, whereas those of the innate resistant patients had less effect. These results may suggest that such L-ficolin is more readily effective at neutralising HCV entry. However, it was more likely that the neutralisation is also the activity of other immune factors such as antibodies and lectins, for example MBL (Brown et al., 2010). To avoid interference by these factors, an ELISA could be performed to study interaction of L-ficolin with virus glycoproteins and using an L-ficolin-specific antibody.

Analyses were restricted somewhat by the small numbers of samples available. Ideally, the sample size of the TCD cohort should be increased, as more patients exposed to the single source virus exist. The anti-L-ficolin mAb GN4-capture ELISA should be used to quantify L-ficolin, as the AcBSA-capture ELISA is influenced by the affinity of L-ficolin variants. The samples could also be genotyped for the +6359 and +6424 SNPs, and other SNPs, to reveal the L-ficolin variant of each patient and identify a possible correlation with response to HCV infection and neutralisation effect. Any correlations could be used to predict susceptibility to and outcome of HCV infection, and improve prognosis.

4.4.3 The Ethnicity Cohort of Chronic HCV Patients

Ethnicity has been associated with HCV infection outcome and T-cell response (Sugimoto et al., 2003). The ethnicity cohort of chronic HCV patients would allow differences between ethnicity-based differences in L-ficolin serum concentrations and SNPs to be determined in the situation of HCV infection.

It should be noted that ethnicities were identified using the forenames and surname of each patient, as the ethnicity of each patient was not formally recorded. While this method of assortment was inevitably prone to error, due to the possible change of surnames through marriage and the existence of mixed-race individuals who may have characteristics of both ethnicities, it is likely that within such a small cohort such issues were uncommon. Further experimentation should use cohorts with logged ethnicity data, as well as stronger control of other factors such as age, sex and disease progression.

No significant differences were identified between the ethnicities in terms of L-ficolin concentrations or AcBSA binding activity, nor was there a significant or clear correlation between L-ficolin concentration and AcBSA activity. To expand findings, similar quantification should be carried out using healthy European and Asian controls, and even HCV patients with differing responses such as responders to treatment. Samples should be increased and made equal for each HCV genotype.

Some samples were able to bind to AcBSA stronger than others, regardless of L-ficolin concentration. This was hypothesised to be a result of the mutations at residues 236 and 258 of L-ficolin, encoded by the +6359 and +6424 SNPs in exon 8, which are known to affect ligand binding. In Chapter 2 it was shown that the L-ficolin variants encoded by these minor allele SNPs – T236M and A258S – have increased and decreased AcBSA-binding activity, respectively, whereas the opposite was true for GlcNAc-binding (Hummelshøj et al., 2005). Successful genotyping of exon 8 is required to establish a correlation of SNPs with binding levels, however PCR amplification of exon 8 requires more optimisation. Alternative primers showing more specificity for *FCN2* exon 8 could be designed, and a positive control should be used with each PCR reaction.

FCN2 SNPs have been found to differ between ethnicities. Using healthy samples, Hummelshøj et al. (2008) found that *FCN2* minor allele frequencies differed between ethnicities. For example, +6359T – encoding T236M – was found in 4% of Japanese samples (n = 50), 25% of Danish samples (n = 60), 37% of Ghanaian samples (n = 50) and 45% of samples from Mozambique (n = 50). For +6424T – encoding A258S – the same populations had frequencies of 14%, 10%, 17% and 25%. Ojurongbe et al. (2012) on the other hand found no difference in homozygous+6424T minor allele genotype frequency between healthy Brazilian (5% genotype frequency; n = 176), Nigerian (11% frequency; n = 180), Vietnamese (5% frequency; n = 172) and European (5% frequency; n = 165) samples.

In terms of disease, there have been associations between L-ficolin SNPs and concentrations. For instance, the L-ficolin T236M mutation has been linked to higher L-ficolin concentrations and protozoan visceral leishmaniasis infection (Mishra et al., 2015). The minor +6424T SNP was associated with protection against CMV reinfection (de Rooij et al., 2011), while the major +6424G SNP was associated with higher L-ficolin concentrations and immediate clearance of HBV infection (Hoang et al., 2011). These studies often use cohorts of the same nationality. The importance of ethnicity in disease outcome with relation to *FCN2* SNP associations has not been investigated.

Frequencies of the +6359 and +6424 SNPs, and others such as promoter SNPs -986, -602 and -4, of the ethnicity cohort used in this chapter should be compared with frequencies of healthy patients and other HCV patient groups. As started in Chapter 2, data should also be obtained to show the effects of L-ficolin T236M and A258S mutations of binding of HCV glycoproteins and neutralisation of HCV entry. If a particular SNP is more prevalent in a HCV patient group, its binding activity could correlate to HCV infection outcome. Identification of such a SNP could aid in prediction of HCV infection outcome, disease progression and an optimal L-ficolin for use in treatment.

Chapter 5 L-Ficolin Interaction with HCV Envelope Glycoproteins

5.1 Aims

N-linked glycans are of particular interest in microbial research, as they are present on the surfaces of several pathogens, encompassing viruses, bacteria, fungi and protozoans (Kilpatrick and Chalmers, 2012). They are recognised by significant immune components such as antibodies and lectins. Although it is known that L-ficolin displays specificity for acetylated monosaccharides, present in *N*-linked glycans, the specific configurations and arrangements of these ligands are also essential for L-ficolin interaction (Krarup et al., 2008). This prevents L-ficolin activity against healthy host cells via their *N*-linked glycans.

These specific acetylated monosaccharide configurations remain unknown. The presence of microheterogeneous and largely high-mannose *N*-linked glycans on HCV E1E2 allows specific interaction by L-ficolin (Hamed et al., 2014).

The aim of this chapter is to characterise how L-ficolin interacts with the HCV virus, by clarifying if specific *N*-linked glycans on the HCV E1E2 glycoproteins are essential for L-ficolin interaction. A glycan knockout (GKO) library was made using multiple random mutations at HCV E2 glycosylation *N*-linked sites. Investigating the interaction of these GKO E2 proteins with L-ficolin would help better understand and predict L-ficolin interaction with HCV, as well as determine its usability against other pathogens.

5.2 Materials and Methods

5.2.1 Glycan Knockout Library

Using an optimised protocol (Sugrue, 2007) a plasmid library of the JFH-1 strain (genotype 2a) of HCV was constructed by the VRG, containing E1E2 glycan knockouts. Using a QuikChange II site-directed mutagenesis kit (Stratagene), E2 glycosylation site amino acid sequences of NXS/T were mutated to DXS/T to prevent glycosylation. The negatively-charged amino acid aspartate was elected to substitute asparagine at the glycosylation sites as they are of similar size, and aspartate is known to prevent *N*-linked glycosylation (Yasuda et al., 1999).

The GKO HCV E1E2 fragments were amplified using Q5® Hot Start High-Fidelity DNA polymerase as described in Chapter 2.2.2.3 and the JFH primer pair with an annealing temperature of 55°C. Fragments were DpnI-digested, cloned into the pcDNA™3.1D/V5-His-TOPO® vector and transformed into *E. coli* NEB 10-β cells, followed by plating on LB agar with ampicillin overnight. Successful ligation was detected by colony PCR using the T7F and BGHR primers (Table 5.1). The purified plasmids were sequenced using the T7F and BGHR primers, and the GKO library was compiled (Table 5.2). The primers used in this chapter are shown in Table 4.1.

5.2.2 Virus Pseudoparticle Production and Characterisation

Virus pseudoparticle production, infectivity assay and neutralisation assay were performed as per Chapters 2.2.3.1 and 2.2.4. The JFH-1 (genotype 2a) strain of HCV was used for pseudoparticle production, and originated from Wakita et al. (2005).

Table 5.1 – List of Primers Used in This Chapter

Forward represents 5' to 3'. Reverse represents 3' to 5'.

Primer Name	Orientation	Primer Sequence (5'→3')	Size/ bp
JFH	Forward	CACCATGGGTTTCCCCTTTTCTATC	22
	Reverse	TTATGCTTCGGCCTGGCCCAACAAG	20
T7F	Forward	TAATACGACTCACTATAGGG	20
BGH-R	Reverse	TAGAAGGCACAGTCGAGG	18

5.3 Results

5.3.1 Specific E1E2 Glycan Knockout HCV Pseudoparticles Retained Infectivity

To test the effect of specific E1E2 glycan knockouts on L-ficolin interaction, pseudoparticles representing the JFH-1 strain (genotype 2a) of HCV were used, as their entry into Huh7 cells is known to be neutralised by L-ficolin (Hamed et al., 2014). The library of 10 JFH-1 clones with mutated glycosylation sites used in this Chapter is detailed in Table 5.2.

HCV pseudoparticles using the GKO plasmids for glycoproteins needed to maintain a level of infectivity to measure the effect of L-ficolin activity. As several HCV E1E2 glycosylation sites are known to be essential for HCV entry (Goffard et al., 2005), the E1E2 glycan knockout HCVpp library was expressed to test their effect on HCVpp entry into Huh7 cells.

When producing pseudoparticles, the optimal ratio of viral glycoprotein plasmid to backbone/reporter plasmid(s) often varies with each plasmid used. Therefore, this ratio first required optimisation to produce infectivity with the highest luminescence values. This was necessary to determine the greatest effects that GKO mutations have against infectivity.

A checkerboard experiment was performed using all combinations of the pNL4-3.Luc.R$^-$E$^-$ or the phCMV5349/pTG126 backbone/reporter plasmids with the consensus HCV JFH-1 glycoprotein plasmid. phCMV5349 and pTG126 plasmids were used in unison at equimolar amounts. The luminescence of each pseudoparticle was determined in an infectivity assay (Figure 5.1).

The use of 2000 ng of the backbone/reporter plasmids produced infectious pseudoparticles. The use of 500 ng of HCV JFH-1 glycoprotein plasmid with the phCMV5349/pTG126 backbone plasmids produced the highest luminescence values.

Table 5.2 – The HCV JFH-1 E1E2 Glycan Knockout Clone Library

Plasmids containing the HCV (JFH-1 strain; genotype 2a) E1E2 glycoprotein fragments with specific glycan knockout (GKO) mutations were sequenced. Wild-type asparagine (N) residues were mutated to aspartate (D) at random NXS/T glycosylation sites to prevent N*-linked glycosylation. The percentage values of conservation of asparagine (N) at each E2 glycosylation site in HCV genotype 2a viruses, such as JFH-1, were taken from Helle et al. (2007).*

HCV E1E2 GKO Fragment	HCV E2 Glycosylation Site (% Conservation of N at Site)										
	N1 (100%)	N2 (100%)	N3 (95.2%)	N4 (100%)	N5 (90.5%)	N6 (100%)	N7 (100%)	N8 (95.2%)	N9 (100%)	N10 (100%)	N11 (100%)
GKO1	N	N	N	N	D	N	D	N	D	N	N
GKO2	D	N	D	N	D	N	D	N	N	N	N
GKO3	D	N	N	N	D	N	D	N	D	N	N
GKO4	D	D	D	D	D	D	D	N	N	N	D
GKO5	D	D	D	D	D	D	D	N	D	N	D
GKO6	D	N	D	N	D	N	D	N	N	N	N
GKO7	D	D	N	N	D	N	D	N	D	N	D
GKO8	D	D	D	D	D	N	D	N	D	N	D
GKO9	D	D	N	D	D	N	D	N	D	N	D
GKO10	D	D	D	N	D	D	D	N	D	N	D

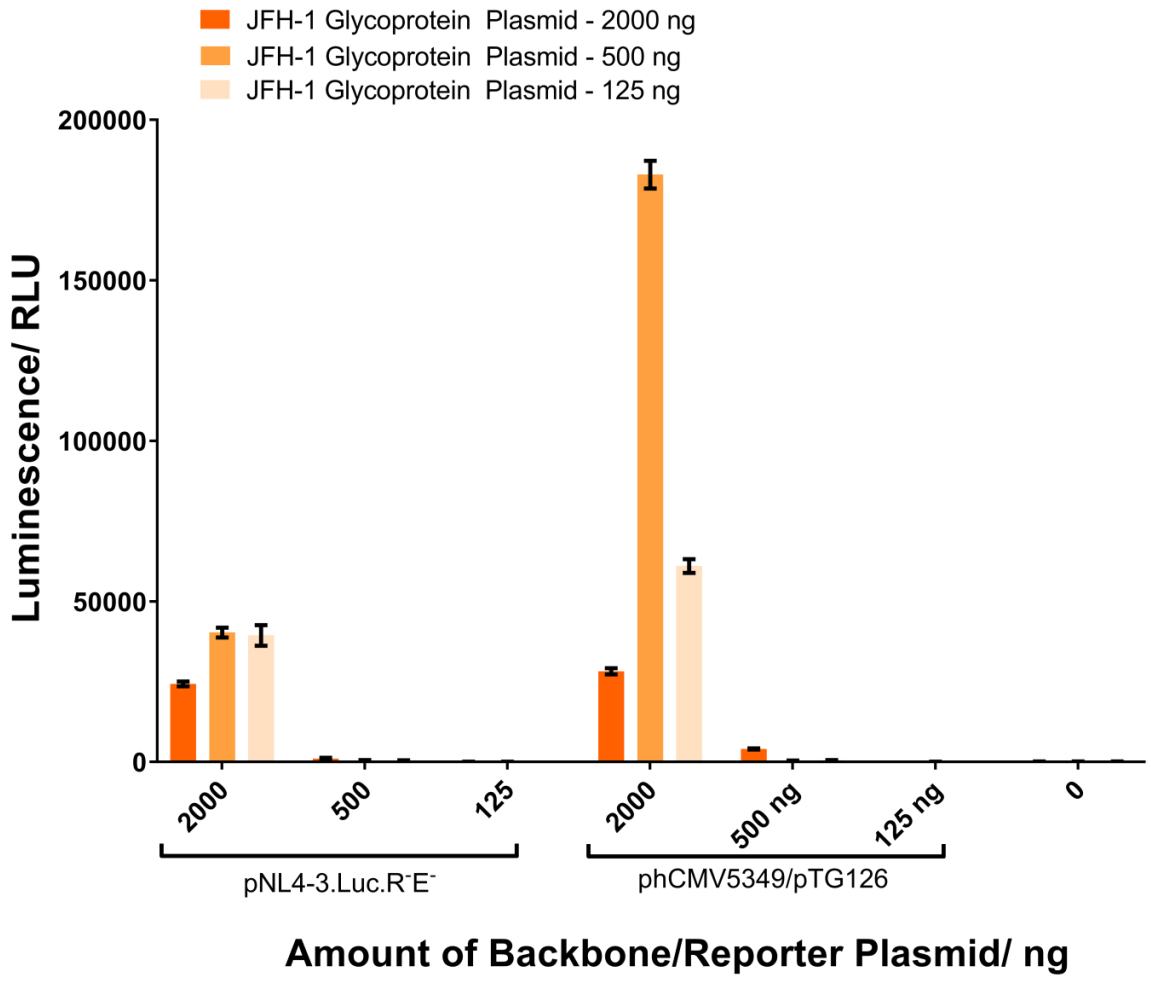

Figure 5.1 – Optimisation of Plasmid Ratio for Production of Infectious HCV JFH-1 Pseudoparticles

Two backbone/reporter plasmids – pNL4-3.Luc.R$^-$E$^-$ and phCMV5349/pTG126 – were tested to produce the most infectious pseudoparticles expressing the JFH-1 (genotype 2a) strain of HCV E1E2 glycoprotein. By combining several combinations of plasmid ratios, 2000 ng of phCMV5349/pTG126 backbone/reporter plasmid with 500 ng of JFH-1 plasmid gave the highest luminescence values upon infection of Huh7 cells.

Using 2000 of phCMV5349/pTG126 plasmids and 500 ng pcDNA™3.1D/V5-His-TOPO® plasmids expressing HCV E1E2 GKO glycoproteins, five assays were performed to compare the infectivity of each GKO pseudoparticle with the wild-type HCV JFH-1 strain pseudoparticle (100% infectivity). The infectivities of each GKO HCVpp was compared to the ΔE pseudoparticle control (0% infectivity), in which no glycoprotein plasmid was used, using one-way ANOVA and Dunnett's test analysis. Only the pseudoparticles produced using the HCV E1E2 GKO1 (4.1% mean infectivity; $p = 0.0060$) and GKO6 (3.8% infectivity; $p = 0.0138$) glycoprotein plasmids had significant infectivities (Figure 5.2). While the GKO2 pseudoparticle had slight infectivity, it was not statistically significant (1.8% infectivity; $p = 0.5615$). The infectivity of all other pseudoparticles was abolished, as it was comparable to the ΔE pseudoparticle control ($p = 0.9999$). As a percentage of infectivity was retained, the GKO1, GKO2 and GKO6 pseudoparticles would be used for further experimentation.

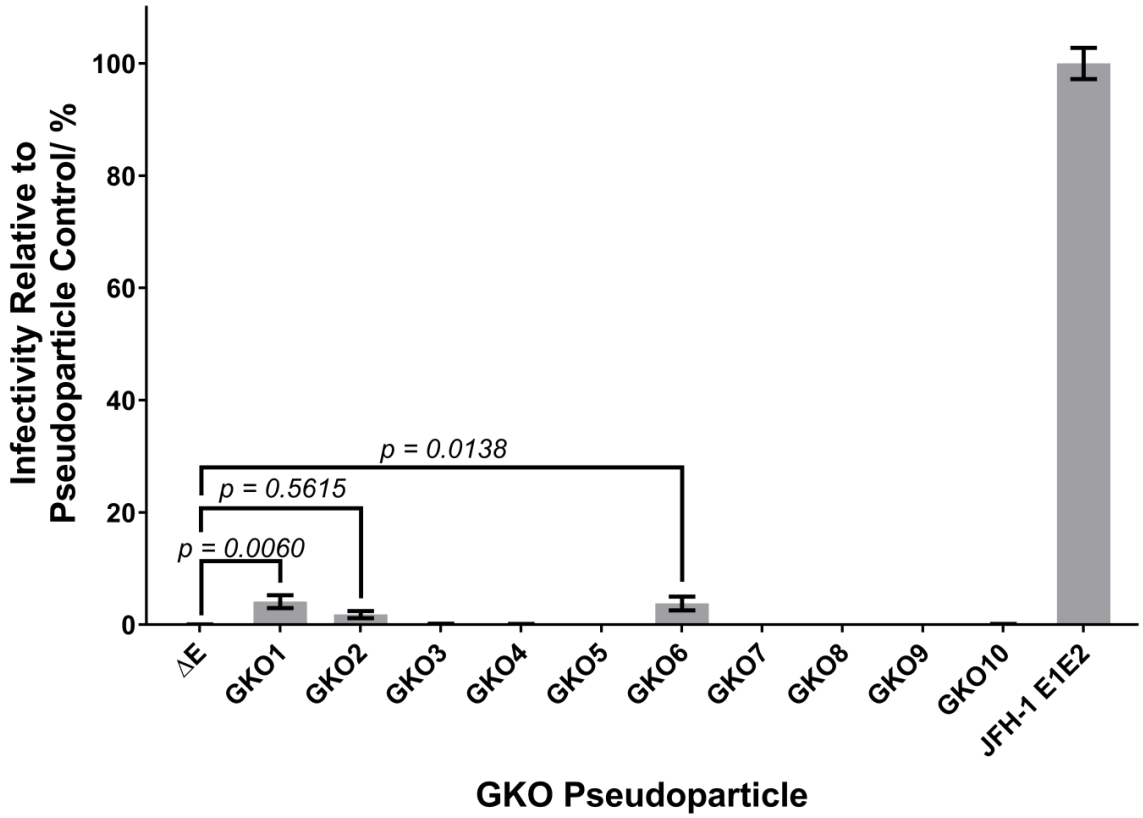

Figure 5.2 – Infectivities of HCV JFH-1 Pseudoparticles Containing Random Mutated E2 Glycosylation Sites

Entry of pseudoparticles expressing the glycan knock out HCV E1E2 glycoproteins into Huh7 cells was investigated in triplicate (n = 5). Infectivity was normalised to the luminescence of wild-type JFH-1 strain HCVpp (100%) and a ΔE negative control, in which no glycoprotein plasmid was used in expression (0%). Certain glycosylation sites, or combinations of glycosylation sites, are essential for infectivity. Infectivities were compared to the ΔE negative control using one-way ANOVA and Dunnett's test analysis, with a p value threshold of significance of 0.05. HCV pseudoparticles with the GKO1 (p = 0.0060) and GKO6 (p = 0.0138) E1E2 glycoproteins retained significant infectivity, whereas GKO2 HCVpp maintained a non-significant level of infectivity (p = 0.5615). All other GKO HCV pseudoparticles could not enter Huh7 cells (p = 0.9999). Error bars represent standard error of mean.

5.3.2 L-Ficolin Neutralisation of Infectious GKO HCV Pseudoparticles

With the presence of infectious GKO HCVpp confirmed, a neutralisation assay was performed in order to investigate the effect of the GKO mutations on L-ficolin neutralisation of HCV entry. It was expected that if L-ficolin was unable to prevent entry of a particular infectious GKO HCVpp into Huh7 cells, then one or more of the mutated glycosylation sites of the GKO HCVpp would be involved in L-ficolin interaction.

Recombinant wild-type L-ficolin (569 µg/mL total protein as determined by BCA assay) was used, with a negative control (227 µg/mL total protein) produced using no expression plasmid. Infectivities were normalised to the infectivity of each respective uninhibited GKO HCVpp. Analysis between samples was performed using an unpaired t-test with Welch's corrections. The assay was performed once due to limited amounts of active L-ficolin.

Preliminary results showed a level of L-ficolin neutralisation of all GKO HCVpp infection, however there was no statistically significant difference between entry with L-ficolin or the negative control for GKO1 ($p = 0.1878$), GKO2 ($p = 0.2948$) or GKO6 ($p = 0.0671$) pseudoparticles (Figure 5.3).

With the current dataset, a specific glycosylation site responsible for L-ficolin interaction cannot be determined. It is possible that one of the glycosylation sites essential for infectivity is involved in interaction, therefore its role in L-ficolin binding cannot be determined in this assay. It is equally plausible that L-ficolin interaction may be able to bind any *N*-linked glycan on the E2 glycoprotein.

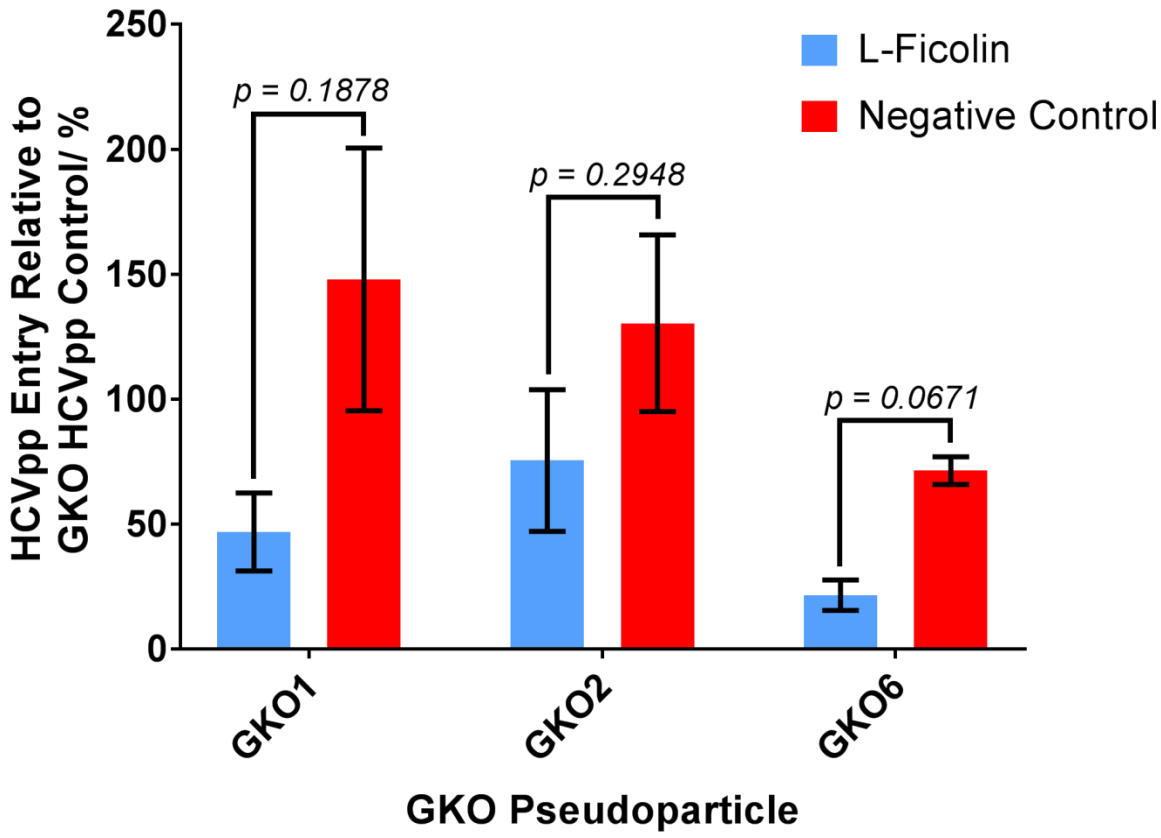

Figure 5.3 – Neutralisation of GKO HCV Pseudoparticle Entry into Huh7 Cells by L-Ficolin

Recombinant wild-type L-ficolin and a mock-transfection negative control (containing no L-ficolin) were used in triplicate to neutralise entry of HCV pseudoparticles with HCV E1E2 glycoprotein glycan knockouts (GKO) into Huh7 cells (n = 1). Samples were compared using t-test analysis with Welch's corrections (p value threshold of significance = 0.05). Although L-ficolin was unable to block GKO HCVpp entry to a higher extent than the negative control, there was no statistically significant difference between neutralisation by the samples. These results cannot determine if any specific glycosylation sites are required for L-ficolin interaction. Error bars represent standard error of mean.

5.4 Discussion

Several *N*-linked glycans of the HCV E1E2 glycoprotein have been shown to play different roles in the HCV life cycle (Figure 5.4). For example, research using HCVpp discovered that the E2 glycosylation sites E2N8 and E2N10 are essential for chaperone-independent folding and thus E1E2 incorporation into the envelope. E2N2 and E2N4 glycans directly affect HCV entry (Goffard et al., 2005). E1N2, E2N1, E2N5, E2N6 and E2N11 may modulate entry, either at the receptor-binding or fusion stage. A similar study using HCVcc revealed that E1N1, E2N7 and E2N10 were important for glycoprotein folding and heterodimerisation. Furthermore, E2N1, E2N2, E2N4, E2N6 and E2N11 were implicated in shielding neutralising antibody epitopes, the former four of which are located near the CD81-binding site of E1E2 (Helle et al., 2010).

The infectivity assay in this project supported some of these findings. In agreement with Goffard et al. (2005), GKO mutants in this project containing at least one N>D mutation at E2N2 and E2N4 – glycoproteins which are directly essential for HCV entry – were not infectious.

An exception to this is the GKO3. GKO3 HCVpp also possessed wild-type E2N2 and E2N4, however it was not infectious. The only difference between the mutations in GKO3 and infectious GKO1 pseudoparticles is the wild-type E2N1 in GKO1. Although the infectious GKO2 and GKO6 pseudoparticles had mutated E2N1 sites, these results may imply a non-essential role of the E2N1 glycosylation site in infectivity. Furthermore, the wild-type E2N11 site was found in all infectious GKO HCVpp with the exception of GKO3, whereas the mutant form was present in non-infectious GKO HCVpp. These results may suggest interdependence between certain glycosylation sites to enable infectivity, potentially due to small changes in E2 conformation after mutation. Historically, the E2N1 and E2N11 sites are thought to have roles in HCV entry modulation (Goffard et al., 2005).

In summary, this study supports the evidence that E2N2 and E2N4 were essential for HCV infectivity, whereas E2N1 and E2N11 also have potential roles in entry.

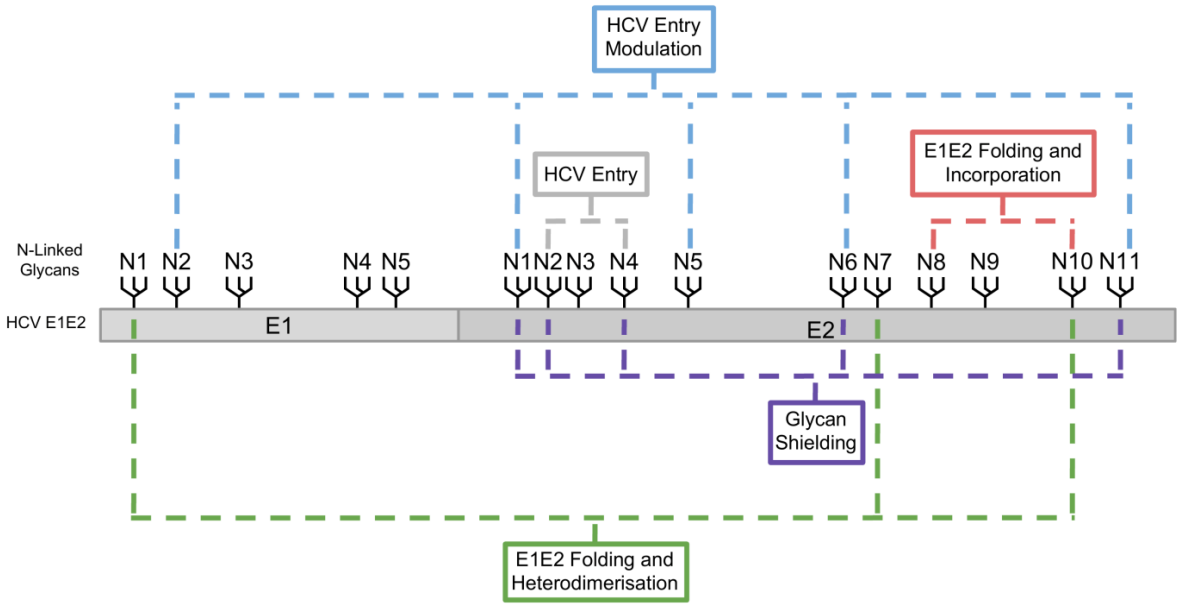

Figure 5.4 – Significant Glycosylation Sites of the HCV E1E2 Envelope Glycoprotein

Several glycosylation sites (N) of the E1 and E2 glycoproteins have essential roles in the HCV life style. For example, E2N2 and E2N4 are essential for HCV entry, and E2N1 and E2N11 are involved in HCV entry modulation, as supported by the results in this study. Information derived from Goffard et al. (2005) and Helle et al. (2010).

5.4.1 Future Work

Due to limited resources in this project, the assays require more repeats. The L-ficolin sample used in this Chapter had a low concentration of oligomeric active protein, and as a consequence gave results with large error margins, therefore the results should be treated as preliminary. Furthermore, as shown in Chapter 2 and 3, no significant neutralisation data was obtained using L-ficolin or ficobody samples in this project. Each assay should be repeated using an L-ficolin sample with a greater concentration and activity, and a HCV JFH-1 pseudoparticle control should also be used. GKO HCVpp could be added to the Huh7 stably-transfected with the *FCN2* gene, as higher L-ficolin concentrations are produced and secreted, and have shown neutralisation of virus pseudoparticle entry (Jalal et al., 2019b).

Once activity is established, a two-fold dose curve could be performed to compare the half maximal inhibitory concentration (IC_{50}) of L-ficolin against each GKO HCVpp. Differences in IC_{50} could help determine important glycosylation sites for L-ficolin interaction. HCVcc of infectious GKO preparations could be produced to test their infectivity and interactions with L-ficolin in more authentic environments.

As the infectivity of the GKO HCVpp limits study, ELISAs could be performed to test the specific interaction of the E1E2 glycoproteins with L-ficolin. Western blots specific to HCV E1E2 glycoproteins and MLV gag should be performed to confirm that receptor binding is affected by the GKO mutations, rather than pseudoparticle production. Preliminary ELISA testing using GNA lectin to capture glycoproteins required optimisation. An E1E2-specific antibody may be better to capture the lysate of the GKO HCVpp.

In the future, more GKO mutant combinations could be engineered for similar testing. The single GKO mutants studied in Goffard et al. (2005) could also be used, however L-ficolin interactions may too complex to attribute to one glycosylation site. This information could be used to predict the applicability of L-ficolin against certain viruses based on their glycosylation profiles, and help to understand how L-ficolin binds such a wide variety of ligands.

Chapter 6 General Discussion and Future Work

Enveloped RNA viruses have major economic and health burdens worldwide, with significantly varied factors such as routes of transmission and infection, progression of disease, and immune evasion strategies. Antiviral treatments can be most effective when their target is essential, conserved and widespread. One such factor for many enveloped viruses is glycosylation.

L-ficolin is a significant component of the human immune system, with varied immune roles and a far-reaching array of specificities towards several ligands, in particular acetylated compounds. Due to its documented interaction with various glycosylated microorganisms – from bacteria to fungi to viruses – the basis of this project is to understand the contribution of L-ficolin to recognition of pathogenic viruses, thus improving our knowledge of the innate immune response to viruses and contribute to new approaches to antiviral treatment.

6.1 General Summary

6.1.1 The L-Ficolin Variants

The main hypothesis at the beginning of this project was that the previously described non-synonymous SNPs in exon 8 of the *FCN2* gene, encoding the T236M and A258S mutations, would affect L-ficolin binding of viruses, and thus affect neutralisation of virus entry. This could identify an optimal form of the L-ficolin protein to be used in antiviral treatment. While it was demonstrated that the variants do affect binding to an acetylated compound, AcBSA, issues in L-ficolin production hindered discovery of differences in binding to viral glycoproteins, using HCV and EBOV as enveloped virus models. Nevertheless, data showed that the variants bind AcBSA in different patterns than to another ligand, GlcNAc, perhaps indicating that no particular variant is superior for activity against all viruses.

6.1.2 Generation of a Novel 'Ficobody' Chimaeric Molecule

The favourable qualities of the structure and immune activities of L-ficolin led to the hypothesis that its activity could be improved by combining it with another immune protein – the D03 nanobody. Thus the recombinant ficobody protein was designed, linking D03 to the C-terminus of L-ficolin. Data showed that ficobody had far stronger AcBSA and virus glycoprotein binding activity than L-ficolin. Further characterisation should be performed to confirm the superiority of ficobody in neutralisation of virus entry when compared to L-ficolin and D03, and to test improvements in its half-life *in vivo*.

6.1.3 L-Ficolin Serum Concentrations

The concentrations of L-ficolin are known to influence susceptibility to and outcome of several infectious diseases. To characterise such effects of HCV infection, two cohorts were studied. One cohort showed that L-ficolin concentrations inversely correlated with disease progression, with chronic HCV patients having the lowest L-ficolin concentrations. This contradicted previous findings. Another cohort showed no difference in L-ficolin concentrations or AcBSA-binding between European and Asian ethnicities. These data sets represent starting points for research with more conclusive data. For example, the exon 8 SNPs of the patients could be genotyped to identify possible correlations with HCV disease outcome or ethnicity.

6.1.4 Functionally Essential Glycosylation Sites of the HCV Envelope Glycoprotein E2

This Chapter aimed to characterise the glycosylation sites of the HCV E1E2 glycoprotein that are required for L-ficolin interaction, with the aims of beginning to understand how it is able to bind such a vast array of microorganisms. It could also help to predict L-ficolin interaction with other viruses. While no specific glycosylation sites of interaction were identified in this project, data supported previous findings of the essential nature of certain HCV E1E2 glycosylation sites in HCV entry and modulation.

6.2 Future Optimisation of L-Ficolin and Ficobody Expression

The protein expression protocol used in this project requires further optimisation. The low concentrations of protein obtained could be symptomatic of a faulty cell line. With higher yields, further purification steps could be imposed, such as ion exchange chromatography, ligand (such as CysNAc or GlcNAc) affinity chromatography or size-exclusion chromatography to isolate active, oligomeric L-ficolin. However, L-ficolin concentration and stability may be affected.

6.2.1 Alternative Tags

In this study a His_6-tag was used. To increase yields, optimisation may be required by altering the tag used on the construct to enhance expression levels and to allow the use of affinity chromatography with higher specificity towards L-ficolin and ficobody, and less towards media components.

Various considerations should be taken into account when selecting a tag for purification. The positioning of the tag, at the N-terminus or C-terminus, is important (Structural Genomics et al., 2008). Although the use of an N-terminal was determined to be optimal for His_6-tagged L-ficolin, the same may not be true for other tags. However, as the C-terminus is involved in protein activity, it is likely that the N-terminus is the ideal location, without the sacrifice of the equally-essential oligomerisation capacity of the L-ficolin. Tag removal is not ideal due to the risk of protein damage; however it may be a necessary compromise for the efficient production and purification of the protein.

An alternative approach could be the use of alternative transition metal ions such as Cu^{2+}, Fe^{3+} or Zn^{2+} to purify the His_6-tagged protein. However a common problem when using insect and mammalian cell expression systems, as is observed in the present study, is highly promiscuous purification due to the abundance of histidine residues in these cell lines (Kimple et al., 2013). The same applies for biotin tags – which are purified using avidin – as mammalian cells may contain other biotinylated proteins that would be co-purified (Kimple et al., 2013).

Epitope tags such as FLAG-tag require extreme pH levels during elution which may affect protein structure or activity, thus its use is limited particularly in terms of large-scale expression. Calmodulin binding peptide – a small tag with only mild elution requirements and no need for tag removal – may be a more suited choice (Kimple et al., 2013; Zhao et al., 2013).

Certain fusion protein tags, such as the small ubiquitin-like modifier (SUMO)-tag can increase expression levels (Kimple et al., 2013). However, such large tags could affect L-ficolin folding and stability. Furthermore, following SUMO-specific purification, the SUMO-tag would require cleavage followed by nickel affinity chromatography, thus conserving the same problems that have been witnessed in this study. Typically, this tag is used in *E. coli* systems, which would not give L-ficolin its native glycosylation profile and thus could affect its activity.

6.2.2 Alternative Expression Vectors

Alternative expression vectors could also be explored, to discover the optimal transcriptional promoter to use. While viral promoters are often used such as CMV and SV40 (used in this study in the pcDNA™3.1D/ V5-His-TOPO® vector), non-viral promoters such as EF-1 can also be used (Khan, 2013).

6.2.3 Optimal Storage of Samples

Storage should also be used wisely, for example freezing small aliquots at -80°C to avoid excessive freeze-thawing (Structural Genomics et al., 2008). In this study, samples were typically stored at -20°C and used between 5 – 10 times over the course of a month.

6.2.4 Alternative Cell Lines

The use of alternative human cells for expression, or an alternative system of HEK 293T cells which better allow up-scaled volumes of expression, would be needed. Mammalian – preferably human – cell lines would be required to

maintain the correct post-translational modification profiles of the L-ficolin protein, for more accurate downstream characterisation (Khan, 2013).

Several mammalian cell lines for protein production exist, such as the human HeLa cell line and the hamster CHO cell line, however the human cell line HEK 293 cell line is most commonly used due to its high success rate of transfection using the cost-effective PEI transfection reagent (Khan, 2013). On the other hand, CHO cells are typically used in large-scale industrial protein production (Bandaranayake and Almo, 2014).

A potentially optimal compromise for the production of L-ficolin is the use of HEK 293S, a suspension cell line that uses serum-free media and can be up-scaled (Bandaranayake and Almo, 2014). Serum-free media would avoid contamination of samples following nickel affinity chromatography. However, this cell line lacks the SV40 large T-antigen of the HEK 293T cell line necessary for strong protein expression. Alternatively the HKB11 cell line could be used, which combines the efficient transfection and protein secretion characteristics of other cell lines (Bandaranayake and Almo, 2014). The CAP-T or PER.C6 cell lines could also be used, the latter of which is a high-output system that also uses serum-free media (Bandaranayake and Almo, 2014).

Recently, the VRG produced a Huh7.5 cell line designed to over-express L-ficolin, with concentrations up to 7 µg/mL, however growth of this cell line depends on media containing FBS (Jalal et al., 2019b). Geno et al. (2015) detailed how standard L-ficolin expression and purification protocols using GlcNAc or CysNAc affinity chromatography is laborious and produces low yields. To overcome these issues, L-ficolin was continually expressed using CHO cells stably transfected with *FCN2* cDNA – a cell line named huf2E – in a CELLine Bioreactor. DMEM with 10% FBS and a selective antibiotic were used. L-ficolin concentrations were found to be 20 – 40-fold higher than L-ficolin produced by standard protocols in CHO cells, at concentrations of approximately 1 mg/mL. Samples purified by GlcNAc affinity chromatography contained little contaminant. Such an improved protocol removes the need for concentration and perhaps even dialysis, depending on the use of the protein. Bioreactors are also suitable for up-scaling of expression.

6.2.5 Handling of L-Ficolin Samples

Further advice offered by Geno et al. (2015) include the use of CysNAc for elution, the addition of 10% glycerol to supernatants prior to concentration by centrifugal filter units in order to avoid L-ficolin precipitation, and the use of 10% FBS in media for proper oligomerisation. However, higher order oligomers were observed in concentrated samples without glycerol the present study. Nevertheless, yields could be improved using this guidance.

6.3 Future Characterisation of L -Ficolin Variants and Ficobody

In the future, a human ficolin-2 ELISA kit (Hycult Biotech HK336) which quantifies L-ficolin concentrations in the same way as an anti-L-ficolin mAb GN4-capture ELISA, albeit using a tetramethylbenzidine substrate and a stop solution, can be purchased. This could produce more consistent results. The consistent use of a higher MW protein ladder such as HiMark™ Pre-stained Protein Standard (30 - 460 kDa; Cat. LC5699; Thermo) during SDS-PAGE and western blotting will confirm the presence of active dodecameric L-ficolin with each expression.

Once L-ficolin production is optimised, several characterisations could be performed. Tryptic digests followed by MALDI-TOF-MS/MS would identify any contaminating components within the sample.

ELISAs could be performed to elucidate the effect of the L-ficolin T-236M and A258S mutations on binding of several other ligands and other enveloped virus glycoproteins, such as HIV-1, HBV and IAV. The ELISA protocol could be updated in which L-ficolin is immobilised and lysate interaction is detected, rather than the opposite way round.

In the study of ficobody, parallel D03 characterisation and mutations of the ficobody molecule are required to fully characterise the activity of this protein.

6.4 The Therapeutic and Prophylactic Potential of L-Ficolin

The accumulation of research of the structural, immune and clinical roles of L-ficolin has made this protein a viable candidate in the potential treatment of several infectious diseases (Mason and Tarr, 2015).

6.4.1 Potential Prognostic and Diagnostic Uses for the Single Nucleotide Polymorphisms and Serum Concentrations of L-Ficolin

Serum L-ficolin concentrations have been identified a marker of acute and chronic HCV infection (Hu et al., 2013b; Liu et al., 2009), demonstrating a potential use for L-ficolin concentrations in the diagnosis of HCV infection and severity. Correlations in *FCN2* haplotypes and SNPs could be applied as biomarkers to predict natural susceptibility to and outcome of several infectious diseases, in order to plan a more individualised course of treatment for each patient, with ethnicity-correlated differences taken into account. Characterising the activity of the L-ficolin mutants encoded by the exon 8 SNPs could provide insight into the biochemical basis of differences in susceptibility to infection.

On the other hand, although *FCN2* polymorphisms clearly affect serum concentration, the ranges of serum concentrations associated with each polymorphism and haplotype often overlap, and therefore may not have significant or reliable clinical use (Kilpatrick and Chalmers, 2012).

6.4.2 Potential Applications of L-Ficolin and Ficobody in Immunotherapy

A role of L-ficolin in innate resistance to viruses could identify an optimal form of the protein for future use in immunotherapy. Similar to the success of RCLs, ficobody has potential as an optimal humanised protein with a wide specificity for several ligands and viruses, in addition to effective immune activities.

Due to the high degree of genetic conservation at the *N*-linked glycosylation sites and the essential nature of *N*-linked glycans in the life cycle of HCV – and likely other enveloped viruses – the E1 and E2 proteins are considered appealing therapeutic and vaccine targets (Hamed et al., 2014). Furthermore, several pathogens possess conserved acetylated compounds on their extracellular surfaces, making them viable targets for treatment and infection prevention by L-ficolin (Hamed et al., 2014), due to its wide range of ligand specificities and binding sites (Garlatti et al., 2007).

Lectins play a significant role in the clearance of HCV, and there are no documented HCV strains or mechanisms of evasion to directly avoid the anti-HCV effects of the ficolins (Tarr et al., 2012). Indeed, other lectins such as MBL (Brown et al., 2010), cyanovirin-N (Helle et al., 2006) and griffithsin (Meuleman et al., 2011) are known to inhibit HCV entry through interaction with the virus' *N*-linked glycans. Furthermore, exogenous administration of recombinant L-ficolin elicited a protective effect against IAV *in vivo* in mice (Pan et al., 2012). Hamed et al. (2014) discovered that L-ficolin was able to neutralise entry of several HCV genotypes, some of which were resistant to the protective action of broadly-neutralising antibodies. It is possible that L-ficolin can be exploited for the therapy and prevention of HCV and other pathogenic infections (Hamed et al., 2014). It is noteworthy that recombinant rather than serum-purified L-ficolin would likely be used in potential therapy, due to its relative ease and lower cost of synthesis.

6.4.2.1 Potential Use of L-Ficolin in Active Immunotherapy

Active immunotherapy is used to stimulate an immune response to infection. L-ficolins could be used in vaccines as adjuvants in combination with broadly neutralising antibodies, in order to make the vaccines sufficiently immunogenic. An example of this is the co-administration of a recombinant complement receptor fused with an antibody Fc region in monoclonal antibody therapy against cancer cells, which triggered complement system activity – typically suppressed by tumour cells – and improved survival of mice *in vivo* (Boross and Leusen, 2012; Elvington et al., 2012).

While antibody therapy – like the example above – is therapeutic and vaccination is prophylactic, both involve the action of antibodies and thus can be strengthened by the stimulation of the complement system. However, the degree to which antibodies interact with and depend on the complement system varies with each antibody, for example it was found that MBL prevented rather than enhanced the HIV-1 neutralising activity of the neutralising antibody 2G12 (Marzi et al., 2007). Moreover, the level at which the lectin pathway of complement activation and its interaction with the humoral immune system play in the protection against infectious disease varies with each pathogen and each strain. For example it has been proposed that differences in E1E2 glycosylation between HCV genotypes affect the efficiency of neutralisation by lectins, with genotypes that are more heavily glycosylated being more susceptible to neutralisation (Brown et al., 2010). Therefore, the potential strength of the immune response that can be achieved by the use of each candidate antibody or complement component, or by co-administration, should be assessed (Boross and Leusen, 2012; Fuchs et al., 2011).

Current tests of the neutralising effects of L-ficolin are carried out mostly in cell lines and some animal models, rather than humans, therefore the clinical use of the protein cannot be determined until tested in human patients. Interference of the immune system, in particular the complement system, is extremely complex and may lead to potentially harmful and excessive immune activity, therefore strict caution and research should be undertaken in order to assess the levels of toxicity that could be caused by the treatment (Boross and Leusen, 2012; Liu et al., 2013).

6.4.2.2 Potential Use of L-Ficolin and Ficobody in Passive Immunotherapy

L-ficolin has the potential to be used in passive immunotherapy, in which the components do not actively trigger an immune response such as the complement system to fight the infection. Instead, the protein would be introduced into individuals for the inhibition of HCV entry into cells, thus preventing HCV infection and replication (Hamed et al., 2014). This would

avoid excessive stimulation of the complement system. Avoidance of complement activation by L-ficolin or ficobody could be achieved by the site directed mutagenesis of the Lys57 residue of the FBG domain, which is responsible for MASP interaction and interaction with phagocytic receptors (Lacroix et al., 2009). Further research of the immune activity of such an L-ficolin mutant should be undertaken in the future (Lacroix et al., 2009).

Preliminary clinical trials of MBL replacement therapy for MBL-deficient patients using plasma-purified MBL have been attempted, and resulted in normal, long-term complement activation and opsonisation activities with no obvious adverse or autoimmune effects (Valdimarsson, 2003). The production and purification of safe, active and functional therapeutic MBL is feasible, but requires optimisation (Laursen, 2003). Therapy using recombinant MBL avoids ethical issues and allows cost-effective large-scale production (Jensenius et al., 2003), and phase I trials proved recombinant MBL to be tolerable, safe and effective in the restoration of MBL activity in MBL-deficient patients, with mild to no adverse effects (Petersen et al., 2006). Such findings would prove useful if L-ficolin was developed for similar use.

Comparable therapeutic approaches for other recombinant lectins have been researched. For example, recombinant MBL has therapeutic potential against EBOV infection as it neutralises the virus *in vitro* and *in vivo* via complement activation (Michelow et al., 2011), phagocytosis, and direct inhibition of glycoprotein interaction with the DC-SIGN/L-SIGN receptor (Ji et al., 2005). Specific mutagenic engineering of human SP-D enhanced IAV binding and clearance and murine survival *in vivo* (Crouch et al., 2011). Porcine SP-D neutralises a wider range of IAV infections more potently than recombinant human SP-D *in vitro* and *ex vivo*, however it may be immunogenic in humans, therefore further development is necessary (Hillaire et al., 2014). In fact, current research is being undertaken by the VRG to discover the virus neutralising potential of ficolin orthologues, such as murine ficolin A and B and porcine ficolin-α and -β (Matsushita and Fujita, 2001), under the hypothesis that ficolins that have not co-evolved with a human virus such as HCV will be more effective at neutralising infection.

In conclusion, polymorphisms in innate immune genes often show complex associations in infectious diseases. For example, Azad et al. (2012) reviewed the many identified associations of SNPs in the genes of various innate immune factors with tuberculosis infection. Associations were found with SNPs in the DC-SIGN, MBL, and several interleukin genes. The high frequencies of the +6359C>T and +6424G>T SNPs in the *FCN2* gene encoding the T236M and A258S L-ficolin mutations suggest that an associated selective advantage of the mutations exists. The fact that the T236M had superior binding to AcBSA but inferior binding to GlcNAc suggests that the mutations affect ligand binding and perhaps specificity. If the same observation applies to binding of pathogens, this may influence response to infection by different viruses. For example, this would explain why the +6424T SNP is associated with protection against CMV re-infection (de Rooij et al., 2011) yet is contrarily associated with predisposition to *Coxiella burnetii* pneumonia (van Kempen et al., 2017). Characterisation of L-ficolin variant binding and neutralisation of several enveloped viruses, and determining a similarity between the viruses, could identify molecular bases for any correlations. Such a correlation could be exploited to predict susceptibility to and outcome of specific virus infections. The same applies with *FCN2* SNPs associated with L-ficolin concentrations. Furthermore, a correlation could be exploited to identify the optimal variant of L-ficolin to treat specific viral infections. Such optimal constructs could be used as scaffolds for novel ficobody constructs using differing virus-specific nanobodies.

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Appendix

Table A – List of Methods Used to Optimise Protein Yields in this Study

Optimisation		Finding
Transfection	Amount of Plasmid	125 ng – 10000 ng of plasmid tested. Strongest bands in western blot when ≥2000 ng used.
	Transfection Reagent	Using a scaled up version of the manufacturer's protocol, a 3:1 ratio of FuGENE 6:plasmid produced the most protein. However, this was not cost-effective when compared to the PEI transfection reagent.
	Stable Transfection	HEK 293T cell line was stably-transfected with C-terminal His_6-tagged L-ficolin plasmid using blasticidin selection. Produced active L-ficolin with AcBSA-binding and minor HCVpp-neutralising capacities. However, its need for FBS for growth complicated sample purity.
Expression and Processing	HEK 293T Cell Seeding Density	Seeding densities of 2.5 – 3.0 x 10^6 in 10 cm diameter plates gave the best results.
	Media FBS Content	Less FBS gave purer samples but impacted cell growth.
	Centrifugation	Increasing the molecular weight cut off of the centrifugal filter unit from 10 kDa to 100 kDa did not rid the sample of the persistent FBS-related 50 kDa contaminant.
	Dialysis	Slide-A-Lyzer™ dialysis cassettes were used overnight, though results were not improved.

	Media	Use of Opti-MEM without FBS prevented contamination, but did not increase L-ficolin yields.
	Expression Volumes	Upscaled to 100 mL and 200 mL, producing larger amounts of protein.
Purification	FLAG-Tagged Purification	This was not pursued after a low yield was produced.
	GlcNAc Affinity Chromatography	Produced relatively pure L-ficolin samples with AcBSA-binding activity, however not particularly high yields. In a neutralisation assay, the negative expression control had no activity; a significant improvement when compared to nickel affinity purification.
Neutralisation Assay	Pseudoparticle Backbone/Reporter Plasmid Selection	MLV (phCMV5349/pTG126) and HIV-1 (pNL4-3.Luc.R-E-). The higher luminescence values of the HIV-1 plasmid in conjunction with HCV and EBOV glycoprotein plasmids allowed for easier distinction of neutralisation effects.

```
FCN2                  -6  GAAGAGATGGAGCTGGACAGAGCTGTGGGGGTCCTGGGCGCTGCCACCCTGCTGCTCTCT
FCN2-WT               -6  AACACCATGGAGCTGGACAGAGCTGTGGGGGTCCTGGGCGCTGCCACCCTGCTGCTCTCT
FCN2-6359C>T          -6  AACACCATGGAGCTGGACAGAGCTGTGGGGGTCCTGGGCGCTGCCACCCTGCTGCTCTCT
FCN2-6424G>T          -6  AACACCATGGAGCTGGACAGAGCTGTGGGGGTCCTGGGCGCTGCCACCCTGCTGCTCTCT
FCN2-6359C>T/6424G>T  -6  AACACCATGGAGCTGGACAGAGCTGTGGGGGTCCTGGGCGCTGCCACCCTGCTGCTCTCT
FCN2-D03              -6  TACACCATGGAGCTGGACAGAGCTGTGGGGGTCCTGGGCGCTGCCACCCTGCTGCTCTCT
D03                       ------------------------------------------------------------

FCN2                  55  TTCCTGGGCATGGCCTGGGCT------------------CTCCAGGCGGCAGACACCTGT
FCN2-WT               55  TTCCTGGGCATGGCCTGGGCTCATCATCACCATCACCATCTCCAGGCGGCAGACACCTGT
FCN2-6359C>T          55  TTCCTGGGCATGGCCTGGGCTCATCATCACCATCACCATCTCCAGGCGGCAGACACCTGT
FCN2-6424G>T          55  TTCCTGGGCATGGCCTGGGCTCATCATCACCATCACCATCTCCAGGCGGCAGACACCTGT
FCN2-6359C>T/6424G>T  55  TTCCTGGGCATGGCCTGGGCTCATCATCACCATCACCATCTCCAGGCGGCAGACACCTGT
FCN2-D03              55  TTCCTGGGCATGGCCTGGGCTCATCATCACCATCACCATCTCCAGGCGGCAGACACCTGT
D03                       ------------------------------------------------------------

FCN2                  97  CCAGAGGTGAAGATGGTGGGCCTGGAGGGCTCTGACAAGCTCACCATTCTCCGAGGCTGT
FCN2-WT              115  CCAGAGGTGAAGATGGTGGGCCTGGAGGGCTCTGACAAGCTCACCATTCTCCGAGGCTGT
FCN2-6359C>T         115  CCAGAGGTGAAGATGGTGGGCCTGGAGGGCTCTGACAAGCTCACCATTCTCCGAGGCTGT
FCN2-6424G>T         115  CCAGAGGTGAAGATGGTGGGCCTGGAGGGCTCTGACAAGCTCACCATTCTCCGAGGCTGT
FCN2-6359C>T/6424G>T 115  CCAGAGGTGAAGATGGTGGGCCTGGAGGGCTCTGACAAGCTCACCATTCTCCGAGGCTGT
FCN2-D03             115  CCAGAGGTGAAGATGGTGGGCCTGGAGGGCTCTGACAAGCTCACCATTCTCCGAGGCTGT
D03                       ------------------------------------------------------------

FCN2                 157  CCGGGGCTGCCTGGGGCCCCTGGGCCCAAGGGAGAGGCAGGCACCAATGGAAAGAGAGGA
FCN2-WT              175  CCGGGGCTGCCTGGGGCCCCTGGGCCCAAGGGAGAGGCAGGCACCAATGGAAAGAGAGGA
FCN2-6359C>T         175  CCGGGGCTGCCTGGGGCCCCTGGGCCCAAGGGAGAGGCAGGCACCAATGGAAAGAGAGGA
FCN2-6424G>T         175  CCGGGGCTGCCTGGGGCCCCTGGGCCCAAGGGAGAGGCAGGCACCAATGGAAAGAGAGGA
FCN2-6359C>T/6424G>T 175  CCGGGGCTGCCTGGGGCCCCTGGGCCCAAGGGAGAGGCAGGCACCAATGGAAAGAGAGGA
FCN2-D03             175  CCGGGGCTGCCTGGGGCCCCTGGGCCCAAGGGAGAGGCAGGCACCAATGGAAAGAGAGGA
D03                       ------------------------------------------------------------

FCN2                 217  GAACGTGGCCCCCCTGGACCTCCTGGGAAGGCAGGACCACCTGGGCCCAACGGAGCACCT
FCN2-WT              235  GAACGTGGCCCCCCTGGACCTCCTGGGAAGGCAGGACCACCTGGGCCCAACGGAGCACCT
FCN2-6359C>T         235  GAACGTGGCCCCCCTGGACCTCCTGGGAAGGCAGGACCACCTGGGCCCAACGGAGCACCT
FCN2-6424G>T         235  GAACGTGGCCCCCCTGGACCTCCTGGGAAGGCAGGACCACCTGGGCCCAACGGAGCACCT
FCN2-6359C>T/6424G>T 235  GAACGTGGCCCCCCTGGACCTCCTGGGAAGGCAGGACCACCTGGGCCCAACGGAGCACCT
FCN2-D03             235  GAACGTGGCCCCCCTGGACCTCCTGGGAAGGCAGGACCACCTGGGCCCAACGGAGCACCT
D03                       ------------------------------------------------------------

FCN2                 277  GGGGAGCCCCAGCCGTGCCTGACAGGCCCGCGTACCTGCAAGGACCTGCTAGACCGAGGG
FCN2-WT              295  GGGGAGCCCCAGCCGTGCCTGACAGGCCCGCGTACCTGCAAGGACCTGCTAGACCGAGGG
FCN2-6359C>T         295  GGGGAGCCCCAGCCGTGCCTGACAGGCCCGCGTACCTGCAAGGACCTGCTAGACCGAGGG
FCN2-6424G>T         295  GGGGAGCCCCAGCCGTGCCTGACAGGCCCGCGTACCTGCAAGGACCTGCTAGACCGAGGG
FCN2-6359C>T/6424G>T 295  GGGGAGCCCCAGCCGTGCCTGACAGGCCCGCGTACCTGCAAGGACCTGCTAGACCGAGGG
FCN2-D03             295  GGGGAGCCCCAGCCGTGCCTGACAGGCCCGCGTACCTGCAAGGACCTGCTAGACCGAGGG
D03                       ------------------------------------------------------------

FCN2                 337  CACTTCCTGAGCGGCTGGCACACCATCTACCTGCCCGACTGCCGGCCCCTGACTGTGCTC
FCN2-WT              355  CACTTCCTGAGCGGCTGGCACACCATCTACCTGCCCGACTGCCGGCCCCTGACTGTGCTC
FCN2-6359C>T         355  CACTTCCTGAGCGGCTGGCACACCATCTACCTGCCCGACTGCCGGCCCCTGACTGTGCTC
FCN2-6424G>T         355  CACTTCCTGAGCGGCTGGCACACCATCTACCTGCCCGACTGCCGGCCCCTGACTGTGCTC
FCN2-6359C>T/6424G>T 355  CACTTCCTGAGCGGCTGGCACACCATCTACCTGCCCGACTGCCGGCCCCTGACTGTGCTC
FCN2-D03             355  CACTTCCTGAGCGGCTGGCACACCATCTACCTGCCCGACTGCCGGCCCCTGACTGTGCTC
D03                       ------------------------------------------------------------

FCN2                 397  TGTGACATGGACACGGACGGAGGGGGCTGGACCGTTTTCCAGCGGAGGGTGGATGGCTCT
FCN2-WT              415  TGTGACATGGACACGGACGGAGGGGGCTGGACCGTTTTCCAGCGGAGGGTGGATGGCTCT
FCN2-6359C>T         415  TGTGACATGGACACGGACGGAGGGGGCTGGACCGTTTTCCAGCGGAGGGTGGATGGCTCT
FCN2-6424G>T         415  TGTGACATGGACACGGACGGAGGGGGCTGGACCGTTTTCCAGCGGAGGGTGGATGGCTCT
FCN2-6359C>T/6424G>T 415  TGTGACATGGACACGGACGGAGGGGGCTGGACCGTTTTCCAGCGGAGGGTGGATGGCTCT
FCN2-D03             415  TGTGACATGGACACGGACGGAGGGGGCTGGACCGTTTTCCAGCGGAGGGTGGATGGCTCT
D03                       ------------------------------------------------------------

FCN2                 457  GTGGACTTCTACCGGGACTGGGCCACGTACAAGCAGGGCTTCGGCAGTCGGCTGGGGGAG
FCN2-WT              475  GTGGACTTCTACCGGGACTGGGCCACGTACAAGCAGGGCTTCGGCAGTCGGCTGGGGGAG
FCN2-6359C>T         475  GTGGACTTCTACCGGGACTGGGCCACGTACAAGCAGGGCTTCGGCAGTCGGCTGGGGGAG
FCN2-6424G>T         475  GTGGACTTCTACCGGGACTGGGCCACGTACAAGCAGGGCTTCGGCAGTCGGCTGGGGGAG
FCN2-6359C>T/6424G>T 475  GTGGACTTCTACCGGGACTGGGCCACGTACAAGCAGGGCTTCGGCAGTCGGCTGGGGGAG
FCN2-D03             475  GTGGACTTCTACCGGGACTGGGCCACGTACAAGCAGGGCTTCGGCAGTCGGCTGGGGGAG
D03                       ------------------------------------------------------------
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FCN2                  517  TTCTGGCTGGGGAATGACAACATCCACGCCCTGACCGCCCAGGGAACCAGCGAGCTCCGT
FCN2-WT               535  TTCTGGCTGGGGAATGACAACATCCACGCCCTGACCGCCCAGGGAACCAGCGAGCTCCGT
FCN2-6359C>T          535  TTCTGGCTGGGGAATGACAACATCCACGCCCTGACCGCCCAGGGAACCAGCGAGCTCCGT
FCN2-6424G>T          535  TTCTGGCTGGGGAATGACAACATCCACGCCCTGACCGCCCAGGGAACCAGCGAGCTCCGT
FCN2-6359C>T/6424G>T  535  TTCTGGCTGGGGAATGACAACATCCACGCCCTGACCGCCCAGGGAACCAGCGAGCTCCGT
FCN2-D03              535  TTCTGGCTGGGGAATGACAACATCCACGCCCTGACCGCCCAGGGAACCAGCGAGCTCCGT
D03                        ------------------------------------------------------------

FCN2                  577  GTAGACCTGGTGGACTTTGAGGACAACTACCAGTTTGCTAAGTACAGATCATTCAAGGTG
FCN2-WT               595  GTAGACCTGGTGGACTTTGAGGACAACTACCAGTTTGCTAAGTACAGATCATTCAAGGTG
FCN2-6359C>T          595  GTAGACCTGGTGGACTTTGAGGACAACTACCAGTTTGCTAAGTACAGATCATTCAAGGTG
FCN2-6424G>T          595  GTAGACCTGGTGGACTTTGAGGACAACTACCAGTTTGCTAAGTACAGATCATTCAAGGTG
FCN2-6359C>T/6424G>T  595  GTAGACCTGGTGGACTTTGAGGACAACTACCAGTTTGCTAAGTACAGATCATTCAAGGTG
FCN2-D03              595  GTAGACCTGGTGGACTTTGAGGACAACTACCAGTTTGCTAAGTACAGATCATTCAAGGTG
D03                        ------------------------------------------------------------

FCN2                  637  GCCGACGAGGCGGAGAAGTACAATCTGGTCCTGGGGGCCTTCGTGGAGGGCAGTGCGGGA
FCN2-WT               655  GCCGACGAGGCGGAGAAGTACAATCTGGTCCTGGGGGCCTTCGTGGAGGGCAGTGCGGGA
FCN2-6359C>T          655  GCCGACGAGGCGGAGAAGTACAATCTGGTCCTGGGGGCCTTCGTGGAGGGCAGTGCGGGA
FCN2-6424G>T          655  GCCGACGAGGCGGAGAAGTACAATCTGGTCCTGGGGGCCTTCGTGGAGGGCAGTGCGGGA
FCN2-6359C>T/6424G>T  655  GCCGACGAGGCGGAGAAGTACAATCTGGTCCTGGGGGCCTTCGTGGAGGGCAGTGCGGGA
FCN2-D03              655  GCCGACGAGGCGGAGAAGTACAATCTGGTCCTGGGGGCCTTCGTGGAGGGCAGTGCGGGA
D03                        ------------------------------------------------------------

FCN2                  697  GATTCCCTGACGTTCCACAACAACCAGTCCTTCTCCACCAAAGACCAGGACAATGATCTT
FCN2-WT               715  GATTCCCTGACGTTCCACAACAACCAGTCCTTCTCCACCAAAGACCAGGACAATGATCTT
FCN2-6359C>T          715  GATTCCCTGATGTTCCACAACAACCAGTCCTTCTCCACCAAAGACCAGGACAATGATCTT
FCN2-6424G>T          715  GATTCCCTGACGTTCCACAACAACCAGTCCTTCTCCACCAAAGACCAGGACAATGATCTT
FCN2-6359C>T/6424G>T  715  GATTCCCTGATGTTCCACAACAACCAGTCCTTCTCCACCAAAGACCAGGACAATGATCTT
FCN2-D03              715  GATTCCCTGACGTTCCACAACAACCAGTCCTTCTCCACCAAAGACCAGGACAATGATCTT
D03                        ------------------------------------------------------------

FCN2                  757  AACACCGGAAATTGTGCTGTGATGTTTCAGGGAGCTTGGTGGTACAAAAACTGCCATGTG
FCN2-WT               775  AACACCGGAAATTGTGCTGTGATGTTTCAGGGAGCTTGGTGGTACAAAAACTGCCATGTG
FCN2-6359C>T          775  AACACCGGAAATTGTGCTGTGATGTTTCAGGGAGCTTGGTGGTACAAAAACTGCCATGTG
FCN2-6424G>T          775  AACACCGGAAATTGTTCTGTGATGTTTCAGGGAGCTTGGTGGTACAAAAACTGCCATGTG
FCN2-6359C>T/6424G>T  775  AACACCGGAAATTGTTCTGTGATGTTTCAGGGAGCTTGGTGGTACAAAAACTGCCATGTG
FCN2-D03              775  AACACCGGAAATTGTGCTGTGATGTTTCAGGGAGCTTGGTGGTACAAAAACTGCCATGTG
D03                        ------------------------------------------------------------

FCN2                  817  TCAAACCTGAATGGTCGCTACCTCAGGGGGACTCATGGCAGCTTTGCAAATGGCATCAAC
FCN2-WT               835  TCAAACCTGAATGGTCGCTACCTCAGGGGGACTCATGGCAGCTTTGCAAATGGCATCAAC
FCN2-6359C>T          835  TCAAACCTGAATGGTCGCTACCTCAGGGGGACTCATGGCAGCTTTGCAAATGGCATCAAC
FCN2-6424G>T          835  TCAAACCTGAATGGTCGCTACCTCAGGGGGACTCATGGCAGCTTTGCAAATGGCATCAAC
FCN2-6359C>T/6424G>T  835  TCAAACCTGAATGGTCGCTACCTCAGGGGGACTCATGGCAGCTTTGCAAATGGCATCAAC
FCN2-D03              835  TCAAACCTGAATGGTCGCTACCTCAGGGGGACTCATGGCAGCTTTGCAAATGGCATCAAC
D03                        ------------------------------------------------------------

FCN2                  877  TGGAAGTCGGGGAAAGGATACAATTATAGCTACAAGGTGTCAGAGATGAAGGTGCGACCT
FCN2-WT               895  TGGAAGTCGGGGAAAGGATACAATTATAGCTACAAGGTGTCAGAGATGAAGGTGCGACCT
FCN2-6359C>T          895  TGGAAGTCGGGGAAAGGATACAATTATAGCTACAAGGTGTCAGAGATGAAGGTGCGACCT
FCN2-6424G>T          895  TGGAAGTCGGGGAAAGGATACAATTATAGCTACAAGGTGTCAGAGATGAAGGTGCGACCT
FCN2-6359C>T/6424G>T  895  TGGAAGTCGGGGAAAGGATACAATTATAGCTACAAGGTGTCAGAGATGAAGGTGCGACCT
FCN2-D03              895  TGGAAGTCGGGGAAAGGATACAATTATAGCTACAAGGTGTCAGAGATGAAGGTGCGACCT
D03                        ------------------------------------------------------------

FCN2                  937  GCCTAG--------------------------------------------------------
FCN2-WT               955  GCCTAG--------------------------------------------------------
FCN2-6359C>T          955  GCCTAG--------------------------------------------------------
FCN2-6424G>T          955  GCCTAG--------------------------------------------------------
FCN2-6359C>T/6424G>T  955  GCCTAG--------------------------------------------------------
FCN2-D03              955  GCCGGTGGGTCAGGCGGATCGATGGCGGAAGTCCAACTGCAAGCGTCGGGTGGTGGTCTG
D03                   -21  ----------------------ATGGCGGAAGTCCAACTGCAAGCGTCGGGTGGTGGTCTG

FCN2                       ------------------------------------------------------------
FCN2-WT                    ------------------------------------------------------------
FCN2-6359C>T               ------------------------------------------------------------
FCN2-6424G>T               ------------------------------------------------------------
FCN2-6359C>T/6424G>T       ------------------------------------------------------------
FCN2-D03             1015  GTCCAACCGGGTGGCTCCCTGCGTCTGTCGTGTACGGCATCTGGTTTTACGGATGACTAT
D03                    40  GTCCAACCGGGTGGCTCCCTGCGTCTGTCGTGTACGGCATCTGGTTTTACGGATGACTAT
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FCN2                        ------------------------------------------------------------
FCN2-WT                     ------------------------------------------------------------
FCN2-6359C>T                ------------------------------------------------------------
FCN2-6424G>T                ------------------------------------------------------------
FCN2-6359C>T/6424G>T        ------------------------------------------------------------
FCN2-D03              1075  TACGCAATTGGCTGGTTTCGTCAGGCTCCGGGCAAAGAACGCGAAGGTGTCAGCTGCATC
D03                    100  TACGCAATTGGCTGGTTTCGTCAGGCTCCGGGCAAAGAACGCGAAGGTGTCAGCTGCATC

FCN2                        ------------------------------------------------------------
FCN2-WT                     ------------------------------------------------------------
FCN2-6359C>T                ------------------------------------------------------------
FCN2-6424G>T                ------------------------------------------------------------
FCN2-6359C>T/6424G>T        ------------------------------------------------------------
FCN2-D03              1135  ACCAACTTTGATGGCGGTACCTATTACGCGGACAGCGTTAAATCTCGTTTCACCATGAGT
D03                    160  ACCAACTTTGATGGCGGTACCTATTACGCGGACAGCGTTAAATCTCGTTTCACCATGAGT

FCN2                        ------------------------------------------------------------
FCN2-WT                     ------------------------------------------------------------
FCN2-6359C>T                ------------------------------------------------------------
FCN2-6424G>T                ------------------------------------------------------------
FCN2-6359C>T/6424G>T        ------------------------------------------------------------
FCN2-D03              1195  CGCGATAACGCCAAAAATACGGTGTATCTGCAAATGAATTCCCTGAAACCGGAAGATACC
D03                    220  CGCGATAACGCCAAAAATACGGTGTATCTGCAAATGAATTCCCTGAAACCGGAAGATACC

FCN2                        ------------------------------------------------------------
FCN2-WT                     ------------------------------------------------------------
FCN2-6359C>T                ------------------------------------------------------------
FCN2-6424G>T                ------------------------------------------------------------
FCN2-6359C>T/6424G>T        ------------------------------------------------------------
FCN2-D03              1255  GCGGTTTATTACTGCGCGGCCGACAAAGGTCTGTGTAGCTGGCTGCGTGCGGGTGGTAAA
D03                    280  GCGGTTTATTACTGCGCGGCCGACAAAGGTCTGTGTAGCTGGCTGCGTGCGGGTGGTAAA

FCN2                        ------------------------------------------------------------
FCN2-WT                     ------------------------------------------------------------
FCN2-6359C>T                ------------------------------------------------------------
FCN2-6424G>T                ------------------------------------------------------------
FCN2-6359C>T/6424G>T        ------------------------------------------------------------
FCN2-D03              1315  GTCACGTTCGGTTCCTGGGGTCAAGGCACGCAAGTCACGGTTTCATCGGCGGCCGCACTC
D03                    340  GTCACGTTCGGTTCCTGGGGTCAAGGCACGCAAGTCACGGTTTCATCGGCGGCCGCACTC

FCN2                        ------
FCN2-WT                     ------
FCN2-6359C>T                ------
FCN2-6424G>T                ------
FCN2-6359C>T/6424G>T        ------
FCN2-D03              1375  GAGTGA
D03                    400  GAGTGA
```

Figure A – DNA Sequence Alignment of Constructs Used in This Study

The N-terminal His_6-tagged L-ficolin variants were sequenced using the T7F and BGHR primers. The DNA sequences were aligned with the wild-type FCN2 *sequence (GI Number 61744444, Accession Number NM_004108.2) from the NCBI Gene Database (first line). Sequence is written 5' to 3'. Numbers represent the first base of the line, with +1 representing the first base of the start codon. The 5' Kozak sequence is underlined, with the start codon highlighted in yellow. The His_6-tag (red) and GGSGGS (green) linker sequences are also highlighted. The +6359 (blue) and +6424 (pink) bases of interest in this study, influencing residues 236 and 258, respectively, are also highlighted.*

```
L-Ficolin              1  MELDRAVGVLGAATLLLSFLGMAWA------LQAADTCPEVKMVGLEGSDKLTILRGCPG
L-Ficolin-WT           1  MELDRAVGVLGAATLLLSFLGMAWAHHHHHHLQAADTCPEVKMVGLEGSDKLTILRGCPG
L-Ficolin-T236M        1  MELDRAVGVLGAATLLLSFLGMAWAHHHHHHLQAADTCPEVKMVGLEGSDKLTILRGCPG
L-Ficolin-A258S        1  MELDRAVGVLGAATLLLSFLGMAWAHHHHHHLQAADTCPEVKMVGLEGSDKLTILRGCPG
L-Ficolin-T236M/A258   1  MELDRAVGVLGAATLLLSFLGMAWAHHHHHHLQAADTCPEVKMVGLEGSDKLTILRGCPG
Ficobody               1  MELDRAVGVLGAATLLLSFLGMAWAHHHHHHLQAADTCPEVKMVGLEGSDKLTILRGCPG
D03                       ------------------------------------------------------------

L-Ficolin             55  LPGAPGPKGEAGTNGKRGERGPPGPPGKAGPPGPNGAPGEPQPCLTGPRTCKDLLDRGHF
L-Ficolin-WT          61  LPGAPGPKGEAGTNGKRGERGPPGPPGKAGPPGPNGAPGEPQPCLTGPRTCKDLLDRGHF
L-Ficolin-T236M       61  LPGAPGPKGEAGTNGKRGERGPPGPPGKAGPPGPNGAPGEPQPCLTGPRTCKDLLDRGHF
L-Ficolin-A258S       61  LPGAPGPKGEAGTNGKRGERGPPGPPGKAGPPGPNGAPGEPQPCLTGPRTCKDLLDRGHF
L-Ficolin-T236M/A258  61  LPGAPGPKGEAGTNGKRGERGPPGPPGKAGPPGPNGAPGEPQPCLTGPRTCKDLLDRGHF
Ficobody              61  LPGAPGPKGEAGTNGKRGERGPPGPPGKAGPPGPNGAPGEPQPCLTGPRTCKDLLDRGHF
D03                       ------------------------------------------------------------

L-Ficolin            115  LSGWHTIYLPDCRPLTVLCDMDTDGGGWTVFQRRVDGSVDFYRDWATYKQGFGSRLGEFW
L-Ficolin-WT         121  LSGWHTIYLPDCRPLTVLCDMDTDGGGWTVFQRRVDGSVDFYRDWATYKQGFGSRLGEFW
L-Ficolin-T236M      121  LSGWHTIYLPDCRPLTVLCDMDTDGGGWTVFQRRVDGSVDFYRDWATYKQGFGSRLGEFW
L-Ficolin-A258S      121  LSGWHTIYLPDCRPLTVLCDMDTDGGGWTVFQRRVDGSVDFYRDWATYKQGFGSRLGEFW
L-Ficolin-T236M/A258 121  LSGWHTIYLPDCRPLTVLCDMDTDGGGWTVFQRRVDGSVDFYRDWATYKQGFGSRLGEFW
Ficobody             121  LSGWHTIYLPDCRPLTVLCDMDTDGGGWTVFQRRVDGSVDFYRDWATYKQGFGSRLGEFW
D03                       ------------------------------------------------------------

L-Ficolin            175  LGNDNIHALTAQGTSELRVDLVDFEDNYQFAKYRSFKVADEAEKYNLVLGAFVEGSAGDS
L-Ficolin-WT         181  LGNDNIHALTAQGTSELRVDLVDFEDNYQFAKYRSFKVADEAEKYNLVLGAFVEGSAGDS
L-Ficolin-T236M      181  LGNDNIHALTAQGTSELRVDLVDFEDNYQFAKYRSFKVADEAEKYNLVLGAFVEGSAGDS
L-Ficolin-A258S      181  LGNDNIHALTAQGTSELRVDLVDFEDNYQFAKYRSFKVADEAEKYNLVLGAFVEGSAGDS
L-Ficolin-T236M/A258 181  LGNDNIHALTAQGTSELRVDLVDFEDNYQFAKYRSFKVADEAEKYNLVLGAFVEGSAGDS
Ficobody             181  LGNDNIHALTAQGTSELRVDLVDFEDNYQFAKYRSFKVADEAEKYNLVLGAFVEGSAGDS
D03                       ------------------------------------------------------------

L-Ficolin            235  LTFHNNQSFSTKDQDNDLNTGNCAVMFQGAWWYKNCHVSNLNGRYLRGTHGSFANGINWK
L-Ficolin-WT         241  LTFHNNQSFSTKDQDNDLNTGNCAVMFQGAWWYKNCHVSNLNGRYLRGTHGSFANGINWK
L-Ficolin-T236M      241  LMFHNNQSFSTKDQDNDLNTGNCAVMFQGAWWYKNCHVSNLNGRYLRGTHGSFANGINWK
L-Ficolin-A258S      241  LTFHNNQSFSTKDQDNDLNTGNCSVMFQGAWWYKNCHVSNLNGRYLRGTHGSFANGINWK
L-Ficolin-T236M/A258 241  LMFHNNQSFSTKDQDNDLNTGNCSVMFQGAWWYKNCHVSNLNGRYLRGTHGSFANGINWK
Ficobody             241  LTFHNNQSFSTKDQDNDLNTGNCAVMFQGAWWYKNCHVSNLNGRYLRGTHGSFANGINWK
D03                       ------------------------------------------------------------

L-Ficolin            295  SGKGYNYSYKVSEMKVRPA*-------------------------------------
L-Ficolin-WT         301  SGKGYNYSYKVSEMKVRPA*-------------------------------------
L-Ficolin-T236M       301  SGKGYNYSYKVSEMKVRPA*-------------------------------------
L-Ficolin-A258S       301  SGKGYNYSYKVSEMKVRPA*-------------------------------------
L-Ficolin-T236M/A258 301  SGKGYNYSYKVSEMKVRPA*-------------------------------------
Ficobody             301  SGKGYNYSYKVSEMKVRPAGGSGGSMAEVQLQASGGGLVQPGGSLRLSCTASGFTDDYYA
D03                  -27  -------------------------MAEVQLQASGGGLVQPGGSLRLSCTASGFTDDYYA

L-Ficolin                 ------------------------------------------------------------
L-Ficolin-WT              ------------------------------------------------------------
L-Ficolin-T236M            ------------------------------------------------------------
L-Ficolin-A258S            ------------------------------------------------------------
L-Ficolin-T236M/A258      ------------------------------------------------------------
Ficobody             361  IGWFRQAPGKEREGVSCITNFDGGTYYADSVKSRFTMSRDNAKNTVYLQMNSLKPEDTAV
D03                   34  IGWFRQAPGKEREGVSCITNFDGGTYYADSVKSRFTMSRDNAKNTVYLQMNSLKPEDTAV

L-Ficolin                 ------------------------------------------
L-Ficolin-WT              ------------------------------------------
L-Ficolin-T236M            ------------------------------------------
L-Ficolin-A258S            ------------------------------------------
L-Ficolin-T236M/A258      ------------------------------------------
Ficobody             421  YYCAADKGLCSWLRAGGKVTFGSWGQGTQVTVSSAAALE*
D03                   94  YYCAADKGLCSWLRAGGKVTFGSWGQGTQVTVSSAAALE*
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Figure B – Amino Acid Sequence Alignment of Constructs Used in This Study

The amino acid sequences were aligned with the wild-type L-ficolin *sequence (GI Number 61744444, Accession Number NM_004108.2) from the NCBI Gene Database (first line). Sequence is written from N-terminus to C-*

*terminus. Numbers represent the first residue of the line, with +1 representing the start codon (yellow). The His_6-tag (red) and GGSGGS (green) linker sequences are also highlighted. The 236 (blue) and 258 (pink) residues of interest in this study are also highlighted. * = stop codon.*

Publications

The work contained within this thesis has contributed towards the following published research articles and reviews:

- **Journal of Innate Immunity, 2014. Vol. 6 (5): p. 676 – 684.**

Title: Recombinant Human L-Ficolin Directly Neutralizes Hepatitis C Virus Entry.
Authors: Hamed, M.R., Brown, R.J., Zothner, C., Urbanowicz, R.A., Mason, C.P., Krarup, A., McClure, C.P., Irving, W.L., Ball, J.K., Harris, M., Hickling, T.P. and Tarr, A.W.

- **Molecules, 2015. Vol. 20 (2): p. 2229 – 2271.**

Title: Human Lectins and Their Roles in Viral Infections.
Authors: Mason, C.P., and Tarr, A.W.

- **The Journal of General Virology, 2016. Vol. 97 (9), p. 2265 – 2279.**

Title: Novel Functional Hepatitis C Virus Glycoprotein Isolates Identified using an Optimized Viral Pseudotype Entry Assay.
Authors: Urbanowicz, R.A., McClure, C.P., King, B., Mason, C.P., Ball, J.K., and Tarr, A.W.

- **Journal of Medical Microbiology, 2019. DOI: 1099/jmm.0.000935 (Electronic Publication Ahead of Print).**

Title: Expression of Human Ficolin-2 in Hepatocytes Confers Resistance to Infection by Diverse Hepatotropic Viruses.
Authors: Jalal, P.J., Urbanowicz, R.A., Horncastle, E., Pathak, M., Goddard, C., Saeed, A., Mason, C.P., Ball, J.K., Irving, W.L., McClure, C.P., and Tarr, A.W.

- **Virology, 2019. DOI: 10.1016/j.virol.2019.02.002 (Electronic Publication Ahead of Print).**

Title: Elevated Serum Activity of MBL and Ficolin-2 as Biomarkers for Progression to Hepatocellular Carcinoma in Chronic HCV Infection.
Authors: Jalal, P., King, B., Saeed, A., Adedeji, Y., Mason, C. P., Ball, J., Irving, W., McClure, P., and Tarr, A. W.