

The interface between adaptive and innate neutralisation of virus entry: competition, addition or synergy?

Supervisors: Dr Alexander Tarr and Prof. Patrick Tighe

Sophie Wartnaby, BSc

14327964

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Abstract

A complex interplay exists between the innate and adaptive immune systems when responding to viral infections. In particular, complement activity is required to both directly and indirectly eradicate virus infection. The aim of this study was to identify the effect of complement on antibody-mediated virus neutralisation, using Hepatitis C Virus (HCV) as a model. Hepatitis C Virus causes 1.5 million new infections globally every year, with the majority of cases progressing to chronic disease. There is often an asymptomatic acute phase of infection, meaning that early infections are not diagnosed. Complement haemolytic screening (CH50) assay of the classical pathway highlighted there was a significant impairment of complement activity in 75% of chronically infected HCV sera. We confirmed this depletion in complement activity was a genuine result and not due to storage conditions of the sera. The effect of complement on the neutralisation potential of human monoclonal (mAbs) and polyclonal antibodies (HCV-infected IgG), was assessed using an infection model utilising HCV pseudoparticles and human hepatoma cells. It was discovered that mAbs and polyclonal anti-HCV IgG preparations had increased neutralisation potential in the presence of complement, indicating an additional mechanism for the complement cascade in limiting virus infection. This is important for developing immunisation strategies to prevent the spread of HCV and limiting chronic infection. Using this assay, we developed a complement activity depletion model which illustrates the possible outcomes upon HCV infection. This informative model is crucial to understanding how complement function reduces the repercussions of HCV infection and whether complement replacement therapy is necessary alongside DAAs.

Abbreviations

Å – Angstrom Unit
Ab – Antibody
AIS – Adaptive immune response
AIS – Adaptive immune system
AMPS – Antimicrobial peptides
ATP – Adenosine triphosphate
BSA – Bovine serum albumin
C1 – Complement protein 1
CRP – C reactive protein
DAA – Direct-acting antivirals
DAF – Decay accelerating factor (CD55)
DEPC – Diethyl pyrocarbonate treated water – RNA free water
DMEM – Dulbecco's modified Eagle's medium.
FBS – Fetal Bovine Serum
GPI – Glycosylphosphatidylinositol
HCV – Hepatitis C Virus
HCVpp – Hepatitis C virus pseudoparticles
HEKS – Human embryonic kidney cells
Huh – Hepatocyte-derived carcinoma cell
IFN – Interferon
IgG – Immunoglobulin G
IgM – Immunoglobulin M
IL - Interleukin
ISG – Interferon stimulating gene
MAC – Membrane attack complex
MASP 1/2 – Mannose binding lectin serine protease
MAVS - Mitochondrial antiviral-signalling protein
NAb – Neutralising antibodies
NK cells – Natural killer cells
NLR – NOD-like receptor
NOD - nucleotide-binding and oligomerization domain
PAMPS – Patten-associated molecular patterns
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PKR – Protein kinase R
pNPP – p-Nitrophenyl Phosphate
PRR - Pattern-recognition receptor
q-PCR – Real-time polymerase chain reaction
RLRs – RIG-I-like receptors
RLU – Relative Light Unit
RNA – Ribonucleic acid
SRBC – Sheep red blood cells
TBS-Tween– Tris buffer solution & Tween
TLR – Toll-like receptors

1 Introduction and background

1.1 The immune system

The mammalian immune system is an ancient mechanism that is imperative for host survival. Molecules produced by the host work collaboratively to form a defence network against invading pathogens, which if given the opportunity, will cause detrimental disease. The immune system is comprised of the innate and adaptive systems that can be distinguished by their unique responses to the invasion of a pathogen. Both systems have co-evolved a complex interplay to provide the best protection for the host. The immune system's main function is to respond to infection although it also has prominent roles in prohibiting tumour growth and repairing damaged tissue, across the body (Abbas, Lichtman and Pillai, 2020). Defects in either system ultimately leads to autoimmune diseases, allergies, cancer development, as well as giving the opportunity of pathogens to cause disease.

1.2 Innate Immune system

Innate immunity provides a rapid response to the invasion of a pathogens. It relies on germline-encoded PRRs to recognise pathogen-associated molecular patterns (PAMPS) which are mediated by Toll-like receptors (TLRs) (Kawai and Akira, 2010). These transmembrane proteins (e.g., TLRs) can identify PAMPs in the various forms of lipids, proteins and nucleic acids, which are associated with all different microbes (Akira, Uematsu and Takeuchi, 2006). The innate immune is considered a non-specific reaction, however, TLRs recruit adaptor molecules which provide specific responses to the identified microbe. The receptors can discriminate between pathogen classes to recruit specific immune components (Barbalat *et al.*, 2009). Ficolins and pentraxins are another class of PRRs which bind to microbial cell surfaces for opsonisation. These secreted PRRs can also activate the classical and lectin pathways (Iwasaki and Medzhitov, 2010). The final class of PRRs are regarded as cytosolic and include retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs). RLRs can detect viral RNA and NLRs are intracellular sensors which detect microbial products (Iwasaki and Medzhitov, 2010).

1.2.1 First line defence of the innate immune – The Epithelial Barrier

Epithelial barriers are overlooked as an important feature of the innate immune system. They provide a direct interface between the host and the external environment (Svirshchevskaya *et al*, 2012). Epithelial cells can form tightly packed mechanical barriers as well as secreting antimicrobial peptides (AMPs). Cationic AMPs like defensins, cathelicidin and anionic AMP dermcidin act as natural antibiotics, to disrupt bacteria and viruses. These peptides can be constantly secreted or upregulated during various external and internal stimuli (Herman and Herman, 2019). Epithelial cells can be classed as 'non-professional' phagocytes acting as a first line defence for the human body.

1.2.2 Cells of the innate immune system

A key component of the innate response is the 'professional' phagocytes which include neutrophils, monocytes and monocyte-derived macrophages (Lim, Grinstein and Roth, 2017). Neutrophils are short-lived, terminally differentiated cells which are released from the bone marrow into the peripheral blood (Ma, Zhang and Zhu, 2021). They are recruited to the site of infection via proinflammatory cytokines and the interaction with complement component C5a (Galani and Andreakos, 2015). Increased viral replication and subsequent mortality has been associated with a decrease in neutrophils in neurotropic mouse hepatitis virus infection (Zhou *et al*, 2003). As the most abundant cell type, their response is largely considered beneficial for clearance of virus infection. Working alongside neutrophils, monocyte-derived macrophages are recruited during pathogenic microenviroments (Zhang, Yang and Ericsson, 2021). Both external stimuli and cytokine microenvironment contribute to macrophage function and phenotype. In turn, the phenotype affects the onward activation of immune response and viral outcome (Banete *et al.*, 2022). The M1 proinflammatory phenotype has phagocytic abilities and recruits T and B cells via cytokine and chemokine release. Whereas the M2 phenotype is more diverse with the main role of resolving inflammation (Ross, Devitt and Johnson, 2021). Both cell types work closely with other innate components to inhibit viral replication and stimulate further responses to eradicate the infection.

1.2.3 Complement

Complement is another integral part of the immune which forms a crosslink between in the innate and adaptive immune. The system is comprised of over 30 proteins which work together to opsonise pathogens and trigger further inflammatory response. Innate immune cells can present pathogen complexes through complement proteins and complement receptors (Tarr, Urbanowicz and Ball, 2012). The complement system is known for its substantial role in removing pathogens from the blood, however, it is often overlooked that it also responds to cancer and tissue injury.

The complement cascade is a tightly controlled system which ensures the response is localised and does not impinge uninfected areas (Merle et al, 2015). Over activation of the complement system can lead to chronic inflammation and cancer development, while deficiencies can increase susceptibility to infection. Therefore, the body requires a fine balance. Complement molecules are produced primarily in the liver and continuously circulate in your blood. Most of these proteins lie in a passive state until activation upon pathogen entry, which stimulates the complement cascade. Three pathways allow for the activation of the complement cascade; classical (**Figure 1**), lectin (***Figure 2***)and alternative (***Figure 3***) which ultimately leads to the activation of the adaptive immune system.

1.2.3.1 Classical Pathway

The classical pathway is recognised as one of the most important pathways during infection. Brown *et al* (2002), identified classical activation as the dominant pathway during innate immunity to *Streptococcus pneumoniae* and loss of this pathway caused rapid progression to septicaemia. Furthermore, human acute-phase reactant C-reactive protein (CRP) protected mice from *S. pneumoniae*, though only in the presence of complement C3 and C4(Mold, Rodic-Polic and Du Clos, 2002).

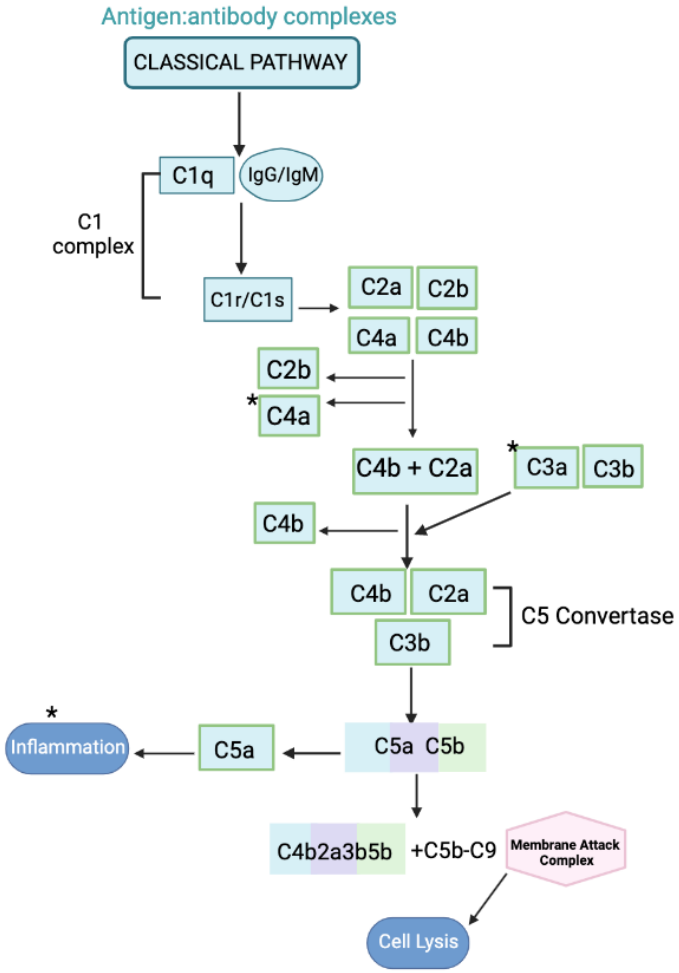


Figure 1: Classical complement pathway
The stimuli and outcome for the classical complement pathway. The pathway starts independently with an antigen:antibody complex (blue boxes), before following the same process as the lectin pathway (blue boxes with green outline). All three athways converge (represented by multiple colours) at C5 with the end goal of forming the membrane attack complex (MAC) to induce cell lysis. Adapted from Dunkelberger and Song (2010) and Mellors et al (2020).

Classical complement is led by C1, C2 and C4 with the common terminal pathway of C5-C9. This aims to trigger the formation of the membrane attack complex (MAC) which forms cytotoxic pores on the surface of the pathogen. The pathway starts with the C1 complex, which is comprised of three subcomponents, C1q, C1r and C1s. C1q acts as a scaffold for C1r/C1s and binds to the Fc region of complement fixing antibodies, also known as IgG and IgM (Dunkelberger and Song, 2010). This activates the two serine proteases, C1r and C1s, to cleave C4 and C2. The cleaved products are size specific. C4b and C2a are the larger by-products and form a C4bC2a complex on pathogenic surfaces (Dunkelberger and Song, 2010). This complex is a serine protease known as C3 convertase and is imperative for the continuum of the classical pathway. C3 convertase cleaves C3 into anaphylatoxin C3a and opsonin C3b (Mellors *et al*, 2020). C3b can covalently bind carbohydrates due to a thioester

interacting with hydroxyl nucleophilic groups (Law and Dodds, 1997). The thioester domain moves ≈100 Å which allows the interaction with antigenic surfaces (Nishida, Walz and Springer, 2006). This process tags the microbes as 'foreign' to allow recognition of other innate and adaptive cells to act. Alike C3, C4 also contains an internal thioester bond on C3b, upon cleavage. This can form covalent amide or ester linkages with the plasma membrane of the pathogen and associated antibodies, allowing it to become an opsonin (Law and Dodds, 1997). C3b forms the C5 convertase (C4b2b3b) to allow the cleavage of C5 into C5a and C5b (Mellors *et al*, 2020). C5b acts as the building block for the membrane attack complex (MAC). Both convertases are imperative to the functioning of the cascade and the elimination of pathogens. In the case of misfunctioning or inadequate convertases, there are no other substitutes for the cascade, resulting in no MAC formation and therefore no cell lysis. The absence or depletion of a specific complement component can be determined using a haemolytic complement activity assay. This screening assay assesses the lysis ability of sera, using sensitised sheep red blood cells (SRBC) coated with haemolysin. Complement activation (via the classical pathway) is proportional to cell lysis, therefore the complement quantity can be quantified (Costabile, 2010). The CH50 value is based upon the dilution factor of serum to obtain 50% lysis of cells.

1.2.3.2 Lectin Pathway

The lectin pathway can be initiated by five different humoral pattern recognition molecules including mannose-binding lectin (MBL), M-, L- and H-ficolin and collectin 11. These molecules work closely with serine proteases in the form of proenzyme (zymogens) (Megyeri *et al*, 2013). Zymogens like MASP-1 and MASP-2 circulate in a biologically inactive form until bound with the pattern recognition molecules. Upon activation, MASPs cleave C2 and C4 and the by-products form the C3 and C5 convertase (Beltrame *et al.*, 2015). This activation pathway has elements conserved with the classical pathway, eventually leading to the terminal pathway and MAC complex formation.

Across most literature, it is reported that MASP-2 can autoactivate to cleave proteins C4 and C2, producing the C3 convertase complex (Gál *et al.*, 2005). This is not a common pathway due to the low circulating quantity of MASP-2. On the other hand, Héja *et al*, (2012)

proposes the new model in which MASP-1 autoactivates and cleaves the zymogen MASP-2. MASP-1 predominantly cleaves C2 and MASP-2 solely cleaves C4, allowing the formation of the C3 convertase. MASP-2 is considered to be essential in cleaving C4 and shows to disable the lectin pathway in MASP-2 depleted serum at high serum concentrations (Møller-Kristensen *et al.*, 2007).

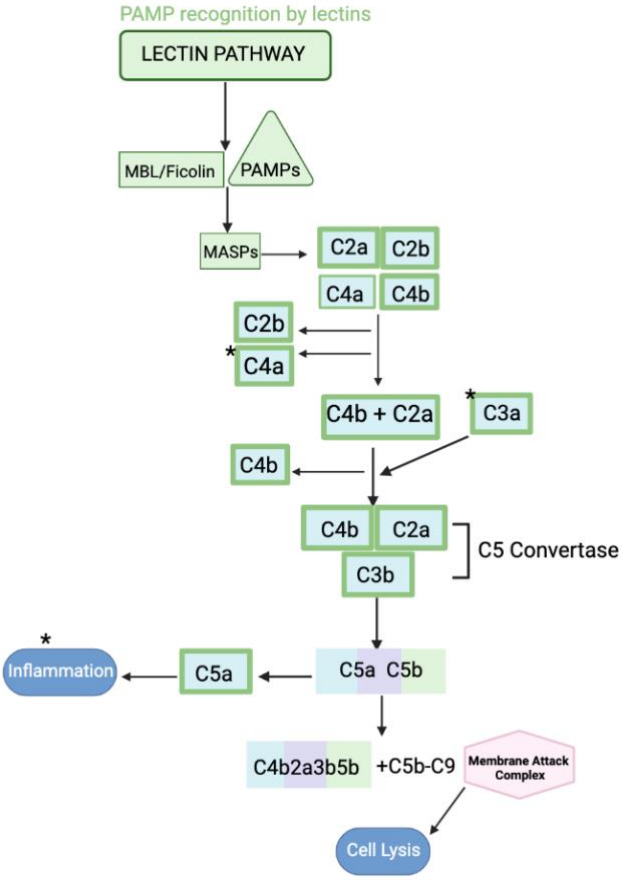


Figure 2: Lectin Complement Pathway
The pathway starts via PAMP recognition by lectins which in turn activates MASPS, causing the cleavage C2 and C4. This is the point at which the pathway aligns with the classical pathway (blue boxes with green outline). All pathways converge (represented by multiple colours) at C5 with the end goal of forming the membrane attack complex (MAC) to induce cell lysis. Adapted from Dunkelberger and Song (2010) and Mellors et al (2020).

1.2.3.3 Alternative Pathway

Dissimilar to the classical and lectin pathway, the alternative pathway is slowly activated by the spontaneous hydrolysis of C3(H_20) producing C3b (). Through the actions of Factor D, C3 binds to Factor B causing the cleavage and formation or Bb and Ba (Dunkelberger and Song, 2010). This cleavage gives rise to the alternative pathway convertase, C3(H_20)Bb which contributes to the amplification loop of C3 cleavage (Thurman and Fremeaux-Bacchi, 2023).

The AP C3 convertase is stabilised by Properdin (Factor P), the only known positively charged regulator of the complement system (Blatt, Pathan and Ferreira, 2016). On the other hand, one of the main negative regulators of the AP is Factor H. Wu *et al* (2009), provided insight to the crystal structure of Factor H and C3b which demonstrated the destabilisation of C3 convertase by competition and the proteolytic degradation of C3b by protease factor 1. Furthermore, low expression of factor H and continuous AP activation is associated with disease progression in diagnoses like lupus nephritis (Wang *et al*, 2012). It has been established that the alternative pathway is the largest contributor to the activation of C5 and can substantially reduce the effects of the terminal pathway activation (Harboe and Mollnes, 2008).

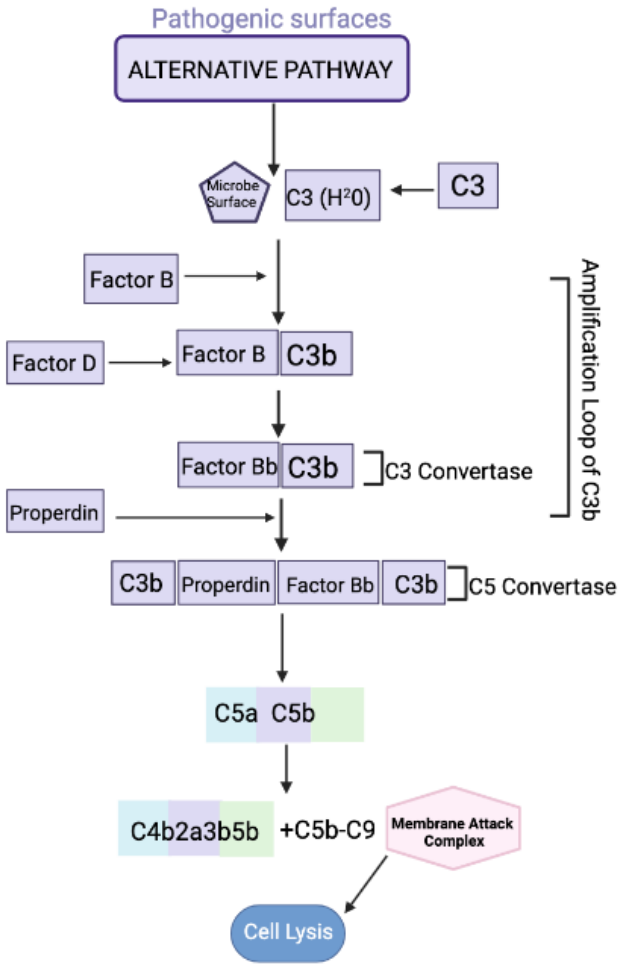


Figure 3: Adaptive complement pathway
The adaptive complement pathway is the dissimilar to the other pathways and producing a different C3 and C5 convertase. The alternative pathway joins the common pathway (represented by multiple colours) at C5 with the end goal of forming the membrane attack complex (MAC) to induce cell lysis. Adapted from Dunkelberger and Song (2010) and Mellors et al (2020).

1.3 Adaptive Immune System

The adaptive immune system (AIS) is an antigen specific response which is often initiated by the actions of the innate immune system. The antigens are recognised by lymphocytes in the form of T-cells and B-cells which are highly specific and have individual responsibilities when mounting an immune response (Delves *et al*, 2017, p53). T-cells require the binding of an antigen-presenting cell to proliferate from naive T-cells to effector T cells (capable of immune response). Effector cells produce memory T-cells which hold an immunological memory in the case of re-exposure to the antigen (O'Leary *et al.*, 2006) This process is imperative for vaccine efficacy as it allows the host to provide the fast, efficient responses upon reinfection (Marshall *et al.*, 2018). The specific cell response is controlled by the secretion patterns of cytokines (soluble cell to cell communication molecules) which in turn is stimulated by the pathogen microenvironment (Vazquez, Catalan-Dibene and Zlotnik, 2015). Cytokines have a broad range of responsibilities; from activating macrophages and the death of pathogens (IFN-γ) to aiding B-cells produce antibodies (Delves *et al*, 2017, p63). B-cells are able to secrete numerous antibodies once activated and differentiated. Antibodies play a vital role in the immune system with their ability to bind to most pathogen surfaces at high affinities (Sela-Culang, Kunik and Ofran, 2013). In attachment to the antigen, the antibody is responsible for the recruitment of other immune cells and tiggering pathways. The antibody acts as a bridge between the pathogen and classical complement pathway, neutrophils, macrophages, and antigen-dependent cellular cytotoxicity (Delves *et al.*, 2017, p58). The constant region of the antibody binds to the future immune effectors and the variable region binds to the antigen (Delves *et al.*, 2017).

Neutralising antibodies, which protect the healthy cells from pathogen entry, are the best prospect for the adaptive immune to disarm virus entry. Through the use of cell receptors, viruses enter the host cell to replicate and produce virions, which are secreted out the cell to infected neighbouring cells. By binding to the 'free' virus and blocking entry, the consequent replication would not occur (Overbaugh and Morris, 2012). The production of antibodies can be broken down into different subgroups. Polyclonal antibodies, in the form of IgG purified from infected sera, are derived from multiple B cells so can bind to different epitopes on the same antigens (Lipman *et al.*, 2005). On the other hand, monoclonal antibodies are originated from one B cell and are highly specific to only one epitope. A

process of fusing B cells and immortal myeloma cells to form hybridoma cells lines can ensure the immortal replication of the specific monoclonal antibody (Mitra and Tomar, 2021). Monoclonal antibodies are one of the most successful biotherapeutic drugs due to their affinity for a specific target and the lack of interaction with other proteins (Quinteros *et al.*, 2017). Pattern recognition receptors (PRRs) can activate transcription factors to induce both T and B cell responses. Secreted PRRs and mannose receptors cannot induce the adaptive immune themselves (Palm and Medzhitov, 2009). PRRs are one of the main stimuli which inform the adaptive immune how to respond, yet this relies on the innate immune activation.

1.3.1 Regulation of the immune system

All elements of the innate immune system require appropriate control to ensure pathogens are eliminated but there is no consequence for host cell health. This also includes the correct identification of non-self-cells and how the effector proteins are responding. Therefore, to avoid autologous tissue damage, there are proteins inhibiting specific aspects of the complement pathway.

The amplification loop within the alternative pathway offers detrimental repercussions if not adequately controlled. Factor H is a conserved plasma glycoprotein and negative regulator which inhibits the amplification loop within the alternative pathway. Factor H controls the loop via destabilisation of C3 convertase (Wu *et al*, 2010). The N-terminal complement control proteins domains 1-4, harbour the C3b-binding site. The C-terminal possesses domains which allow it to bind to C3b/C3d and sialic acids with the ability to attach to host surfaces under complement attack (Morgan *et al.*, 2011). However, there are factor H-related proteins which may enhance complement activation indirectly by inhibiting Factor H from binding but also directly (Józsi, 2017). It is believed pathogenic microorganisms have developed the ability to bind to factor H and factor H-related proteins to use them for their host protection function (Józsi, 2017).

Another inhibitor which is often acquired by the pathogen is the decay-accelerating factor (DAF) called CD55. The glycosylphosphatidylinositol (GPI)- anchored membrane protein is available on many different cell types (Miwa and Song, 2001). It operates by shortening the half-life of available C3 convertase to interrupt the complement pathway. DAF also acts within the alternative pathway by dissociation of Bb from fixed C3b, inhibiting the formation of a working C3 convertase (C3bBb) (Ejaz *et al*, 2012). During normal microenvironments CD55 is beneficial for the host though it can be incorporated into the Zika viral envelope and contribute to the lack of sensitivity to human complement (Malekshahi *et al.*, 2021). Neutralising antibodies successfully blocked CD55 and increased cell sensitivity to complement. Using neutralising Abs to block CD55 could be beneficial where complement activity is being muted due to the upregulation of CD55 in the presence of infection. CD55 has many benefits for immunotherapy and the general regulation of the complement pathways.

CD59 is a GPI-anchored cell surface receptor with the main function of inhibiting the MAC complex. This membrane-bound protein is one of the final inhibitors in the cascade and last resort in preventing damage to uninfected human cells (Couves *et al.*, 2022). CD59 has been found to indirectly inhibit MAC polymerisation through the consistent helical formation of transmembrane b-hairpins of C9. Keeping C9 is a transitory state can prevent binding of additional monomers (Couves *et al*, 2022). CD59 can also simultaneously deflect the cascading B-hairpins of C9 and engage with C8a to prevent complement-mediated cytotoxicity.

1.3.2 Complement protein 3

Complement protein 3 (all C3 components) could be considered the most important complement component within the innate and adaptive immune systems, with more than 1mg/mL circulating in the blood (Zarantonello *et al*, 2023). Independent of how the system or pathway is initiated or amplified, there is always involvement of a C3 component. The identification of a functional homolog of vertebrate complement 3 (CrC3), in an ancient fossil of horseshoe crab, illustrates how ancient the component is and possibly the reason for its widespread involvement (Zhu *et al*, 2005).

There are numerous studies which have focused on the implications of C3 deficiency. There are often detrimental effects associated which highlights the protective role C3 possesses. C3 absence can be correlated with a diverse range of issues. C3 can regulate $CD4^+$ and $CD8^+$ T-cell responses, which have been shown to be valuable to control acute influenza virus infections. C3 deficient mice were highly susceptible to primary infection and showed a delayed viral clearance (Kopf *et al*, 2002). On the other hand, C3 has been implicated to have a role in neurodegenerative diseases. Microglia use C3 to induce phagocytosis, to defend against pathogens and clear amyloid b cerebral plaques in Alzheimer's disease (Fu *et al*, 2013). Therefore, a C3 deficiency would contribute to the lack of clearance of the plaque deposits.

Depletion in C3 or C3 convertase can inhibit all action of the classical and lectin pathway. Miller *et al* (2012), demonstrated that minimal total haemolytic activity was due to C3 depletion. It has also been shown that recurrent pyogenic infections like *S.pneumoniae* and *Neisseria meningitidis* are a consequence of C3 deficiency. It can also be associated with memory B-cell response and T-cell development, though deficiencies in C3 are rare (Ghannam *et al.*, 2014)

1.4 Virus of interest – Hepatitis C

Hepatitis C Virus (HCV) is a small, blood-borne virus that primarily affects the liver and is unique in its ability to cause a dichotomous outcome (viral clearance – acute infection, viral persistence – chronic infection) (Kemming, Thimme and Neumann-Haefelin, 2020). The World Health Organisation currently estimates that 58 million people worldwide have chronic HCV infections, with 1.5 million new infections emerging every year (World Health Organization, 2022). HCV genome encodes a single polyprotein that is processed into three structural proteins, p7 and six non-structural proteins (Velázquez-Moctezuma *et al.*, 2019). These non-structural proteins include the E1/E2 glycoproteins which are often the targets of research. Extensive research has shown that neutralising antibodies (NAbs) to E1/E2 offer protection from infection, yet the high genetic diversity of HCV means this is not easily achieved (von Hahn *et al.*, 2007). The innate immune must work quickly and efficiently to respond to ~10^{12} HCV virions being produced every day, as well as stimulating the adaptive

immune response (Shin, Sung and Park, 2016). Most patients require direct-acting antiviral (DAA) treatment targeting NS3, NS5A, and NS5B proteins to reduce viraemia and clear infection.

1.5 How the immune responds to Hepatitis C virus infection

1.5.1 Acute HCV infection

Due to the initial asymptomatic nature of HCV, acute infections are hard to diagnose, and treatment normally occurs during the chronic phase of infection. Acute cases can be identified through routine testing of high-risk individuals, a known encounter of HCV transmission and some cases of symptoms.

Hepatocytes (parenchymal liver cells) are the primary target for HCV infection but, only 1-54% of cells are affected, with half the liver undergoing normal function conditions. The clusters of affected hepatocytes were seen to correlate with viral load but there were no associations with HCV genotype (Wieland *et al.*, 2014). HCV can establish infections through cell surface receptors (tetraspanin CD81, SR-B1, OCLN and CLDN1), as well as through cell-to-cell transmission (Park and Rehermann, 2014). Upon entry to the cell, the RNA genome is processed into the HCV structural and non-structural proteins, packaged into viral particles and released (via exocytosis) from hepatocytes into the blood. During the replication phase, Retinoic-acid-inducible gene 1 (RIG-1) like receptors (RLRs) are quickly activated by the ribonucleotides in the 3'UTR region of the HCV genome (Horner and Gale, 2013). Virus entry is also recognised by RNA-dependent protein kinases R (PKR). The processes together recognise the HCV genome, followed closely by endosomal compartment toll-like receptor 3 (Wang *et al*, 2009). RIG-1 and PKR receptors oligomerise, forming filaments with mitochondrial antiviral signalling protein (MAVS), whereas TLR3 uses the adapter molecule Toll/IL-1 receptor domain (Horner and Gale, 2013). This in turn activates the signalling cascades of INF production and proinflammatory cytokine release (Park and Rehermann, 2014). Type 1 and Type 3 IFNs stimulate interferon stimulated genes (ISGs) which supress HCV replication (Shin, Sung and Park, 2016). However, the viral protease NS3/4A can cleave and inactivate MAVS, blocking interferon-stimulated gene induction and IFN production. HCV utilises this mechanism to evade the immune and establish chronic infection (Cheng,

Zhong and Chisari, 2006). These effectors are still produced, with 50% of HCV patients displaying ISG expression in hepatocytes (Wieland *et al*, 2014). It is assumed ISGs and IFNs could clear HCV infection which suggests they are not working to full efficiency due to virus interference.

Alongside the cell receptors and interferons, the viral load is influenced by the potency of innate cells like natural killer (NK) cells. NK cells are effectors cells of the innate immune. HCV replication can be controlled by IFN-y secretion from NK cells, as confirmed by Kokordelis *et al* (2014), showing a positive correlation within HCV infected samples. NK cells possess the ability to perform different roles depending on the infection. NK cells within acute (self-limiting) infection were efficient in inhibiting HCV replication *in vitro* due to the activated phenotype (Kokordelis *et al*, 2014). During acute infection, CD94/NK group 2 member A (NKG2A), NK inhibitory receptor expression is decreased and NKG2D expression is increased (Pelletier *et al.*, 2010; Shin, Sung and Park, 2016). This receptor environment increases the effector functions and NK cell degranulation independent of how the infection progresses (acute clearance or chronic infection) (Pelletier *et al.*, 2010; Shin, Sung and Park, 2016). NK cells release lytic granule contents during degranulation; designed to be cytotoxic to the target cell they adhere to. Such release of perforin and granzymes are the trademark of NK cell activation (Murugin *et al.*, 2011). This response can be muted by HCV E2 glycoprotein binding to the NK cell CD81 receptor (Tseng and Klimpel, 2002). Consequently, less cytotoxic granules would be released, reducing the threat of lysis to the HCV infected cells or virus particle. This is an efficient immune evasion method for the virus.

NK cell degranulation can also be correlated with HCV-specific T cell response (Pelletier *et al*, 2010). HCV-specific T cells have been identified in most patients around 6-8 weeks post infection. This response is severely delayed despite the signalling cascades and innate immune activation that occurs soon after initial infection. Irrespective of this, the production of HCV-specific T cells reduces viral titres (Shin, Sung and Park, 2016). $CD8^+$ T cells are heavily involved in the spontaneous clearance of HCV infection during the acute phase. Such strong responses of HCV-specific $CD8^+$ T cells are not seen within chronic infections. Despite the same initial activation, there is a depletion of HCV-specific $CD8^+$ T cells seen in chronic infection (Kemming, Thimme and Neumann-Haefelin, 2020).

Furthermore, neutralising antibodies (Nabs) are associated with the clearance of acute infections as well as a reduction of chronic phase transition. NAbs are at low-titres in chronic infections compared to the high-titre seen during clearance of acute infections (Dowd *et al*, 2009). HCV-specific antibodies can neutralise virus entry into hepatocytes by interacting with the E1E2 glycoproteins yet the high mutation rate of HCV challenges this. The virus uses amino acid substitutions in the hypervariable region 1 of E2 to evade the neutralising antibodies produced by the host (Dowd *et al.*, 2009). Therefore, spontaneous clearance of acute infection is reliant on the broadness of HCV-specific $CD8^+$ T cell response and neutralising antibody production. HCV-specific $CD8^+$ T cells are reliant on $CD4^+$ T cell action, with a weakened response seen in their absence (Thimme *et al*, 2001).

1.5.1.1 Spontaneous Resolution of HCV infection

Spontaneous clearance of HCV infection is possible during the acute phase, despite only occurring in 20-30% of individuals (Kemming, Thimme and Neumann-Haefelin, 2020). The adaptive immune system has a considerable role in this resolution of infection. HCV clearance has been associated with a rapid development of a broad neutralising antibody response, providing support for the benefits of a vaccine (Osburn et al, 2015). Similar to other virus infections, HCV clearance is also dependent on multi-specific T cell responses (Kemming, Thimme and Neumann-Haefelin, 2020). Upon reinfection, liver resident $CD8^+$ T cells contributed to rapid acquisition of virus-specific cytolytic activity (Shoukry *et al*, 2003). Depletion of $CD8^+$ T cells before reinfection, prolonged virus replication, and clearance of infection was not achieved until of $CD8^+$ T cells were recovered (Shoukry *et al*, 2003). This response to infection was independent of $CD4^+$ T cells, highlighting the significance of $CD8^+$ T cells during acute infection. Our knowledge regarding acute phase infection and reinfection has been advanced due to these studies on chimpanzees. They have been extensively used, as the only animal able to be HCV infected. Previous research highlighted that HCV-specific antibody titres might decrease 10-20 years after clearance of the virus (Rehermann and Nascimbeni, 2005). Shoukry *et al* (2003) identified high frequencies of memory T cells targeting HCV proteins in chimpanzees and cells were present for seven years after infection. Another study showed there were differences in the maintenance of HCV-specific antibodies between disease severity. The results showed 69.2% of patients had anti-HCV antibodies 9-10 years post infection. Only 15.4% were from cleared infection and

82.7% were from chronically infected patients (Dittmann *et al*, 1991). It would be understandable that a sustained response, for a longer period of time, as seen in chronic infections, may generate a larger quantity of antibodies. Therefore, the higher quantity would still be detectable 9-10 years later. This time frame could suggest a vaccine course may be applicable, especially for people in high-risk environments (drug users or clinical patient facing roles).

1.5.2 Chronic HCV infection

Patients who cannot clear the virus within the first six months progress into the chronic phase of infection. Prolonged infection brings greater risks of developing liver inflammation, fibrosis or cirrhosis with an estimated 33% of patients developing fibrosis within three years (Ryder, 2004). Liver cirrhosis (scarring of the liver tissue) is associated with HCV infection and can cause decompensated liver disease. Another consequence of HCV infection is the significant risk for developing hepatocellular carcinoma (HCC). HCC risk is reduced with the successful clearance of HCV through DAA treatment, nevertheless chronic infection risk is still 1.8%-2.5% annually (Luna-Cuadros *et al*, 2022). DAA treatment does not provide protection against HCV reinfection therefore, the risk of hepatic injury increases. A prophylactic or therapeutic HCV vaccine would be required to prevent the damage and following consequences of infection.

The levels of ISGs in acute and chronic infections are similar, however there are distinct differences in the level of $CD8^+$ T cells. The quantity of $CD8^+$ T cells responding is lower in chronic infections and has been shown to deplete rapidly. (Shin et al, 2016). Furthermore, the rapid response of broadly NAbs is associated with clearance of the virus. It could be assumed this is also diminished allowing for viral persistence into the chronic phase (Osburn *et al.*, 2014).

NK cells contribute to the innate immune system and have a strong role in viral clearance. They are found to have a polarised phenotype during chronic HCV infection (Ahlenstiel *et al*, 2010). Chronically infected HCV patients exhibit NK cells which express tumour necrosis factor-relate apoptosis-inducing ligand (TRIAL) and cell cytotoxicity receptors (NKp22 and NKp46) (Ahlenstiel *et al*, 2010). Alongside this, the subunit of the IL-2 receptor CD122, had higher expression in chronic patients than in healthy controls. The subunit is key for NK cell

activation, therefore higher expression would increase the NK cell population in the liver. In turn this would cause widespread degranulation and more damage to the hepatocytes. Overall, this highlights that HCV chronic infections cause significant and pronounced damage to the liver and one pathway may be through NK cell cytotoxicity (Ahlenstiel *et al.*, 2010).

Complement components have also been shown to contribute to the clearance of HCV infection. The literature is limited but there are reports that the complement activity of the classical pathway is depleted in chronically infected HCV patients (Kolopp-sarda and Miossec, 2020). The reliability of the study is improved by the large cohort of patients. Therefore, chronically infected HCV patients (a large proportion due to the asymptomatic nature of the virus), could develop problems associated with the depletion of complement. Other complement pathways may compensate for the loss of the classical pathway however, in terms of effect, the innate activation would be less in this instance.

1.5.3 Complements response to HCV infection

Complement components are assumed to be involved in the immune response regarding HCV infection. Complement proteins work alongside other innate immune cells to identify and respond to virus infection. A strong overlap between the innate and adaptive immune system leads to the expectation that they work together to eradicate infection. Research has investigated the effect of supplemented complement on neutralisation (measured as 50% reduction of pseudotype virus plaque formation in this study) regarding immunoglobulin G1 monoclonal antibodies (mAbs) with HCV infection (Meyer *et al*, 2002). The results indicate that complement assisted neutralisation of the virus via the classical pathway, as C4 deficient sera led to a loss in neutralisation (Meyer *et al*, 2002). Deficiencies in Factor-B, a protein implicated in the alternative pathway, did not change the neutralisation, once again indicating the reliance on the classical pathway. Not only did the neutralisation titres of the mAbs increase, some HCV-infected sera showed improved neutralisation ability in the presence of supplemented complement (Meyer *et al*, 2002). Additionally, complement has been shown to engage with human monoclonal antibodies to exhibit complement-dependant phagocytosis of immune complexes formed in HCV-infected sera, with a variety of genotypes (Eren *et al*, 2006). Phagocytosis by neutrophils only

occurred with the presence of HCV virus and HCV specific monoclonal antibodies, as no activity was seen in heat inactivated serum or non-relevant antibody (Eren *et al*, 2006). Further reports suggest phagocytosis by neutrophils can be associated with complement receptor 1 and/or iC3b-mediation (Boero *et al*, 2023). Literature also implies IgG or fibronectin may be associated with complement mediated phagocytosis, once again highlighting the interconnection between the two immune systems (Boero *et al*, 2023).

The role of complement activating and benefiting the production of the antibody response has been thoroughly investigated, however antibodies can also influence complement activation in the classical pathway. Complement mediated cytotoxicity involves antibodies from the adaptive immune activating the complement cascade to lyse target pathogenic cells. Optimal positioning of the antibody crystallisable fragment (Fc) and C1 complement complex (C1q) deploy a complement pathway (Wang *et al*, 2020). Eren *et al* (2006), showed that human monoclonal antibodies could induce complement mediated phagocytosis but failed to induce cytotoxicity. With regards to therapeutics, it is beneficial for the human monoclonal antibodies to neutralise the virus without cytotoxic effects for the hepatocytes (Eren *et al,* 2006)

Despite complement's contribution to the immune response against pathogens, viruses can exploit the complement system and use the complement proteins to their advantage. HCV has evolved to use different complement functions to evade the immune. The most consequential hijacking is HCV effect on the MAC complex. MAC complex forms on pathogenic cells, disrupting the phospholipid bilayer leading to cell lysis (Tegla *et al.*, 2011). This functioning of the MAC complex is imperative for effective clearance of pathogens from the host. Genotype 2a HCV infection can inhibit the MAC complex through downregulating complement component C9 at transcriptional levels (Kim *et al*, 2013). More specifically, the HCV core demonstrated a 95% reduction of C9 mRNA expression, with HCV proteins, NS2 and NS5A, showing somewhat of an effect too (Kim *et al*, 2013). Muting the expression of C9 means there is less risk of death for the virus. The HCV core protein was also implicated in reducing expression of C9 in chronically infected liver biopsy specimens as well as sera (Kim *et al*, 2013). These results are crucial in showing this is a true physiological effect, not limited to laboratory cell lines (Huh7.5 cells). Cytokine release of γ IFN can improve C9

promoter activity. However, this effect was also significantly reduced by HCV core proteins (Kim *et al*, 2013). Hence, efficient activation of the classical, lectin and alternative pathway can be halted by the HCV core proteins effect on C9. This significantly reduces the effectiveness of the complement pathways as there is no substitute for C9, resulting in no formation of the MAC complex and less removal of HCV infection.

Aims and objectives of this study

The literature shows there is an inter-dependent interaction between the innate and adaptive immune. We understand that complement activity can benefit the action of antibodies and vice versa. However, we do not have a comprehensive understanding of the contribution of complement responses to limiting HCV infection. There has been a strong focus in previous research on the adaptive immune response, and while some immune modulating therapeutic interventions have been described, the complement system is under-studied. The current clinically utilised therapeutic DAAs do not prevent damage caused by HCV nor offer protection from reinfection. Therefore, it is necessary to develop a more thorough knowledge of the effect of complement to see if this could contribute to better patient outcomes. Limited research exists suggesting that activation of the classical pathway and subsequent complement effects are depleted in chronic HCV infection. Thus, the primary objective is to develop greater understanding of how the neutralizing antibody and complement proteins coordinate to prevent Hepatitis C virus entry.

Aims:

1. Develop methods for the assessment of complement activity in sera isolated from HCV-infected individuals.
2. Assess the effect of storage conditions of clinical specimens on the activity of complement.
3. Develop a greater understanding of the complement activity in HCV-infected patients.
4. Determine if the presence of active complement proteins influences the activity of neutralizing antibodies targeted to the HCV structural proteins.

2 Materials and methods

Unless otherwise stated, all routine biological chemicals were obtained from Merck, Poole UK or Thermo Fisher Scientific, Loughborough UK.

2.1 HCV sera samples

A panel of 14 HCV-infected sera were originally selected. These represented chronically infected individuals who were PCR positive with a high viral load and had undergone genotype testing. Six samples were selected from a 2010 cohort (Tarr *et al.*, 2011) and eight from 2021 cohort of HCV testing at Queens Medical Centre. Four HCV-infected sera were also tested from the BOSON clinical trial (Foster *et al*, 2015), to identify the complement present during HCV treatment. The final panel included 11 HCV-infected sera from patients within the acute phase of infection. Throughout this research there were five non-HCV-infected individuals which represented the healthy controls. Clinical samples were provided with ethical approval (MREC/98/3/55) by the Trent Clinical HCV Cohort.

2.2 Cell lines

Huh7 cells are an immortal hepatoma cell line which are permissive to infection assays (JCRB0403,Nakabayashi *et al.*, 1984). HEK293T (contain SV40 Large T-antigen) are human embryonic kidney-derived neuronal cells used for pseudoparticle production (ATCC:CRL-3216, DuBridge *et al.*, 1987). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with nonessential amino acids, L-glutamine and fetal bovine serum (FBS). Cells were maintained in an incubator at 37°C and supplied with 5% CO_2

2.3 Cell Passage

Both cell lines were passaged with either 72 h (Monday-Thursday) or 96 h (Thursday-Monday) growth time. All cell work was carried out in a class 2 microbiological safety cabinet. At 80% confluence, old media was aspirated and cells were washed with PBS. Both cell lines were trypsinized and recovered using DMEM. The cells were spun at 300G for 7 minutes and resuspended in 10ml DMEM. Once counted on a haemocytometer, cells could

be seeded at the desired density. Huh7 cells were typically seeded at 1.1X10^6 and HEK293T at 0.9 X 10^6 per 75cm^2 flask.

2.4 HCVpp Transfection and Neutralisation Assay

Hepatitis C Virus pseudoparticles (HCVpp) were developed as previously described by Tarr et al (2007). These include E1E2 glycoproteins (UKN1A20.8) from Genotype 1a, as this is the reference genotype used in HCV research. Pseudoparticles with solely the HIV-1-based packing plasmids (pNL4.3) were also produced as a negative control. Huh7 cells were used as targets in infection assays to explore the neutralisation potential of the serum or antibodies against the pseudoparticles. As seen in Figure 4, HEK293T cells were seeded into 100-mn Corning® Primaria™ cell culture dishes at 1.5 X 10^6 cells in DMEM and incubated overnight. Medium was changed to opti-MEM and cells transfected with PNL gag-pol, luciferase and E1E2 glycoprotein plasmids. Plasmids were previously incubated with polyethylenimine (PEI) for 1 h. PEI condenses DNA into positively charged particles that bind to anionic cell surfaces, allowing more efficient transfection of plasmids. This also increases the chances of all plasmids entering the cell evenly. After 6 h medium was replaced with fresh DMEM for optimum growth conditions. Twenty-four hours before HCVpp were ready, Huh7 cells were seeded into a white bottom 96 well plate at 1X10^5 per well and incubated overnight. After 72 h medium containing HCvpp was filtered through 0.45um syringe filter and stored at +4°C for a maximum of two weeks. Media was aspirated from 96-well plate and HCVpp preparations were added in triplicates and incubated at 37°C and supplied with 5% CO_2. HCVpp was aspirated after 4 h and DMEM was replaced. After 72 h the cells were lysed (1:4 dilution of Promega Luciferase Cell Culture Lysis 5X reagent and distilled water) and placed on a rocker for 10 minutes, followed by 15 seconds on an orbital shaker. Luciferase activity was measured using a BMG Labtech FluoStar Optima at 3600 gain.

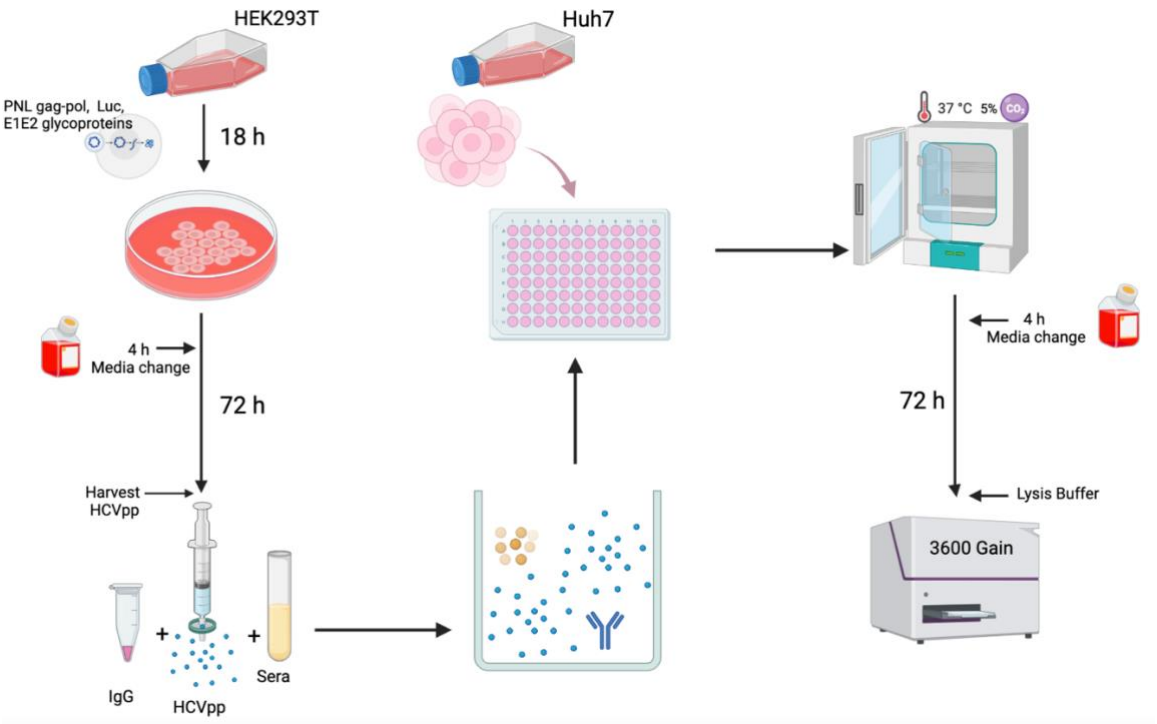


Figure 4: Protocol for HCVpp transfection and neutralisation infection assay.
Using HEK293T cells for transfection and Huh7 cells for infection. Neutralisation potential measured using luciferase. Generated with reference to Chumbe et al (2022).

2.4.1 Sera and HCV-infected patient IgG

Neutralisation assays were performed as previously described using similar conditions as HCVpp assay. The effect of serum components was analysed by preparing a serum dilution in a V bottom well plate. The dilution factor was a ratio of serum to pseudoparticles. 1 µL of serum to 100 µL of pp (1:100), continuing down 1:50, 1:25, 1:12.5. Serum and HCVpp preparations were incubated together for 30 minutes before being added to Huh7 cells in triplicates. Luciferase activity was assessed after 72 h.

2.4.2 Initial pre-optimisation assay

This assay was first carried out with a two-fold dilution of serum from 1:4 to 1:128, to align with the CH50 assay. However, upon optimisation the above dilutions (1:100-1:12.5) were our final method. All other aspects of the protocol were kept the same.

2.4.3 Producing IC50 curves for IgG preparations

For this experiment the cells were seeded and incubated, as per the neutralisation assays above. In a V-shaped bottom plate, purified patient IgG was spiked into heat inactivated serum. This was a 5-fold dilution series starting at 50µg/ml. Healthy patient IgG preparations were used as a negative control. HCVpp preparations were added to each well, incubated for 30 minutes and added into triplicates to the Huh7 cells. Luciferase activity was assessed after 72 h.

2.5 CellTitre-Glo® 2.0 Cell Viability Assay

Complement mediated cell lysis was assessed using the CellTitre-Glo® 2.0 Cell Viability Assay (Promega,#G9681). This homogenous method can determine the number of viable cells in culture by quantifying the ATP produced. Cells were seeded into an opaque walled 96-well plate at 10,000 per well (Connelly et al, 2012) and incubated overnight. Fresh and frozen serum were added into different conditions. Fresh medium was replaced after 4 h and cells were incubated for 72 h. Cells were lysed in a 1:1 mixture of medium and CellTitre-Glo® reagent and contents mixed on an orbital shaker for 2 minutes. Plate was incubated at room temperature for 10 minutes to stabilise the luminescence signal. Luminescence was measured using a BMG Labtech Fluorostar Optima. Upon optimisation of the assay, the CellTitre-Glo® reagent can be used at a 1:4 dilution to avoid saturation when reading.

2.6 CH50 Assay

A haemolytic complement assay was used to quantify the activation of the classical and alternative complement pathways (CH50 Test, HaemoScan, #K002)

2.6.1 Erythrocyte suspension

The erythrocyte suspension was prepared by diluting 0.14ml of erythrocyte concentrate into 0.71ml of dilution buffer into a 1.7ml Eppendorf. The solution was centrifuge for 5 minutes and the supernatant was aspirated. The suspension was diluted and centrifuge until the supernatant was colourless. This was ensuring all previously lysed cells had been removed. The erythrocyte pellet was resuspended in 5mL of dilution buffer. In a flat bottom plate, 25µL of the erythrocyte suspension was mixed with 75µL of demineralized water, in

triplicates. The plate was read on the BMG Labtech Microreader at OD415, with an expected value between 0.700 and 0.900. A value between this figure suggested the erythrocyte suspension contained the appropriate number of cells to proceed. We ensured the above erythrocyte suspension is sufficient for 96 wells (Optimised plate layout to use all wells seen in Table **2-1**).

2.6.2 Serum and plate protocol for CH50 assay

A series of two-fold serial dilutions were prepared starting from 1:4 through to 1:128 using PBS. This included all test serum, control serum and two reference plasmas. Reference plasmas were reconstituted with 250µL of distilled water. 50ml of lysis buffer acted as a positive control and 50µL of dilution buffer was used for the negative. Upon completed serial dilutions, 100µL of erythrocyte suspension was added to every well. The plate was sealed with foil topper and incubated for 30 minutes at 37 degrees. After the incubation, 100µL of stop solution was added to all wells, plate was sealed and spun in plate centrifuged at 400G for five minutes. 120µL was transferred into PCR plate and the samples were heat-inactivated at 60 degrees for 10 minutes. This deactivates the live virus in the samples. Upon completion, 100µL of each sample was transferred into a flat bottom plate and read at OD415 on the BMG Labtech Microreader.

Table 2-1 : **Suggested plate layout for CH50 assay to maximise use of plate.**
Allows the testing of six serum samples in duplicates alongside all controls necessary.

1:4	1:8	1:16	1:32	1:64	1:128	1:4	1:8	1:16	1:32	1:64	1:128
Sample 1						Sample 5					
Sample 2						Sample 6					
Sample 3						Control	Serum				
						Ref	Low				
Sample 4						Ref	Normal				
						N	N	N	P	P	P

2.7 Expression analysis

2.7.1 RNA Purification

Cells were passaged as described above. Once resuspended and counted, 10 million cells (as per protocol) were aliquoted and centrifuged at 300G for 5 mins. All media was removed and the pelleted cells were frozen at -25 until use. A Qiagen RNeasy Mini Kit (#74104) was used to carry out purification of total RNA from animal cells using spin technology protocol. The protocol was followed except for the use of a Hybaid Ribolyser to homogenise the lysate. Total RNA was quantified using a NanoDrop spectrophotometer.

2.7.2 cDNA synthesis

Takara EcoDry Premix with random hexamers allows efficient first-strand cDNA. Each tube contains lyophilized master mix which was resuspended with 18µL of RNase-free water. 5µg/ml of mRNA is necessary for the reaction so 2.2µL of HEK293T mRNA was added to the first reaction and 1.8µL of Huh7 mRNA to the second. The solution was pipetted well to mix. The samples were incubated at 42°C for 60 minutes. The reaction was stopped by heating to 70°C for 10 minutes. (Ausubel et al, 1995).

2.7.3 Primer design

Primers were designed to amplify the CD46, CD55 and CD59 mRNA sequences however, only CD55 and CD59 was taken forward in this experiment. To ensure the primers were functional we also used Wang et al (2017), primers which had shown to successfully amplify CD55 and CD59 in breast cancer cells. GAPDH was the housekeeping gene used for comparison of expression. Primers were designed over exon boundaries using NCBI Primer Blast. To account for all variants, we finalised the following primers as follows: CD55 forward, 5’ TTTCAGGCAGCTCTGTCCAG 3’ and reverse 5’ AACTGTTGGTGGGACCTTGG 3’ ; CD59 forward, 5’ CGAGGCTGGAAGAGGATCTTG 3’ and the reverse 5’ TTGCAGTCAGCAGTTGGGTT 3’. This work was carried out by fellow colleagues (Referred to hereon as MW and GS) who designed the following primers: CD55 forward, 5’TTCCAGTCGGTACTGTTGTG 3’ and CD55 reverse, 5’ TTTCGTATTTCTCCCGGAT; CD59 forward, 5’ AGCGATTCTCGTGTCTCAGCCTC 3’ and reverse, 5’CCCATTGTGATTGTCCACAGAACTAC 3’. All standard DNA oligo primers were obtained from

ThermoFisher Scientific in a dry powder formulation. Upon arrival the primers were resuspended with DEPC-treated water to 10µM and stored at -25°C.

2.7.4 q-PCR

qPCR of both HEK293T and Huh7 cDNAs was performed using X2 BIO Sygreen Master Mix (PCR Biosystems). This included DNA polymerase, magnesium ions, free nucleotides and SYBR green dye. Initial experiments used a 10-fold serial dilution of the cDNA from 1:10 to 1:1000 with DEPC-treated water as the negative control. The samples were amplified for 40 cycles with one cycle consisting of 95˚C for 2 min, 60˚C for 30 sec. Amplified samples were placed in 4°C fridge overnight before being loaded onto gel electrophoresis, to confirm product length. This experiment was repeated using all CD55 and CD59 primers alongside existing laboratory GAPDH primers.

2.7.5 Gel electrophoresis

A 2% agarose gel was prepared with a 1X TAE buffer. 5µL of amplified qPCR product was combined with 1µL of FastDigest Green buffer 10X loading dye (ThermoFisher Scientific). 4µL of the DNA ladder (Thermo Scientific GeneRuler DNA Ladder Mix) is loaded into the first well and 5µL (1µL spare) of each PCR mixture was added into the adjacent wells. The gel was run at 90 volts for 36 minutes. Photographed on the G:Box F3.

2.8 Immunostaining of complement inhibitory proteins

2.8.1 Coverslip preparation

Cover slips were treated with 1M hydrochloric acid and washed with DMEM (pH indicator) until the acid neutralised. HEK293T cells were seeded onto the coverslip at 0.5 X 10^6 and Huh7 were seeded 0.45 X 10^6 into a 6-well Corning cell culture plate. The coverslips were incubated at 37 ^oC, 5% CO_2 for 24 h. The cells were fixed with 4% formaldehyde formalin and placed individually into each well of a 6-well plate and stored in a 4°C fridge.

2.8.2 Immunostaining protocol

The coverslips were acclimatised to room temperature before being immersed in 5% bovine serum albumin in PBS, to block nonspecific immunoglobulin binding sites. After 1 h in a moist chamber, the coverslips were washed in PBS bath, ensuring the cell side remained facing up. The coverslips were incubated with the anti-CD55 primary antibody at a 1:50 dilution in 5% BSA (79T/36) or 1:100 dilution of Anti-CD59 monoclonal antibody (Rabbit IgG Mab, Stratech). The controls for both cell lines were incubated with 5% BSA. Following 1 h in the moist chamber, the primary antibody was aspirated, coverslips were rinsed in PBS and allowed to air dry. 0.4ml of FITC goat anti-mouse IgG (whole molecule) AlexaFluor 488 was added to 200 mL of 5% BSA for a 1:500 dilution. 50mL was added to each coverslip and incubated for 1 h, before being rinsed in PBS and left to air dry. 2 µL of DAPI stock solution was diluted into 50 µL of PBS then added to the coverslips for a maximum of 2 minutes. After a final rinse in PBS the coverslips were fixed with mounting media onto a slide.

2.8.3 Microscopic evaluation

Both cell lines were examined with a Zeiss inverted widefield florescence microscope using a X10, X40 and X60 objectives. Settings were maintained across all samples so a direct comparison could be generated. AlexaFluor has 488nm excitation and emission 525nm. DAPI was 350nm excitation and 440nm emission.

2.9 IgG and serum binding assay

2.9.1 IgG dilution preparations

All IgG preparations were carried out in a V-bottom 96-well gamma radiated plate. An eight point 3-fold dilution starting at 100 $\mu g.mL^{-1}$. Our final method used 3% BSA to dilute all IgG preparations.

2.9.2 IgG Binding ELISA

NUNC Maxisorp plates were coated with 5 $\mu g.mL^{-1}$ GNA (*Galanthus nivalis* agglutinin) in coating buffer (Carbonate-Bicarbonate) at a 1:1000 dilution and incubated at 4 overnight. Plates were blocked with 3% Bovine Serum Albumin (BSA) for 2 h. Plates were washed with TBS-Tween, lysates containing HCV glycoproteins E1/E2 were diluted 1:5 with 3% BSA and

incubated for 90 minutes. Cell lysate from an untransfected HEK293T cell was used as a negative control (mock). After three washes, the patients IgG was added in duplicates to both E1/E2 and the mock, before incubating for 1 h. After washing, anti-goat IgG secondary antibody at a 1:2000 dilution with BSA was added and incubated for 1 h. Binding was detected at 450nm after the addition of PNPP substrate (Thermo Fisher Scientific). EC50 values for each patient samples were calculated using Prism 9.4.1.

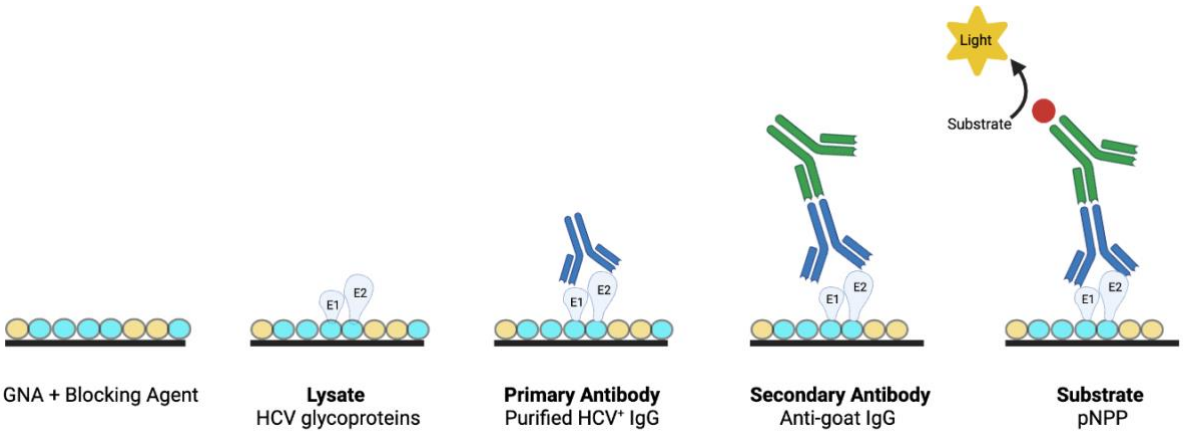


Figure 5: IgG binding ELISA
Schematic to demonstrate the ELISA binding assay. Plates were coated with Galanthus nivalis agglutinin (GNA:Blue) and blocked using 3% BSA (Yellow). Between each stage, the ELISA plate was washed three times with TBS Tween (0.1%). Lysate containing E1E2 glycoproteins and lysate from cells only (mock control) was added. Purified IgG from HCV infected patients was incubated on the plate before using an anti-goat IgG secondary antibody. Binding detected using PNPP substrate.

2.9.3 Blocking Optimisation

Originally used 5% milk powder was sued to block and dilute antibodies, however upon optimisation of the blocking reagent, 3% BSA was taken forward as a blocking agent of choice. This experiment was carried out as stated above using a healthy control IgG sample at 50µg.mL^{-1}. 5% milk powder, 3% whey, The blocking solution and 3% BSA were all tested with the same conditions.

2.9.4 Serum and IgG binding ELISA

For this experiment, the plate was coated, blocked and lysate added as stated above. Serum and IgG preparations were carried out in a V-Shaped-Bottom plate. The serum was added in a two-fold dilution series starting at 1:4 through to 1:128. The IgG EC50 value was supplemented into each dilution point. The serum and IgG were added to the ELISA plate simultaneously therefore, the mixtures were made up half volume, double concentration.

Once completed these were added in duplicates to the E1/E2 and mock wells. The following secondary antibody, PNPP lysate and detection were used as stated above.

2.10 Statistical analysis

Statistical analysis of results was carried out in Prism 9.4.1. To analyse the CH50 assay data we fit non-linear regression curve (sigmoidal dose-response – variable slope). From this we could calculate the $LogEC_{50}$ which represented the CH50 value. To compare between the conditions, we used an unpaired T-Test and for samples with a large standard deviation we used a Welch's T Test. Results were considered statistically significant if the values were below the threshold 0.05 ($p<0.05$).

3 Results

3.1 The action of complement within chronically infected HCV patients

3.1.1 Introduction to the CH50 test

To analyse the interaction between the complement and antibodies, it was essential to first determine the quantity of complement activity present in the samples. A complement haemolytic activity screening assay was performed to assess the complement components of the classical pathway. Serum was incubated with sensitized sheep erythrocytes and haemolysis occurred upon activation of the complement system. This can be quantified as the free haemoglobin is directly proportional to complement activation. It was then possible to calculate the dilution factor of serum to obtain 50% lysis of erythrocytes (CH50). This assay is sensitive to the reduction or absence of any complement protein so will give a valid indication of the complement activity in the HCV-infected serum.

3.1.2 Chronic HCV samples

A total of eight patients formed the first cohort (2010) of samples which were chosen from a previous study which had performed antibody profiling (Tarr *et al.*, 2011). The antibody data allowed us to have a baseline knowledge of the function of the serum which would be used in conjunction with the CH50 assay results. Samples were selected that were HCV positive as determined by RT-PCR and exhibited a high HCV viral load at the time of sampling. It was important to ensure all patients within this cohort were chronically infected (infection greater than 6 months) and represented a range of genotypes (1,2,3,4; including different subtypes).

3.1.3 CH50 assay of chronically infected HCV patients – 2010 Cohort

First, samples from the 2010 cohort were analysed and two distinctive groups could be identified (**Figure 6**). Five out of eight patients had an undetermined CH50 value, due to a negligible amount of complement activity. However, three patients maintained functioning complement activity. Out of these samples, C21.6 (CH50 of 4.845) and C21.1 (CH50 of 8.63),

both situated lower than a healthy patient (H22.3 = CH50 of 14.72). However, C21.3 exhibited a high quantity of complement activity with a CH50 value of 42.25 which was considerably higher than the normal reference or healthy patient sample (represented by H22.1, H22.2 and H22.3). There was a statistically significant difference (P=0.02, T-test) between the two groups of those who maintained complement activity and those with complement deficiencies upon HCV infection. This data strongly suggests that in these samples, chronic HCV infection causes a dysfunction in complement proteins contributing to lack of completion function. To confirm that the statistical tests provided an accurate indication of the comparison between CH50 values calculated for each sample, the CH50 values were compared at a single fixed dose (1/8 dilution), compared using t-tests. Comparison of the mean effect of each sample was found to be correlated with the CH50 value **Appendix 1**

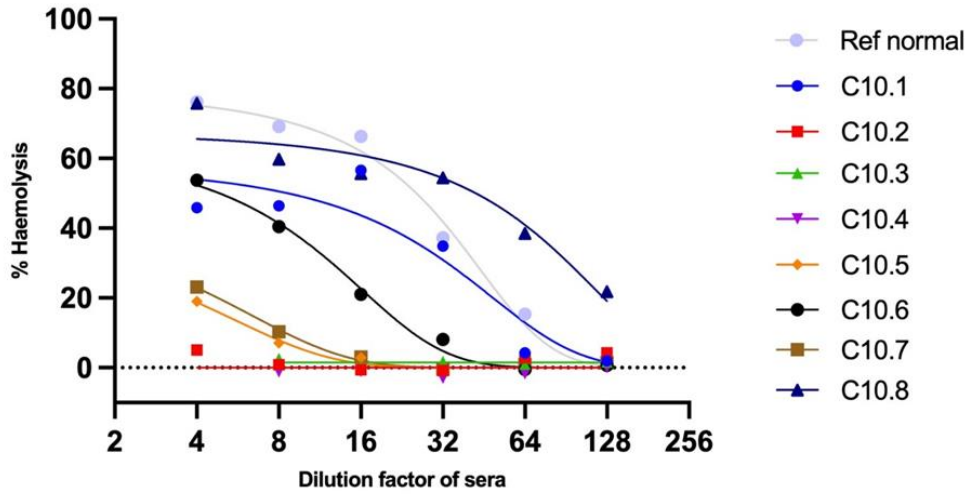


Figure 6: Depletion in complement levels seen in some chronically infected HCV patients.
CH50 assay of eight chronically infected HCV patient samples from 2021. Identifying the same depletion in some chronically infected HCV patients as seen in 2010 cohort. Samples which had depleted levels and exhibiting an absence in a complement protein; C10.2, C10.3, C10.4, C10.5, C10.7. CH50 values for these patients could not be determined. Three samples-maintained complement function C10.1 (8. 63), C10.6 (4.84) and C10.8 (42.25). Data was analysed and CH50 values (in brackets) were obtained via a non-linear regression fit. The CH50 value calculated is the dilution factor of sera needed to obtain 50% lysis of sheep red blood cells. The normal reference was within range and produced a CH50 value of 25.58. There was a significant difference between these two groups of samples (P=0.02).

3.2 Method Optimisation to interrogate complement activity

3.2.1 Storage conditions of serum samples

The identification of low and normal CH50 levels in 2010 patient sera led us to question whether some sample deteriorated during storage. The samples had been stored in a -70°C freezer for over twelve years, thus it was important to identify whether storage affected the composition and activity of the complement in these samples. Complement components are considered more structurally unstable and prone to degradation than antibodies and therefore requiring freezing for maintenance in serum. However, there is limited research as to the effects of long-term storage on complement activity. The 2010 cohort samples have also been used within multiple assays which requires thawing. Most reagents have an advisory to thaw-freeze the products as little as possible, but once again the publications regarding complement proteins within sera is minimal. Therefore, several experiments were carried out to ensure to samples were still suitable.

3.2.1.1 The effect of storage conditions on complement composition within serum samples.

CH50 assays were used to identify the differences in complement activity with varying storage conditions. We had previously confirmed that fresh non-HCV infected serum had complement activity within normal range. Therefore, any depletion in complement would be easily recognised. There was a significant difference (P=0.0168, T-test) in percentage haemolysis between the serum kept at +4°C compared to -70°C, for 91 days (**Figure 7**). This showed that storage at +4°C degraded the complement proteins to the point at which they were no longer functioning to haemolyse the red blood cells. The data highlights that once frozen at -70°C, the complement within sera was stable as no depletion was seen for up to three months. Alongside this, we assessed whether the presence of HCV-infected purified IgG changed the lysis potential of the complement. This was to ensure there was no confounding effects in future experiments where serum and antibodies were utilized. Additional IgG was found to have no effect on complement mediated cell lysis.

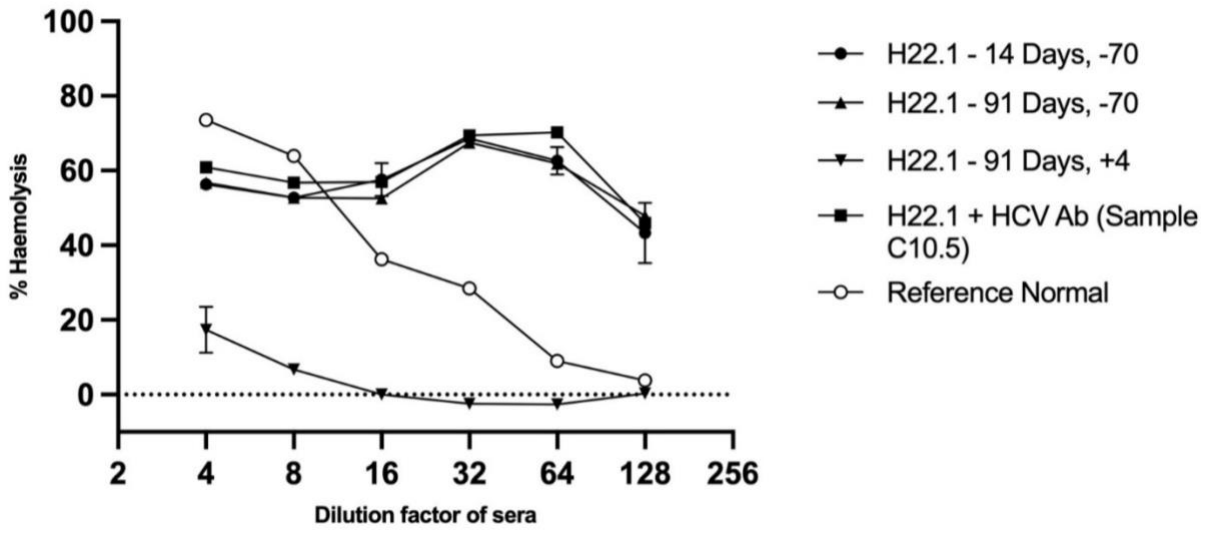


Figure 7: The storage temperature of sera affects the complement molecule composition and the ability to lyse cells.

A CH50 assay was performed on healthy, non-HCV-infected sera that had been aliquoted and stored at difference temperatures for a different number of days. Storage of sera at 4°C saw a significant depletion (P=0.0168) in complement mediated lysis and CH50 values could not be determined. Sera frozen at -70°C did not change composition, independent of time frozen. The addition of IgG does also not affect the complement levels. The normal reference was situated correctly. Data was analysed and CH50 values were obtained via a non-linear regression curve.

The storage conditions were repeated with different healthy serum to ensure the results were seen universally and were not specific to one sample. The healthy sera (H22.1) highlighted complement proteins degraded within three months at +4˚C (**Figure 8**). Therefore, to identify a more accurate timeline of depletion of complement the CH50 assay was performed at one and three months. It was expected for the complement activity to have depleted after one month at +4°C however, this was not the case. The highest quantity of complement was seen in the +4°C sample but showed large error bars. At three months the complement had degraded and a CH50 value could not be determined. There was a significant difference (p=<0.0001) in CH50 values between the sera frozen at -70 for three months and the sera stored in the fridge at +4°C for three months. This data further confirms complement components deplete after three months at +4°C.

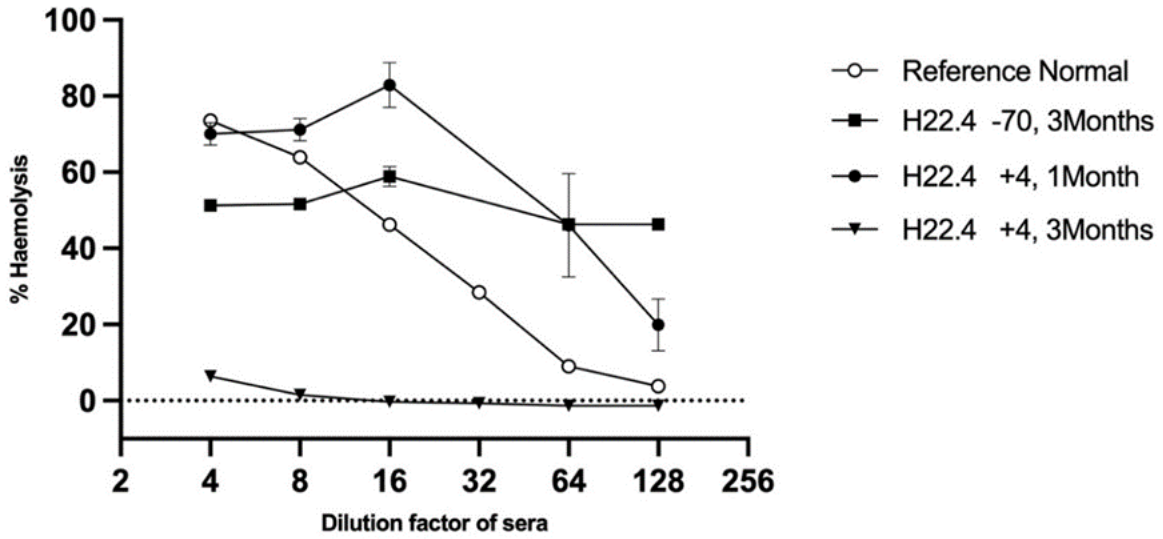


Figure 8: Complement levels of sera depleted after being stored at 4°C for three months.
A CH50 assay of healthy, non-infected-HCV sera which has been stored in different conditions. The condition +4, indicates the sera being stored in the fridge at 4°C. Complement levels are within normal range after three months at 4°C but completely depletes after three months. Significant difference (p=<0.0001) between the samples stored at -70°C and +4°C for three months. Data was analysed and CH50 values were obtained via a non-linear regression curve.

3.2.1.2 *Complement degradation within sera during storage at +4°C*

It has been shown that storage temperature can affect the stability of complement molecules. Having demonstrated that storage at +4°C caused complement activity to deplete beyond detection limit of the CH50 assay, samples from a previous clinical trial (the BOSON trial, Foster *et al*, 2015)) which had been stored at 4°C for 3-5 months were tested for complement activity (**Figure 9**). We originally intended to assess how the complement activity responds after treatment however, the results indicated that there was degradation of complement activity in all samples. This provides significant evidence that storage at 4°C, for over three months, is not suitable for serum samples.

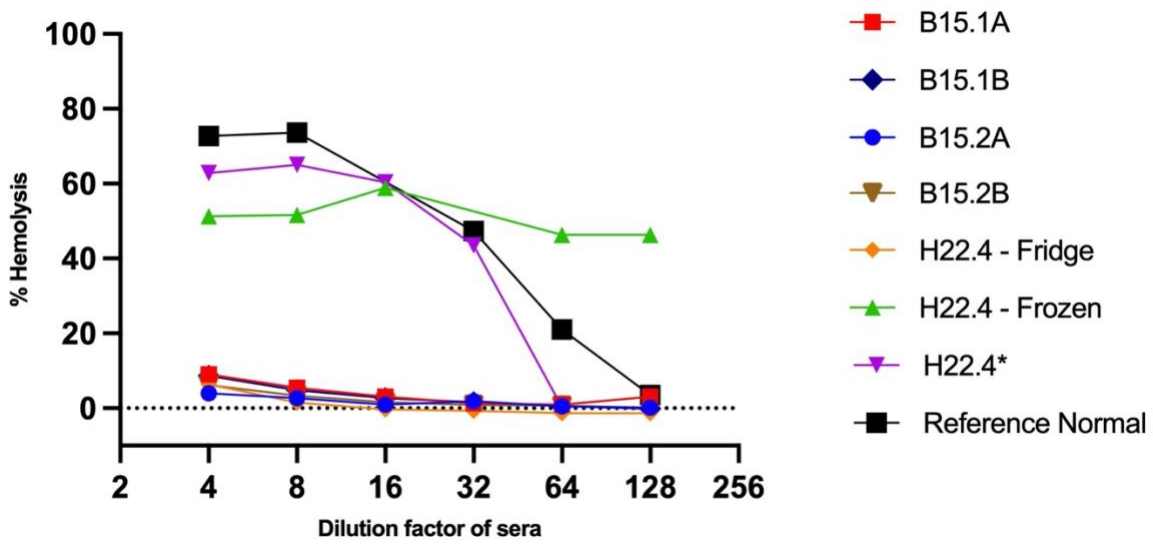


Figure 9: Storage of sera at 4°C causes complete degradation of complement proteins.
CH50 assay of 4 samples from the BOSON clinical trial (Foster et al, 2015) (B15.1A, B15.1B, B15.2A, B15.2B) and healthy non-HCV-infected sera (H22.4). Confirmation that storage of sera at 4°C causes degradation of the complement proteins and the absence of cell lysis. Healthy sera (H22.4 and H22.4) maintained normal complement levels when stored at -70°C. H22.4* represents healthy sera with IgG removed via agarose beads. Data was analysed and CH50 values were obtained via a non-linear regression curve.*

3.2.1.3 *Identifying changes in complement composition during freeze-thawing of sera.*

Our previous data concluded that storage at -70°C was suitable for sera and the functioning of complement. However, the 2010 samples had also undergone multiple freeze-thaw cycles during the Tarr *et al* (2011) study. This could contribute to compositional changes to the complement proteins and the lack of functioning (low CH50 values).

The aim of this assay was to establish whether the number of thaw-freezing cycles had a detrimental effect on the complement activity in the serum. Therefore, fresh healthy patient serum samples were freeze-thawed four times before assaying them. Limited differences between the complement activity after one and four cycles were observed (**Figure 10**). To confirm this, samples from the 2010 cohort which had been thawed over 10 times, were shown to have high complement activity.

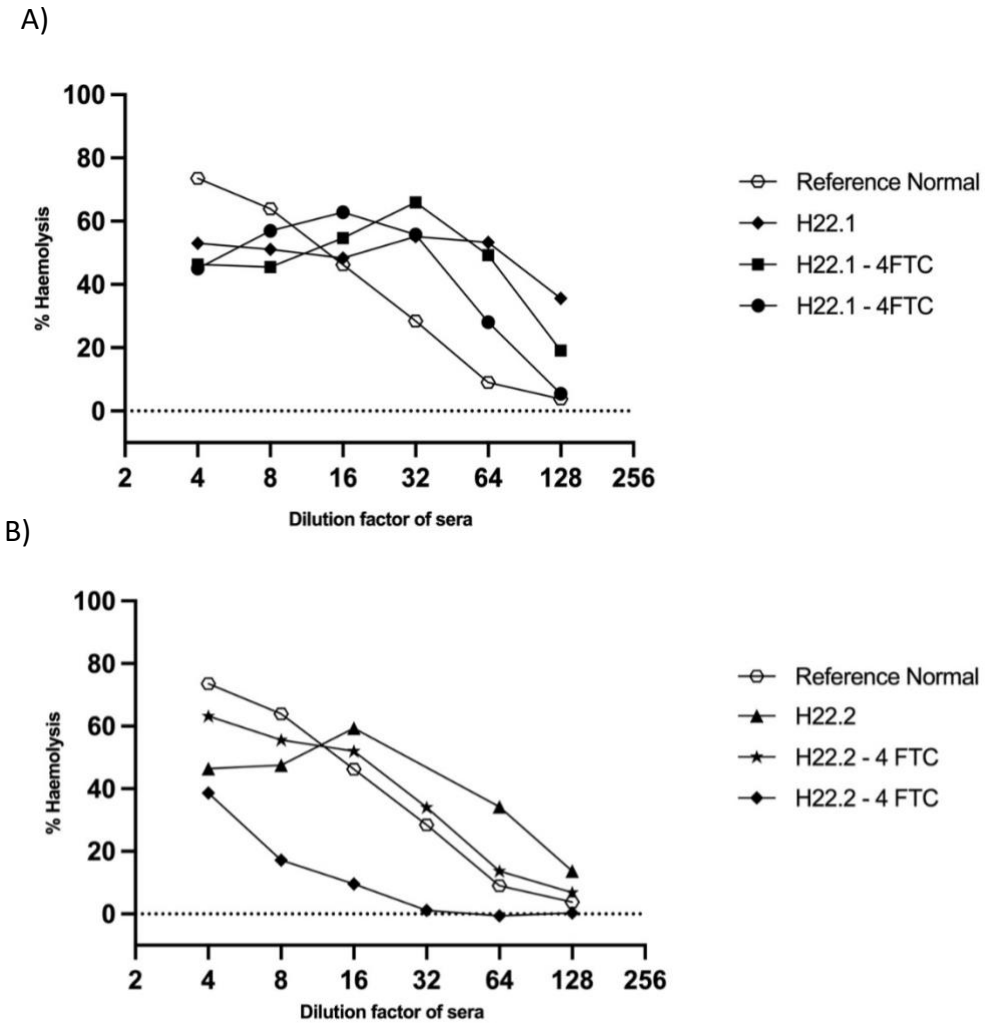


Figure 10: Four freeze thaw cycles does not impact the composition of complement.
CH50 assay performed on two healthy, non-HCV-infected samples to establish whether changes occur to the *composition of complement after four freeze-thaw cycles. Samples were frozen from fresh and thawed-freeze*d *four times*. A) Healthy sample 1 (H22.1) shows there is no difference in CH50 values between the fresh sample and freeze-thawed sample. B) Healthy sample 4 (H22.4) shows both the fresh sample and one four freeze-thaw cycle sample follow the normal reference. The diamond sample is *considered an a*nomaly *based on H22.1 and 2010 cohort data*. FTC: Freeze-thaw cycle. *Data was analysed and CH50 values were obtained via a non-linear regression curve.*

3.2.2 CH50 assay of chronically infected HCV patients – additional cohort

The previous data allows us to be confident that long term storage of the HCV sera (2010 cohort) was appropriate for maintaining complement functioning. Additionally, there were limited effects seen for freeze-thawing samples (2010 samples were not thawed greater than four times). To further validate the reduction in CH50 levels in chronic patients, we selected recent samples from the HCV testing bank. Six HCV-infected samples were tested, which had all been stored at -70°C and never thawed (once frozen in test facilities). Samples included a range of genotypes (1a, 1b, 2b, 3a) and were chronically infected (infection over six months). Therefore, the aim of this CH50 assay was to confirm whether the depletion in complement activity was associated with chronic HCV infection. Five of the patient samples (C21.1, C21.2, C21.4, C21.5, C21.6) differed significantly to the healthy controls (**Figure 11**). These samples show a depletion in complement activity to an extent to which the CH50 value cannot be determined. To obtain these low results, it only requires the absence or inactivity of one complement protein. C21.3 is the only patient sample which has maintained complement activity of a healthy patient, despite being chronically infected. There was a significant difference (P=0.0006) between C21.3 (maintained complement activity during infection) and C21.4 (complement depleted during infection). This data is consistent with the 2010 cohort data, whereas most chronically infected HCV patients have a depletion in complement activity, however some patients can maintain it.

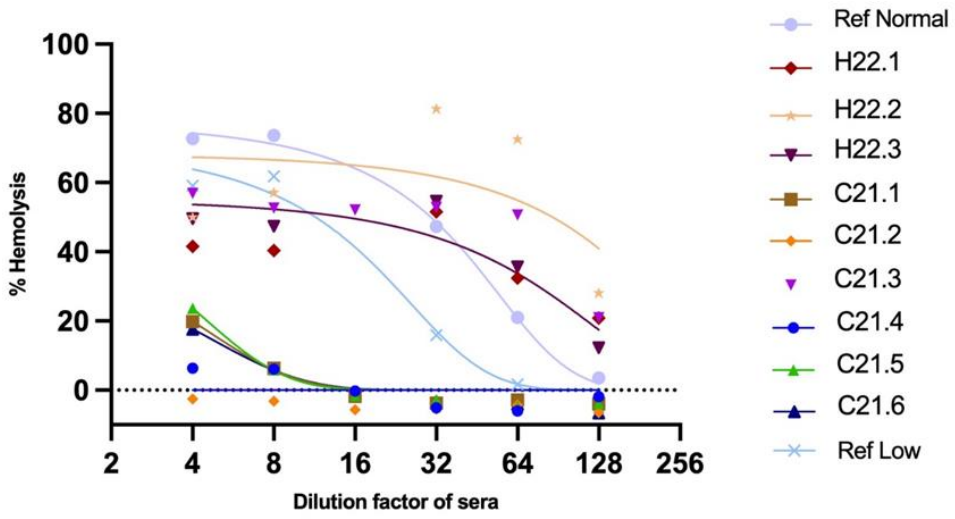


Figure 11: A depletion in complement levels seen in another cohort of HCV patients.
CH50 assay of six HCV-infected sera samples (C21.1, C21.2, C21.3, C21.4, C21.5, C21.6) and three healthy control sera (H22.1, H22.2, H22.3). Quantifying the amount of complement present in the samples and calculating the dilution factor of sera to obtain 50% lysis of sheep red blood cells (CH50), via the fit of non-linear regression fit. Five HCV-infected patients had depleted levels in complement. C21.3 was the only sample to maintain complement levels within the normal range. Both the normal and low references were situated correctly with CH50 values of 30.52 (Normal) and 11.43 (Low).

3.3 The influence of complement components on antibody response to HCV infection

3.3.1 Introduction to neutralisation assays

The previous assay work has provided knowledge on the complement levels within chronically infected HCV patients. It is important to understand the functioning of complement proteins in conjunction with other components circulating during infection. Pseudotyping allows us to safely manipulate and mimic the conditions of the virus setting. Antibodies and innate molecules have the independent ability to neutralise the virus. However, there is limited knowledge on their interaction and whether they synergise each other's actions to improve neutralisation against the virus.

3.3.2 The impact of complement on cell viability

Prior to the neutralisation assays, it was necessary to establish the effect of serum/complement on Huh7 cells, to distinguish the true results. There is a possibility the complement components would lyse the cells, so the negative results would be due to dead cells not neutralisation of virus entry.

There are several cell viability assays which allow identification of the effect of external stimuli on the health and viability of cells. Due to availability and ease, our first method used trypan blue staining to identify the proportion of healthy and dead Huh7 cells. We found this method to be inconsistent and biased to the field of view being analysed. To improve this method, we aimed to find an assay that would confirm if our cells were healthy and metabolically active. It was also beneficial to for this method to work alongside other cell assay techniques so they could be completed simultaneously.

To test for cell viability, fresh serum was added to Huh7 cells and a CellTitre-Glo® 2.0 Cell Viability Assay (Promega, #G9681) was performed (**Figure 12**). The high RLU was a good indication that there was limited cell death. It was important to identify whether the potency of the serum would be affected by freezing. However, we saw no differences in cell viability when comparing fresh sera to frozen. This assay also assessed whether a high

concentration of serum would increased cell death. The concentrations represent those later used in a neutralisation assay and results confirmed there were no issues with cell viability using these concentrations of serum.

We also tested to see if there were any differences in cell viability in the presence of complement and antibodies. Upon closer analysis, there were variances between the conditions however, these were not significant compared to the positive control. We decided no further investigation was necessary and that the cell viability was suitable to continue.

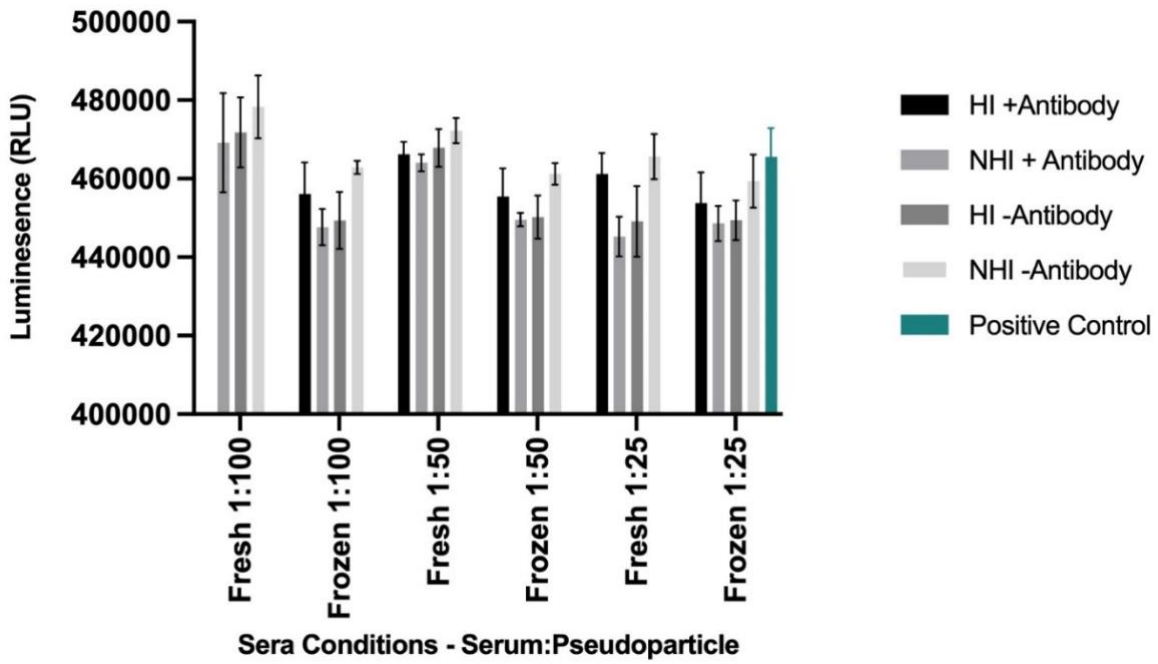


Figure 12: There is no significant difference in cell viability for different sera, antibody and pseudoparticle conditions.
Cell viability assay carried out to identify whether cell death was being caused by the added serum. There are limited differences between the serum:pseudopartiicle concentrations. There are differences between the conditions, but this is no significant against the positive control. The overall high luminescence reading suggests the cells are metabolically active and viable for future assays.

3.3.3 Identification of CD55 and CD59 on Huh7 and HEK293T cells

Having performed a cell toxicity assay, we needed to understand why the effect of the complement was being muted. We identified proteins with a strong influence on the regulation of the complement cascade, with specific focus on two transmembrane proteins, CD55 and CD59. The glycoprotein CD55 is also known as a decay-accelerating factor (DAF) which inactivates C3 convertases by preventing their assembly. CD59 is a MAC-inhibitory protein whereby it binds to C8 or C9 preventing the integration of these molecules and the formation of the membrane attack complex.

3.3.3.1 Determining the expression levels of CD55 and CD59 on Huh7 and HEK293T cells

According to data retrieved from the Human Genome Atlas, there was low expression of CD55 and CD59 in Huh7 cell lines. Therefore, confirmation of the expression level within the laboratory cell lines, would inform us whether there was complement inhibition affecting future experiments. When testing at three different dilutions of cDNA (1:10, 1:100, 1:100) there was significant expression of CD55 and CD59 detectable in Huh7 cells (***Figure 13*****,** ***Figure 14*****A**). A specific pure product was confirmed by the melt peak curves which were all situated in a close range. Alongside this, a RT-qPCR was performed without primers to ensure that the expression results were not due to non-specific amplification. Anomalous curves suggested there was contamination within the samples, however these were repeated with new reagents, and this was no longer seen. Going forward we chose to use the 1:10 dilution of cDNA as this gave a suitable representation of expression. Having confirmed there was high expression of both CD55 and CD59 in Huh7 cells, it was important to know whether this was seen in HEK293T cell, as these are used for pseudotype production. Using the same procedure, we produced HEK293TcDNA and performed a RT-qPCR with all the above primers (***Figure 13*****,** ***Figure 14*****B**). The expression of CD55 and CD59 was also high, with a similar Ct value as in the Huh7 cells (**Error! Reference source not found.**). Despite identifying high expression based on the Ct value, we needed to cross reference the relative expression with the housekeeping gene GAPDH. The ubiquitous expression of GAPDH allowed us to assume how regularly our gene of interest was being expressed.

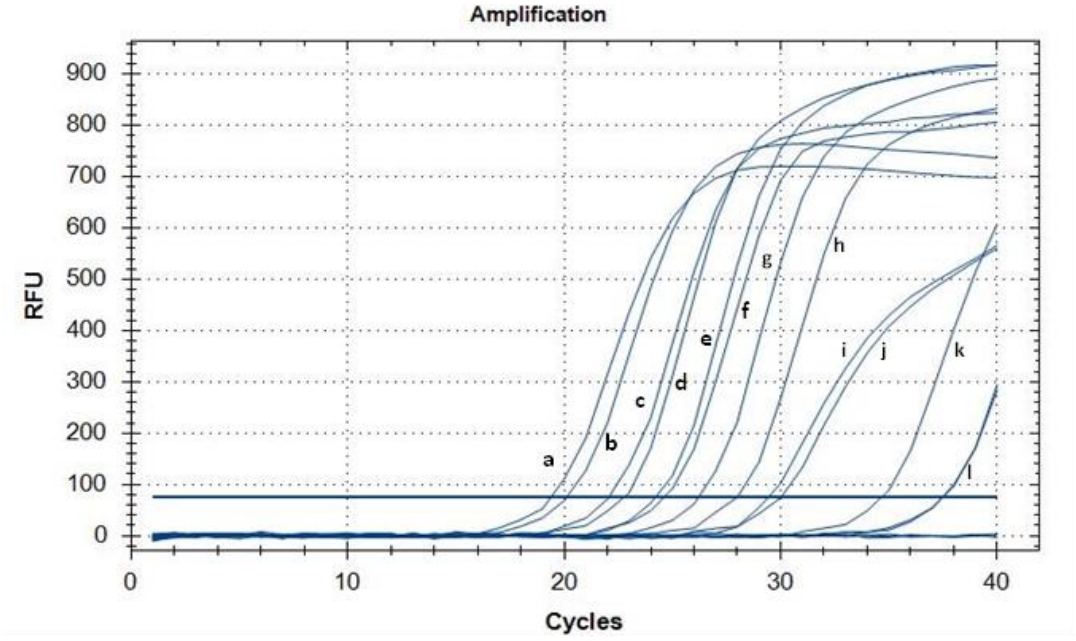


Figure 13: RT-qPCR analysis of CD55 expression in Huh7 cells and HEK293T cells, with comparison to the housekeeping gene GAPDH.
a) Huh7 GAPDH b) HEK293T GAPDH c) Huh7 SW d) Huh7 GS e) Huh7 WA F) HEK293T SW G) HEK293T GS H) HEK293T WA i) HEK293T SW Negative j) Huh7 SW Negative k) Huh7 GAPDH Negative l) Huh7 WA Negative.

Table 3-1: **Cq values obtained from q-PCR of CD55 and CD59 expression in Huh7 cells and HEK293T cells.** Below contains all four primers used to amplify with comparison to the housekeeping gene GAPDH. Cq: Quantification Cycle.

	CD55		**CD59**	
Primer	**Huh7**	**HEK293T**	**Huh7**	**HEK293T**
WA (Wang et al, 2017)	25.88	27.99	26.12	26.93
SW	23.11	24.60	25.04	26.99
MW	N/A	N/A	29.21	26.52
GS	24.05	26.20	N/A	N/A
Average Cq Value	23.34	26.26	26.79	26.81
GAPDH	20.74	20.11	20.46	19.49
Ratio of GADPH: average Cq of primers	**1:1.12**	**1:1.30**	**1:1:30**	**1:1.37**

The quantification cycle (Cq) value indicates the position of the amplification curve respective to the cycle axis. There is a threshold to which the fluorescence is seen to be above the background noise. The Cq value is the point at which amplification crosses this threshold. Comparisons of the Cq values can be limited due to differences between runs and machines.

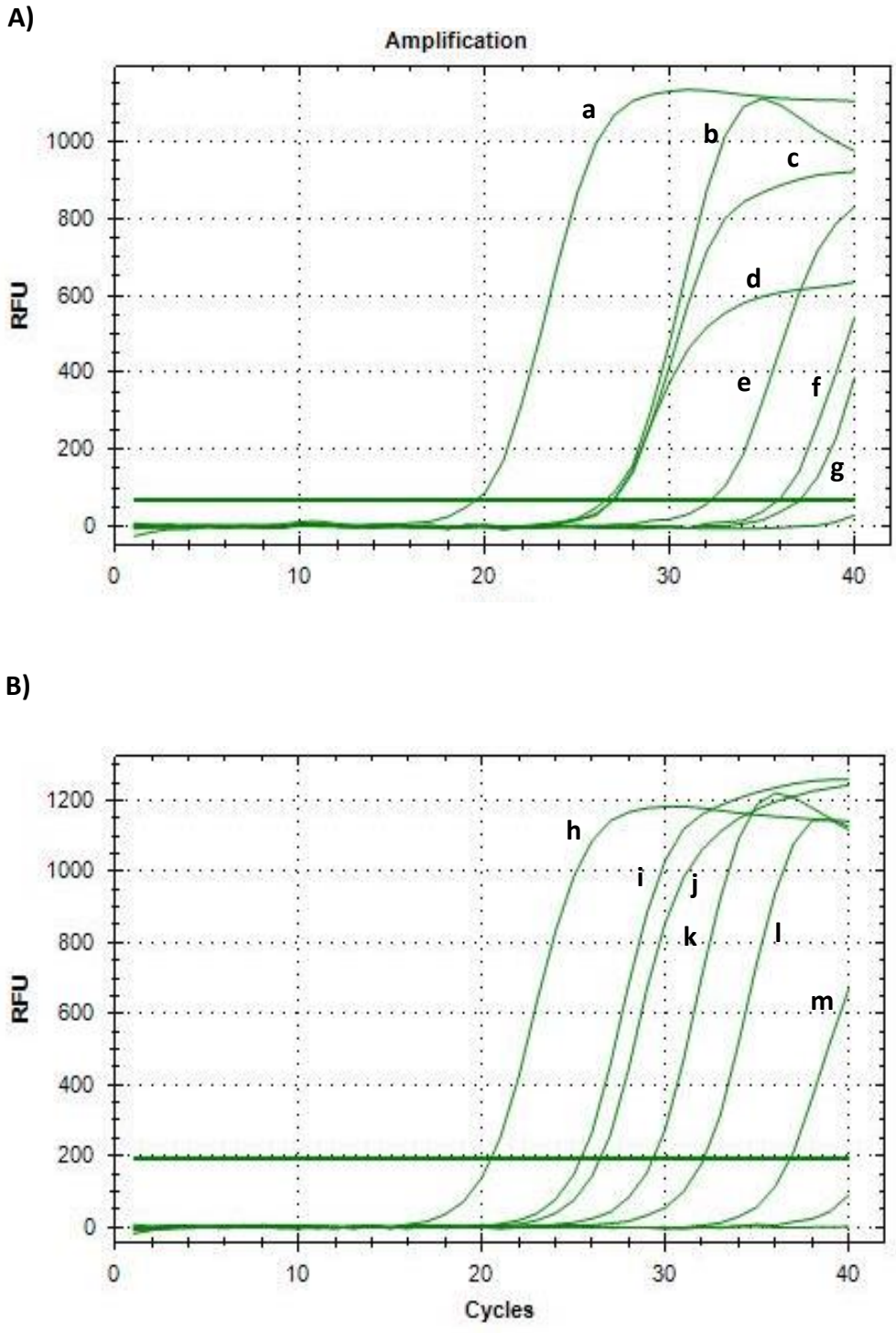


Figure 14: q-PCR analysis of CD59 expression in Huh7 cells and HEK293T cells, with comparison to the housekeeping gene GAPDH.

A) q-PCR results for all primers with Huh7 cells. Cq values are within the brackets. a:GAPDH positive b:MW positive c:SW positive d:WA positive e:GAPDH negative f:WA negative g:SW negative.

B) q-PCR results of all primers with HEK293T cells, performed on a different run. h:GAPDH positive i:SW positive j:WA positive k:MW positive l:GAPDH negative m:WA negative.

3.3.3.2 Immunostaining of CD55 and CD59 proteins on cell surface of Huh7 and HEK293T cells

It was necessary to identify whether the expression level of CD55 and CD59 was proportional to protein production on the cell surface. Using Zeiss Axio Imager at 60X magnification, both CD55 and CD59 could be seen on the cell surface of Huh7 and HEk293T cells (**Figure 15**).

For CD55 protein, the DAPI staining was successful in all controls. There are clear localised regions of high fluorescence of where the protein is residing. The different fields of view hinder a comparison of total protein on the surface. Among the CD59 negative samples there was high level of background noise. We are confident this was background fluorescence where the antibody has bound to another non-specific protein. Despite this, there was still a clear difference when compared to the positive control, where regions (spots) of high fluorescence is sporadically located throughout the membrane.

We have identified that both CD55 and CD59 are not equally expressed onto the surface of the cells. It can be seen that some cells have an abundance of protein, with regions of high fluorescence, yet some have very little. This can be seen across both cell lines. It could be assumed that both proteins are being actively expressed onto the cell surface and offering protection to the cells against complement mediated cell lysis.

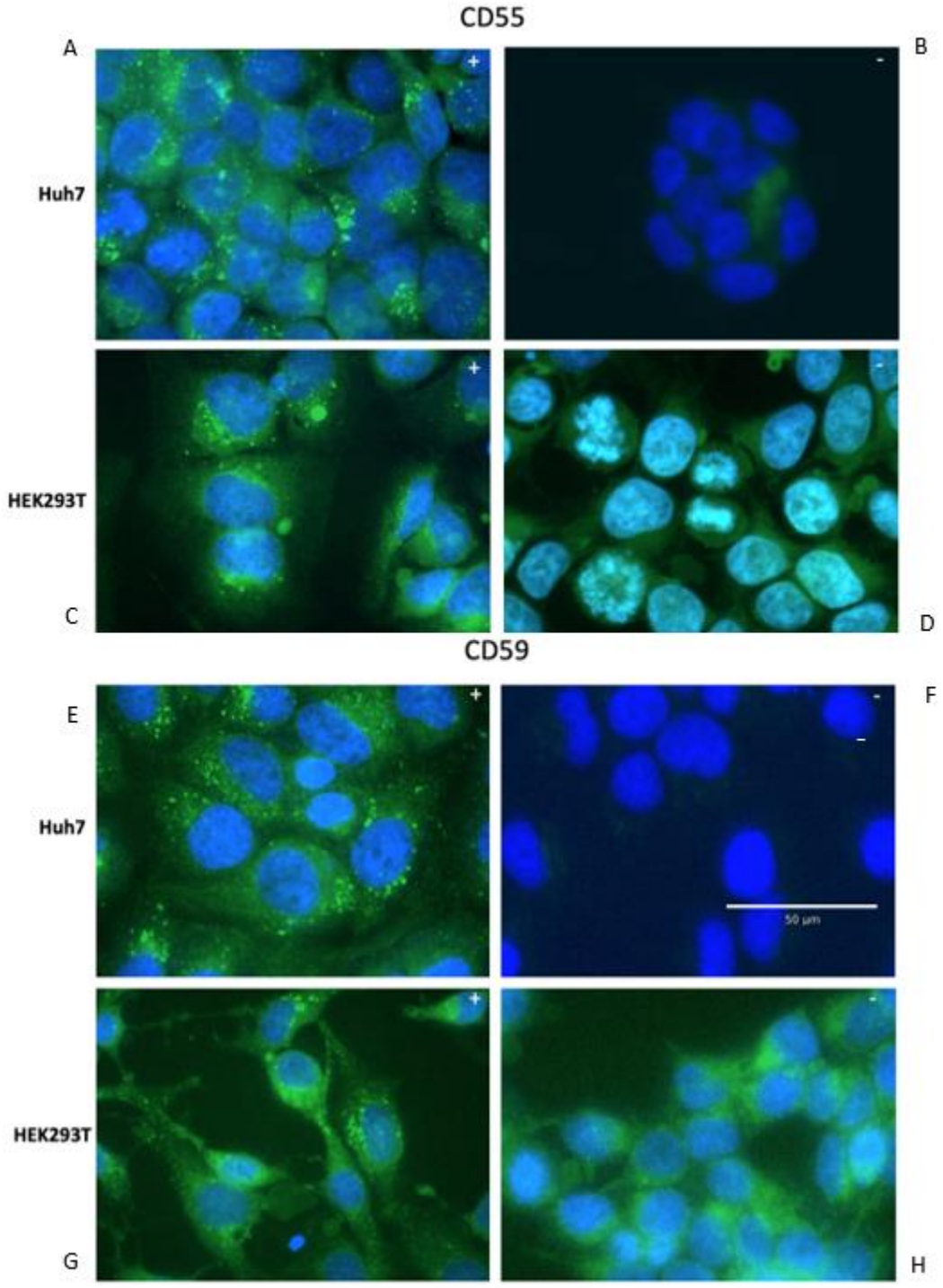


Figure 15: Detection of CD55 and CD59 with immunostaining in Huh7 and Hek293T cells.
CD55 and CD59 stained with Alexafloor488 and counterstained with DAPI in blue. A) Positive control of CD55 in Huh7 cells. B) CD55 negative control in Huh7 cells. C) Positive control of CD55 in Hek293T cells. D) CD55 negative control in Hek293T cells. E) Positive control of CD59 in Huh7 cells. F) CD59 negative control in Huh7 cells. G) Positive control of CD59 in Hek293T cells. HI) CD59 negative control in Hek293T cells

3.3.4 Validating the heat inactivation method to ensure degradation of complement

The last method optimisation included ensuring the heat inactivation approach would successfully inhibit complement function. Future experiments would highlight the interaction between complement and antibodies, using complement absent conditions. To validate the heat inactivation method, CH50 assays were performed on serum which had been in a heated block at 60°C for 10 minutes (**Figure 16**). Two HCV-infected patients which maintained complement activity throughout infection were chosen. In both samples there was a significant difference in the CH50 values between complement present (C21.8: 42.25) and complement absent (Undetermined), due to heat inactivation. Alongside these values, the dilutions curves on the graphs highlight the stark difference in the quantity of complement present. This allowed us to be confident in taking forward this method.

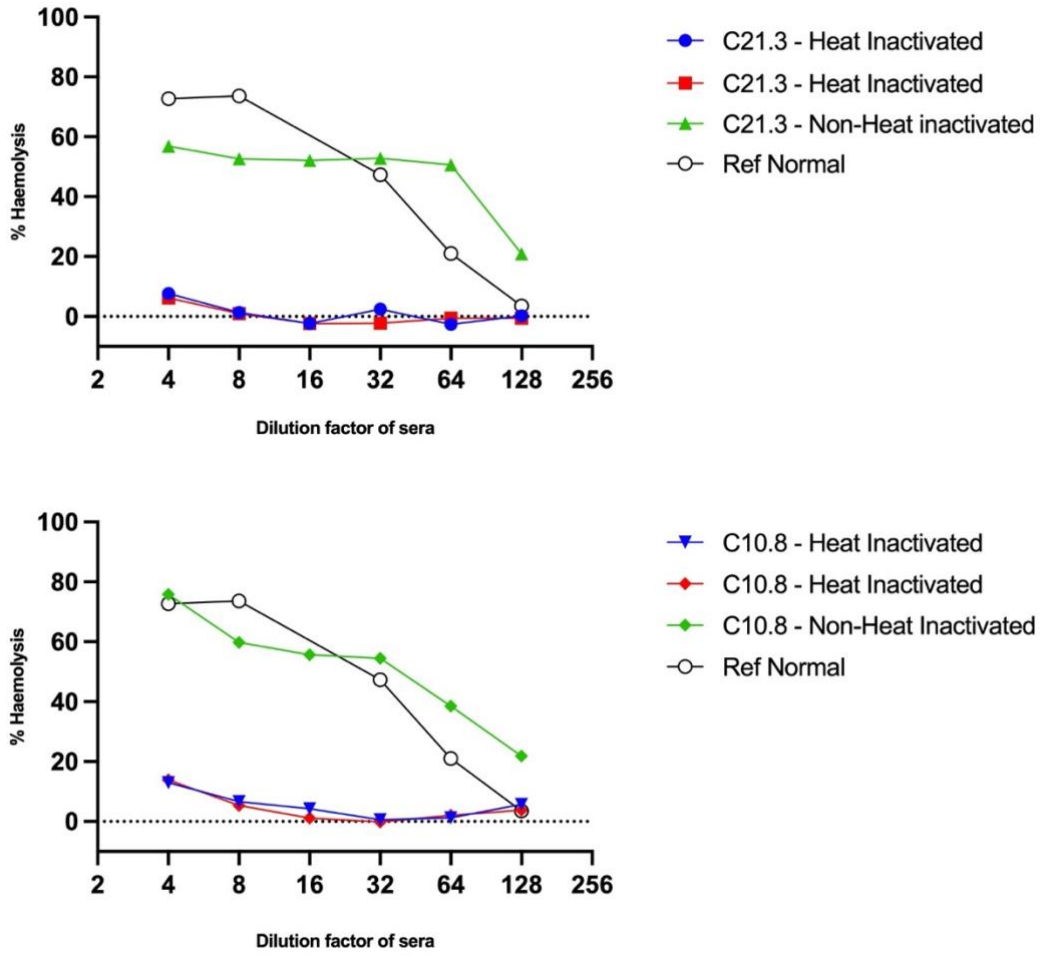


Figure 16: Heat inactivation of sera successfully deactivates the lysing ability of complement proteins.
Two HCV-infected sera from different cohorts, were heat inactivated at 60°C for 10 minutes and complement quantified using CH50 assay. A) Sera C10.3 showing a substantial difference in complement activity between fresh sample (Green) and heat inactivated (Red and Blue). B) Sera C21.8 also following the same trend. Data was analysed and CH50 values were obtained via a non-linear regression curve.

3.3.5 Neutralisation potential of IgG with supplemented complement

Prior work identified that complement components were not affecting cell viability (due to CD55/CD59 protection) and the heat inactivation method was successful. This optimisation of the method allowed us to be confident moving forward with the neutralisation assays.

Patient serum samples that were found to have depleted complement, would not produce results. Therefore, it was applicable to use healthy patient serum to reconstitute complement into these assays. All IgG preparations were purified from HCV-infected patient serum. Using these preparations gave us an indication how the antibodies interact with complement molecules. We could determine whether there were different neutralisation potentials between the antibodies extracted from patients who maintained complement to those who have depleted levels.

Having already assessed the quantity of complement in the healthy sera sample, we could assume how much complement was acting at each dilution point and how this affected the neutralisation potential of the antibody. Therefore, we set up the neutralisation assay using the same dilution curve and quantities of serum as seen in the CH50 assay (two-fold dilution curve starting at 1:4 (25 µL serum) through to 1:128). We found this did not produce reproducible results. There was large variation between replicates, with some samples imitating values of the negative control.

We investigated all possible variables which included the health of cells and functioning of pseudoparticles. New batches of cells were thawed from liquid nitrogen and were handled by different personnel. Pseudoparticles was analysed using a western blot to ensure correct expression of plasmid. Both variables were excluded from having an effect. Our last adaption altered the concentration of sera being added to the cells. Our previous research highlight that at the serum dilution factor of 1:100 (1 µL serum: 100 µL pseudoparticle), 1:50 (2 µL of serum: 100 µL pseudoparticles) and 1:25 (4 µL of serum: 100 µL pseudoparticle), cell viability was maintained. However, the CH50 assay dilutions used much higher quantities of serum. It was concluded that the concentration of serum was too high and killing the cells. Therefore, we adapted the dilution curves to maintain cell viability and analyse the effect of complement components on antibody neutralisation of HCV entry.

Five polyclonal antibodies, in the form of purified IgG from HCV-infected serum, were combined with healthy non-HCV infected serum (**Figure 17**). Heat inactivation of the healthy serum allowed us to manipulate the presence of complement. The heat inactivated serum represents complement absent condition (Blue) and the unmanipulated serum represents complement present (Red) (**Figure 17**). The antibody concentration was fixed at each individual EC50 value and serum was added in a dilution factor of serum to 100µL pseudoparticles per well.

The results prominently showed there were variances in neutralisation potential between different polyclonal antibodies in the presence of serum. There were stark variations in the dilution curves and the effect of complement. Sample C10.1 demonstrated a considerable increase in neutralisation potential in the presence of complement, suggesting complement assisted the neutralisation of HCV entry (**Figure 17G**). On the other hand, both conditions of C10.2 were of similar neutralisation potential until the serum concentration increased, in which complement seemed to improve neutralisation (**Figure 17H**). A common theme throughout all polyclonal samples, was that the presence of complement improved neutralisation of HCV entry (independent of how much of a sizeable change). All samples produced neutralisation potential greater than the EC50 value when complement was present. This trend was not seen when complement was absent, with neutralisation around the EC50 or less. From this we could assume the presence of complement was an advantage to the action of the antibodies. Alongside this we assessed the effect of complement alone (H22.4*) on the neutralisation of HCV entry (**Figure 17I).** It was identified that the complement increased neuralisation of the virus. Furthermore, the positive and negative samples were as expected.

Another focus of this assay was to identify the dose effect of complement. As we increase the proportion of serum to pseudoparticle, this would increase the concentration of complement and we expected to see a difference in the neutralisation potential. C21.2 had a dose dependent effect on neutralisation of HCV entry (as serum increased, neutralisation increased) (**Figure 17D**). However, C21.3 and C10.1 showed limited differences in

neutralisation potential across all serum dilution when complement is present (**Figure 17F** and G). This could be the maximal neutralisation potential.

We were interested in the specific interactions between antibodies and complement, so we choose three monoclonal antibodies (Mab 1:7, AR3A and AR4A) which are known to target different regions on the E1E2 glycoproteins (**Figure 17**). AR3A and AR4A target epitopes that are conserved across genotypes, so information regarding their interaction would be useful. Firstly, we saw a difference between the complement presence versus absent conditions. It followed the same trend as the polyclonal antibodies, with complement increasing the neutralisation of virus entry, with all points under the EC50 value. In the complement absent condition, AR3A showed a decrease in neutralisation as the concentration of serum increases(**Figure 17B**). It is possible another serum protein was interacting with the antibody, or it requires complement for neutralisation. This trend could also be seen subtly in MAb 1:7 and AR4A.

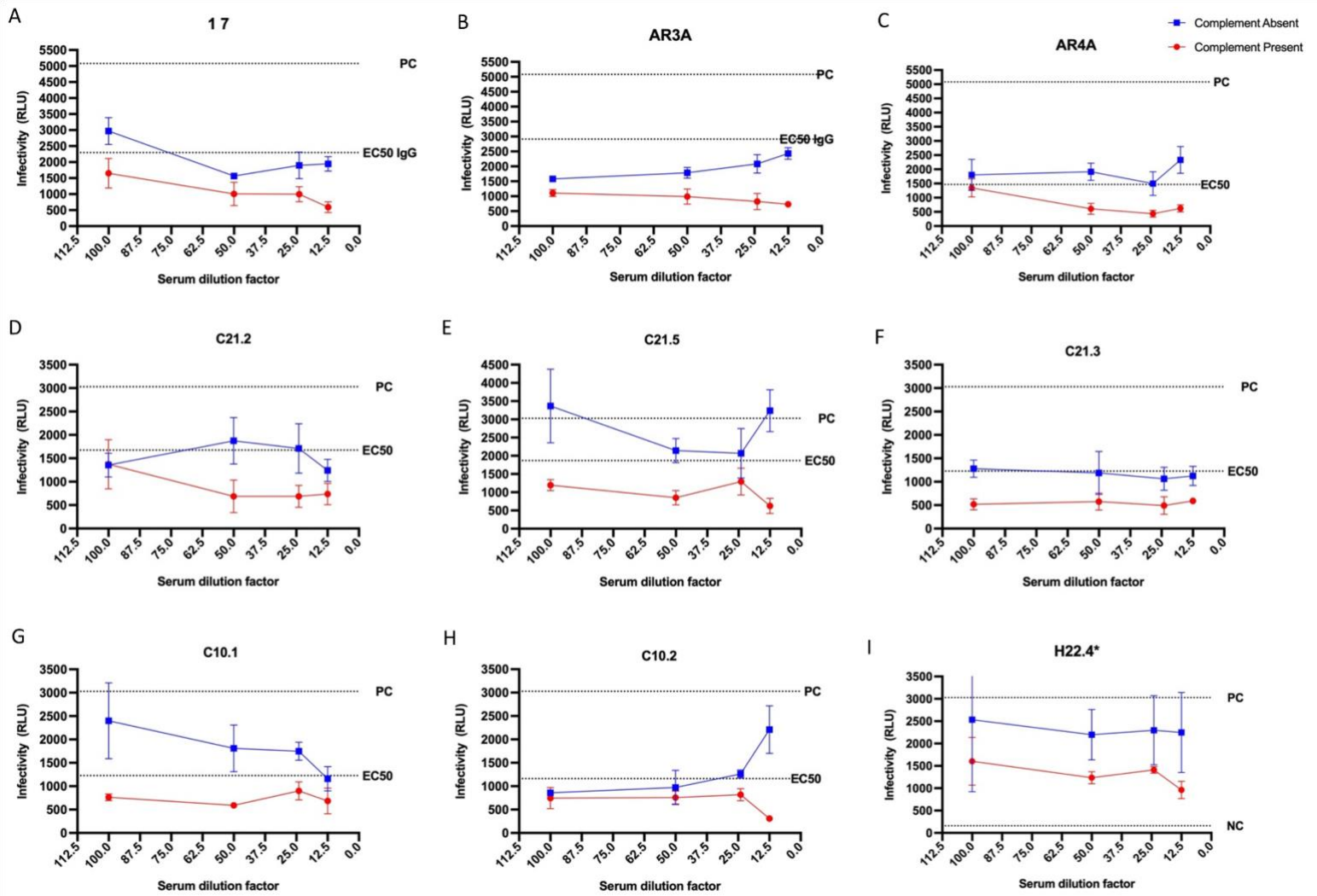


Figure 17**: **Neutralisation assay to assess the effect of complement on the neutralisation potential of antibodies.
*Three monoclonal antibodies (Mab 1 7, AR3A, AR4A) and five IgG preparations (purified from HCV-infected patients – C21.2, C21.5, C21.3, C10.1, C10.2) were added at their antibody binding EC50. Healthy sera was added either with complement present (RED) or complement absent via heat inactivation (BLUE). A)Mab 1 7 B)AR3A C) AR4A D)C21.2 E)21.5 F) C21.3 G)C10.1 H)C10.2 I)H22.4**

3.3.6 Analysis of antibody binding to E1E2 glycoproteins in the presence of serum proteins

The impact of serum on antibody was net assessed utilising an in-house ELISA. The aim of these ELISA assays was to assess the effect of serum, more specifically complement, on antibody binding to E1E2 glycoproteins. We wanted to identify how they interact. It is possible that antibody binding is enhanced by the presence of complement components however, they could also be competing for the same binding site. Healthy, non-HCV-infected sera (H22.4) was supplemented into the assay to represent normal conditions. This serum has been previously analysed in the above CH50 assays to confirm complement activity within the normal range (**Figure 9**).

The initial results highlighted high binding from the healthy control (H22.4). We suspected this to be due to the patient IgG within the sera sample. As the patient was non-HCV-infected, this would indicate non-specific binding to the E1E2 glycoproteins or other proteins present in the lysate. We optimised the blocking procedure and established which blocking solution would diminish the majority of background noise, produced by the control sera.

Figure 18 illustrates a significant reduction in binding when IgG in diluted in a blocking solution compared to TBS Tween (0.1%). In both serum conditions the negative controls were successful and showed that the absence of one antibody causes no binding. Slight differences can be seen between H22.1 and H22.4 samples, as some blocking solutions reduced binding more effectively than others. However, it can be concluded that 3% BSA was successful in blocking the majority of non-specific binding for both serum samples. BSA is a readily available product which meant it was a suitable to take forward. Once repeated within the full ELISA protocol, there was no binding for either H22.1 or H22.4 when compared to HCV-infected purified IgG.

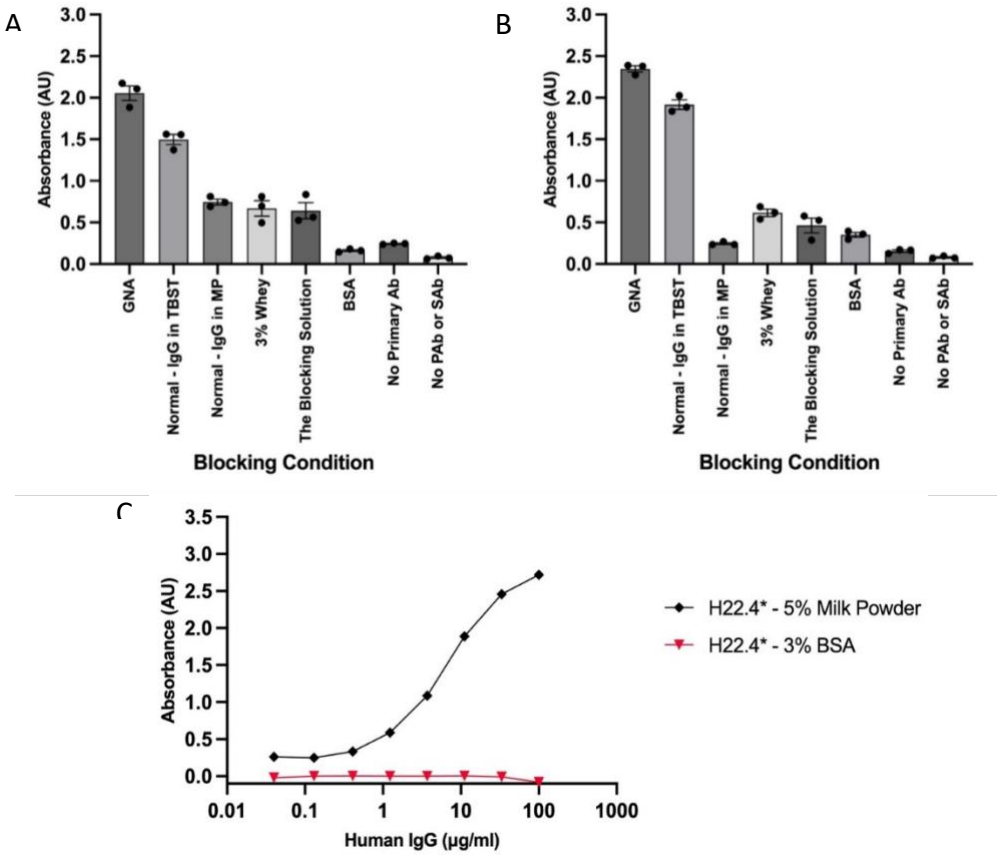


Figure 18: 3% BSA was an appropriate blocking agent for non-specific binding.
IgG binding ELISA with two healthy, non-HCV-infected sera with different blocking agents used for IgG dilution and 2 h blocking window. A) H22.1 Sera with 3% BSA most effective. B) H22.4 sera with 3% BSA most effective. BSA: Bovine serum albumin. C) Assay highlighting that BSA was sufficient to block the non-specific binding of the IgG

3.3.6.1 Binding curves of HCV patient IgG to establish EC50 values

An IgG binding assay was carried out using purified IgG from HCV-infected patients. Using a dilution curve, we could calculate the concentration of antibody which gives half-maximal binding (EC50). The original starting concentration was from 50µg.mL^{-1} in a 5-fold dilution (**Figure 19**). The results highlighted this would not produce an accurate EC50 values as the top end of the curves had not begun to plateau. The only antibody which was different was mAb 1 7 which would be expected to bind at greater affinity, as a monoclonal antibody to E1E2.Therefore, we increased the starting concentration to 100 µg.mL^{-1} and used a 3-fold dilution curve. The results from the 2010 cohort plateaued at both ends of the curve, allowing us to assume an accurate EC50. Conversely, some of the 2021 cohort serum did not start to plateau, in which a higher concentration could have been used. The calculated EC50 values were still taken forward despite the curves not reaching a top end plateau. This was because we only required the antibodies to be constant binding around 50%

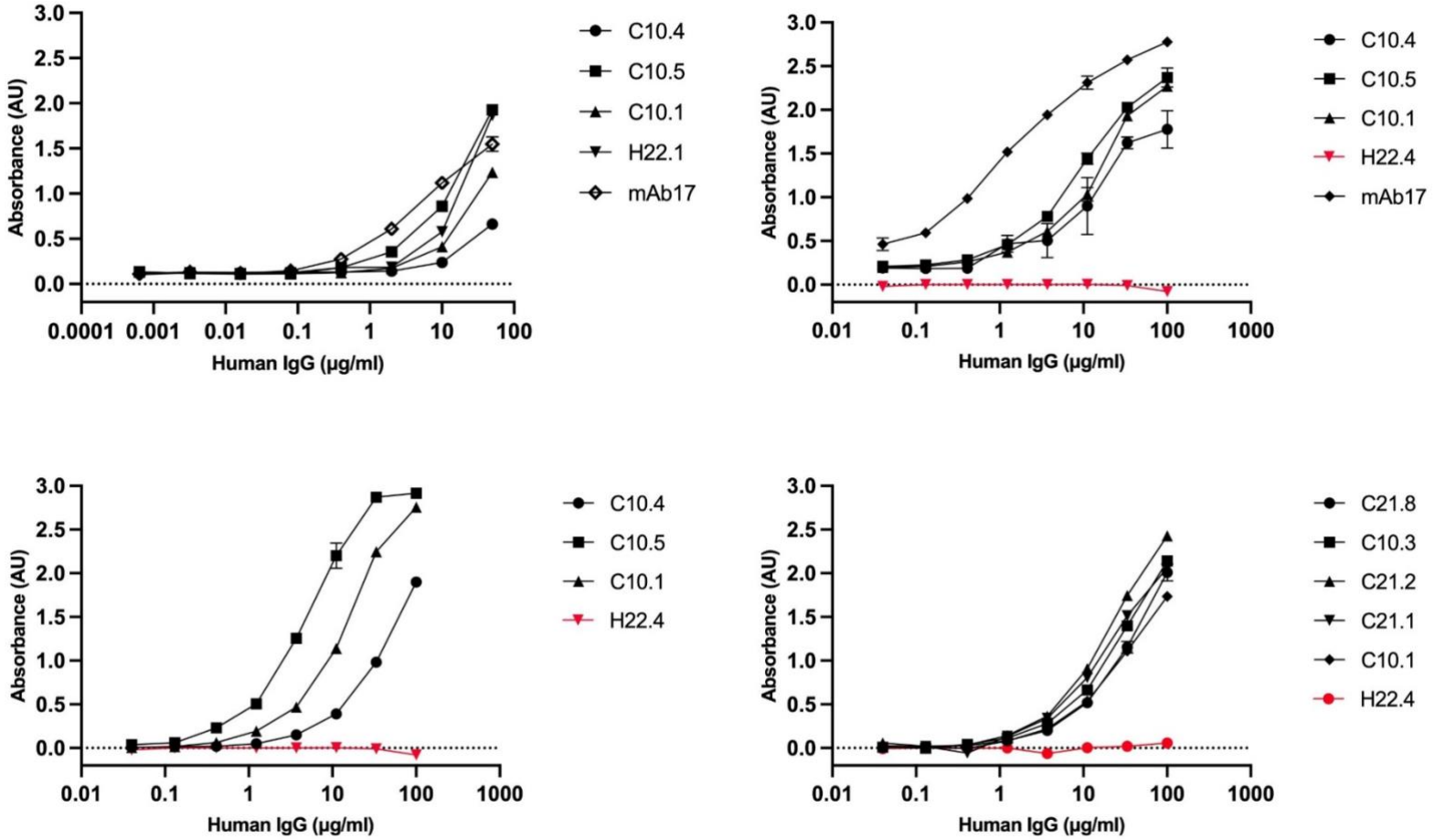


Figure 19: Antibody binding ELISA to E1E2 glycoproteins with purified IgG samples from the 2010 and 2021 cohort of HCV-infected patients.
A) 5-fold dilution curve from 50g/ml of IgG. This did not plateau at the top end and did not produce accurate EC50 values. B) Repeated ELISA with a 3-fold dilution starting at 100ug/m confirming blocking solutions for non-specific binding of control sera (RED). C) Repeated 3-fold dilution at 100ug/ml. D) Produced binding curves for the remaining samples with effective blocking of control sera. EC50 values were calculated as the concentration of IgG needed to obtain 50% of binding to the E1E2 glycoproteins. EC50 values were taken forward into future ELISA assay.

3.3.6.2 Binding of HCV patient IgG in the presence of serum

This assay is using the previously established EC50 of the HCV-infected patient purified IgG and supplementing this into complement present serum. Using the same dilution curve as the CH50 assay, we can assume to amount of complement present alongside a fixed quantity of antibodies. This manipulation allows us to study the effect of serum components (specifically complement) on antibody binding to E1E2 glycoproteins.

To further minimise the non-specific binding, we extracted the IgG from the serum using Protein-G agarose beads. Figure 17, illustrates that sera H.22* (sera H22.4 with (IgG depleted) still maintained complement activity within the normal range. We see a sudden decrease in complement from 1:16/32 but this is not a concern for future work.

Moving forward with the assay we observed high levels of non-specific binding to the mock lysate. The mock lysate only includes proteins naturally occurring produced by the HEK293T cells. This non-specific binding was observed during the high concentrations of 1:4 to 1:16 dilution of serum. Previously established ELISA methods were not suitable in reducing this background, so we needed to adapt this. By blocking the plate with 3% BSA and producing the serum and IgG dilution curves with 3% BSA instead of TBST, we saw a reduction in the non-specific binding. The mock lysate acts as the negative control which is subtracted from all E1E2 lysate results. Therefore, if this signal is high then the results are skewed, and you cannot see the true results. Due to the high mock lysate signal, the graph was being inverted. Due to time constraints, this was not investigated further.

3.4 Complement response during acute HCV infection and spontaneous recovery.

3.4.1 Model of disease progression and complement depletion

Having shown that during chronic HCV infection, complement activity depleted, it was valuable to investigate when the dysfunction occurs. Samples A1 through to A4 represent different disease progressions with chronic infection, acute infection, and infection clearance. Despite the small sample size, it gives us the first understanding of complement depletion upon HCV infection. All samples were gained from a previous study therefore, we were limited (AS.3 and AS.4 we do not have samples from before infection). **Figure 20**, shows a predicted model of disease progression, due to our analysis of the chronic samples, only showing a snapshot of the infection. We could assume that prior to HCV infection the patients have normal complement activity, represented as the red, yellow and green lines. However, each line diverts as we are unaware as to when the depletion in complement occurs. The purple line represents a patient which experiences complement deficiency which may have made them susceptible to HCV infection. All disease progressions could be represented throughout the population of HCV infection.

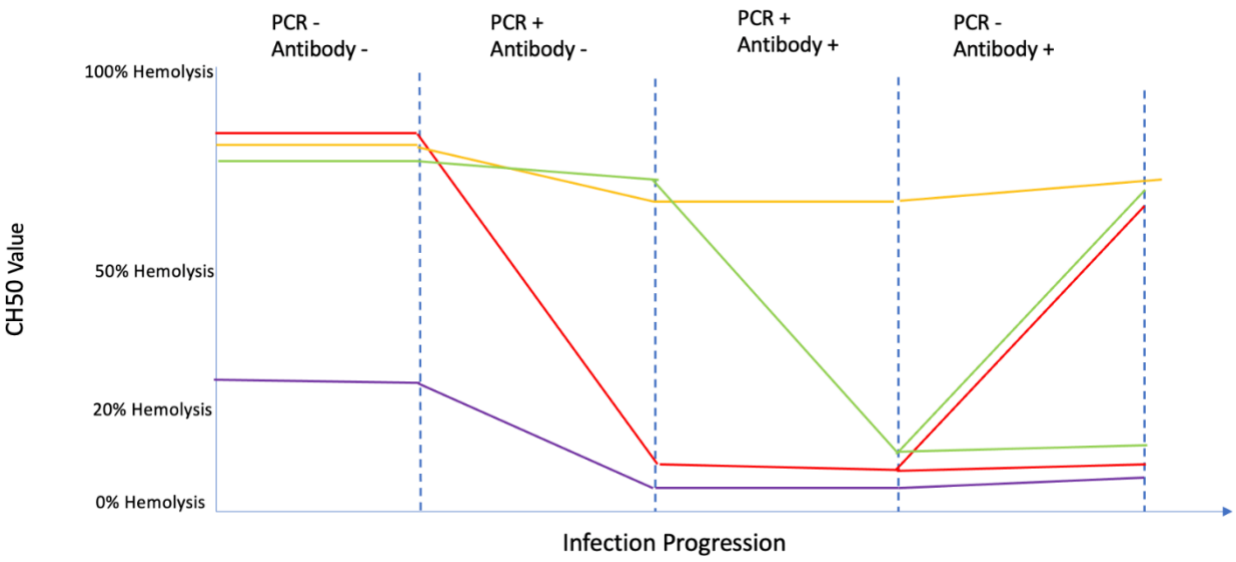


Figure 20: Model of complement depletion throughout HCV disease progression.
Colours represent the different patient outcomes that could be expected. Red – Complement level decreases immediately after infection, this either improves or remains depleted once infection cleared. Yellow – Patient maintains 'normal' complement levels throughout infection. Green – Patient complement depletes once seroconverted. Purple – Patient had low complement which makes them susceptible to HCV infection. Complement remains low.

3.4.2 Acute Progression – Pre-Infection and prolonged Infection of AS.1

Sample AS.1 showed pre infection, acute phase infection and chronic infection for one year (**Figure 21**). Sample AS.1A was taken prior to HCV-infection and displayed a normal curve with a CH50 value of 3.87. Despite being lower than the healthy patient, this was not significantly different. Once the patient was PCR positive and seroconverted there was an increase in complement activity with a CH50 value of 6.70. This could be expected as the immune system is reacting to infection. However, long term chronic infection of 22 months, as seen in sample AS.1C, demonstrates a significant depletion of complement (P=0.0021, compared to PCR negative). A CH50 value could not be determined.

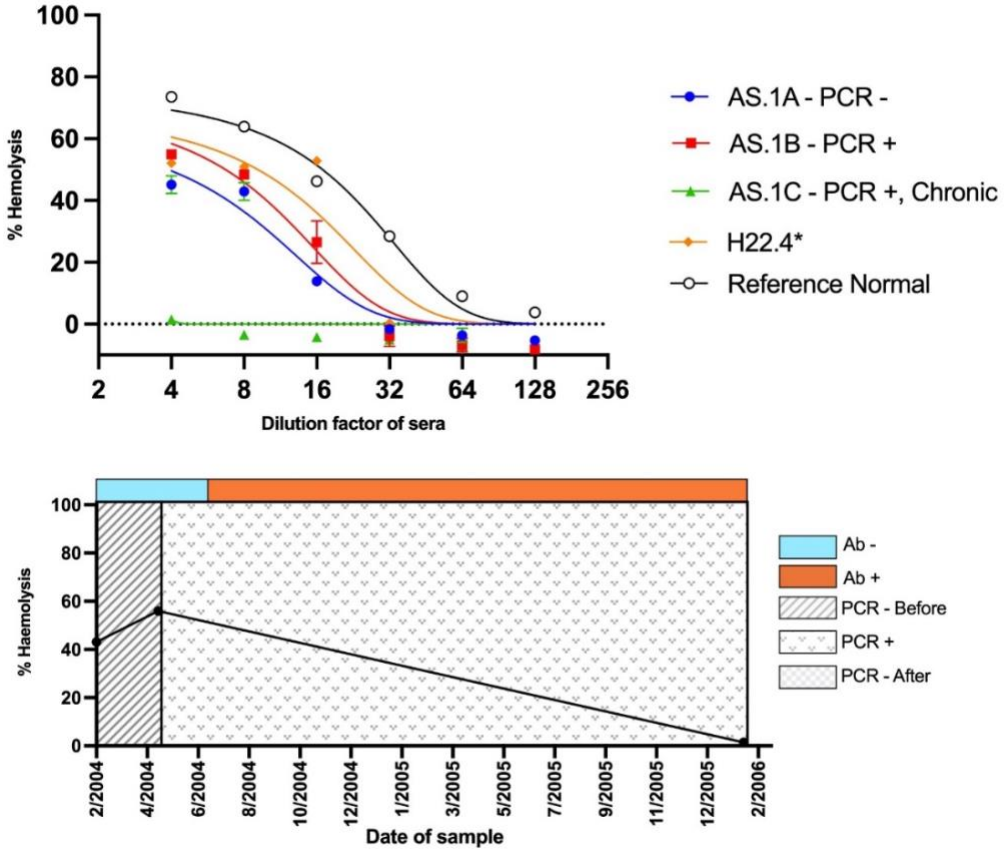


Figure 21: Complement quantification and timeline of an acute and chronic HCV infection
A) Three samples collected from one HCV infected patient. AS.1A was taking before infection (3.87). AS.1B was taken when the patient was PCR + for HCV (6.70). AS.1C is a chronic infection sample, 22 months after initial infection (Undetermined). CH50 assay quantified complement levels and CH50 value (dilution factor of sera for 50% lysis of cells) shown in brackets. H22.4: 9.12, Ref Norm: 16.19. B) The three samples plotted on a timeline. Pattern boxes represent the PCR status and the blue/orange boxes show the antibody status*

3.4.3 Acute Progression – Pre-Infection, During Infection and Clearance of AS.2

AS.2 samples follow a similar trend to AS.1, with a decline in complement activity as HCV infection progresses (**Figure 22**). AS.2A was taken two months after initial infection during the acute phase (PCR positive, Ab negative). An elevated level of complement was identified which could be expected as the innate immune is responding to initial infection. The CH50 value (13.07) was lower than the reference sample (16.19) due the gradient of the slope. After a further two months the patient seroconverted to be PCR positive, Ab positive. During this period we seen a significant depletion (P=0.0043) in complement activity which produces a CH50 value of 4.43. One month later the patient was PCR negative, upon clearance of the virus by DAA therapy, yet a further depletion was seen (CH50 value of 0.85). This data suggests there was a spike in complement activation upon initial infection, followed by a decrease in complement as infection progresses. It could be assumed the patient would have become complement deficient (similar to AS.1), if infection had not been cleared.

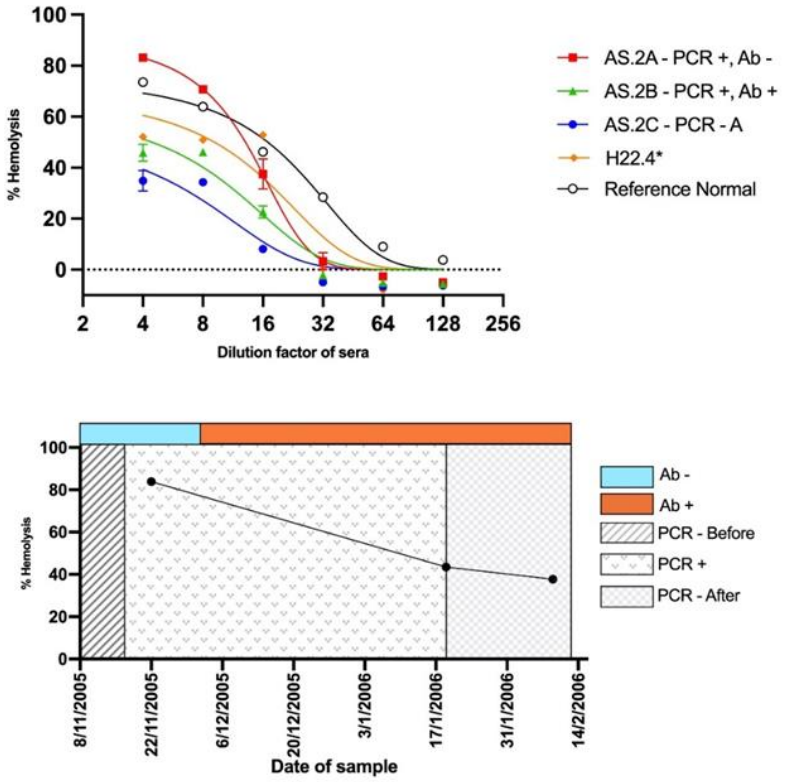


Figure 22: Complement quantification and timeline of an acute HCV infection and clearance.
A) Three samples collected from one HCV infected patient. AS.2A was two months after initial infection (13.07). AS.2B is seroconverted after another two months (4.43). AS.2C is PCR negative with clearance of the virus (0.85). CH50 assay quantified complement levels and CH50 value (dilution factor of sera for 50% lysis of cells) shown in brackets. H22.4*: 9.12, Ref Norm: 16.19. B) The three samples plotted on a timeline. Pattern boxes represent the PCR status, and the blue/orange boxes show the antibody status.

3.4.4 Acute Progression – Infection and clearance of AS.3

Interferon therapy was available to patients to aid clearance HCV infection. We tested samples which were during infection and post clearance of infection via interferon therapy. Treatment was initiated at the time of the first sample (AS.3A) which could contribute to the complement activity residing within normal range (**Figure 23**). Sample AS.3A was one month after the first positive PCR test. We also identified this sample was Ab positive so can assume initial infection was 6-8 weeks prior, due to the estimated time of seroconversion. There was little variance between the PCR positive and the PCR negative samples. There were also no significant differences between the CH50 values, with a small range of 2.49. We excluded the first point of AS.3C to ensure a good regression fit.

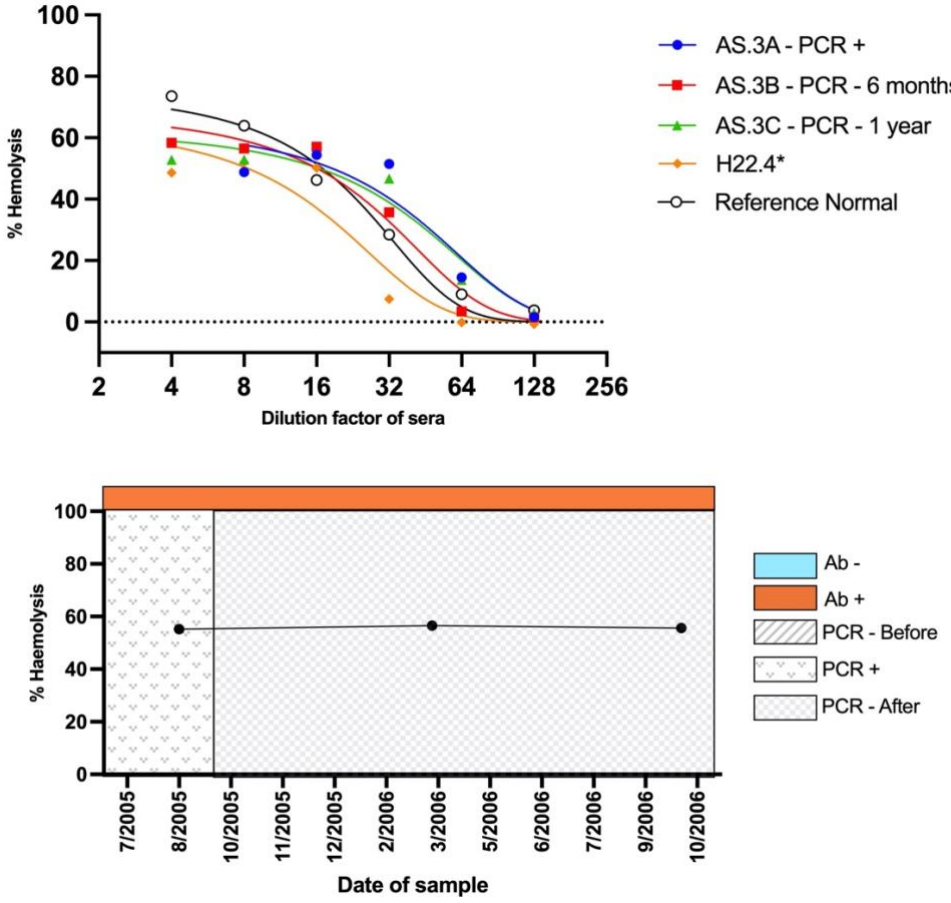


Figure 23: Complement quantification and timeline of an acute HCV infection and clearance.
A) Three samples collected from one HCV infected patient. AS.3A was one month after initial infection (18.57). AS.3B is 6 months after HCV clearance (16.08). AS.3C is one year after clearance (16.40). CH50 assay quantified complement levels and CH50 value (dilution factor of sera for 50% lysis of cells) shown in brackets. H22.4*: 9.12, Ref Norm: 16.19. B) The three samples plotted on a timeline. Pattern boxes represent the PCR status and the blue/orange boxes show the antibody status.

3.4.5 Acute Progression – Infection and clearance of AS.4

Having investigated the effect of initial infection on complement activity, it was necessary to understand the outcome once HCV infection has been cleared. We aimed to identify whether the complement can recover to similar quantities as before infection or whether the deficiencies remain until medical intervention. We were fortunate to find patient AS.4 who was PCR positive with a PCR negative (infection cleared) sample seven months after. It was evident that during infection the patient was complement deficient, with an undetermined CH50 value. Nevertheless, upon clearance (with DAA therapy) complement activity started to increase. The first PCR negative result was in two months after AS.4A, in which the complement regains function over the next five months (time at which AS.4B sample was taken). The PCR negative sample does not show complete replacement of complement (CH50 value of 8.28) however, it does show complement activity has improved and is functional. We cannot confirm at which point complement activity recovers or whether this is a gradual or immediate increase.

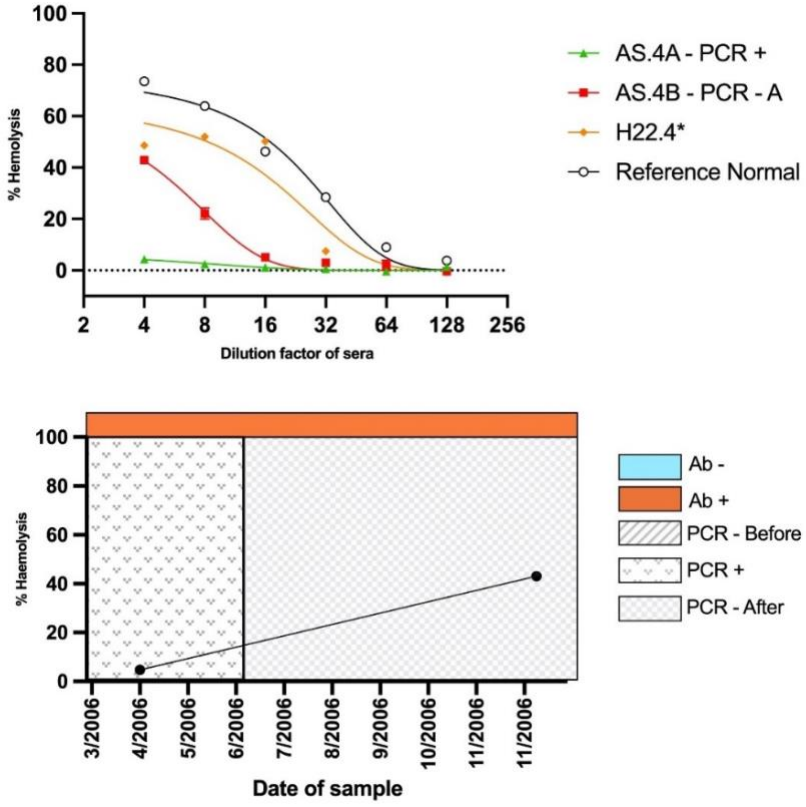


Figure 24 : Complement quantification and timeline of an acute HCV infection and clearance.
Three samples collected from one HCV infected patient. AS.4A is PCR +, seven months after initial infection (undetermined). AS.4B is 6 months later, PCR negative, recovered complement level (2.72). CH50 assay quantified complement levels and CH50 value (dilution factor of sera for 50% lysis of cells) shown in brackets. H22.4: 9.12, Ref Norm: 16.19. B) The three samples plotted on a timeline. Pattern boxes represent the PCR status and the blue/orange boxes show the antibody status.*

4 Discussion and conclusions

The aims of this study were to better understand the complex interplay between innate and adaptive immunity, with a specific focus on the interactions in response to HCV infection. Our initial experiments set out to establish experimental systems and evaluate the impact of potentially confounding effects within our research. We established the appropriate storage conditions of sera and ensured cell cytotoxicity was mitigated. Once we were confident in our experimental set up, we assessed the activity of complement, via activation of the classical pathway, in HCV samples from the acute and chronic phases of infection. This provided us with an understanding of complement activity present during infection and indicate that there may be consequences of altered complement activity. Furthermore, HCV pseudotype neutralisation assays were conducted to identify whether complement would improve or reduce antibody-mediated neutralisation. This interaction was further analysed with ELISA binding assays. The results build one of the first models of complement depletion throughout HCV infection.

One of the findings from this research was the identification of complement depletion within the classical pathway in chronically infected HCV patients. Complement activity depleted significantly, to which a CH50 value (dilution factor of sera to obtain 50% haemolysis of erythrocytes) could not be calculated. The CH50 assay demonstrates there is a lack of MAC complex formation and therefore an absence or significant impairment of one or more complement proteins. To be able to identify which specific pathway or protein is affected multiple assays need to be performed. Classical pathway deficiency is assumed with a decreased CH50 activity and normal alternative pathway haemolytic assay (AP50) value. However, the chronic HCV samples had a diminished CH50 activity which would suggest terminal pathway deficiency if paired with a AP50 value which is 10% below the normal range (El Sissy *et al*, 2019). A limitation of our research is that we could not identify the specific complement component(s) that were deficient, due to only carrying out a CH50 assay. Some samples had low but detectable classical complement activity which would indicate a sub-total deficiency in C5, C5 or C7, once again highlighting the value of conducing both assays simultaneously. Dumestre-Perard *et al*, (2002), also demonstrated that chronically infected patients have low CH50 values compared to healthy controls.

Past literature has focused on specific complement proteins and their loss of function in the presence of HCV. Inhibition of the classical and lectin pathway has been demonstrated in other flaviviruses such as the West Nile virus and Dengue virus (Avirutnan *et al*, 2010) Flavivirus NS1 protein binds to C1 and C4 in complex, recruiting complement-specific proteases to degrade C4 to C4b, protecting itself from complement neutralisation (Avirutnan *et al*, 2010). It could be assumed HCV may also bind and effect the functioning of C3 or C4 complement components during infection. Past literature demonstrated cleavage of C4 by HCV NS3/4A proteins, both *in vitro* and in cell culture using Huh7.5.1 cells (Mawatari *et al.*, 2013). Cleavage of C4 halts the activity of the classical and lectin pathways which causes a significant decrease in the level of virus neutralisation (50% reduction of pseudotype virus plaque formation) (Meyer *et al*, 2002). These results were further confirmed using a mutant NS3 (amino acid substitution) and an inhibitor of NS3/4A which both exhibited no cleavage of C4 (Mawatari *et al*, 2013).

Grazoprevir is an approved HCV DAA therapy, which inhibits the action of the NS3/4A proteins. The use of this inhibitor would show whether the cleavage of C4 causes a depletion in complement activity. Elbasvir is another DAA often used in conjunction with grazoprevir, which inhibits the HCV NS5A protein, that is shown to significantly decrease the expression of C9 and inhibit the activity of the C9 promoter (Kim *et al*, 2013). Inhibition of C4, C9 and the C9 promoter would cause detrimental effects to the complement system with interruptions to the classical and lectin pathway (C4) and limit all cell lysis due to no formation of the MAC complex (C9). Interestingly, patients with chronic kidney disease and chronic HCV infection genotype 1, had improved levels of C3 and C4 after a course of Graxoprevir and Elbasvir (Ogawa *et al*, 2018). Despite our results showing no differences in complement activity between genotypes, we cannot confirm all genotypes will improve C3/4 function during treatment. Furthermore, this specific combination therapy is directed at genotypes 1 and 4 so further limits the generalizability of the results.

Kim *et al* (2014), used hepatoma cells infected with cell cultured virus as well as liver patient specimens to identify the inhibition of classical C3 convertase activity in HCV infected patients. Decreased expression of C3 convertase and a low iC3b level reduces the cell-

mediated immunity as an outcome (Kim *et al*, 2014). iC3b is composed of can also function as an opsonin on pathogenic surfaces so inhibition of this is another loss of function. On the other hand, one of the largest complement deficiency studies looked at specific genetic and clinical characteristics of classical complement deficiencies. Undetectable CH50 activity was associated with low levels of one of the C1qrs complex or C2 protein (El Sissy *et al.*, 2019). Many studies focus on the activity of a specific complement protein. There are often differences in proteins between hosts, which reduces the precision of this method. C4 is highly polymorphic and differs between isotypes, so this would need to be considered. Using a large cohort of patients with a wide range of assays, would confirm the results of specific studies. Enzyme-linked binding assays are a powerful alternative as the amount of protein can be quantified even in conditions where the protein is non-functional. It can be discriminated if a protein is present at normal quantities, but the action is inhibited, or if the protein is depleted through degradation.

It is important to determine the specific complement deficiencies in HCV infections. The obvious applications of this research are informing research into future therapeutics and vaccine design. It would also be able to demonstrate if susceptibility to specific coinfections are likely. Four unrelated patients that had complete deficiency in C3 all presented with *S. pneumoniae* infections (Ram, Lewis and Rice, 2010). As deficiency of C3 components have been implicated in HCV infections, susceptibility to *S. pneumoniae* might be increased. Symptoms for *S. pneumoniae* infections include fever, cough, sensitivity to light and sleeplessness, which might be overlooked when acute HCV infection occurs (Centers for disease control and prevention, 2022). Mollah and Tam (2023), also highlighted there can be inadequate opsonisation of pathogen due to defects in complement 3b or its cleavage product C3ib, which can cause frequent sinopulmonary infections (Streptococcus pneumonia) and encapsulated bacterial infections (*Neisseria meningitidis* and *S pneumoniae*). Furthermore, El Sissy *et al*, (2019), emphasised the risk of pneumococcal infections in classical pathway deficiencies. Clinicians examining patients with acute and chronic HCV reported that co-infections with HCV infection are rarely observed. It is plausible that the redundant nature of the complement cascade goes some way to compensating for specific complement component deficiency. Dumestre-Perard *et al*, 2002), demonstrated the possibility for the lectin pathway to mediate complement response in the

presence of an inactive classical pathway. One hypothesis is that the other pathways can compensate complement activity during acute phase of infection but are not sufficient during chronic infections.

Our investigations led us to assess the point at which depletion in the complement activity occurs in chronically infected patients. We were fortunate to have access to acute samples from both pre- and post- infection. Our most significant result identified that before infection complement activity was within the normal range, but upon infection and seroconversion, the complement activity depleted. A patient who progressed to long-term chronic infection was determined to have undetectable levels of complement activity, whereas a patient who cleared infection did not experience such depletion. It could be suggested that HCV is inhibiting C3 action, and after clearance no further decline is observed. The negligible haemolytic activity found in the chronic patients could be secondary to C3 deficiency (Miller *et al*, 2012). Alongside this, we identified whether complement activity recovered after clearance of HCV infection. The results highlighted while PCR positive complement activity was diminished but once HCV RT-PCR negative for a period of months, complement activity started to recover. We could assume the inhibition of C3 components is while HCV is present, so once cleared the function returns, the cascade becoming operational and complement activity can be detected. This data clearly highlights how HCV infection has a substantial impact on complement activity during infection. However, this impact is not seen across all patients.

During our complement activity analysis (both acute phase infection and chronic phase CH50 assays) we observed patients who maintained complement activity, within the range of reference samples and healthy non-HCV infected sera. We questioned whether this innate immune response was due to characteristics of the specific infection. Lower viral loads could cause less inhibition of complement expression and host protein cleavage, accounting for the maintenance of complement activity. However, it was identified that there was no correlation between viral load and complement depletion within the chronically infected samples. Of note, the acute sample AS.3 displayed unusual characteristics. At the time of the sampling of the RT-PCR positive sample, interferon treatment had been administered and had effectively reduced HCV viral load, to which

complement activity could recover. However, the complement activity recovered quickly to within normal ranges, illustrating that they may have maintained this activity throughout acute infection. Earlier samples would need to be tested to form any conclusions.

It is important to recognise that naturally occurring polymorphisms in HCV NS3/4A could affect complement activity. Resistance associated substitutions in NS3/4A lead to negative HCV treatment outcomes and drug resistance (Zhou *et al*, 2016; Matthew *et al*, 2020). It is possible these polymorphisms can also affect the enzymatic activity of NS3/4A, altering the effect on the function of complement proteins. Therefore, the discrepancies in complement depletion may be influenced by viral polymorphisms during HCV infection. Patra, Ray and Ray, (2019), suggested that spontaneous clearance of infection is associated with a prompt induction of innate immunity. Therefore, it can be hypothesised that the patients who maintained complement activity, elicited an early potent innate response which mitigated the initial threshold for establishing chronic infection. Furthermore, the heightened innate response would ensure a strong adaptive immune response. This adaptive immune response may offer the protection, minimising damage requiring a lower complement response, in which the circulating complement level maintains.

Despite the small acute samples cohort, the results start to form a model (as seen in Figure 20 of complement activity during disease progression, which is currently not known but is critically important. The acute sample which showed recovery of complement activity would still be classified as low (10% below normal) CH50 activity (De Latour *et al*, 2015) and could be still at risk to problems associated with deficiencies. Further investigation could identify how prolonged the depletion is. Not only would chronically infected patients have diminished levels of complement activity for years but patients who have cleared infection may also suffer prolonged complement deficiency. This is a consequence of infection that DAAs may not recover. While DAA are successful in clearing infection in 95% of patients, they may not repair cellular damage caused by infection. Furthermore, this could also be damage caused by co-infections. DAAs do not prevent reinfection in which it would be desirable to have fully functioning complement activity to clear infection before further damage it caused (Li *et al*, 2018). All acute patients tested had normal complement activity before infection and couldn't clear infection without therapeutic assistance. Therefore, if

complement activity, especially C3 or C9, is low then the chance of clearing infection without permanent damage is minimal. Future research would need to assess a large cohort of patients who are undergoing HCV treated and monitor the recovery of complement activity. If this trend is seen across most infections, complement replacement therapy may be a potential therapeutic. Numerous cases of HCV infection and reinfection are seen in high-risk environments (Falade-Nwulia *et al*, 2018; Walters *et al*, 2023); therefore, it would be important to ensure patients return to normal complement activity to mitigate future complications and save resources.

There is potential for developing therapeutics agents which recover the significantly low complement activity via the classical pathway. Specific inactivation of a single protein will knock out the entire CP cascade. Substitute therapeutic molecules could be synthesised to counter the interactions blocked by the presence of HCV replication. The synthetic complement could act in the pathway to achieve complement activity. For this to be effective, a single complement protein, for example the C3, would need to be implicated across most patients as the source of complement deficiency. As detailed above, there are many components associated with HCV infection as well as the virus's ability to use complement to evade the immune response. An alternative approach may be a personalised complement replacement therapy, similar to MBL replacement therapy. Both therapies could be used in conjunction with DAAs to aid eradication of the virus infection. Our results identified the complement depletion (CH50 decline) within the acute phase of infection, therefore substitute therapy could assist the spontaneous clearance directly or indirectly by producing a stronger response of neutralising antibodies. Complement replacement therapy may be beneficial to patients who cannot recover complement activity, despite PCR negative. Furthermore, this could also be a short-term treatment to offer protection against confections. (Frakking *et al*, (2009) tested the safety of MBL replacement therapy and found there to be no related adverse reactions. However, there is a fine balance in complement levels. Dysregulation and overstimulation can have adverse effects due to the inflammation, therefore this would need to be extensively researched.

Within this research we identify the presence of complement activity in a variety of serum samples however, it was important to investigate the role of complement in antibody-

mediated virus neutralisation. Meyer *et al (*2002), demonstrated an increase in neutralisation of two human immunoglobulin G1 monoclonal antibody with the addition guinea pig complement. We hypothesised there would be a positive correlation between complement activity and neutralisation, as we increased the quantity of sera (containing complement proteins) there would be greater neuralisation of virus entry. Given that antibodies are naturally produced in the presence of complement, it would be logical that the presence of complement would be beneficial. When performing these experiments it was unknown whether there would be an additive or synergistic effect of complement on antibody binding and function. To obtain a broad understanding, both polyclonal HCV-infected IgG and HCV-specific monoclonal antibodies were used, with the supplementation of healthy sera (complement activity confirmed via CH50 assay and normal reference sample. Using heat inactivation method, complement activity was diminished in half the samples, allowing direct comparison of the effect of complement on antibody-mediated neutralisation. The neutralisation potential of all MAbs (Mab 1:7, AR3A and AR4A) improved in the presence of complement. This is consistent with past literature which identifies the positive relationship between complement activity and antibodies (Meyer *et al*, 2002). This effect was also observed in polyclonal antibodies (IgG from HCV-infected sera). There were varying effects within the samples, in which some samples showed a dose dependent effect (increasing serum (complement) quantity was proportional to increase neutralisation potential). Overall, these data confirm that the presence of human complement components may improve antibody mediated neutralisation, a significant progression for understanding their relationship and clinical relevance. Yet, it is plausible other heat-liable components of the serum may also contribute to this effect.

To expand these studies further, future work would perform similar experiments using cell cultured models of HCV replication, and conducting these sets of experiment using HCV-infected sera. These experiments require Level 3 containment facilities in which to culture the virus. These experiments would further demonstrate the effect of depleted complement activity and consequent lack of complement activity on antibody-mediated neutralisation. It is of utmost importance to understand whether the relationships and interactions were different with virus particles instead of pseudotypes. All research results require the validation before it can be generalisable to patient HCV infections.

Data retrieved from the Human Genome Atlas indicates low expression of CD55 and CD59 in Huh7 and HEK293T cells. Comparisons of the quantification cycle values (indicates the position of the amplification curve respective to the cycle axis, at the point to which amplification crosses the threshold for background noise), can be limited due to differences between runs and machines. Assessing the average Cq values, we can assume there was higher expression of CD55 in uninfected Huh7 cells compared to HEK293T cells. GAPDH, a housekeeping gene, was used for comparison of relative expression. High expression was seen with a ratio of 1:1.12 (GAPDH:CD55 in Huh7 cells). CD55 can be upregulated on HCV infected hepatocytes as a mechanism for immune evasion (Mazumdar *et al*, 2013).

CD59 was expressed at a lower level than CD55, however it was still significant enough to be a factor within the assays. The ratio of GAPDH mRNA expression to CD59 mRNA expression was 1:1.30. Human hepatoma cells can get protection from complement attack via cytokine-mediated up-regulation of CD55 and CD59 (Spiller et al, 2000). A greater expression improves the chances of HCV selectively incorporate CD59 aid protection against cell lysis of the MAC complex (CD59 inhibits MAC complex formation) (Amet et al, 2012). Therefore, our research aimed to identify the expression levels with HEK293T cells which are used for pseudotype production. CD59 was expressed the least within HEK293T cells however, the ratio of 1:1.37 (GAPDH:CD59) illustrates high expression. Despite the CD59 primers being performed in different runs, the average Cq values show equal expression levels between the cell lines and the ratios to GAPDH were close range.

Immunostaining confirmed the high expression of both proteins in Huh7 and HEK293T cells was proportional to the protein expressed on the cell surface. The proteins have a protective function to inhibit the action of complement and could have muted the full potential of the healthy sera within the neutralisation assay. To minimise this effect, Huh7 cells could be initially incubated with CD55 blocking peptide, to ensure all sites were occupied and all complement would be contributing to the neutralisation of the virus.

Having demonstrated the positive effect complement has on antibody-mediated virus neutralisation, we aimed to investigate the binding properties of them both working in

conjunction. The initial ELISA binding curves allowed us to evaluate the binding properties and calculate EC_{50} values for each HCV IgG preparation. The results illustrate there are differences in the binding potential of each IgG to E1E2 glycoprotein however, this may not the case for different viral antigens. When producing the pseudotypes and the cell lysate for the ELISA assay, we use a plasmid encoding for genotype 1 glycoproteins (clone UKN1A20.8). Genotype 1 has the greatest prevalence in the world (49.1 % in 201) and is the most widely used in HCV research therefore, for our findings to be translational, this is our chosen genotype (Petruzziello et al, 2016). Our cohort is represented with genotypes 1,2,3 and 4 (including different subtypes), which each exhibit different binding properties when used with genotype 1 E1E2 glycoproteins. Due to the similarities in genotype 1 and 3 any genotype 3 IgG still had good binding affinity. However, the lowest binding potential was exhibited by genotype 2 IgG. We would also expect to see the effect of genotype specific binding properties, which were seen in drug-protein interactions (Khalid et al, 2020). The IgG would have greater neutralisation capabilities against the genotype it was interacting with inside the human host, this was considered during analysis. Genotype has a substantial clinical relevance with regards to treatment, as various genotypes are seen to react differently to DAA therapy (Yan and Wang, 2017). Therefore, future neutralisation and ELISA assays could utilise the genotype specific to the HCV-infected sera.

To successfully reduce the damage caused by HCV infection, risks associated with reinfection and therapeutic vaccine design efforts, the innate immune response needs to be full understood. There has been a great effort to understand which complement proteins HCV interacts with and the outcome of this. Furthermore, the innate immune has been proficiently researched to understand the complement response to viral infections. However, there is limited research profiling the downregulation of complement and how this contributes to HCV clearance and eradication during chronic infections. Data suggests that control of HCV infection is associated with more rapid development of a broadly nAb response (Osburn *et al*, 2014). This process of rapid development may be reliant on the speed at which it activates the adaptive pathway. Therefore, understanding the true interactions is essential. Our results highlighted there is a decline in a complement activity, via the classical pathway, during acute infections. A reduced immune response would downregulate the activation of the AIS and possibly nAb response. On the other hand, HCV

mainly implicates the classical pathways through C1-C4 inhibition (Mawatari *et al*, 2013; Kim *et al*, 2014), in which the lectin pathway could compensate of this loss. A low CH50 value for chronically HCV-infected sera correlated with a low specific C4 binding (Dumestre-Perard *et al*, 2002) Specific C4 binding is a valuable measurement of the ratio of haemolytic C4/C4 protein time by 100. A reduction in specific C4 binding shows complete activation therefore, with a non-functional classical pathway, this gives evidence the lectin is still causing the breakdown of C4. During infection, some monoclonal and polyclonal antibodies can cause complement activation (Goldberg and Ackerman, 2020). Therefore, stimulating a stronger adaptive activation could in turn keep continuous activation of the complement activity. If fast nAb response is correlated with clearance of infection, the generation of broad anti-HCV nAB prior to infection, may offer the immune system an advantage over the virus (Osburn *et al*, 2014). This was independent of the infection viral genotype, providing further evidence for the role of nAb in controlling HCV infection and the potential benefit of generating broad anti-HCV nAb responses by vaccination (Osburn *et al*, 2014). HCV-specific antibodies have been identified to be circulating up to 7 years after infection and upon reinfection, liver resident CD8+ T cells contributed to rapid acquisition of virus-specific cytolytic activity (Shoukry et al, 2003). This further supports the production of a HCV vaccine, with the requirement of a vaccine course to maintain antibody titres. Vaccine trials have failed due to HCV ability to escape the T cell response; therefore, we require knowledge of other mechanism which can mitigate HCV (Velázquez-Moctezuma *et al.*, 2019). Complement has been found to enhance the neutralising potential of antibodies induced by a vaccine (Li *et al*, 2017). We could hypothesise that additional innate activation and complement activity could ensure a successful response to an HCV therapeutic vaccine. Administering the vaccine before infection, during a point when most hosts would have 'normal' complement activity, may improve the production of antibodies in response to the vaccine. In turn, producing a greater immune response to HCV and limiting the progression of disease. A great deal of research is required to fully understand complements interaction with antibodies with regards to a vaccine however, complement does seem to be key for future success.

4.1 Limitations and future work

There were a few limitations identified as we progressed this project. Some were technical, while others occurred due to the number and nature of samples that were available. Technically, the main limitations of the research proved to be the non-specific binding signal with the complement and antibody ELISAs. Our experiment used lysate from HEK293T cells expressing the HCV glycoproteins. To control for non-specific binding, we used a control lysate which were solely expressed the proteins naturally occurring in HEK293T cells. We expected to have high background signal due to the high concentrations of sera, which were anticipated to contribute to non-specific binding. However, we took many approaches to mitigate this effect. Adaptions to the blocking solution and dilutant did not sufficiently reduce the non-specific binding. It was necessary to use the control lysate, but this was affecting us from perceiving the true results. To eliminate this factor, we would need to extract the E1E2 glycoproteins using GNA coated agarose beads, in which we could be confident this is the only protein present in the binding assay. Additionally, this removes the need to control for non-specific binding of the serum and the results would show the true relationship of binding between the complement proteins and antibodies. This assay would demonstrate whether the proteins compete for binding or whether one causes conformational changes to the binding site, to aid future binding. This data would supplement the neutralisation data. Due to time restrictions of the MRes, this experiment could not be carried out however it would be a valuable experiment for future work.

Obtaining a comprehensive range of samples from specific individuals is important when identifying new trends of infection. This was a limiting factor for this research, as all samples available were biobanked sera that were not specifically collected for this study. Due to the complex host-viral interactions, and the extensive genetic plasticity exhibited by HCV, the HCV genotype can be a major factor in predicting the patient's response to treatment (Yan and Wang, 2017) and pathogenesis (reference). Because of this, it will be important in future work to include representatives of all genotypes in the serum samples analysed, as well types of virus, including the types of pseudotypes utilised in neutralization experiments. The viral populations can be considered as quasispecies, the individual members of which display differences in the transmission and the pathogenesis (Pawlotsky, 2003). HCV infection occurs in a higher proportion of males than females (Csete, 2008).

However, gender-specific risk factors have been confirmed for virological non-response of infection, enhancing the need for suitable distribution of both genders throughout the samples (Schott *et al.*, 2007). Ethnicity can also be associated as an HCV risk factor with more Asian and Hispanic patients developing cirrhosis and HCC incidence than Caucasians (Le *et al*, 2017). HCV infection can be influenced by host factors, leading to differences in immune responses. The samples collected were considerably carefully to offer the best representation, yet lacked full diversity, due to being repurpose from previous HCV studies.

Another limitation was the small sample size with used for the experiments. As an exploratory study, the results provide important evidence that can be investigated further, but it is difficult to generalise our findings to the whole HCV-infected population and derived models of complement activity during HCV infection. Samples were chosen to represent the different stages of infection, pre or post infection, as well as infection outcomes of clearance or chronic infection. Due to the variation in sample collection because of the retrospective nature of sampling, a full clinical history could not be ascertained. This issue was compounded due to the short timelines of the study. A greater selection of samples from several patients would be required to allow accurate trends to be confirmed. Furthermore, the cohort of acute phase samples was small, which limits our ability to generalise these findings. Most acute phase infections are often overlooked due to the asymptomatic nature, in which most patients only receive treatment once chronically infected, when symptoms present.

Development of this research to further improve our understanding of the effects of complement deficiency on patients requires the design of a new study. This study would need to longitudinally assess patients throughout acute and chronic HCV infections, collecting regular serum samples to assess complement activity while monitoring all symptoms. With a large cohort, trends of component deficiencies and subsequent infections could be resolved. This would inform possible therapeutics needed alongside HCV treatment to address the complement deficiencies associated with infection.

A further limitation proved to be the storage of previously studied samples. There were many samples used in the BOSON clinical trial (Foster *et al*, 2015) that would potentially allow us to interrogate the change in complement activity during antiviral therapy, representing the different patterns of complement activity during and after treatment. The samples were stored in the fridge at 4°C for over three months, which clearly impacted on their suitability for this study. It is clear from the results presented here that future work must ensure that all samples have been stored appropriately, ideally stored at -70°C to maintain function of complement (**Figure 9**). Therefore, these valuable samples were excluded from the data. To overcome this limitation, future work would require ethic approval to obtain fresh serum samples, to achieve confidence in the results based on appropriate storage and handling.

A major focus for future work is to further develop these findings and trends using authentic virus particles. HCV pseudotypes offer a safer and more manipulable method for virus interrogation, yet the full interactions and effects cannot be assumed to accurately model the surface of the HCV particles. While it is known that Hepaciviruses do not have the classical virion structure displayed by other Flaviviruses, and can incorporate host cell proteins into these infectious particles, it is likely that the retroviral particles displaying HCV structural glycoproteins do not completely mimic the natural particles.

While this research focussed exclusively on HCV, it is possible that the findings can be extrapolated to other virus infections, particularly those that infect hepatocytes. Low C3, C4 and CH50 activity has been identified in patients with Hepatitis B Virus (HBV) related-acute-on-chronic liver failure, in which C3 levels is correlated to greater mortality (Zhang *et al.*, 2016). These results follow trends seen within HCV infection. Future work would establish whether the targeted site of infection correlates with complement depletion. Both HCV and HBV infection target the liver, the site of most complement production, thus initial progression would be to confirm the HCV findings within HBV samples. Leading on from this, it would be important to see whether the different forms of complement depletion are associated with chronic virus infection with a different target site. Lymphocytic choriomeningitis mammarenavirus (LCMV) infection in mice is the standard model virus for investigating chronic viral infections and the immune response to infection, affecting the

brain and retina (Bonthius, 2012). Identifying the presence of complement activity depletion in a virus that does not infect the liver would provide a useful comparison to identify if these complement-altering effects are limited to virus-host interactions in hepatocytes. On the other hand, if complement activity was fully functional, this would spotlight the effect on the liver and provide significant knowledge for future HCV vaccines and therapeutics.

Within this research a range of methods have been developed which will allow further investigations into other viruses and the effect on the innate immune system. While HCV infection can be functionally cured by the new generation of DAAs, the consequences of HCV on the innate sensing remains to be determined.

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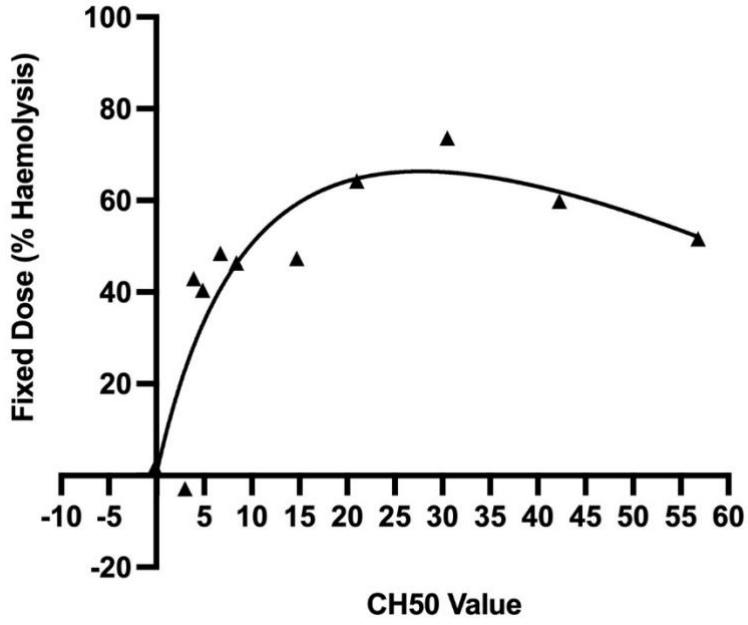
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Appendices



Appendix 1: Correlation between fixed dose percentage haemolysis and CH50 values.
Highlighting the relationship between the percentage haemolysis at a fixed dose (1/8) against the calculated CH50 value. Correlation can be assumed. Low/undetectable amounts of haemolysis (low complement) is mirrored with a low CH50. If there is high quantities of complement present, there is high percentage haemolysis which correlates with a high (low dilution factor) CH50 value. This relationship allowed us to be confident in our fixed dose statistical analysis. The model of the single interaction binding curve is due to the EC50 values better differentiating at the top points.