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Therapeutic outcomes, immunological responses to treatment and vaccine
preventative strategies for Hepatitis C in HIV seropositive individuals

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Philosophy (PhD)

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Declaration

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Summary of thesis

HIV and Hepatitis C virus (HCV) represent significant causes of morbidity and mortality worldwide. Because of shared routes of transmission co-infection is common, affecting an estimated 2.3 million people worldwide. Recent advances in treatment for both HIV and HCV look set to significantly alter the natural history of both diseases and offer long-term hope for patients. The advent of direct-acting antiviral (DAA) therapy for HCV has markedly improved therapeutic outcomes for patients engaged in care.

Despite, excellent tolerability and high rates of sustained virological response (SVR) with these new agents, they are costly, associated with drug-drug interactions, do not prevent against re-infection and are not 100% effective. Additionally, HCV viral resistance may represent a challenge in the future. DAA therapy will have a limited impact on the burden of HCV-related disease on a population level unless barriers to HCV education, screening, evaluation and treatment are addressed and treatment uptake improves. At present, no commercial vaccine exists for prevention of HCV.

The aims of this thesis were threefold. Firstly, I looked to examine therapeutic outcomes for patients with HIV/HCV co-infection treated for HCV from 2001 to 2016. A significant difference in mortality and liver-related morbidity was observed between patients who obtained and didn't obtain a SVR with interferon-based treatment regimens. Outcomes for people who inject drugs (PWIDs) were similar to non-PWIDs. An increase in treatment uptake and SVR rates was seen with the new DAA regimens.

Secondly, further understanding of the complex interaction between HCV and the immune system is likely to yield benefits in terms of therapeutic and preventative strategies. We looked to examine alterations in the IFN- α JAK-STAT signaling pathway in primary immune cells of co-infected patients as a result of HCV treatment with a Telaprevir-based regimen. We found that STAT1 protein levels are reduced from primary immune cells in patients with HIV/HCV co-

infection and Telaprevir-based regimens resulted in on-treatment restoration of STAT1 levels in these patients

Finally, towards a preventative strategy for HCV in HIV patients, we looked to examine the safety and immunogenicity of novel vaccine candidates (AdCh3NSmut1 and MVA-NSmut) delivered in a prime-boost regimen. We showed that AdCh3/MVA vaccination in HIV-infected patients is safe and induced T-cell responses in the majority of antigenic pools tested by ELISpot assay in all individuals.

This work has chronicled the improvements in therapeutic outcomes for HCV therapy in co-infected patients and shown the benefits of obtaining a SVR in HIV/HCV co-infected patients. I have outlined a previously undescribed phenomenon in HIV/HCV co-infected patients where IFN-containing DAA therapy has resulted in a significant increase in STAT1 protein from primary immune cells. This work has also evaluated the safety and immunogenicity of a novel vaccine strategy in HIV-positive patients. The vaccine strategy has been shown to be safe and highly immunogenic.

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Glossary of commonly used abbreviations:

AdCh	Chimpanzee Adenovirus
AdHu	Human Adenovirus
AE	Adverse Event
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine aminotransferase
ART	Antiretroviral therapy
AST	Aspartate aminotransferase
BA	Beta Actin
CD4	A specific receptor molecule on a T lymphocyte helper cell
ConA	Concanavalin A
CTL	Cytotoxic T Lymphocyte
DAAs	Direct-acting antiviral agents
dH ₂ O	Distilled water
EAP	Early Access Program
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Environmental Protection Agency
ESLD	End Stage Liver Disease
FBC	Full Bloods Count
FEC	Flu/EBV/CMV
FP	Fowlpox
GMO	Genetically Modified Organism
GUIDE	Department of GU Medicine and Infectious Diseases
HAART	Highly Active Antiretroviral Therapy
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HC	Healthy Control
HCV	Hepatitis C Virus
HCC	Hepatocellular Carcinoma
HCl	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HPRA	Health Products Regulatory Authority
HPSC	Health Protection Surveillance Centre
II	Integrase Inhibitor
IFN	Interferon
IP-10	IFN- γ induced protein 10
JAK	Janus Kinase
LFTs	Liver Function Tests
MELD	Model for End-Stage Liver Disease
MHC	Major Histocompatibility Complex
MVA	Modified Vaccinia Ankara
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NS	Nonstructural
PCR	Polymerase Chain Reaction
PEACHI	Prevention of HCV and HIV-1 co-infections
PI	Protease Inhibitor

pSTAT	Phosphorylated STAT
RP	Renal Profile
RPR	Rapid Plasma Reagin
RVR	Rapid Virological Response
SAE	Serious Adverse Event
SOCS	Suppressor of Cytokine Signaling
SOP	Standard Operating Procedure
STAT	Signal Transducer and Activator of Transcription
SUSAR	Suspected Unexpected Serious Adverse Reaction
SVR	Sustained Virological Response

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Chapter 1 Introduction

1.1 Hepatitis C Virus

During the 1970s, when serological tests became available for Hepatitis A virus (HAV) and Hepatitis B virus (HBV), it became evident that another agent caused the majority of cases of transfusion-associated hepatitis [1]. Efforts to identify the aetiological agent through culture, electron microscopy and serological means were unsuccessful, leaving investigators frustrated for many years. A key finding, using chimpanzee models, in the mid-1980s revealed that the causative agent may be a small, enveloped RNA virus [2]. Further work in the late 1980s identified a virally encoded antigen associated with non A, non B hepatitis [3] leading to molecular cloning of the complete viral genome [4] and the virus being named Hepatitis C virus (HCV). HCV was the first virus identified by a direct molecular approach, without tissue culture, electron microscopy or serology. This discovery introduced a new dimension in viral research and innumerable new viruses have been discovered by this or similar methods since.

HCV belongs to the Hepacivirus genus of the Flaviviridae family. It is an enveloped virus with an icosahedral capsid that contains a 9.6 kb-long, single-stranded, positive sense genomic RNA. The genomic RNA is translated into a viral polyprotein which is cleaved by cellular and viral-encoded proteases to generate the capsid protein (C); the two glycoproteins E1 and E2 that are expressed as heterodimers on the virion surface; p7 a small protein belonging to the viroporin family of ion channel proteins, which is essential for particle formation; viral proteases NS2 and NS3, and the non-structural proteins NS4A, NS4B, NS5A and NS5B, which are required for viral RNA replication. Collectively, these proteins contribute to various aspects of HCV life cycle, including viral attachment, entry and fusion, HCV RNA translation, posttranslational processing, HCV replication, virus assembly and release.

HCV infects only humans and chimpanzees. It can be classified into distinct genotypes (1-6), whose distribution varies both geographically and between risk

groups [5, 6]. Each of the six main genotypes of HCV is equally divergent from the others, differing at 31 to 34% of nucleotide positions on pairwise comparison of complete genomic sequences, leading to approximately 30% amino acid sequence divergence between the encoded polyproteins [6]. These approximately equidistant genetic groups each contain a variable number of more closely related, genetically and epidemiologically distinct subtypes. These subtypes are found to differ from each other by 20-25% at the nucleotide level [6].

Of the variants circulating in the Western world, 1a, 1b, 2a, 2b, 3a, 4a, and 6a are the most common subtypes identified. The distribution of HCV subtypes reflects their recent epidemic spread. For example, genotypes 1b, 2a and 2b are prevalent in elderly populations throughout Europe and are frequently linked to past blood transfusions, whereas genotypes 1a and 3a typically infect injecting drug users. Genotype 4a is most frequently found in the Middle East [7]. A distinct pattern of viral diversity is observed in South East Asia and sub-Saharan Africa, where infections with individual genotypes predominate over large geographical areas, within which there is substantial genetic diversity. This pattern of diversity is the aftereffect of the recent epidemic spread of HCV into new risk groups, superimposed on top of the much older endemic circulation of the virus in South East Asia and sub-Saharan Africa [7].

Within an individual, HCV exists as a swarm of closely related but distinct viral strains or 'quasi-species.' This is because of the high mutation rate of HCV due to a lack of proof-reading capacity in the HCV RNA dependent RNA polymerase. Sequence diversity varies across the genome and is greatest in the E1, E2 and NS2 genes (coding for the envelope glycoproteins and the NS2 protease respectively) with ~40% variability between genotypes. By contrast, the non-structural polypeptide spanning from NS3 to NS5b is more conserved [6].

Pathophysiology and Natural History

HCV is one of the major aetiological agents for chronic viral hepatitis. The term chronic viral hepatitis is used to describe sustained hepatocellular necrosis and inflammation, frequently with fibrosis, that lasts longer than 6 months. Chronic viral hepatitis are classified histologically by the degree of hepatocellular necrosis and inflammation (grade) and the fibrosis (stage) [8]. Like the other hepatitis viruses, HCV is not cytopathic. Instead, the presence of virus-infected cells results in an on-going host-mediated cytolytic T cell response that is ineffective in achieving adequate clearance of all virus-infected cells. Persistent infection can progress to chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC). It is generally a slowly progressive disease characterised by persistent hepatic inflammation, leading to the development of cirrhosis in approximately 10–20% of patients over 20–30 years of HCV infection [9].

The natural history of HCV is variable [10]. A proportion (20-25%) of individuals will spontaneously clear HCV infection, but approximately 80% of acutely infected individuals will develop chronic infection. Primary (acute) infection is usually asymptomatic, although some patients may present with jaundice. The risk of reinfection remains a possibility after clearance of acute hepatitis C. Host factors that have been suggested to correlate with a higher frequency of chronicity include male gender, older age, ethnicity, mild/unapparent acute infection, immunological compromise and certain HLA haplotypes [10].

Variability in progression of chronic HCV remains poorly understood. Extreme differences in outcomes between patient cohorts have been described. For example, high rates of progression to cirrhosis (30% at 11 years) were seen in a group of patients who received contaminated immune globulin [11], compared to very low rates of progression to cirrhosis in patients who received contaminated anti-D Rh immunoglobulin (0-2% at 17-20 years) at childbirth [12, 13]. In a recent systemic review of 111 studies of prognosis of chronic HCV, it was found that rates of progression from histological fibrosis stage to stage varied (nonlinear progression) and the overall progression to cirrhosis after 20

years was 16% [14]. Excess alcohol consumption is felt to increase the risk of end stage liver disease [15]. Once cirrhosis is established, the risk of HCC is approximately 1-4% per year [16].

Spontaneous clearance of chronic HCV is rare. The incidence rate of spontaneous clearance in a large Scottish patient cohort was recently estimated at 0.36/100 years of patient follow up [17]. Clearance was associated with certain host and viral factors. Positive associations include female sex, younger age at infection, co-infection with HBV and a low HCV viral load [17]. Intravenous drug use was negatively associated with spontaneous clearance.

HCV, in addition to direct liver disease, is also associated with several extrahepatic manifestations [18]. Mixed cryoglobulinaemia may develop in patients. This is a small vessel vasculitis that can have presentations ranging from cutaneous, neurological and visceral involvement to glomerulonephritis. It has also been associated with B cell non-Hodgkin lymphoma. HCV patients have higher rates of insulin resistance, diabetes and atherosclerosis, which may lead to increased cardiovascular morbidity and mortality. Fatigue and cognitive impairment are neurological manifestations of HCV [18]. Patients with HCV have impairment in quality-of-life scores [19]. The mechanisms causing these extrahepatic effects are likely multifactorial and may include endocrine effects, HCV replication in extrahepatic cells [20, 21], or a heightened immune reaction with system effects. Effective viral suppression induces reversal of most extrahepatic manifestations [18].

Another clinically important issue is that of co-infection with other hepatitis viruses. Patients co-infected with chronic HBV have an accelerated course of HCV disease [22] and super-infection with HAV can result in a substantial risk of fulminant hepatitis and death [23]. All patients with HCV infection should be tested for other hepatotropic viruses [24] and non-immune patients should be vaccinated.

Transmission

HCV is transmitted by blood and now occurs primarily through injecting drug use, and less frequently, through sex with an infected partner, occupational exposure, and maternal-foetal transmission. Co-infection with HIV seems to increase the risk of sexual transmission of HCV [25, 26]. In some cases, no risk factors can be identified [27]. Transfusion-related HCV infection is rare now since the introduction of routine screening of blood for HCV antibodies, and subsequent molecular testing, in the early 1990s.

Figure 1-1 details the most likely risk factor for HCV acquisition in Ireland for the years 2007 – 2016.

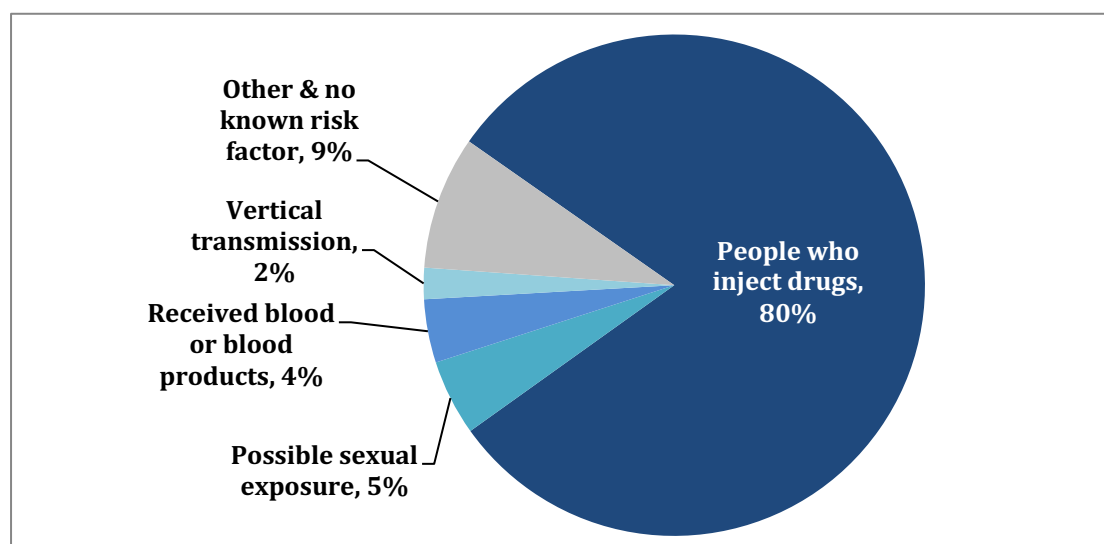


Figure 1-1 Risk factors for acquisition of HCV in Ireland

HPSC data for most likely risk factor (%) of acquisition for cases of hepatitis C notified 2007-2016 (where data available, n=5255, 51%, accessed HPSC.ie, 23/4/17)

Diagnosis

The diagnosis of acute and chronic HCV infection is based on the detection of HCV RNA by a sensitive molecular method (lower limit of detection typically <15 international units [IU]/mL). Anti-HCV antibodies are detectable by enzyme immunoassay (EIA) in the vast majority of patients with HCV infection, but EIA results may be negative in early acute hepatitis C and in profoundly immunosuppressed patients. Following spontaneous or treatment-induced viral

clearance, anti-HCV antibodies persist in the absence of HCV RNA but may decline and finally disappear in some individual [28].

HCV reinfection has been described after spontaneous or treatment-induced HCV clearance, essentially in patients at high-risk of infection e.g. people who inject drugs. Reinfection is defined by the reappearance of HCV RNA at least 6 months after a SVR and the demonstration that infection is due to a different HCV strain (a different genotype or distantly related strain by phylogenetic analysis if the genotype is the same).

The diagnosis of chronic HCV infection is typically based on the detection of both anti-HCV antibodies and HCV RNA, which generally occurs in the presence of biological or histological signs of chronic hepatitis. Since, in the case of a newly acquired HCV infection, spontaneous viral clearance is very rare beyond 4 to 6 months of infection [17], the diagnosis of chronic HCV infection can be made after that time period.

Assessment of disease liver severity is recommended prior to therapy for HCV. Identification of patients with advanced fibrosis or cirrhosis is of particular importance, as the choice of treatment regimen and post-treatment prognosis are dependent on the stage of fibrosis. A liver biopsy is considered the gold standard for staging of liver disease. A liver biopsy is generally considered a safe procedure, but complications of liver biopsy can include pain and bleeding and, typically, a biopsy requires admission to a hospital as a day case.

However, considerable recent evidence suggests that non-invasive methods, including liver stiffness measurement and panels of biomarkers of fibrosis, can be used to assess liver fibrosis and the presence of portal hypertension [29]. Both methods perform well in the identification of cirrhosis or no fibrosis, but they perform less well in identifying intermediate degrees of fibrosis [29]. These non-invasive methods have now largely superseded biopsy when making therapeutic decisions for the majority of patients. Liver biopsy may be indicated

in cases of contradictory results with non-invasive markers and in cases of known or suspected mixed aetiologies e.g. metabolic syndrome or autoimmunity.

Prior to treatment initiation, the HCV genotype, including genotype 1 subtype (1a or 1b), should be assessed. This will determine the choice of therapy, among other parameters. At the time of writing, systematic testing for HCV resistance prior to treatment is not recommended [30]. No standardised tests for the resistance of HCV to approved drugs are available as purchasable kits at present and resistance testing relies on labour-intensive in-house techniques based on deep sequencing or population sequencing (Sanger sequencing) [31].

Treatment

Recent advances in therapeutics have revolutionised the management of patient identified with chronic HCV. These advances are discussed in the context of HIV/HCV co-infection later in this chapter. Current guidelines recommend that all treatment-naïve and treatment-experienced patients with compensated and decompensated chronic liver disease related to HCV, who are willing to be treated and who have no contraindications to treatment, must be considered for treatment [24]. Treatment is not recommended in patients with limited life expectancy due to non-liver related co-morbidities.

The primary goal of HCV therapy is to cure the infection, i.e. to achieve a sustained virological response (SVR) defined as undetectable HCV RNA 12 weeks or 24 weeks after treatment completion. The infection is cured in more than 99% of patients who achieve a SVR. A SVR is generally associated with normalisation of liver enzymes and improvement or disappearance of liver inflammation and fibrosis in patients without cirrhosis [24]. Patients with severe liver disease remain at risk of life-threatening complications; however hepatic fibrosis may regress and the risk of complications such as hepatic failure and portal hypertension is reduced. Recent data suggest that the risk of HCC and all-cause mortality is significantly reduced, but not eliminated, in cirrhotic patients who

clear HCV compared to untreated patients and non-sustained virological responders [32, 33].

Prevention

Even though treatment for HCV has existed for several decades, there still remains no commercial vaccine available at present for HCV. Since sequence variation in HCV genotypes has implications for vaccine development; it may be necessary to develop immunogens that induce cross-reactive immune responses in order to achieve protection against multiple genotypes. In addition, development of an effective therapeutic vaccine will need to address viral diversity within infected individuals.

1.2 HCV and the Immune Response

The human immune system represents an extraordinary and complex barrier against environmental threats, such as harmful bacteria and viruses, and comprises many effector systems, cells and molecules, which are present in all the organs of the body and defend it from infection and disease.

Two main mechanisms, the innate and the adaptive immune systems, are effective providers of anti-viral immunity. The innate immune response represents the first non-specific mechanism of defence against pathogenic entry, and it is capable of repeatedly eliminating many potential infections through activation of Pattern Recognition Receptors (PRRs).

In case of failure of the innate response, the more specific adaptive immune mechanism takes over and acts in a more targeted manner, retaining immunological memory. The adaptive immune system is composed of both humoral and cellular responses. In the humoral or antibody response, B cells are activated to produce antibodies (also known as immunoglobulins). Antibodies are large Y-shaped proteins used by the immune system to identify and neutralize foreign materials. T cells are responsible for carrying out cell-

mediated immune responses. CD4⁺ T lymphocytes, also called "helper" T cells, are immune response mediators, and play an important role in establishing and maximising the capabilities of the adaptive immune response. These cells have no cytotoxic or phagocytic activity; and cannot kill infected cells or clear pathogens, but in essence manage the immune response, by directing other cells to perform these tasks. Cytotoxic CD8⁺ T cells are another sub-group of T cells that induce the death of cells that are infected with viruses (and other pathogens) or are otherwise damaged or dysfunctional. Like other viruses, including HIV, HCV has evolved an immune evasion mechanism which allows it to overcome the body's natural anti-viral defences, with major consequences, not only for spontaneous viral clearance, but also for response to conventional therapies.

Dendritic cells (DCs) are an important cell population involved in innate immunity and represent key mediators of anti-viral immunity. They are classified into conventional DCs (cDCs), which target a wide range of pathogens, and plasmacytoid DCs (pDCs), which, in response to viral infection, produce large amounts of Type I IFNs, and have been implicated in controlling HCV infection [34].

Upon viral infection, PRRs of the innate immune system sense the virus as "non-self", via the identification of defined conserved patterns within viral RNA, DNA and/or protein called Pathogens Associated Molecular Patterns (PAMPs). PRRs trigger signalling cascades, leading to transcription of numerous anti-viral genes, including IL-1 β and Type I and III Interferons (IFNs), which are then secreted by the infected cells and activate cytokine-specific signalling pathways [35].

The innate immune response utilises three main classes of PRRs to sense HCV: Toll-Like Receptors (TLRs), the Retinoic Acid-Inducible Gene I (RIG-I)-like Receptors (RLRs), and the Nucleotide oligomerisation domain-Like Receptors (NLRs) [35, 36]. PRRs are highly expressed in bone marrow-derived immune cells, such as the liver-based macrophages (Kupffer cells) and hepatic dendritic cells, but also on resident liver cells [hepatocytes, liver sinusoidal endothelial

cells (LSECs), and hepatic stellated cells] [37]. The HCV NS3/4A protease efficiently cleaves and inactivates important signaling molecules in the sensory pathways that react to HCV PAMPS (reviewed in [38]).

The Interferon Anti-viral System

Interferon (IFN) was identified in 1957 when chick embryos infected with an inactivated influenza virus produced and released an unknown protein into the surrounding fluid that was able to protect non-infected cells against viral infection. This factor was simply named “interferon” as it “interferes” with viral infection [39]. This discovery represented a significant milestone in understanding how the immune system responds to pathogens and in particular viruses. Nearly four decades later, the IFN Janus Kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) signalling pathway has been well characterised, has provided new insights into the anti-viral immune response and the mechanism of many diseases.

IFNs are a multi-gene family of inducible cytokines that are best known for their potent anti-viral properties [40-42], although they also play a critical role in cell growth [43] and have immunomodulatory effects [44]. There are three main classes of IFNs: Type I, Type II and Type III.

Type I IFNs include IFN- α , IFN- β , IFN- ϵ , IFN- κ , and IFN- ω , which are expressed in humans, IFN- δ , which is found in pigs and cattle, IFN- ζ , only expressed in mice, and IFN- τ , only found in cattle [45-47].

Type II IFN is only represented by IFN- γ and it is mainly involved in mycobacterial infections and is only expressed in activated T cell, macrophages and NK cells. Type II interferons are also important in antiviral immunity.

Type III IFNs are classified into four family members: IFN- λ 1, [Interleukin-29 (IL-29)], IFN- λ 2 (IL-28A), IFN- λ 3 (IL-28B), and the newly discovered IFN- λ 4 [48-50]. Until recently, IFN- λ family's role in anti-viral immunity has been mainly

unknown. However, most recently the discovery of a single gene polymorphism (SNP) upstream of the IFNL3 (IL-28B) gene which predicts viral clearance in acute Hepatitis C infection and also response to exogenous IFN treatment, has highlighted the importance of this family of cytokines in HCV infection [51].

IFN- α establishes an anti-viral state via the intracellular JAK-STAT signalling pathway (See Figure 1-2), resulting in upregulation of several hundred IFN-Stimulated Genes (ISGs). Binding of Type 1 IFNs to their corresponding receptor results in auto-phosphorylation and activation of the receptor-associated JAK kinases, and subsequent recruitment of STAT transcription factors, which form homo- or heterodimers and translocate into the nucleus where they bind to specific promoter elements. IFN- α -mediated JAK-STAT signalling involves activation of the receptor-associated tyrosine kinase (TYK)2 and JAK1 and the STAT1-STAT2 heterodimer, which ultimately binds to a third transcription factor, IFN regulatory factor (IRF)9, thus forming the IFN-stimulated gene factor 3 (ISGF3) complex, which, once in the nucleus, binds to specific promoter sites, known as IFN-stimulated response elements (ISREs) that triggers ISG transcription.

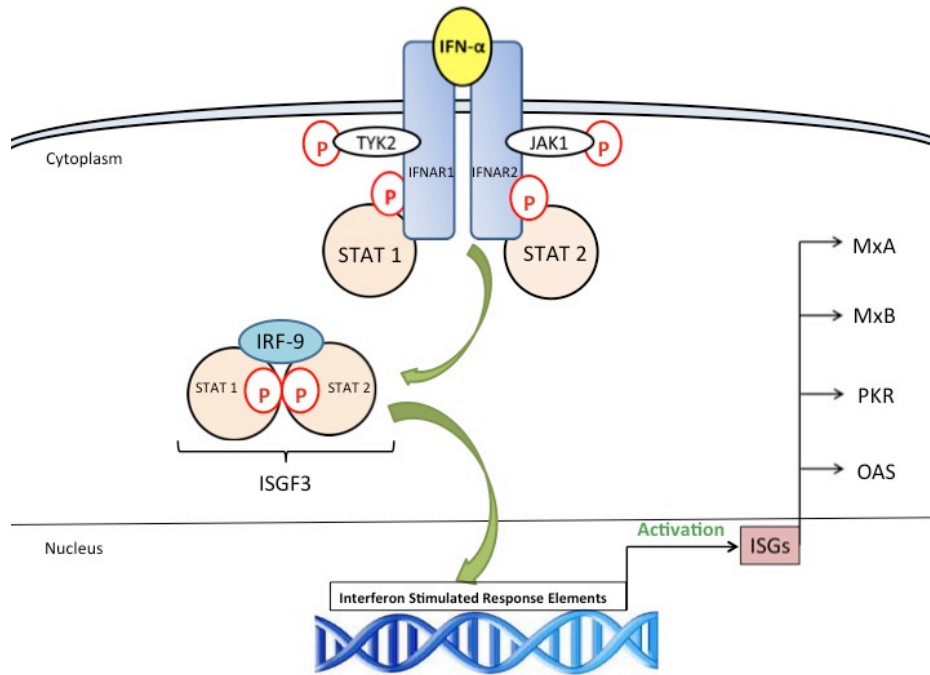


Figure 1-2 The JAK-STAT Signaling Pathway

Secreted Interferon binds to and activates its cognate receptor, which in turn recruits kinases of the Janus kinase family. These kinases phosphorylate STAT1 and STAT2 leading to their heterodimerization and association with IRF9. This complex, termed ISGF3, can translocate to the nucleus where it stimulates transcription of ISGs. ISG mRNA is then translated in the cytoplasm giving rise to potent anti-viral effector proteins. HCV has been reported to subvert these innate immune defense mechanisms at several levels. (Figure – Courtesy of Paul Kelly, Trinity College Dublin)

Recent investigations have been carried out into the status of the JAK-STAT signaling pathway in both primary immune cells and hepatocytes from HCV infected patients. It was found that STAT1 and STAT3 proteins were reduced in all major immune cell populations and hepatocytes from HCV infected patients [52]. Further information is required to fully understand the HCV viral escape mechanisms that protect HCV from IFN-mediated innate immune reactions. Given significant number of patients do not respond to treatment for HCV with pegylated interferon, our understanding of the immunological mechanisms of treatment success or failure for HCV needs to be expanded.

Adaptive Immunity

The hallmark of spontaneously resolved HCV infection, indicated by an undetectable viral load, is a broad and robust HCV specific T cell response. By contrast, a weak and narrowly focused T cell response is observed in chronically

infected individuals [53-55]. It has been shown that clearance of HCV is associated with human leukocyte antigen (HLA) type and the magnitude and quality of CD4+ and CD8+ T cell responses [54, 56, 57]. In resolved HCV infection, functional HCV-specific CD4+ and CD8+ T cells are maintained for decades [58] and substantially reduce the risk of persistent infection upon re-exposure [59], whereas in chronic HCV when viral loads are high, these responses are rarely detectable at all in blood [54]. This may be due to: (i) T cell exhaustion, (ii) downregulation of effector responses by regulatory T cells or T cell priming in the tolerogenic liver environment and (iii) viral antigenic variation leading to escape from T cell responses (Reviewed in [60]).

Additionally, it is increasingly recognised that the liver, the largest organ in the body and also one of the most complex, possesses its own local immune system providing a regional immune-surveillance organisation [61]. This immune network is especially relevant to the gut given its proximity and shared vascular supplies. Regardless of the causative agent of liver disease, once activated, the intrahepatic immune system is felt to play a dual role in host defense and in propagating and/or causing liver disease. The protective role of the intrahepatic cellular immune response for HCV has been well described. As in the peripheral circulation, spontaneous clearance of HCV is associated with activation of intrahepatic cellular immunity. In contrast, as occurs in the majority of cases, chronic infection ensues, and this is associated with evasion of cellular immunity [62]. The family of intrahepatic lymphocyte populations have been broadly characterised in humans and feature a number of cell populations that may play a key role in the pathogenesis and control of HCV infection. For example, gamma delta T cells represent a subgroup of T cells that possess γ - and δ -glycoprotein chains linked by disulfide bonds rather than the 'traditional' $\alpha\beta$ chain. These cells were discovered after isolation of the T-cell receptor γ chain gene in the 1980s [63]. Gamma delta T-cells represent 2-10% of all human T cells in the peripheral blood [64] and occur at higher frequencies in intrahepatic lymphocyte populations [65]. As a result, their contribution to liver disease remains of great interest. Of the total six subsets that occur in humans, two main subsets of $\gamma\delta$ T cells predominate. V δ 1 cells are found in peripheral tissues such

as the gut, kidney, lung and spleen, whereas V δ 2 cells serve as the major portion of circulating $\gamma\delta$ cells in blood [66].

Interestingly, depending on their V δ chain expression, $\gamma\delta$ cells may provide protection or induce tissue damage in the liver. For example, V δ 1 T cells are associated with a higher necroinflammatory score in patients with chronic HCV infection [67]. These findings were replicated in HIV/HCV co-infected patients. A greater frequency of V δ 1 T cells was found in both the liver and peripheral blood in these patients. These findings were associated with a higher degree of necroinflammation compared to patients with other causes of liver disease and changes in peripheral V δ 1 T cells did not normalise with antiretroviral therapy [67]. Further studies of these T cells would be relevant not only to patients with HCV, but also in the setting of other chronic liver diseases such as Hepatitis B and autoimmune liver diseases.

It is also apparent that other T cell populations such as regulatory T (Treg) cells, specifically CD4+CD25+Foxp3+ Treg cells, likely play an important role in the natural history of HCV infection [68]. CD4+CD25+Foxp3+ Treg cells constitute a particular T cell population that can suppress the activation, proliferation, differentiation and effector functions of multiple immune cells, including T and B cells [69, 70], Natural Killer cells [71] and Dendritic cells [72]. Treg cells have been shown to suppress T cells responses, including T cell proliferation and IFN- γ secretion, directed against HCV in chronically infected patients [73]. However, they may also play a protective role in limiting the extent of fibrosis by downregulating immune-mediated mechanisms of liver damage [74]. The mechanisms responsible for these two different roles and whether distinct Treg subpopulations are involved remain unclear [68]. A more detailed understanding of the mechanistic systems of Treg cells and their distinct subpopulations should help further our knowledge of the immunopathogenesis of HCV.

The interaction between HCV and the adaptive immune system is explored further in Chapter 5.

In summary, further understanding of the complex interaction between HCV (and other pathogens) and the immune system is likely to yield benefits in terms of therapeutic and preventative strategies. As HCV and HIV (discussed below), share similar routes of transmission, co-infection rates are common. This is especially true for patient groups such as people who inject drugs (PWID) and haemophiliacs [75, 76]

1.3 Human Immunodeficiency Virus (HIV)

HIV/AIDS was first clinically observed in the early 1980s, with initial reports of acquired cellular immunodeficiency in populations of previously healthy individuals [77, 78]. It was apparent that, if an infection was responsible for this acquired immunodeficiency, sexual, vertical and blood-borne transmission was possible.

In 1983, Dr Luc Montagnier's team at the Pasteur Institute in Paris reported the isolation of a human retrovirus from a patient at risk of acquired immune deficiency syndrome (AIDS) [79]. Subsequently a causal link with human T lymphotropic virus (HTLV-III), later renamed HIV-1 virus was made [80].

HIV is a member of the genus *Lentivirus*, which are transmitted as single-stranded, positive-sense enveloped RNA viruses. Upon entry into the target cell, the viral RNA genome is converted into double-stranded DNA by a virally encoded reverse transcriptase that is transported along with the viral genome in the virus particle. The resulting viral DNA is then imported into the cell nucleus and integrated into the cellular DNA by a virally encoded integrase and host co-factors. Once integrated the virus may become latent, allowing the virus and its host cell to evade the host's immune system. Alternatively, the virus may be transcribed, producing new RNA genomes and viral proteins that are packaged and released from the cell as new virus particles that begin the replication cycle anew. An overview of the life cycle of the HIV virus is shown in below in Figure 1-3.

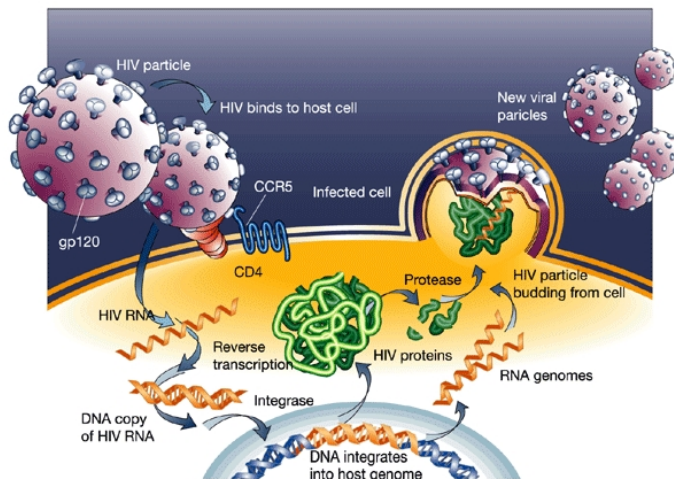


Figure 1-3 HIV life cycle overview.

Binding of the CD4 receptor by the gp120 induces the infection of target cells by HIV. After a conformational change, mediated by the binding of CD4, gp120 is able to bind the co-receptor CXCR4 or CCR5. After the binding, gp41 separates from gp120 and merges with the host membrane. During merging, the genome and enzymes from the core are released in the host cell. Reverse transcriptase copies the viral RNA into double stranded cDNA. Integrase mediates the integration of the viral cDNA into the host genome. New HIV particles will be formed due to the transcription of the integrated viral genome during CD4 + T-cell replication. The newly formed HIV particles assemble and leave the host cell by “budding”, coating itself in host membrane (Source: Weiss 2001[81])

HIV infection is typically acquired through sexual intercourse, exposure to infected blood or perinatal transmission. Risk factors for transmission include high viral load [82], certain sexual behaviours [83], lack of circumcision [84], presence of ulcerative sexual transmitted diseases [82] as well as host and genetic factors [85].

Stages of HIV infection

HIV may be divided into the following stages: viral transmission, acute HIV infection with seroconversion and chronic HIV infection. The natural history of chronic HIV infection, in the absence of antiretroviral therapy, is from asymptomatic or early but symptomatic to acquired immunodeficiency syndrome (AIDS) and advanced HIV infection (CD4 count <50 cells/ μ l).

The acute retroviral syndrome is characterised by fever, lymphadenopathy, sore throat, joint and muscle aches, and headache. However, a small proportion of patients may be asymptomatic. During early HIV infection plasma HIV levels are

typically very high before reaching a steady state level by approximately six months of infection as seen in Figure 1-4.

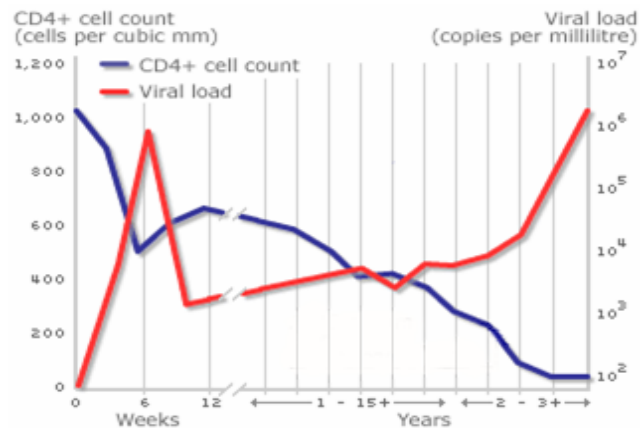


Figure 1-4 Natural history of HIV infection in a patient in the absence of ART detailing trends in CD4 count (blue line) and viral load (red line) over time from viral transmission, acute HIV infection with seroconversion to chronic HIV infection (Source: researchgate.net accessed 22/4/17)

The period of chronic HIV infection following early infection and seroconversion is characterised by a progressive decline in CD4 cell count (normal range 500 - 1500 cells/ μ L) and a stability of HIV viral load levels. This occurs prior to the development of severe immunocompromise. The rate of CD4 cell decline correlates with the level of viraemia in the patient [86].

During this stage, the majority of patients are asymptomatic, although generalised lymphadenopathy is often seen. Also, certain HIV-associated clinical findings such as susceptibility to herpes virus, human papilloma virus, tuberculosis and bacterial pneumonia may be seen despite a CD4 count above 200 cells/ μ L.

AIDS is defined as a CD4 count <200 cells/ μ L or the presence of any AIDS-defining condition regardless of CD4 cell count. AIDS-defining conditions are illnesses that occur more frequently or more severely in immunocompromised hosts. They include mainly infections including *Pneumocystis jiroveci* pneumonia, disseminated *Mycobacterium avium* complex, oesophageal candidiasis and cerebral toxoplasmosis. A wasting syndrome attributed to HIV and certain malignancies including lymphoma and Kaposi sarcoma are also included. In the absence of ART the median survival of patients with advanced HIV infection is 12-18 months [87].

Prevalence and Distribution

The World Health Organisation estimates there were 36.7 million people living with HIV in 2015. During that year there were 2.1 million people newly infected with HIV and 1.1 million deaths related to AIDS. The majority of infections occur in Africa with an estimated 25.5 million people living with HIV in Africa in 2015 (Source: WHO website accessed 10/03/17).

The distribution of the modes of transmission of HIV infection varies from country to country. For example, in resource-limited countries, vaginal sex is responsible for 70-80% of HIV infections [88]. By contrast, during the initial years of the epidemic in the US, injecting drug use and male-to-male sexual contact accounted for approximately 50% of cases [89].

In 2015, there were 485 HIV notifications in Ireland giving a rate of 10.6 per 100,000 population, the highest rate ever reported in Ireland. In 2016, a further increase was seen with 512 notifications. Between 2010 and 2014, HIV diagnosis rates in Ireland were stable but increased by 30% between 2014 and 2015. This increase was mainly confined to HSE East (where a 38% increase in rate was seen). A number of factors contributed to the increase in HSE East, including an improvement to the national surveillance case definition introduced in January 2015 which resulted in improved sensitivity, timeliness and increased number of notifications; an outbreak of HIV among PWID [90]; and an increase in diagnoses among migrant men who have sex with men (MSM).

Figure 1-5 below shows the trend in HIV diagnoses by route of transmission from 2003 to 2016. In the 10 years since 2005, the number of diagnoses among MSM has increased four-fold. The number of cases among heterosexuals has remained stable since 2010 with between 125 and 132 cases per year. The number among PWID increased sharply in 2015 due to an outbreak of HIV among PWID described above.

HIV notifications by probable route of transmission, 2003 to 2016

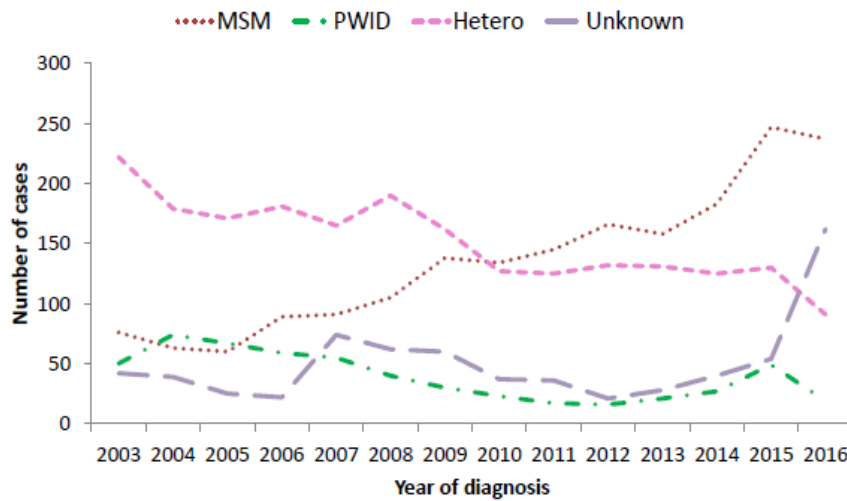


Figure 1-5 Trend in HIV diagnoses by probable route of transmission, 2003 to 2016 (HPSC.ie, accessed 22/4/17)

Recently, efforts were undertaken to describe the demographics and treatment status of HIV-infected adults accessing ambulatory care in Ireland and estimate diagnosed HIV prevalence rates. Nationally, 3,254 patients attended for care over a 12-month period between 2009 and 2010. 80% of the patients attending HIV services were on antiretroviral therapy. The HIV diagnosed prevalence rate is estimated at 1.09/1000 nationally and at 2.25/1000 in the Dublin area for 15-59 year olds [91].

Treatment of HIV

The treatment of HIV infection involves the use of combination antiretroviral therapy (ART). The goals of ART are to reduce HIV-related morbidity and mortality (from infectious and non-infectious causes) and to prevent onward transmission of HIV to others. Maximal viral suppression from ART prevents the selection of drug-resistant mutations and allows for improved immunological function (measured by the CD4 cell count). ART has dramatically altered the natural history of HIV infection and patients without significant comorbidities who are treated appropriately can expect to have a life expectancy similar to the general population [92].

International societies update their recommendations for HIV regularly and currently ART should be offered to all HIV patients, including asymptomatic individuals regardless of their immune status [93].

There are currently more than 25 antiretroviral medications available from six drug classes. There are four classes of antiretroviral drugs typically used in initial regimens. These include: nucleoside (and nucleotide) reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and integrase strand transfer inhibitors (INSTIs). A CCR5 antagonist (Maraviroc) is also available and a fusion inhibitor (Enfuvirtide) which are reserved for patients with multidrug resistant virus.

When determining the most suitable regimen for a patient initiating ART, a number of factors should be taken into account, including co-morbid conditions, degree of organ dysfunction, HIV viral load, HLA-B*5701 status, the impact of factors related to the regimen itself (pill burden and size, drug interactions), drug availability, transmitted drug resistance and cost.

1.4 The Challenge of HIV-1 and HCV co-infection

Since HCV and HIV-1 share similar transmission routes, co-infection is common. Overall, the global prevalence of HIV/HCV co-infection has been estimated at approximately 2.3 million people [94]. The highest burden of disease is in Eastern Europe and Central Asia. This is due to a large HIV-infected population of PWID [94]. A consistently higher prevalence of HCV infection was observed in HIV-infected individuals across all risk groups regions. This was most marked in the PWID population [94].

Although injection drug use (IDU) accounts for the majority of all HCV infections [95], in the past 15 -20 years there has also been a more than 10-fold increase in the incidence of acute HCV infection in HIV-1-seropositive MSM [96]. This is currently reflected in epidemic outbreaks of acute HCV infection in multiple

major European cities [97]. The greatest increase in HIV-1 transmission in recent years has occurred in MSMs, as has sexual transmission of HCV, highlighting the failure of preventative measures aimed at changing behaviour. Within the Swiss HIV Cohort Study the incidence rate of HCV infection in HIV-1 seropositive MSMs increased from 0.23/100 person-years in 1998 to 4.09/100 person-years in 2011, with inconsistent condom use and diagnosis of past syphilis being associated with HCV acquisition [98]. In Ireland, there has been a recent increase in HCV cases identifying as MSM, as outlined in Figure 1-6. Data has shown that HIV-negative MSMs remain at risk of HCV acquisition also [99].

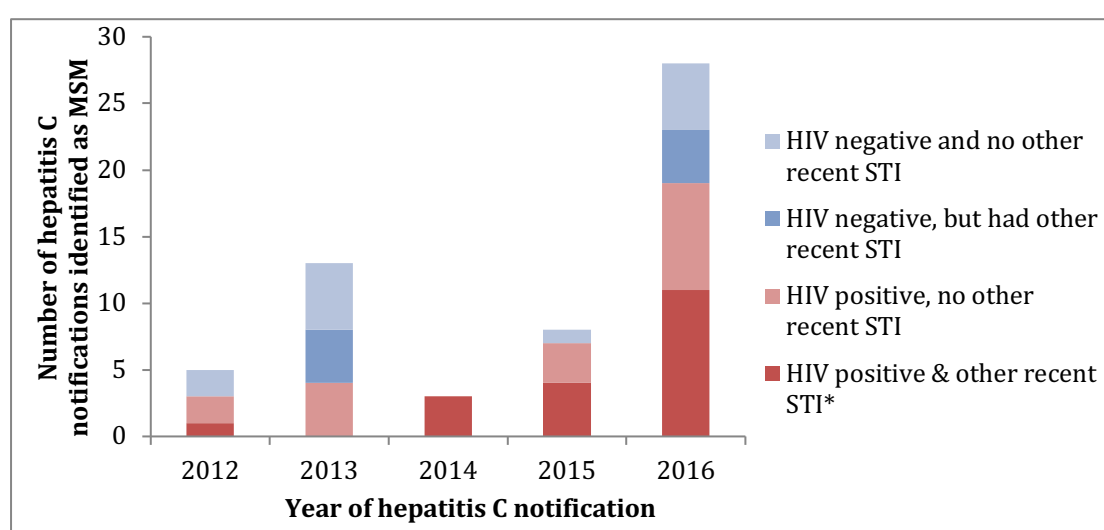


Figure 1-6 Recent increase in hepatitis C cases identified as MSM

*Syphilis, gonorrhoea or chlamydia notified in the same year or in the year prior to the hepatitis C notification (Source: HPSC.ie, accessed 23/4/17)

Historically, the natural course of HCV infection was accelerated in HIV-1 seropositive individuals [100]. HIV-1 seropositive adults have had an increased rate of progression to liver cirrhosis, end-stage liver disease, hepatocellular carcinoma and death. 2-year survival of HIV/HCV patients with decompensated liver cirrhosis is only 50% [101]. HIV-1 seropositive individuals have significantly lower HCV specific T cell responses than mono-infected people, which may underlie their lower rate of spontaneous HCV clearance and historical poor responses to therapy [102].

ART has markedly improved the life expectancy of persons infected with HIV [103, 104]. As a result, there has been a renewed focus on co-morbid diseases,

including viral hepatitis, which may be exacerbated by concurrent HIV infection [104]. Antiretroviral therapy in co-infected patients decreases the likelihood of hepatic decompensation by 28-41% [105]. Therefore, current guidelines recommend initiation of ART in all HIV-HCV co-infected patients. Additionally, recent evidence suggests that with improvements in care for HIV with earlier treatment with safer, less hepatotoxic medications, liver fibrosis progression is increasingly similar to patients with HCV mono-infection [106].

1.5 The need for a vaccine for Hepatitis C

Currently there is no vaccine available for either the prevention (prophylactic vaccination) or the treatment (therapeutic vaccine) of HCV. Until recently, the treatment of HCV with the best available therapy was costly, lengthy, and frequently ineffective and the development of an effective vaccine is felt to be an urgent priority towards eradication of HCV.

Among the estimated 170 million people estimated to be infected with HCV worldwide, at least 8 million live in Europe, with prevalence rates of over 5% in some European countries [107]. It is estimated between 20,000 and 50,000 individuals are affected by Hepatitis C in Ireland [108]. Primary (acute) infection is usually asymptomatic, though sometimes patients may present with jaundice. Unlike hepatitis B virus infection, fulminant liver failure during primary infection is extremely rare. Given its asymptomatic nature, it is possible that the true prevalence of infection is greater than reported.

Persistent HCV is a significant cause of morbidity and mortality, due to progressive liver fibrosis, with 20-50% of patients developing cirrhosis and hepatocellular cancer [27]. Chronic HCV is now a leading cause of liver failure, hepatocellular cancer and liver transplantation, and is among the most common causes of death in patients co-infected with HIV-1 on combination ART [109]. Additionally data shows that individuals infected with HCV are large users of

emergency departments, outpatient and inpatient services, reflecting the public health burden of HCV [110].

As mentioned above, HCV therapies were frequently associated with significant toxicity. Until recently, the mainstay of therapy for over a decade was Pegylated-Interferon- α (PEG-IFN) in combination with ribavirin. Sustained virological responses (SVR; defined as undetectable HCV RNA 24 weeks after treatment termination) were achieved in less than 50% of mono-infected patients with genotype 1 disease, the most common genotype in Western Europe. SVR rates for HIV/HCV co-infected individuals varied from 15-70% according to genotype, HCV RNA level and IL-28B genotype, and were as low as 22% for genotype 1 in a recent international trial [111]. In addition, multiple studies have demonstrated that SVR rates were markedly lower in patients with HIV/HCV co-infection, compared to those with HCV alone [112-114].

Eradication of HCV after therapy with interferon and ribavirin in HIV/HCV co-infected patients has not only been associated with a reduction in liver-related events but also with a reduction in HIV progression and mortality not related to liver disease [115].

The field of HCV therapeutics has evolved rapidly in recent years. Basic science enhancements in HCV cell culture systems and replication assays have led to a broadening of our understanding of many of the mechanisms of HCV replication and, therefore, potential novel antiviral targets. These steps broadly encompass viral attachment, entry, and fusion; viral RNA translation; posttranslational processing; HCV replication; and viral assembly and release [116].

Two direct-acting anti-viral (DAA) agents (Telaprevir and Boceprevir) for HCV received regulatory approval in 2011. Telaprevir and Boceprevir are first generation DAA agents and are classed as NS3/4A protease inhibitors. These compounds target the serine protease NS3/4A, which cleaves the HCV polyprotein at four sites during its replication cycle [117]. However, the additional cost for these medications was more than €25,000 per patient. This

DAA therapy significantly improved response rates in both HCV-mono-infected and HCV/HIV-co-infected patients with genotype 1 disease. However, in the latter, up to 40% still failed to clear HCV infection.

Because mono-therapy with either agent resulted in viral resistance, these agents needed to be used in combination with PEG-IFN and ribavirin. As a result, Telaprevir and Boceprevir-based therapy were associated with more frequent and severe adverse effects, coupled with drug-drug interactions, including with antiretroviral drugs [118]. Despite suboptimal SVR rates with these therapies, favourable treatment outcomes have been associated with short courses of therapy [119], highlighting the fact that our knowledge of positive predictors of response to HCV therapy needs to be expanded.

Universal hope for future and emerging DAA therapy is for an all-oral combination regimen that is highly potent, well tolerated, pan-genotypic, of short duration and with little possibility of the emergence of drug resistance. Ideally drug-drug interactions associated with concomitant HIV antiretroviral therapy and other medication should be minimal. Recent trials assessing these newer DAA agents have shown great promise [120, 121], with SVR rates typically greater than 90% with few side effects.

Current approved DAAs inhibit 3 specific steps in the HCV lifecycle including NS3/4A protease enzyme (as described above), NS5A protein and the NS5B polymerase. The NS5B enzyme is crucial for HCV replication as it catalyses the synthesis of the complementary minus-strand RNA and subsequent genomic plus-strand RNA. There are two types of NS5B polymerase inhibitors: nucleotide inhibitors (active site inhibitors) and nonnucleoside inhibitors (allosteric inhibitors). NS5A inhibitors target a protein that appears to be essential to the replication machinery of HCV and essential in the assembly of new infectious virus particles. The specific functions of this protein have yet to be elucidated. Table 1.1 lists the approved DAAs in Europe in 2016. Other treatment regimens are at the clinical development stage and will likely reach the market within the next two years.

Table 1.1 Approved HCV DAAS in Europe in 2016

Sofosbuvir	Dasabuvir
Sofosbuvir/ledipasvir	Grazoprevir/elbasvir
Sofosbuvir/velpatasvir	Daclatasvir
Paritaprevir/ombitasvir/ritonavir	Simeprevir

Following on from the clinical trials of DAA medications, experience is emerging from prospective cohorts showing all-oral DAA regimens are well tolerated and associated with a high virologic efficacy in cirrhotic HIV/HCV co-infected patients, long considered a population that are difficult to treat [122]. Multiple studies have demonstrated similar SVR rates to those observed in trials of HCV-mono-infected individuals [123].

However the cost of these newer agents will continue to be a deterrent for population-wide applications of these highly effective regimens [124]. A major consideration in treating HCV in individuals with HIV co-infection is the identification and management of drug interactions between DAAs and antiretroviral agents [125]. Additionally, HCV viral resistance may represent a challenge in the future. We have reported a previously undescribed case of treatment-emergent non-structural protein 5A (NS5A) resistance mutations, Q30H and Y93C, leading to a failure of 24-week course of sofosbuvir/ledipasvir and ribavirin therapy for the treatment of HCV genotype 1a in an interferon-experienced, HIV-1 co-infected patient with cirrhosis [126]. Onward transmission of treatment-resistant virus may also pose challenges towards eradication of HCV in the future [127].

Anticipated advances in HCV therapy will have a limited impact on the burden of HCV-related disease on a population level unless barriers to HCV education, screening, evaluation and treatment are addressed and treatment uptake improves [128].

The development of a T cell-based vaccine that prevents HCV infection, or induces effective T cell responses during treatment and impacts on HCV viral load could therefore be of major benefit to patients and would have a substantial impact on morbidity, quality of life and treatment costs.

1.6 Aims of the thesis

The aims of this thesis were threefold. Firstly, I looked to examine therapeutic outcomes for patients with HIV/HCV co-infection who were treated for HCV from 2001 to 2016. I looked to assess outcomes for a variety of evolving treatment options for HCV and report liver-related morbidity and mortality data in these patient groups.

Secondly, as further understanding of the complex interaction between HCV and the immune system is likely to yield benefits in terms of current and future therapeutic and preventative strategies, I sought to gain a further understanding of the innate immunological consequences of HIV/HCV co-infection in a cohort of patients. I looked to examine alterations in the IFN- α JAK-STAT signaling pathway as a result of initiation of HCV treatment in these patients.

Finally, in the context of HCV eradication, I looked to examine the safety and immunogenicity of novel vaccine candidates for the prevention of HCV in HIV seropositive individuals.

A brief overview of Chapters 2-6 is outlined below:

Chapter 2 outlines the materials and methods used in the thesis.

Chapter 3 reports the results of therapeutic outcomes of HIV/HCV co-infected patients treated for HCV from 2001-2016 and highlights advances in therapeutics and ongoing challenges in this field.

Chapter 4 details alterations in the IFN- α JAK-STAT signaling pathways in primary immune cells from HIV/HCV co-infected patients who underwent treatment for HCV with a Telaprevir-based regimen.

Chapter 5 further discusses the rationale behind a HCV vaccination strategy and chronicles the development and previous evaluation of the candidate HCV vaccines, the regulatory process involved at a national level, a summary of the inclusion and exclusion criteria and a description of the baseline characteristics of the patients involved in the study.

Chapter 6 details the primary (safety) and secondary (immunogenicity) outcomes in the study of the novel vaccine candidates.

Chapter 7 contains a final discussion of the results of the previous chapters, coupled with an outline of future, planned research avenues.

Chapter 2 Materials and Methods

2.1 Procedure for Human PBMC Isolation, Freezing and Thawing

Introduction

Peripheral blood is the primary source of lymphoid cells for investigations of the human immune system. Its use is facilitated by centrifugation with density gradient media such as Lymphoprep™ or Ficoll-Hypaque™ - a simple and rapid method of purifying peripheral blood mononuclear cells (PBMC) that takes advantage of the density differences between mononuclear cells and other elements found in the blood sample.

Mononuclear cells and platelets collect on top of the layer of density gradient medium because they have a lower density; in contrast, red blood cells (RBC) and granulocytes have a higher density than the density gradient medium and collect at the bottom (Figure 2-1). Platelets are separated from the mononuclear cells by subsequent washing or by centrifugation through a foetal bovine serum (FBS) cushion gradient that allows penetration of mononuclear cells but not platelets.

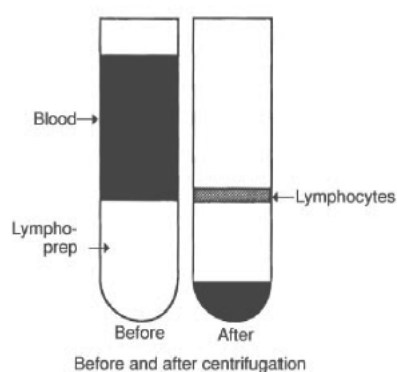


Figure 2-1 Separation of blood components on a Lymphoprep™ gradient

Equipment

Table 2.1 Table of equipment for PBMC separation

Refrigerated centrifuge with swinging bucket rotor
-80°C freezer
Certified class II microbiological safety cabinet ('hood')
Cell counting equipment – Trypan Blue, disposable plastic counting chamber, microscope
Automated cell counter e.g. Muse Cell Analyser
Set of micropipettes
Sterile individually wrapped 5 mL, 10 mL and 50 mL serological pipettes
Sterile plastic Pasteur pipettes (typically 3mL / 4mL)
Sterile polypropylene conical centrifuge tubes, 15 mL and 50 mL volumes
Electric Pipette-Aid
Sterile 2.0 mL polypropylene cryovials with screw cap and O-ring
Freezing container, for example 'Mr Frosty' by Nalgene®, catalogue number C1562-1EA

Table 2.2 Table of reagents for PBMC separation

Reagent ^a	Supplier	Catalogue No.
Sterile D-PBS	Gibco	14190-136
RPMI 1640	Gibco	11875-093
FBS (or FCS) ^b	Sigma	F9665
200mM L-Glutamine	Gibco	25030-024
100X Penicillin/Streptomycin solution	Gibco	15140-122
Lymphoprep	Axis Shield	NYC-1114547
DMSO	Sigma	D2650
DNase I	Sigma	DN25

^a Listed reagent or equivalent was used

^b A single lot of FBS/FCS (Sigma, Lot No: 074M3399) was used in the study

Reagent Preparation

All reagents were prepared in a Class II hood using aseptic technique. See Appendix 2 for details on reagent preparation.

Density Gradient Separation

Fresh heparinised blood was placed into appropriately labelled 50 ml sterile conical centrifuge tubes. 50 mL Falcon tubes were labelled with the subject number. The appropriate volume of Lymphoprep was aliquoted into each Falcon tube, allowing 20-25 mL Lymphoprep per 20 mL blood. 20 mL blood was slowly layered over the Lymphoprep solution into each tube. To maintain the Lymphoprep/blood interface, the centrifuge tube was held at a 45° angle and the blood pipetted along the side of the tube.

The Falcon tubes were centrifuged for 20 min at $900 \times g$, at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$, with no brake. Using a sterile pipette, the upper layer that contains the plasma and most of the platelets was carefully and slowly removed and discarded. Using another sterile pipette, the mononuclear cell layer was transferred to another 50 mL centrifuge tube. Pasteur pipettes were used for this step. The cells were washed by adding excess sterile R0 media (~3 times the volume of the mononuclear cell layer) and centrifuging for 10 min at $900 \times g$, 18° to 20°C .

The supernatant was removed, and the cells re-suspended in sterile R0 by gently tapping the tube with an index finger, and the wash was repeated once, at $200 \times g$, to remove most of the platelets.

The mononuclear cells were initially re-suspended in 10 mL R10, pipetting gently up and down to get a single cell suspension. Once re-suspended, the cells were topped up with R10 to a volume equivalent to the original volume of blood.

The cell concentration was determined using both an automated cell counter (Muse Cell Analyser) and manual haemocytometer method. When using the manual method, 10 μL cell suspension was mixed with 10 μL Trypan Blue and

the mixture was aliquoted into counting chamber. The optimal cell concentration for counting generated 50-100 cells per counting field.

The number of PBMC per mL blood was determined by using the following method: number of live cells in field $\times 10^4$ ($\times 2$ if have used trypan blue at 1:1 ratio). The viability was determined by Trypan Blue exclusion using the manual haemocytometer method (Viability = (number of live cells / total number of cells) $\times 100$).

Blood obtained from a healthy donor typically yields $1-2 \times 10^6$ mononuclear cells per ml blood. 60-70% are lymphocytes, with viability $> 95\%$. Platelet count is $< 0.5\%$ total platelet content of original blood sample. If there was excess erythrocyte contamination, pure mononuclear cell populations were obtained either by subjecting the cells to a second cycle of gradient separation as described above, or by lysing the erythrocytes with red cell lysis solution (such as 'ACK Lysing Buffer' by Gibco, Cat. No. A1049201). A cell count was repeated following red cell lysis.

Freezing Procedure

Cells were frozen in 1 mL aliquots of $10-12 \times 10^6$ cells/mL. The volume of Freezing Mix required to freeze the cells at the appropriate concentration and the number of cryovials needed was determined. Labels included the subject ID code, study number, visit number, sample date and the concentration of cells. The required number of cryovials were pre-chilled.

After counting the cell suspension, the cells were centrifuged at $200 \times g$ for 7 minutes at $2-8^\circ\text{C}$. The Freezing Mix was prepared as follows: 1 mL DMSO to 4 mL RPMI. The Freezing Mix was made daily as required and stored at $2-8^\circ\text{C}$ or on ice until time of use. The supernatant was removed, and the pellet re-suspended in cold FCS at $2-8^\circ\text{C}$ ($500 \mu\text{l}$ per 10×10^6 cells). The Freezing Mix was added so the cells were re-suspended in a solution of FCS to Freezing Mix in a 1:1 ratio, resulting in an approximate cell concentration of $10-12 \times 10^6$ cells/ml. 1 mL cell

suspension in freezing medium was dispensed into each cryovial. The cryovials were placed into a freezing container, e.g. Mr Frosty, and then the container was placed in a -80 °C freezer. The complete process of cell re-suspension in FM, aliquoting, vial placement into Mr Frosty and transfer of Mr Frosty units into -80° C freezer was performed in less than 10 minutes and conducted on ice.

Within 1-3 days at -80 °C, the cryovials were transferred from the Mr Frosty units into standard freezer boxes within a liquid nitrogen container.

Thawing Procedure

10mL of TM per sample to be defrosted was pre-warmed in a water bath set at 37°C (\pm 1°C). One 15 mL falcon tube was labelled per sample. The PBMC cryovial(s) were removed from liquid nitrogen and placed on dry ice until ready to thaw. It was recommended to not thaw more than four cryovials at a time due to time and handling constraints.

Cells were thawed quickly with gentle agitation in a water bath set at 37°C (\pm 1°C), until a visible ice chunk remained in the cryovial. Each thawed cell suspension was gently transferred to the appropriately labelled 15 mL tube.

The vial(s) were rinsed with 1mL TM, adding the TM drop wise to the thawed cells. 10 mL TM was added to each tube slowly and drop-wise; the tubes were swirled gently to mix, thus avoid shear forces. The tubes were centrifuged at room temperature for 5 minutes at 200 \times *g*. The supernatant was discarded and the bottom of the tube was gently tapped to re-suspend the cell pellet. The cell pellet was re-suspended in 1 mL R10 initially, pipetting up and down to get a single cell suspension. Once re-suspended, a further 9 mL R10 was added for a final volume of 10 mL cell suspension. The centrifugation procedure listed above was repeated. The cells were counted using the trypan blue exclusion method.

The cells were spun down and re-suspended in sterile R10 to give a final concentration of 4×10^6 cells/mL. The cells were rested at 37°C for 2 hours.

The cells were re-counted using trypan blue exclusion after resting and adjusted accordingly to give a final concentration of 4×10^6 cells/mL.

2.2 ELISpot Technique

Introduction

The activation and expansion of antigen-specific T lymphocytes is a characteristic of primary viral infections. Upon challenge, CD8⁺ cytotoxic T lymphocytes (CTL) recognise antigenic peptides in conjunction with MHC class I molecules, leading to the lysis of viral-infected cells as well as the secretion of Interferon- γ (IFN- γ). In addition, CD4⁺ T helper lymphocytes recognise antigenic peptides in conjunction with MHC class II molecules, also leading to the secretion of IFN- γ , which in turn affects other aspects of the immune system. The number of antigenic-specific precursor T cells available at the time of challenge determines the magnitude of the immune response and may ultimately affect the course of infection.

The ELISpot technique was first reported in 1983 when Czerkinsky et al described a method to enumerate the frequency of B hybridoma cells secreting an antigen specific immunoglobulin [129]. The technique was developed further in 1998 when Czerkinsky outlined the development of an ELISpot assay measuring the frequency of T lymphocytes secreting a specific lymphokine [130]. Further development of the technique included description of a dual-colour ELISpot assay [131] allowing simultaneous assessment of two different types of cell producing antigenically distinct products and the use of computer imaging to detect and analyse spots.

An ELISpot assay capable of detecting IFN- γ -producing T cells in a sample of peripheral blood mononuclear cells (PBMCs) can be utilised to estimate precursor frequency. The PBMCs are serially diluted and placed in microplate wells coated with anti-human IFN- γ antibody. They are cultured with antigen overnight, resulting in re-stimulation of the precursor cells and secretion of IFN- γ . The cells are washed away, leaving the secreted IFN- γ bound to the antibody-coated wells in concentrated areas corresponding to the location of the displaced cells. The captured IFN- γ is detected with biotinylated anti-human IFN- γ

antibody followed by an alkaline phosphatase-conjugated anti-biotin antibody. The addition of insoluble alkaline phosphatase substrate results in dark spots in the wells at the sites where the cells are located, leaving one spot for each T cell that secreted IFN- γ . The number of spots per well is directly related to the precursor frequency of antigen-specific T cells.

Equipment

Table 2.3 Table of equipment for ELISpot assay

Biological Safety Cabinet
Humidified CO ₂ incubator set at 37°C and 5% CO ₂
Set of micropipettes and corresponding sterile pipette tips
Electronic 12-channel pipette or multi-stepper
Sterile individually wrapped 5 mL, 10 mL, 25 mL and 50 mL serological pipettes
Sterile polypropylene conical centrifuge tubes, 15 mL and 50 mL volumes
Sterile culture bottles (if required), 250 mL volume
Sterile reagent reservoirs
Electric Pipette-Aid
Sterile culture plates or plastic reservoirs
96-well plates with PVDF membrane (Millipore MSIPS4510)
Microscope with 20X objective
ELISpot plate reader (AID ELISpot Reader classic)

Materials

Table 2.4 Table of reagents for ELISpot assay

Reagent*	Supplier	Catalogue No.
Sterile D-PBS (1X)	Gibco	14190-136
IFN- γ human ELISpot kit	MABTECH	3420-2A
RPMI 1640	Gibco	11875-093
200mM L-Glutamine	Gibco	25030-024
100X Penicillin/Streptomycin solution	Gibco	15140-122
Foetal Bovine Serum (FBS)	Sigma	F9665
PHA	Sigma	L1668
Concanavalin A	Sigma	C5275
Dimethyl sulphoxide (DMSO)	Sigma	D2650
TWEEN® 20	Sigma	P2287
Bovine Serum Albumin	Sigma	A4919
BCIP/NBT Substrate Solution	Pierce	34042

* Use listed reagent or equivalent

Reagent Preparation

See Appendix 2 for details on reagent preparation.

ELISpot plate preparation

The following steps were performed in a biological safety cabinet using aseptic technique and appropriate precautions. The required numbers of 96-well ELISpot plate(s) were labelled appropriately, including the subject ID, visit day and date. Each well of the ELISpot plate was pre-wetted with 15 μ l of 35% ethanol in H₂O. If coating more than one plate, then each plate was pre-wetted and coated separately. The ethanol was discarded into a waste trough immediately. The plate was blotted onto a paper towel gently and washed 4 times with 200 μ l/well sterile PBS using a multi-channel pipette and sterile pipette tips and trough.

The volume of coating solution required was determined by multiplying the number of plates to be coated by 7.5 ml (this gave the volume of D-PBS required). The required volume of anti-human IFN- γ monoclonal antibody clone 1-D1K stock solution was determined by dividing the volume of D-PBS required by 200. The appropriate volume of sterile D-PBS and the appropriate volume of anti-human IFN- γ monoclonal antibody clone 1-D1K was dispensed into a 50 ml centrifuge tube. This resulted in a coating solution at 5 $\mu\text{g}/\text{ml}$ of the antibody. The tube was capped tightly and mixed by inversion. Using a multi-channel pipette, 75 μl of coating solution per well was dispensed in each well of the labelled ELISpot plates. The lids were placed on the plates and all plates were placed in a refrigerator (2-8°C) overnight (15-24 hours). Plates were stored with coating solution in the refrigerator for up to 3 days. For storage time greater than overnight, the plates were placed in an airtight container within the refrigerator.

ELISpot procedure

The ELISpot plate coating antibody was discarded from each plate. Each coated plate was washed four to six times with 200 μl sterile D-PBS per well using a multi-stepper or electronic multi-channel pipette. The plate was inverted over a reservoir to decant the wash solution after each wash. 200 μl sterile R10 per well was transferred to each washed plate. The lids were placed on the plates and the plates left in a humidified CO₂ incubator set at 37°C ($\pm 1^\circ\text{C}$) and 5% CO₂ for at least 1 hour.

The blocked plates were then removed from the incubator and inverted to decant the R10 from each well. 50 μl of each antigen/control was transferred from the pre-prepared peptide plate into the appropriate wells of the blocked ELISpot plate (3 wells per antigen tested, including mock, and one or two wells for Con A/PHA control). This step was repeated until all antigen/mock solutions have been plated. A tube containing PMBCs that had been previously isolated, counted and re-suspended at 4×10^6 cells/ml in sterile R10 for each test subject was gently swirled to ensure adequate mixing of the PMBCs. 50 μl PMBC

suspension from each subject was dispensed into the appropriate number of wells of the plate so that each subject has three wells per antigen (at 200,000 cells/well). In addition, at the Week 9 time point only, 25 μ l PBMC suspension was dispensed into the appropriate wells of the plate to give the same number of replicates at 100,000 cells/well. The lids were placed on all plates and the plates were placed in a humidified CO₂ incubator set at 37°C (\pm 1°C) and 5% CO₂ for 15-20 hours.

Assay Development

A solution of biotinylated mouse anti-human IFN- γ diluted to 0.5 μ g/ml (1:2000 dilution of 1 mg/ml stock) in assay diluent was prepared as follows: The volume of assay diluent required was determined by multiplying the number of assay plates by 6 ml. The volume of diluent calculated above was placed into a 50 ml tube. The volume of antibody stock required was determined by dividing the volume of diluent determined above by 2000. The required volume of antibody stock was pipetted into the diluent. The tube or was capped tightly and mixed by inversion.

The ELISpot plates were removed from the incubator and washed with 200 μ l PBS/Tween 20 wash buffer (WB) for a minimum of 6 washes. After this step, all cells are washed away, so benchtop manipulation was acceptable.

The plates were blotted dry on a paper towel. 50 μ l of diluted biotinylated antibody was transferred to each well of the plate(s). Lids were placed on all plate(s) and the plate(s) placed on the benchtop at room temperature for 3 hours \pm 1 hour.

Immediately before the end of the incubation, a solution of alkaline phosphatase (AP)-conjugated anti-biotin antibody, diluted 1:750 in AD, was prepared as follows: The volume of assay diluent required was determined by multiplying the number of assay plates by 6 mL. The volume of diluent calculated above was pipetted into a 50 mL tube. The volume of antibody stock required was determined by dividing the volume of diluent determined by 750. The required

volume of antibody stock was pipetted into the diluent. The tube was capped tightly and mixed by inversion. Each plate was washed a minimum of four times with 200 μ L of WB per well. The plate(s) was blotted dry on a paper towel. 50 μ L of AP-anti-biotin was transferred to each well of every plate. Lids were placed on all plates and the plate(s) were placed on the benchtop at room temperature for 2 hours.

At the beginning of the 2-hour incubation, the bottle of BCIP/NBT stock solution was removed from the refrigerator and placed at room temperature. At the end of the 2-hour incubation, each plate was washed a minimum of four times with 200 μ L WB. The plate(s) was blotted dry on a paper towel. The bottle of BCIP/NBT solution was inverted at least five times to mix. 6 ml BCIP/NBT solution per plate was transferred to an appropriate Falcon tube or bottle with a 0.22 μ m filter. Pre-filtered BCIP/NBT solution was dispensed into a sterile reagent reservoir and 50 μ L transferred to each well of every plate. The plate(s) was incubated at room temperature for 5 minutes, or until distinct dark spots appeared in the positive control wells. To stop colour development, the BCIP/NBT solution was decanted and each plate rinsed three times with tap water. The rubber bottom was removed from all plates and the bottom side of the membrane rinsed with tap water. Each plate was allowed to air-dry overnight.

Analysis

The spots in each well were counted using an ELISpot plate reader (AID ELISpot Reader classic). To calculate the mean number of spot-forming cells (sfc) per 1×10^6 input PBMC, the mean number of spots was multiplied for each set of replicate wells by x ($x = 1 \times 10^6 /$ cells per well input). Example: If 2×10^5 cells/well input, multiply mean spot count by 5 (i.e. $5 = 1 \times 10^6 / 2 \times 10^5$).

Pass/Fail Criteria:

Positive Controls:

All ELISpot assays performed included positive controls, typically ConA (or PHA). At the concentration used, this should have given a totally blue (saturated) appearance to the well. If there were few to no spots in all positive control wells, an assay reagent problem was suspected. All samples on the assay plate(s) in question were re-tested.

Negative Controls:

If test samples plus mock (no antigen) resulted in more than 45 spots per million cells, these samples were reviewed and discussed within the PEACHI consortium. A plan for re-test was discussed if possible and/or necessary.

If media-only wells (no cells) had more than five apparent spots per well, non-specific assay reagent artefacts were suspected. All samples on the assay plate(s) in question were re-tested.

Re-Tests:

If positive controls were valid upon re-test, the earlier test was deemed invalid and the re-test data was accepted as final. If the mock data was still above 45 spots per million cells upon re-test, this confirmed the earlier test. Therefore, the earlier test was deemed valid and was accepted as final.

If the negative control media-only wells were at or below 5 spots per well upon re-test, the earlier test was deemed invalid and the re-test data was accepted as final. All controls had to be acceptable as described above for data to be accepted as final. All re-tests were reviewed and discussed within the PEACHI consortium.

2.3 IP-10 Whole Blood Assay

IFN- γ induced protein 10 (IP-10; CXCL10) is a chemokine produced by innate antigen presenting immune cells – mainly dendritic cells – upon stimulation with IFN- γ . IP-10 is the key driver of a IFN- γ dependent amplification loop supporting a sustained adaptive immune response. The IP-10 receptor CXCR-3 (CD183) is up regulated on pathogen specific effector Cytotoxic T Lymphocytes (CTLs) and Th1-polarised CD4+ T cells. CXCR-3 mediated signalling facilitates the CTL trafficking to the lymphoid compartment.

In comparison to IFN- γ , IP-10 secretion upon pathogen specific stimulation is increased significantly rendering IP-10 a valid alternative for the detection of pathogen specific T cell responses *ex vivo* with increased sensitivity. HCV specific CTL responses tend to be weak in peripheral blood due to the compartmentalisation of the CTLs into the liver parenchyma. Although this compartmentalisation is less likely to happen in HCV vaccine recipients without HCV infection a robust and sensitive assay to detect CTL responses upon vaccination is desirable.

We looked to evaluate a whole blood stimulation assay in conjunction with IP-10, IFN- γ and interleukin 2 (IL-2) detections by Enzyme Linked Immunosorbent Assay (ELISA) in supernatants and the correlation of chemokine / cytokine secretion and respective mRNA cell levels.

Equipment

Table 2.5 Equipment for IP-10 Whole Blood Assay

Biological Safety Cabinet
Humidified CO ₂ incubator set at 37°C and 5% CO ₂
Set of micropipettes and corresponding sterile pipette tips
Sterile Eppendorf 1.5 ml conical tubes
Desktop centrifuge

Materials

Table 2.6 Reagents for IP-10 Whole Bloods Assay

Reagent*	Supplier	Catalogue No.
HCV gen. 1b peptide pools 3.0µg/ml final concentration (stock 300 µg/ml)*	PEACHI (UOXF)	
FEC pool* (Stock 300ug/ml) final concentration 3.0ug/ml	PEACHI (UOXF)	
Concanavalin A (Con A Stock 1mg/mL)* final concentration 10µg/ml	Sigma	C5272
Dimethyl sulphoxide (DMSO)*	Sigma	D2650
DNA/RNA Shield	Zymo Research	R1100-50
1.5 mL Eppendorf tubes (conical)	-	

* HCV peptide pools and negative/positive stimulation tubes were supplied by University of Oxford (UOXF) and were plated out in 1.5 ml Eppendorf tubes (5µL per tube of HCV peptide pools). Further details on the peptide pools are outlined in section 2.4.

“Stimulation tube” refers to 1.5ml Eppendorf tubes containing the appropriate amount of peptide stimulation mixes for each separate stimulation

Reagent Preparation

For preparation of DNA/RNA Shield, DNA/RNA Shield was used undiluted

Preparation of stimulation tubes

5µL each of stock solution of each HCV peptide pools F – M, FEC and ConA (positive controls), and DMSO negative control were pipetted into 1.5 mL Eppendorf tubes.

Set-up scheme:

Table 2.7 Set-up scheme for stimulation tubes for IP-10 Whole Blood Assay

Tube no.	1	2	3	4	5	6	7	8	9
	Negative control + DMSO	Pool F NS3p	Pool G NS3h	Pool H NS4	Pool I NS5A	Pool L NS5B-I	Pool M NS5B-II	Positive control (Con A)	FEC
	5µL DMSO	5µL pool F	5µL pool G	5µL pool H	5µL pool I	5µL pool L	5µL pool M	5 µL ConA	5 µL FEC

Tubes were stored in a freezer at -80°C until use.

Whole blood stimulation set-up

All steps were performed in a biological safety cabinet using aseptic technique.

Preparation of Eppendorf tubes for stimulation

Eppendorf stimulation tubes were stored at -80°C until their use and allowed to thaw at room temperature prior to use. The required numbers of pre-filled 1.5 mL Eppendorf stimulation tubes were appropriately labelled, including the subject trial identification number, visit number and date.

Stimulation set-up

The stimulation was set up within 1-4 hours of blood collection. The heparin tube containing the respective patients' blood was gently mixed by inversion. The cap was removed gently from the heparin tube. 500 µL of whole blood was pipetted into each Eppendorf stimulation tube containing nothing or DMSO alone (negative controls); HCV peptide pools F – M; FEC and ConA (positive controls) as outlined above. The whole blood was mixed with the stimulants by pipetting gently 5 times up and down. The Eppendorf stimulation tubes were transferred

to a humidified CO₂ incubator set at 37°C (± 1°C) and 5% CO₂ for 20 hours; the lids were kept open.

Preparation of serum storage tubes and cell pellet for DNA extraction

The required number of 1.5 mL Eppendorf tubes were appropriately labelled, including the subject ID, visit number, date and tube number/contents. The Eppendorf stimulation tubes were removed from the incubator and the lids were closed.

The Eppendorf tubes were centrifuged for 5 min at 3500 rpm, at room temperature. The Eppendorf tubes were carefully removed to avoid mixing of the plasma and blood cell layer. Using a sterile pipette, the supernatants (approx. 150 µL) were transferred carefully, without disturbing the blood pellet, into the separate, appropriately labelled Eppendorf tube for storage. 0.8ml of RNA Shield was added to the remaining cell pellet. The sample was directly homogenised by pipetting up and down 5-10 times and vortexing. The supernatant and homogenized cell pellet sample in DNA/RNA shield were stored at -80°C until shipment to St Gallen in Switzerland in February 2017 for further analysis. ELISA analysis of the supernatants for IP-10 for all samples was performed in Matthias Hoffman's laboratory in St Gallen.

2.4 Peptide Plate Preparation

Synthetic peptides contained within the HCV proteome and HCV vaccines can stimulate CD4⁺ and CD8⁺ T lymphocyte responses. These responses can be measured through the production of cytokines – such as IFN- γ , by peptide-stimulated PBMC. The definition of exactly which peptides elicit such responses is critical for the elucidation of potentially protective T-lymphocyte responses.

A set of 494 peptides, 15 amino acids (aa) in length, overlapping by 11aa and spanning the open-reading frame from NS3 to NS5B (1985aa) of HCV genotype 1b strain BK (matching the vaccine immunogen) were obtained from BEI Resources (Manassas, VA, USA). Peptides were initially dissolved in dimethyl sulfoxide (DMSO) and arranged into six pools labelled F to M and corresponding respectively to NS3p, NS3h, NS4, NS5A, NS5B I, NS5B II (mean 82, range 73-112 peptides/pool). Pools were used with each single peptide at a final concentration of 3 μ g/mL in ELISpot assays.

Reconstituted peptides were tested for contamination prior to use as this may have caused false positives in subsequent assays. Positive controls used for cytokine production included ConA (Concanavalin A) and FEC (Flu/EBV/CMV), while Mock/DMSO and R10 media were used as a background or negative controls.

Equipment

Table 2.8 Equipment for Peptide Plate Preparation

-80°C freezer
Certified class II microbiological safety cabinet ('hood')
Set of micropipettes
Sterile filtered pipette tips
Vortex
Micro-centrifuge
4°C fridge
Humidified CO ₂ incubator set at 37°C and 5% CO ₂

Materials

Table 2.9 Reagents for Peptide Plate Preparation

Reagent ^a	Supplier	Catalogue No.
RPMI 1640	Gibco	11875-093
FBS (or FCS) ^b	Sigma	F9665
200mM L-Glutamine	Gibco	25030-024
100X Penicillin/Streptomycin solution	Gibco	15140-122
DMSO (Endotoxin-free, sterile)	Sigma	D2650
Round bottomed 96 well plates	Sigma	CLS3799
Titer tops® sterile plate sealers	Sigma	Z688630
Reconstituted peptides		

^a Use listed reagent or equivalent

^b A single lot of FBS/FCS (Sigma, Lot No: 074M3399) was used in the study that had been pre-tested to ensure no background reactivity in the ELISpot assay

Reagent Preparation

All reagents were prepared in a Class II hood using aseptic technique.

See Appendix 2 for details on R10 preparation.

Peptide Plate Preparation

A peptide plate work list was created with the Antigen ID (peptide pool, individual peptide or control antigen), peptide plate column number, row letter, antigen volume, a blank column for operator initials and comments.

The required antigens (peptide pools, individual peptides and control antigens) were made up at 2X the final concentration required. 60 µl of the peptide pool per well of the peptide plate was sufficient for one ELISpot assay. Peptide plates could be freeze-thawed up to three times.

The antigens were aliquoted into round-bottomed 96-well microtitre plates according to the peptide plate template design. Once all the antigens were aliquoted into the peptide plates, the plates were sealed with a Titer Top sterile plate sealer to prevent inter well contamination. The lids were replaced on the plates and the plates frozen until use at -80 °C.

2.5 Serum Separation

Introduction

Blood serum is blood plasma with fibrinogen or other clotting factors and is obtained when whole blood is allowed to clot before centrifugation to remove cellular components. Serum is clearer than plasma because it contains fewer proteins. Proteins are sometimes considered to be interfering substances in assays as they can react with reagents, thereby yielding inaccurate results. Serum is therefore the preferred specimen in clinical testing as the interference that may be caused by using plasma is eliminated.

Method

A tube of whole blood was collected following standard phlebotomy procedures using a serum separator tube. The sample was allowed to clot by leaving the tube in an upright position at room temperature for 30-60 minutes. The tube was then centrifuged for 10 minutes at 1000 x *g*. Using a clean pipette technique, the supernatant was aliquoted into labeled cryovials with subject ID, visit number and date. The vials were then immediately placed into a -80°C freezer.

2.6 Protein Quantification

Protein quantification of cell lysates obtained following PBMC separation was performed using the Bicinchoninic Assay (BCA) Protein Assay Kit (Thermo Scientific). BCA reagent A was mixed with BCA reagent B in a 50:1 ratio and added to a 96 well plate along with protein standards at varying concentrations (0µg, 4µg, 10µg and 20µg) in order to provide standards for the construction of a standard curve. The optical density of each well was then measured using a spectrophotometer plate reader. A standard curve was plotted using Excel Spreadsheet allowing for the subsequent calculation of protein concentration of the unknown samples.

2.7 PAXgene Blood Tube RNA Collection and Storage

The PAXgene Blood Tube is intended for the collection, transport, and storage of blood and stabilization of intracellular RNA in a closed tube and subsequent isolation and purification of intracellular RNA from whole blood for RT-PCR used in molecular diagnostic testing.

Materials

Table 2.10 Materials for PAXgene Blood Tube collection

PAXgene RNA Collection Tube	BD (Catalogue No 762165)
Blood Collection Set	

Method

Blood Collection

The PAXgene Blood RNA Tube was stored at room temperature prior to use and properly labelled with patient identification. If the PAXgene Blood RNA Tube was the only tube to be drawn, blood was drawn into a 'Discard Tube' prior to

drawing blood into the PAXgene Blood RNA Tube so the interior volume of the blood collection set used during phlebotomy could be primed. Otherwise the PAXgene Blood RNA tube was the last tube drawn in the phlebotomy procedure. At least 10 seconds was allowed for a complete blood draw to take place. It was ensured that the blood had stopped flowing into the tube before removing the tube from the holder. The PAXgene Blood RNA Tube with its vacuum is designed to draw 2.5mL of blood into the tube. Immediately after blood collection, the PAXgene Blood RNA tube was gently inverted 8-10 times.

Blood Storage

The PAXgene Blood RNA Tube was stored upright at room temperature (18 - 25°C) for a minimum of 2 hours and a maximum of 72 hours before transferring to a -20°C freezer. The PAXgene Blood RNA Tube was stood upright in a wire rack. After freezing the tubes at -20°C for 24 hours, they were then transferred to a -80°C freezer for long-term storage. Following completion of the clinical trial, the PAXgene tubes were shipped to the University of Oxford for further analysis.

2.8 Western Blotting

Introduction

The western blot is an analytical technique used in immunology to detect specific proteins in a sample of a complex mixture of proteins extracted from cells. This technique uses three elements to accomplish this task. Reducing SDS-PAGE (sodium dodecyl sulphate-Polyacrylamide gel electrophoresis) is the initial step in which a protein is denatured, i.e. it loses its quaternary and tertiary structure, and ran through a polyacrylamide gel using electrophoresis, which will separate proteins based on their molecular weight. Secondly, proteins are then transferred to a polyviniyidene diflouride (PVDF) membrane where, thirdly, a protein of interest can then be probed using a specific antibody for that protein. A secondary antibody is then used which will bind the primary antibody. The protein of interest can then be visualised on a Bio-Rad Gel Image station.

Technique

Gels were initially prepared for polyacrylamide gel electrophoresis (PAGE).

The gel % needed was based on the average mass (M_r) of the protein of interest:

Mr of protein	250+	12-250	40-120	15-40	<15
Separating gel % (mLs)	5%	7.5%	10%	12.5%	15%
Acrylamide	2.5	3.75	5	6.25	7.5
1M Tris-HCl pH 8.8	5.6	5.6	5.6	5.6	5.6
dH2O	6.7	5.45	4.8	3.55	4.2
10% SDS	0.15	0.15	0.15	0.15	0.15

This made a sufficient volume for 2 gels. 75 μ l 10% Ammonium Persulphate (0.1g Persulphate and 1ml H2O) and 15 μ l TEMED (were then mixed and added.

As soon as the AP and TEMED were added, the gel mix was poured between the 2 plates up to the green level (3/4 full). EtOH was then pipetted to cover the gel and the gel was allowed to set (about 15 minutes). When set, blotting paper was used to remove the water. 5% stacking gel was then made as follows:

Stacking gel (mls)	5%
Acrylamide	1
1M Tris-HCl pH 6.8	0.75
dH2O	4.61
10% SDS	0.03

This made sufficient stacking gel for 2 gels. 60µl 10% Ammonium Persulphate and 12µl TEMED was then mixed and added. The stacking gel was poured to the top of the glass and the well combs were pushed in. When set, the combs were removed, the wells washed and the plates with gel placed in between into PAGE apparatus. Running buffer (See Appendix 2 for preparation) was poured between the plates until full and the bottom of the casing was filled to the mark. The samples were then loaded into each well. The SDS-PAGE was run at 200V until the protein reached the end of the gel.

Protein was then transferred from the gel to a PVDF membrane. The PVDF was activated by soaking in methanol for 1 minute and then rinsed in 1x transfer buffer (See Appendix 2 for preparation). Two pieces of filter paper, blotting sponges were soaked in 1x transfer buffer. The transfer was set up as follows: black surface, blotting sponge, filter paper, membrane, gel, filter paper, blotting sponge, white surface. Air bubbles were removed before being run on the transfer apparatus at 100v for 1 hour.

Membranes were blocked to prevent the non-specific binding of antibody in 5% milk solution (5% powdered milk in 1x TBST) for 1 hour. Details on preparation of TBST are listed in Appendix 2.

Membranes were incubated overnight at 4°C with primary antibody diluted 1:1000 in either 5% milk or 3% BSA. The following day, the relevant secondary antibody was added in a dilution of 1:2000 in 5% milk solution and was incubated at room temperature for 1 hour. Primary antibodies used are shown in Table 2.11. The presence of proteins was analysed using the Bio-Rad Gel Doc system.

Table 2.11 Antibodies used in Western Blotting

Antibody	Company
STAT1	Cell Signalling (9176)
STAT3	Sigma (SC482)
pSTAT1	Cell Signalling (9167)
Beta Actin	Sigma (A5441)

2.9 Vaccines

Two investigational medicinal products were used in the vaccine study, namely AdCh3NSmut1 and MVA-NSmut.

AdCh3NSmut1 lot 1 B.0016 was manufactured under Good Manufacturing Practice conditions by Advent s.r.l. (Rome, Italy). AdCh3NSmut1 was supplied as a liquid at the concentration of 5.1×10^{10} vp/ml in buffer A195 (10 mM Tris, 75 mM NaCl, 1 mM MgCl₂, 0.02% PS80, 5% sucrose, 0.1 mM EDTA, 10 mM Histidine, 0.5% ethanol, pH 7.4). The virus suspension was supplied as sterile 0.65 ml aliquots in glass vials.

MVA-NSmut lot 002 08 13 was manufactured under Good Manufacturing Practice conditions by Impfstoffwerk Dessau-Tornau (IDT). MVA-NSmut was supplied as a liquid at the concentration of 5.2×10^8 plaque-forming units (pfu)/ml (lot 002 08 13) in buffer (10 mM Tris, 140 mM NaCl, pH 7.7). The virus suspension was supplied as sterile 0.65mL/vial in 2 ml injection vials, made of clear borosilicate glass, FIOLAX® -klar, WBK 1, DIN ISO 8362/1. The vials were sealed with 13 mm bromobutyl rubber stoppers, grey, FM 457/0, V 9128, SAF 1, DIN ISO 8362-2, aluminium injection closures with a 6mm hole.

After QP release by the manufacturer, the vaccines were shipped to the Clinical BioManufacturing Facility at the University of Oxford where they were labelled and QP released to trial by Dr Eleanor Berrie or authorised deputy. The Chief Investigator co-ordinated shipment of vaccines to the trial sites.

Both vaccines were stored at -70 to -90°C in a locked freezer at the Wellcome Trust-Health Research Board (HRB) Clinical Research Facility in St. James's Hospital, Dublin, Ireland, and the Clinical Trials Unit, Kantonsspital St.Gallen, Switzerland. Storage boxes were clearly labelled and closed to prevent cross-contamination. The vaccines were stored and dispensed by the Pharmacy department at the Wellcome Trust-HRB Clinical Research Facility. Vaccine storage, administration and disposal was documented by the Principal Investigators or delegates in logs held in the Investigator Site File in compliance with GCP and the relevant trial-specific vaccine handling Standard Operating Procedure (SOP) and were monitored by independent site monitors. Adherence to the SOPs was captured in the monitoring reports, which were reviewed, by the Principal and Chief Investigators.

At the end of the vaccine trial all remaining vaccines were shipped back to the supplier.

2.9.1 AdCh3NSmut1 formulation and dose

AdCh3NSmut1 was provided in vials at the concentration of 5.1×10^{10} vp/ml. The dose of AdCh3NSmut1 used in this study was 2.5×10^{10} vp. This dose was obtained by injecting 0.49 ml of the vaccine at 5.1×10^{10} vp/ml (Batch B.0016), without dilution.

2.9.2 MVA-NSmut formulation and dose

MVA-NSmut (lot 002 08 13) was provided at the concentration of 5.2×10^8 pfu/ml in buffer (10 mM Tris, 140 mM NaCl, pH 7.7). The dose of MVA-NSmut used in this study was 2×10^8 pfu. This dose was obtained by injecting 0.38 ml of the vaccine at 5.2×10^8 pfu/ml, without dilution.

2.9.3 Vaccine administration

On vaccination days, vaccines were allowed to thaw to room temperature and were administered within 1 hour. The respective vaccine (AdCh3NSmut1 at day 0; MVA-NSmut at day 56) was administered intramuscularly into the deltoid region of the arm. The investigator wore appropriate universal precautions including disposable gloves, safety glasses and an apron. During administration of the vaccine, medication and resuscitation equipment was immediately available in case of anaphylaxis.

2.9.4 Minimising environmental contamination with Genetically Modified Organisms

As the vaccines were administered by intramuscular injection and the volumes to be injected were small (0.49mL and 0.38mL for the respective vaccines), it was felt unlikely that any vaccine would be released into the environment. However, in order to minimise this risk, the inoculation site was covered with an absorbent dressing after immunisation. The dressing was removed from the injection site after 30 minutes and disposed as Genetically Modified Organisms (GMO) waste by autoclaving at 121°C for at least 15 mins, in accordance with the relevant trial specific SOP, the Environmental Protection Agency regulations and current standard hospital practice.

Examples of vaccine labels are given below in Figure 2-2 according to EUDRALEX (the collection of rules and regulations governing medicinal products in the European Union) annexe 13, volume 4.

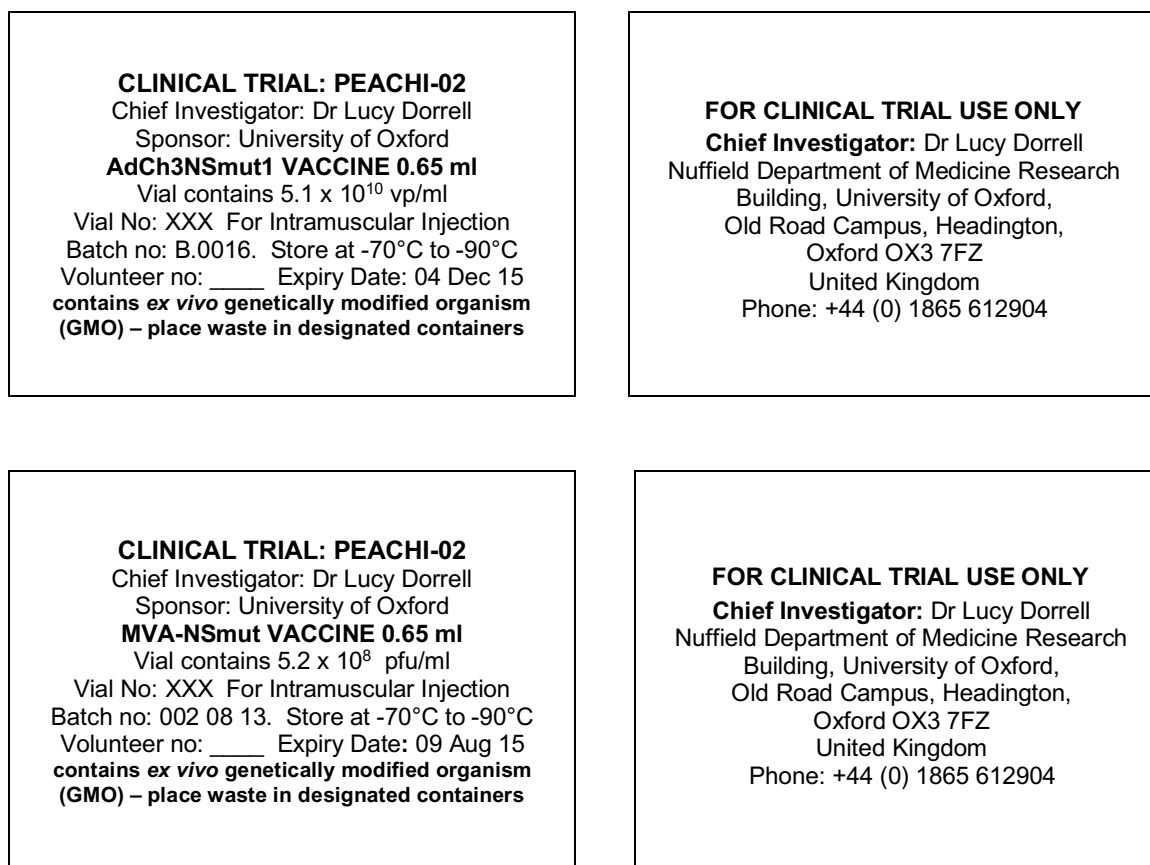


Figure 2-2 Example of vaccine labels according to EUDRALEX annexe 13, volume 4.

2.10 Patients

Descriptions of the patients involved in each of the aims of this thesis (including baseline demographics, clinical details, etc.) are presented in detail in the following results chapters.

Chapter 3 Hepatitis C Therapeutic Outcomes in HIV/HCV co-infected Patients

3.1 Introduction

Chronic HCV infection is estimated to affect approximately 170 million people and is a major cause of cirrhosis and hepatocellular carcinoma worldwide [132]. HCV is also one of the main causes of liver transplantation. Between 350,000 and 500,000 people die of HCV related disease annually [133]. HCV places a significant burden on healthcare resources [134]. Eradication of the virus results in significant reductions in liver-related morbidity and mortality from all causes even in patients with advanced disease [33].

HCV co-infection is common amongst patients with HIV due to shared routes of transmission. Historically, HIV/HCV co-infection has been associated with acceleration of liver disease resulting in increased morbidity and mortality [100]. However, recent evidence suggests that with improvements in care for HIV with earlier treatment with safer, less hepatotoxic medications, liver fibrosis progression is increasingly similar to patients with HCV mono-infection [106].

Until recently, the mainstay of treatment for HCV in co-infected patients was pegylated interferon (PEG-IFN) and ribavirin. Contraindications to PEG-IFN and ribavirin included uncontrolled severe psychiatric disease or epilepsy; pregnancy or couples unwilling to comply with adequate contraception; severe concurrent medical diseases and comorbidities including autoimmune thyroid disease and decompensated liver disease [135]. As a result, many patients were frequently deemed ineligible for this treatment. These contraindications were coupled with poor rates of treatment tolerability due to frequent side effects. Common side effects of PEG-IFN and ribavirin included flu-like symptoms that typically occur after PEG-IFN injections, fatigue, depression, sleeping disorders, skin reactions and dyspnea [136]. Biochemical and haematological side effects included liver enzyme (e.g. ALT) flares, anaemia, neutropenia and thrombocytopenia. It was recommended thyroid function tests (i.e. thyroid

stimulating hormone (TSH) and free thyroxine) be measured every 12 weeks while on therapy [137]. Ribavirin is associated with a risk of teratogenicity and contraception is advised for 6 months beyond treatment completion. Finally, severe or unusual side effects included seizures, autoimmune reactions, neuroretinitis, interstitial lung disease and bone marrow aplasia [136].

PEG-IFN and ribavirin therapy was also associated with suboptimal rates of sustained virological response (SVR) [138]. Previous HIV/HCV cohort groups reported SVR rates of 40-55% for all genotypes for patients treated with PEG-IFN and ribavirin. [114, 139, 140].

As mentioned previously, Telaprevir and boceprevir are first generation direct acting antiviral (DAA) agents and are classed as NS3/4A protease inhibitors. These compounds target the serine protease NS3/4A, which cleaves the HCV polyprotein at four sites during its replication cycle [117]. When prescribed in combination with PEG-IFN and ribavirin, they significantly improved SVR rates in both HCV mono-infection and HIV/HCV co-infection. For genotype 1 HCV infection, clinical trials found that protease inhibitors increase the SVR rate from 45% with standard PEG-IFN and ribavirin-based treatment to as high as 80-90% in selected patients [117].

The field of HCV therapeutics has evolved rapidly ever since. Current approved DAAs inhibit 3 specific steps in the HCV lifecycle including NS3/4A protease enzyme, NS5A protein and the NS5B polymerase. The availability of these new compounds has allowed for IFN-free regimens that have revolutionised the delivery of therapeutic care to patients with HCV, allowing patients previously deemed ineligible to receiving treatment access to regimens with high efficacy and low toxicity.

It is estimated that between 20,000 and 50,000 people are infected with HCV in Ireland [108]. The Department of Genito Urinary Medicine and Infectious Diseases at St James's Hospital, Dublin runs a dedicated Virology Clinic for the

management of patients with infectious hepatitis. At present, approximately 450 HIV/HCV co-infected patients attend the service.

The aim of this section of my research was to assess baseline characteristics and outcomes for all HIV/HCV co-infected patients treated for HCV at the Department of Genito Urinary Medicine and Infectious Diseases (GUIDE) at St James's Hospital from 2001 to 2016.

3.2 Methods

All co-infected patients treated for Hepatitis C were identified from the treatment database from the period 2001 to 2016. Patient data were collected using a standardised data collection form and obtained retrospectively from patient charts and the electronic patient record between January 2015 and March 2017. The hospital information system was examined to identify patients who had died or defaulted from care. Chi-square and Fisher's exact test were used to examine categorical variables. Mann-Whitney U-testing was used to analyse non-parametric continuous variables. Data analysis was performed using SPSS v22 (SPSS Inc., Chicago, IL, USA). Kaplan-Meier curves were generated using GraphPad Prism v5 (GraphPad Software Inc., CA, USA).

3.3 Results

Results of therapeutic outcomes by treatment regimen used and stratified by risk group are presented below.

3.4 Outcomes from patients treated with Interferon-based regimens

All patients who undertook dual therapy HCV treatment received once weekly pegylated interferon- α 2A (Pegasys®; Roche Pharmaceuticals, Reinach, Switzerland) or pegylated interferon- α 2B (Pegintron®; Schering-Plough,

Kenilworth, NJ, USA) and weight based dosing of ribavirin twice daily as described in published reports [141, 142].

During the study period, 168 patients from a co-infected cohort of approximately 450 patients were treated with a dual combination of PEG-IFN and ribavirin. 20 co-infected patients were treated with PEG-IFN, ribavirin and a first generation protease inhibitor. Baseline characteristics of these patients are shown in Table 3.1 and Table 3.2.

Table 3.1 Baseline characteristics of patients commenced on dual treatment with pegylated interferon and ribavirin (n=168)

Mean age, years (range)	37 (22-59)
Male sex [n, (%)]	129 (77%)
Mean CD4 count, mm ³ (range) ^a	501 (155-1417)
Route of Acquisition [n, (%)]	
PWID	118 (70%)
Non-PWID	40 (30%)
Genotype [n, (%)]	
1	70 (42%)
2	10 (6%)
3	83(49%)
4	5 (3%)
Cirrhosis	20%
Antiretroviral therapy	68%
Virally suppressed	93%

^aNormal CD4 count 502-1749 cells/mm³

Table 3.2 Baseline characteristics of patients commenced on triple therapy with pegylated interferon, ribavirin and a direct-acting antiviral (n=20)

Mean age, years (range)	43 (31-61)
Male sex [n, (%)]	17 (85%)
Mean CD4 count, mm ³ (range)	695 (247-1451)
Route of Acquisition [n, (%)]	
PWID	11 (55%)
Non-PWID	9 (45%)
Genotype [n, (%)]	
1a	20 (100%)
1b	0 (0%)
Cirrhosis	35%
Antiretroviral therapy	100%
Virally suppressed	100%

^aNormal CD4 count 502-1749 cells/mm³

Patients were classified as having obtained SVR if they had an undetectable HCV viral load 24 weeks post completion of treatment. In an intention-to-treat analysis the overall sustained virological response for all 188 patients treated with IFN-containing regimens was 62%. Response by genotype and the presence or absence of cirrhosis are listed in Table 3.3. The difference in the receipt of ART between the two groups reflects alterations in the HIV guidelines regarding timing of initiation of ART historically. Of the patients who didn't achieve a SVR, 17 patients relapsed, 35 were partial or non-responders, 19 discontinued due to adverse events and 1 patient was lost to follow up.

Table 3.3 Rates of Sustained Virological Response (SVR) for all patients (n=188) treated with a treatment regimen containing Pegylated Interferon

Group	SVR%
Pegylated Inteferon / Ribavirin (n=168)	
Overall	60% (100/168)
Genotype 1	41% (29/70)
Genotype 2	90% (9/10)
Genotype 3	71% (59/83)
Genotype 4	60% (3/5)
Non-cirrhotic (All genotypes)	67%
Cirrhotic (All genotypes)	29%
Pegylated Interferon / Ribavirin / DAA (n=20)	
Overall	80% (16/20)

When comparing outcomes at the GUIDE clinic for mono-infected and co-infected patients treated with PEG-IFN, ribavirin and a first generation protease inhibitor, no significant difference was seen in outcomes (see Figure 3-1), reflecting the fact that outcomes of treatment with first generation DAA agents combined with interferon therapy are identical in HCV mono-infection and HIV/HCV co-infection.

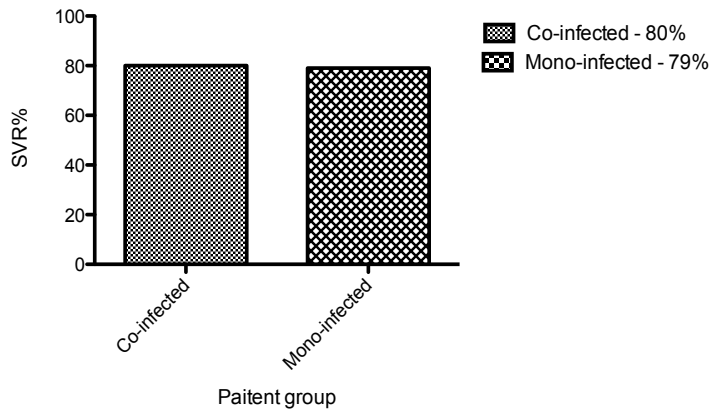


Figure 3-1 Comparison of therapeutic outcomes in HIV/HCV co-infected and HCV mono-infected patients for HCV regimens containing a first generation protease inhibitor.

HIV/HCV co-infected (n=20) and HCV mono-infected (n=19) patients were treated with PEG-IFN, ribavirin and a first generation protease inhibitor. No significant differences in treatment outcomes were seen.

Predictors of a favourable response included obtaining a rapid virological response (HCV viral load not detected by HCV RNA testing at week 4 of treatment), infection with genotype 2 or 3 disease, receipt of a protease inhibitor and the absence of cirrhosis.

Therapeutic response rates for HIV/HCV co-infected patients treated with dual therapy with PEG-IFN were favourable compared with other published cohorts [139, 140]. Management of patients in a specialised viral hepatitis clinic with extensive education, counselling and support prior to and during treatment may have influenced outcomes [143].

We looked to evaluate the common side effects seen with HCV treatment. Anaemia (defined as a Haemoglobin <10g/dL) occurred in 25% of patients. 23% of all patients treated received erythropoietin for anaemia. We have previously shown that haematological support improves outcomes during PEG-IFN therapy [144], as it facilitated maintenance of full-dose PEG-IFN and ribavirin and prevented therapy discontinuation because of haematological side effects. Granulocyte-colony stimulating factor was used for management of neutropenia in 5% of patients. The most common other adverse events reported were flu-like symptoms (>80% of patients), weight loss (30%), psychiatric side effects (20%) and injection site reactions (20%). There were two patient deaths during

treatment. One patient died from pneumonia and another died from hepatocellular carcinoma diagnosed during treatment.

I looked to evaluate the association between SVR, all-cause mortality and liver-related morbidity for all patients treated with PEG-IFN regimens. Time 0 was taken at initiation of therapy. Patients with undetectable HCV RNA 24 weeks post therapy were classified as having obtained SVR, and others were classified as without SVR. Significant reductions were seen in all-cause mortality in patients who achieved SVR compared with patients who did not achieve SVR (Figure 3-2). 5 of the deaths in the non-SVR group were liver-related while none of the deaths in the SVR group were liver-related.

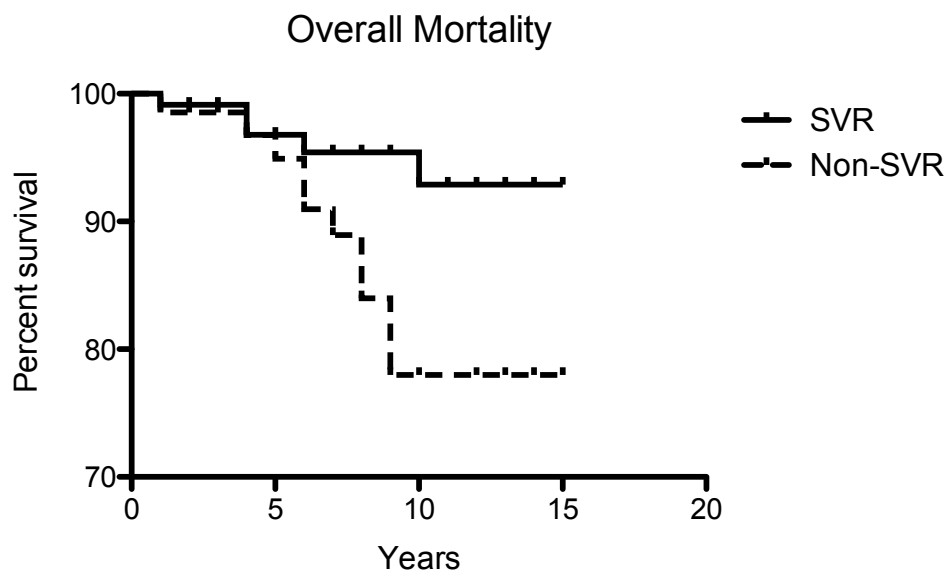


Figure 3-2 Kaplan-Meier curve comparing overall mortality in the SVR (n=116) vs non-SVR (n=72) groups in our cohort of co-infected patients (p<0.05 by Log-rank (Mantel-Cox test))

For the purposes of assessing liver-related morbidity we evaluated a composite of outcomes including the development of decompensated liver disease (ascites, bleeding varices or hepatic encephalopathy), hepatocellular carcinoma or the need for liver transplant. We observed a significant difference in liver-related morbidity between the two groups (**Error! Reference source not found.**).



Figure 3-3 Kaplan-Meier curve comparing liver-related morbidity (development of decompensated liver disease, hepatocellular carcinoma or the need for liver transplant) in the SVR (n=116) vs non-SVR (n=72) groups in our cohort of co-infected patients (p<0.0005 by Log-rank (Mantel-Cox test))

There are a number of limitations in the interpretation of this data. As this analysis was performed retrospectively, I was unable to account for unknown potential confounding factors such as rates of concomitant drug and alcohol use, exposure to other potentially hepatotoxic co-medications or other unrecognised causes of liver pathology such as non-alcoholic fatty liver disease. Additionally, as accurate staging of liver disease (i.e. liver biopsy) was not performed on every patient, it is possible that patients in the non-SVR group had more advanced disease at baseline.

Ideally comparator groups that included matched patient groups with a similar degree of liver disease in the absence of HCV or a group of patients with HIV alone may have been used as controls to both SVR and non-SVR groups. However, in the absence of a national HIV registry or a unique Irish National Patient Identifier, data on these patient groups would frequently be incomplete and subject to bias.

3.5 Outcomes in People Who Inject Drugs (PWID)

PWID represent the majority of the HCV epidemic in the developed world [95]. The majority of new infections develop in active PWID, with this group accounting for more than 80% of new infections in high-income countries [145]. Furthermore, an additional large reservoir of infection exists amongst former PWID who remain undiagnosed. Public health initiatives which aim to reduce the burden of HCV and its attendant complications, or more ambitious strategies to target HCV elimination will therefore be unsuccessful unless they include strategies for engagement and retention of PWID in treatment and follow-up. We evaluated for differences in treatment adherence and response to PEG-IFN and ribavirin in a large urban cohort with and without a history of remote or recent injection drug use. For the purpose of this evaluation, patient data were recruited for patients with both HCV mono-infection and HIV/HCV co-infection.

Patient data were retrospectively reviewed for 1000 consecutive patients – 608 former (no injecting drug use within 6 months of therapy), 85 recent (injecting drug use within 6 months) PWID, and 307 non-drug users who were treated for chronic HCV with PEG-IFN and ribavirin. All patients either attended a large university teaching hospital (St James’s Hospital) or a community-based drug treatment centre and were treated for HCV between 2001 and 2012.

Baseline characteristics of the patients treated are shown below in Table 3.4

Table 3.4 Baseline characteristics of patients treated for HCV (n=1000)

	Former PWID (n=608)	Recent PWID (n=85)	non-PWID (n=307)
Age (years) [mean + SD]	36.2 ± 7.7	35.9 ± 6.6	43.0 ± 11.6
Sex (male) [n (%)]	547 (78.0)	67 (78.8)	196 (63.8)
Genotype [n (%)]			
1	290 (41.8)	42 (49.4)	142 (46.3)
2	29 (4.2)	5 (5.9)	15 (4.9)
3	364 (52.5)	38 (44.7)	131 (42.7)
4-6	10 (1.4)	0 (0)	19 (6.3)
Cirrhosis [n (%)]	83 (12)	0 (0)	43 (14)
HIV Co-infection	114 (16.5)	0 (0)	43 (14)
Log ₁₀ Viral Load – mean (range)	6.53 (2.10-7.84)	6.63 (4.30-7.33)	6.49 (4.06 – 7.64)

Treatment response rates are detailed in Table 3.5.

Table 3.5 Adherence and treatment responses in people who inject drugs (PWID) and non-PWIDs

	Former PWID n (%)	Recent PWID n (%)	Non-PWIDs n (%)	RR (95% CI) in Former versus recent PWID	RR (95% CI) in PWID versus non-PWID
Non-responders	71/608 (11.7)	8/85 (9.4)	46/307 (15)	1.24 (0.62-2.49)	0.74 (0.53-1.05)
Genotype 1 patients	52/248 (21)	8/40 (19)	32/142 (22.5)	1.02 (0.53-1.98)	0.92 (0.63-1.35)
Genotype 3 patients	14/326 (4.3)	0 (0)	7/131 (5.3)	-	0.72 (0.30-1.75)
End of treatment response	454/608 (74.7)	68/85 (82.9)	234/307 (76.2)	0.93 (0.83-1.05)	0.99 (0.92-1.07)
Genotype 1 patients	155/248 (62.5)	28/40 (70)	98/142 (69)	0.78 (0.68-0.90)	0.92 (0.80-1.05)
Genotype 3 patients	270/326 (82.8)	35/38 (94.6)	111/131 (84.7)	0.90 (0.81-0.99)	0.99 (0.91-1.08)
Sustained Viral Response	384/608 (63.2)	60/85 (70.6)	187/307 (60.9)	0.89 (0.77-1.04)	1.05 (0.95-1.17)
Genotype 1 patients	117/248 (47.2)	23/40 (54.8)	69/142 (48.6)	0.82 (0.61-1.10)	1.00 (0.81-1.23)
Genotype 3 patients	241/326 (73.9)	31/38 (83.8)	96/131 (73.3)	0.91 (0.77-1.07)	1.02 (0.91-1.15)
Non-adherence	53/608 (8.7)	5/85 (5.9)	21/307 (6.8)	0.84 (0.33-2.10)	1.23 (0.76-1.99)
Lost to Follow up	30/608 (4.9)	1/85 (1.2)	6/307 (2)	4.19 (0.58-30.4)	2.3 (0.97-5.45)

There was no significant difference in treatment non-adherence between the groups. The overall SVR rate in PWID was not different from non-PWID and there was no significant difference in SVR rates between the groups controlling for genotype. As expected, genotype 1 disease was less responsive to therapy. These results are shown graphically in Figure 3-4.

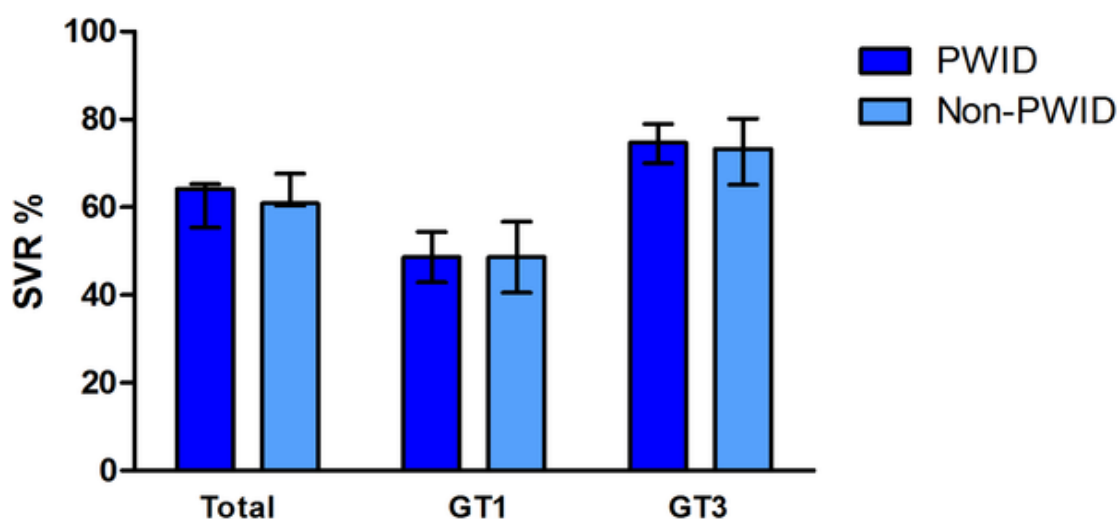


Figure 3-4 SVR rates in PWID vs Non-PWID

No significant difference was observed in SVR rate between PWID vs Non-PWID with respect to overall SVR rate or SVR rate by genotype.

Former and recent PWID had similar adherence rates. Additionally, in the HIV/HCV co-infected cohort the overall SVR rate in this group was 60.5% which is comparable to that of the entire cohort. These data support a public health strategy for HCV treatment and eradication in PWID in the DAA era.

3.6 Decompensated Cirrhotic Early Access Program (EAP)

In December 2014, an Early Access Program was launched making IFN-free DAA medications available for patients with decompensated cirrhosis, a group that were ineligible for treatment with PEG-IFN as these patients had an absolute contraindication to the use of IFN-based regimens. All patients commenced treatment between December 2014 and February 2015. Treatment was with fixed-dose combination of Sofosbuvir/ledipasvir with ribavirin for 12 weeks. Sofosbuvir works as an inhibitor of the HCV NS5B RNA-dependent RNA polymerase. Ledipasvir is a NS5A inhibitor.

Baseline characteristics of the patients treated with this regimen are shown below in Table 3.6.

Table 3.6 Baseline characteristics of HIV/HCV co-infected patients with decompensated cirrhosis treated with an IFN-free DAA regimen as part of the Early Access Program (n=13)

Mean age, years (range)	45 (37-61)
Male sex	11 (85%)
Mean CD4 count, mm ³ (range) ^a	221 (30-457)
Route of Acquisition	
PWID	13 (100%)
Non-PWID	0 (0%)
Genotype	
1a	9 (70%)
1b	2 (15%)
3	2 (15%)
Decompensated cirrhosis	100%
Child-Pugh Class B	46%
Child-Pugh Class C	54%
Antiretroviral therapy	100%
Virally suppressed	100%

^a Normal CD4 count 502-1749 cells/mm³

The overall SVR rate for this patient group was 54% (7/13). Regarding genotype, the cure rate was 64% (7/11) for patients with Genotype 1 disease and neither of the two patients with Genotype 3 disease obtained a SVR. The choice of sofosbuvir/ledispavir for management of Genotype 3 disease has now been superseded by regimens with a higher efficacy.

At 2 years of follow up on these patients, the overall mortality rate was 54%. This reflects the challenges treating this patient group, given the poor overall prognosis for HIV/HCV patients with decompensated liver disease [101]. 2 patients died while on treatment with DAA therapy. High mortality during therapy has been reported on a national level for the Irish EAP [146], especially amongst patients with Child-Pugh C cirrhosis. These findings were mirrored in patients with Child-Pugh C cirrhosis treated with antiviral therapy in the United Kingdom as part of the National Health Service EAP [147]. As a result of these findings, it is advised that clinicians should reflect very carefully on the risk-benefit ratio of therapy compared with rapid referral for liver transplantation evaluation in such patients.

Of the patients who did not obtain a SVR, 2 patients were successfully retreated following relapse and are alive at 2 years of follow up. 4 of the patients who achieved SVR were alive at 2 years. Of the 3 remaining patients who achieved SVR, 1 patient died from decompensated liver disease having been deemed ineligible for liver transplant due to medical co-morbidities. The other 2 patients died of bronchopneumonia and a cerebrovascular event.

Liver transplantation is the treatment of choice for patients with end stage liver disease. Recurrence of HCV infection is ubiquitous in patients who have HCV RNA detectable at the time of liver transplant [148]. In the current era, where there is a shortage of available liver organs, an improvement in liver function following successful treatment of HCV will result in delisting of some patients. If delisted, a patient will keep a diseased liver with the on-going risk of subsequent hepatocellular carcinoma development, further decompensation and death. They also would potentially lose an opportunity to cure both HCV infection and liver disease. This is because HCV treatment is effective in the vast majority of patients treated post-transplant [149]. As such, no definite consensus on the optimal approach to these patients has been reached to date.

Additionally, many patients are deemed ineligible for liver transplant due to a multitude of factors including medical co-morbidities, advanced age,

uncontrolled systemic infection or active alcohol abuse. The primary goal of HCV therapy in patients with decompensated liver disease who are not candidates for a liver transplant is to achieve an improvement in liver function and survival. Several studies have shown high SVR rates in patients who received DAA treatment with decompensated cirrhosis [150, 151]. In up to 50% of patients, a clear therapeutic effect of HCV viral clearance has been demonstrated. This has resulted in significant improvements in bilirubin, INR and albumin values, and as a result, an improvement in Model for End-Stage Liver Disease (MELD) and Child-Pugh scores. Similar results have been replicated in real-world studies [147, 152]. Patients with Child-Pugh B cirrhosis appear to benefit more from viral clearance than those with Child-Pugh C cirrhosis [147]. To date, long-term clinical follow-up data on these patient groups have been lacking. It is important that we should look to identify additional predictors to assess those who are most likely to benefit from HCV therapy. As a result, it is planned on a national level to analyse the two year outcomes on all patients enrolled in the EAP to help answer this question.

3.7 Expansion of DAA availability

Following the EAP, IFN-free DAA therapy was initially made available to patients with advanced liver disease, and eventually made available to all patients with chronic HCV. Baseline characteristics of the 62 HIV/HCV co-infected patients treated to date who have reached SVR12 assessment are presented below in Table 3.7.

Table 3.7 Baseline characteristics of HIV/HCV co-infected patients treated with an IFN-free DAA regimen since January 2016 (n=62)

Mean age, years (range)	47 (24-65 years)
Male sex, [n (%)]	49 (79%)
Mean CD4 count, mm ³ (range) ^a	605 (99-1394)
Route of acquisition, [n (%)]	
PWID	34 (55%)
Non-PWID	28 (45%)
Genotype, [n(%)]	
1	38 (61%)
2	1 (2%)
3	18 (29%)
4	5 (8%)
Non-cirrhotic	26 (42%)
Cirrhosis (compensated), [n(%)]	33 (53%)
Cirrhosis (decompensated), [n(%)]	3 (2%)
Antiretroviral therapy	100%
Viral suppression	100%
Treatment	
Sofosbuvir/ledipasvir	33 (53%)
Sofosbuvir/daclatasvir	18 (29%)
PrOD ^b	11 (18%)

^a Normal CD4 count 502-1749 cells/mm³

^b PrOD - Paritaprevir/ritonavir/ombitasvir and dasabuvir

Results of the overall outcomes of all patients treated to date between 2001 and 2016 are presented below in Table 3.8.

Table 3.8 Treatment outcomes for HIV/HCV co-infected patients by Group 2001-2016

	PEG-IFN/RBV n=168	Telaprevir n=20	EAP n=13	IFN-free DAA n=62
SVR	60% (100/168)	80% (16/20)	54% (7/13)	92% (57/62)
G1	41% (29/70)	80% (16/20)	64% (7/11)	92% (35/38)
G2	90% (9/10)			100% (1/1)
G3	71% (59/83)		0% (0/2)	89% (16/18)
G4	60% (3/5)			100% (5/5)

Of the 5 patients who failed to achieve a SVR, 1 patient relapsed with evidence of emergence of HCV resistance-associated substitutions. 2 patients died on treatment. The final 2 patients have been lost to follow up since completion of HCV treatment, so we have been unable to document whether they have achieved a SVR to date.

3.8 Treatment uptake by year

Figure 3-5 shows the treatment uptake by year since 2001 for co-infected patients in the GUIDE clinic. In recent years DAA therapy was initially only available to selected patient groups including those with cirrhosis and advanced liver disease. However, in early 2017, DAA therapy was made available as a treatment option to all patients with HCV and at the end of the first quarter of 2017, the number of HIV/HCV co-infected patients started on HCV treatment had exceeded the total number treated in 2016. It is anticipated that treatment uptake will continue to increase significantly in 2017.

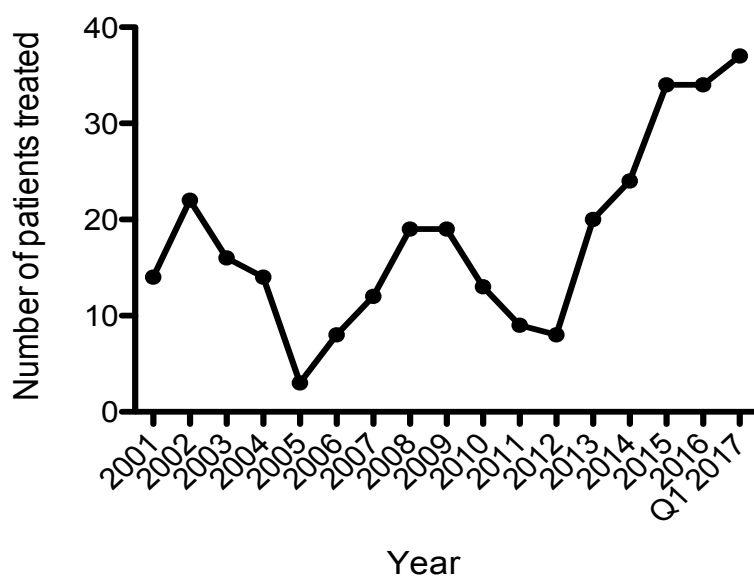


Figure 3-5 Number of HIV/HCV co-infected patients treated for HCV by year since 2001.

A marked increase in treatment uptake has been seen with the introduction of the IFN-free DAA agents. As access to these agents was granted to all patients with HCV in 2017, regardless of fibrosis stage, treatment uptake in the first quarter (Q1) of 2017 has exceeded the total number treated in 2016.

3.9 HCV Reinfections

To date, evidence of HCV reinfection with established reoccurrence of chronic HCV infection post SVR has occurred in 10 patients. With a mean follow up of 5.8 years (Range 1 – 180 months), for all patients who achieved SVR between 2001 and 2016, this equates to a reinfection rate of 11.6/1000 person years of follow-

up. 2 additional patients have had evidence of transient HCV viraemia with spontaneous resolution in the absence of treatment at follow up bloods. 8 of the 10 of the re-infections were attributed to a relapse of intravenous heroin use. The additional 2 cases were presumed to be secondary to cocaine use and MSM contact respectively.

9 of 10 cases occurred post receipt of dual therapy with PEG-IFN and ribavirin. The 10th case occurred after successful treatment with telaprevir-based therapy. To date, no re-infections have been documented with any of the IFN-free DAA agents.

3.10 Conclusion

In summary, the treatment landscape for HCV in Ireland has altered markedly over the past number of years. We have shown that comparable SVR rates are achievable in HIV/HCV co-infected patients compared to HCV mono-infected patients when IFN-based therapies are used. These results hold true for PWID, a group long considered 'difficult to treat'. These data are in line with previously published cohort studies of treatment adherence and outcomes in PWID [153]. I have also demonstrated the benefits of obtaining a cure of HCV in co-infected patients with respect to all-cause mortality and liver-related morbidity.

PEG-IFN is no longer considered standard-of-care and has been replaced by all-oral, well-tolerated short course regimens with high efficacy. Recent availability of DAA agents to all patients with HCV, regardless of fibrosis stage, will allow for higher rates of treatment uptake and SVR rates are anticipated to be close to 100% for patients who complete appropriately prescribed therapy. We have demonstrated that these new DAA agent are associated with very high SVR rates in co-infected patients in a real world setting and universal access to these agents has allowed high rates of treatment uptake with more co-infected patients treated in the first 3 months of 2017 than in all of 2016.

A number of challenges remain. Patients with decompensated liver disease remain a challenge with suboptimal response rates and the possibility that eradication of HCV will not alter the natural history of patients with decompensated disease. There is an on-going debate as to whether patients with decompensated cirrhosis on the transplant list should be treated for their HCV infection prior to liver transplantation or, alternatively, transplanted first and treated promptly after transplantation. Thus far, no consensus has been reached because these two approaches. To date, the two approaches have not been prospectively compared in appropriately powered randomised trials using clinical endpoints. As a result, it is advised that clinicians should reflect very carefully on the risk-benefit ratio of therapy compared with rapid referral for liver transplantation evaluation in such patients while remaining cognisant of the shortage of available livers for transplant.

Universal access to DAA-based HCV therapy will continue to have a limited impact on the burden of HCV-related disease on a population level unless barriers to HCV education, screening, evaluation and treatment are addressed and treatment uptake continues to improve. However, targeted therapeutic strategies focusing on specific risk groups such as co-infected patients and haemophiliacs will likely lead to eradication of HCV in these cohorts.

Finally, although SVR rates for patients treated with these new regimens are favourable they are not 100% effective. Further knowledge of the complex interplay between the immune system and HCV therapeutics will hopefully contribute to our understanding of the immunological determinants that underlie successful HCV viral clearance with therapy and identify patients at risk of treatment failure. This knowledge could be a step towards a personalised approach to HCV therapeutics. Chapter 4 details my work towards this goal.

Chapter 4 Evaluation of the JAK-STAT Signaling Pathway in Patients undergoing treatment for Hepatitis C with a Telaprevir-based regimen

4.1 Introduction

Infection with HCV results in chronic infection in the majority of infected individuals. Primary (acute) infection is usually asymptomatic, although some patients may present with jaundice. Following acute infection approximately 20% of patients will spontaneously clear the virus, while 80% will develop persistent HCV infection. The mechanisms by which some individuals clear the virus and others develop chronic infection are still not yet fully understood, but likely reflect a complex interplay between the host and the virus at the level of the immune response [154].

Persistent HCV infection is a significant cause of morbidity and mortality due to progressive liver fibrosis. Eventually 20-50% of patients will develop cirrhosis. Individuals with chronic hepatitis C and cirrhosis are at increased risk of liver failure, liver transplantation and hepatocellular carcinoma development [132]. Following initial infection, HCV RNA can be detected in plasma within days of exposure, often weeks before liver enzyme levels rise. Peak viraemia occurs in the first 8 to 12 weeks of infection, then drops to lower levels and persists. A number of situations may occur at this time:

- Plasma HCV RNA becomes undetectable within the first few months of infection and remains undetectable indefinitely (viral clearance)
- Plasma HCV RNA is inconsistently detected early in infection and a stable pattern of recovery or persistence is not evident for more than 6 months. Intermittent viraemia may represent re-infection (which is observed in PWID) or escape from an initially successful immune response.

The mechanisms of persistence of HCV remain incompletely understood, but recent studies have revealed important insights into the mechanisms of HCV immune evasion as outlined in Chapter 1. Similarly, a further understanding of

the interplay between the immune system and HCV therapeutics, including identifying immune mechanisms of HCV clearance could be paramount in identifying new therapeutic options and predictors of response to therapy.

4.2 The Interferon Anti-Viral System

IFNs are a multi-gene family of inducible cytokines that are best known for their potent anti-viral properties [40-42]. As outlined in Chapter 1, IFN- α establishes an anti-viral state via the intracellular JAK-STAT signalling pathway (See Figure 1-2). IFN- α receptor binding leads to recruitment and activation of STAT proteins, resulting in upregulation of several hundred ISGs. These ISGs directly interfere with HCV replication [155].

4.3 Aims and Methods of the Study

Given many patients do not respond to PEG-IFN/ribavirin dual therapy, we previously investigated the status of the JAK-STAT pathway in both primary immune cells and hepatocytes from HCV infected patients. We found that STAT1 and STAT3 proteins were reduced in all major immune cell populations and hepatocytes from HCV infected patients [52]. Other groups have identified HCV within immune cells [20, 21], demonstrating the ability of HCV to directly target intracellular protein components of the IFN- α pathway. These are significant findings since STAT1 and STAT3 are essential components of the IFN- α pathway, suggesting that HCV interferes with this anti-viral signaling cascade in a bid to avoid viral clearance.

Until recently, IFN- α had been the backbone of HCV therapy for more than 20 years, and was responsible for a large part of the side effect profile of HCV treatment [156]. Phase I clinical trials with protease inhibitors revealed a greater decline in HCV RNA in patients treated with PEG-IFN/ribavirin and a protease inhibitor, compared to protease inhibitor monotherapy [157].

An improvement seen in SVR rates in previous non-responders to dual therapy with PEG-IFN/ribavirin when they were retreated with the addition of first generation protease inhibitor (PI) suggests that PIs restore the effectiveness of PEG-IFN/ribavirin therapy [158]. The mechanism by which this occurs remains unknown. Since the IFN- α pathway and induction of anti-viral ISGs are essential for successful viral clearance, we hypothesised that in suppressing viral replication directly DAAs may indirectly enable restoration of the IFN- α pathway and that indeed this may be crucial in achieving full clearance of HCV.

Indeed, if restoration of the JAK-STAT pathway occurs during DAA treatment, this may be a useful marker of therapeutic response to DAAs. Therefore, in order to determine if restoration of the IFN- α response might have a role in DAA-mediated HCV clearance, we evaluated STAT1 and STAT3 protein levels in PBMCs from HIV/HCV-infected patients prior to and during therapy with PEG-IFN/ribavirin and Telaprevir.

Cells were harvested in lysis buffer and analysed by immunoblotting using STAT1, STAT3, pSTAT1 and beta-actin. Densitometry was calculated using ImageJ software (v 1.48). Statistical analysis was performed using GraphPad Prism v5 (GraphPad Software Inc., CA, USA) and the Student's t-test, Mann-Whitney U-test, or ANOVA test was used as appropriate.

Blood from patients with HIV/HCV co-infection were studied. All were informed and gave written consent. The study received ethical approval from the Tallaght Hospital / St James's Hospital Joint Research and Ethics Committee, in accordance with the guidelines of the 1975 Declaration of Helsinki.

4.4 Baseline Characteristics of the HIV/HCV co-infected patients

The baseline characteristics of the 11 patients enrolled in this study are listed in Table 4.1 below.

Table 4.1 Baseline characteristics of the HIV/HCV co-infected patients treated with DAA-based therapy in combination with PEG-IFN and ribavirin

Mean age, years (range)	38 (31-47)
Male sex, [n (%)]	10 (91%)
Mean CD4 count, mm ³ (range) ^a	720 (438-1138)
Route of acquisition, [n (%)]	
PWID	4 (36%)
Non-PWID	7 (64%)
Genotype, [n(%)]	
1a	10 (91%)
1b	1 (9%)
Cirrhosis (compensated), [n(%)]	4 (36%)
Antiretroviral therapy	100%
Viral suppression	100%
Telaprevir based treatment	100%

^a Normal CD4 count 502-1749 cells/mm³

4.5 Responses to treatment

The viral load responses to treatment for all patients are plotted on Figure 4-1 below.

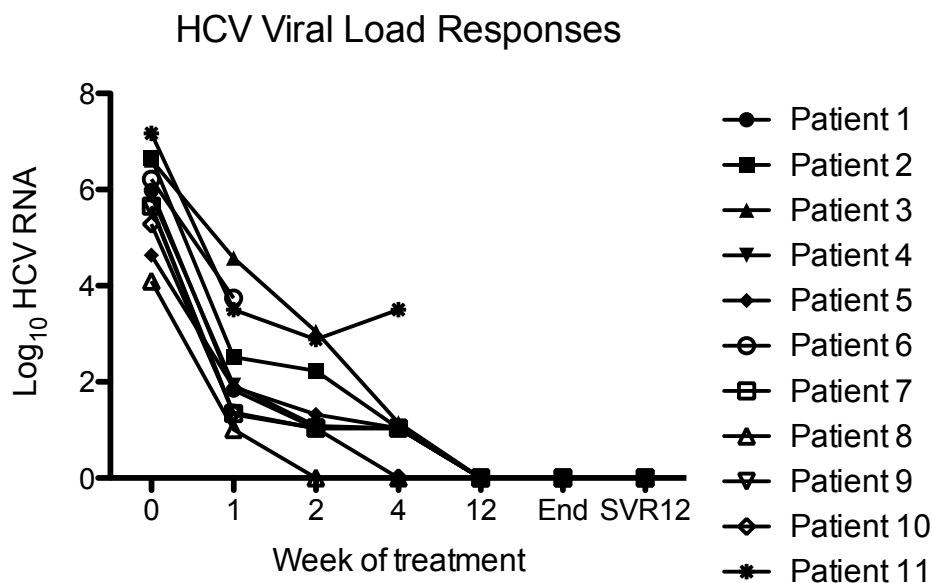


Figure 4-1 HCV viral load responses.

The majority of patients experienced a rapid decline in HCV viral load followed by viral suppression. Patient 11 met the stopping rules for Telaprevir-based treatment.

Of the 11 patients enrolled in the study, 9 (82%) achieved a SVR. Patient 6 discontinued treatment after 1 week due to an intercurrent illness. Patient 11 met the stopping rules for Telparevir-based treatment. The patient had an initial viral decline at weeks 1 and 2, but failed to obtain a HCV viral load of <1000 IU/mL at week 4 and had evidence of viral rebound at this timepoint. As such, treatment was discontinued for this patient. HCV resistance testing revealed the emergence of NS3/4A protease resistance-associated substitutions. This patient was eventually retreated with IFN-free DAA agents and obtained a SVR.

4.6 Results

When baseline levels of STAT1, prior to initiation of Hepatitis C treatment, in PBMCs from co-infected patients were compared to healthy volunteers, we found significantly reduced levels in of STAT1 in the patients (Figure 4-2).

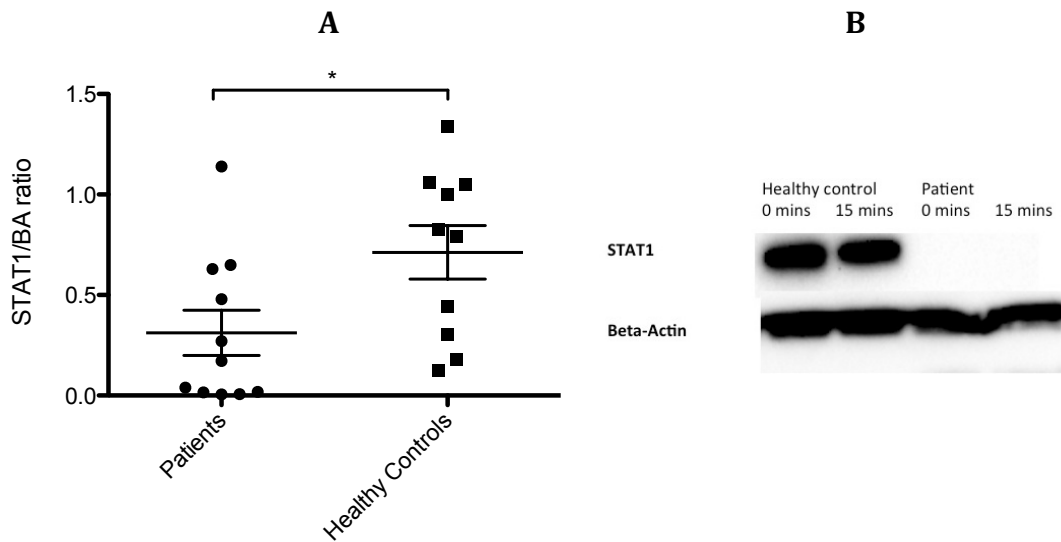


Figure 4-2 STAT1 protein levels are reduced in PBMCs from HIV/HCV co-infected patients.

(A) Ratio of STAT1: house-keeping protein optical density (OD) measured by densitometric analysis of immunoblots of STAT1 and beta-actin (BA) protein levels in PBMCs from HIV/HCV+ patients (n = 11) and healthy (n = 10) individuals. Patients with co-infection had lower levels of STAT1 (p<0.05 Student t test). (B) Lysates from PBMCs of an individual HIV/HCV+ patient and healthy control treated +/- IFN-alpha for 15 min, were analysed by immunoblotting for total for STAT1 and beta-actin.

When we evaluated STAT3 levels from patients and healthy controls (Figure 4-3), there was a spectrum of STAT3 levels in PBMCs from co-infected patients and healthy volunteers. However, while the mean of the HIV/HCV patients was less than the healthy control levels, differences between the two groups were not significant.

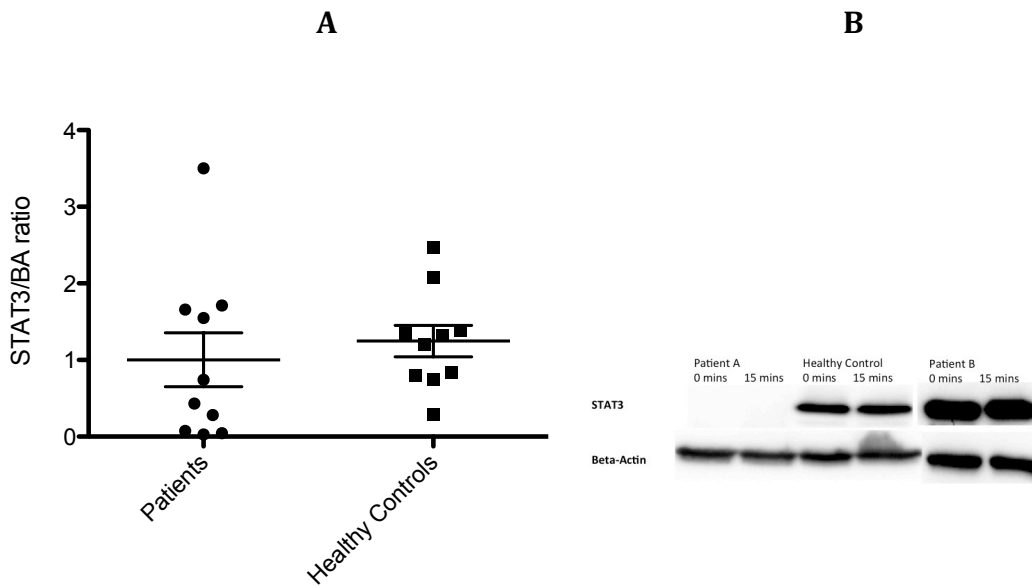


Figure 4-3 STAT3 protein levels are not reduced in PBMCs from HIV/HCV co-infected patients.

(A) Ratio of STAT3: house-keeping protein optical density (OD) measured by densitometric analysis of immunoblots of STAT3 and beta-actin (BA) protein levels in PBMCs from HIV/HCV+ patients (n = 10) and healthy (n = 10) individuals. **(B)** Lysates from PBMCs of 2 individual HIV/HCV+ patients and a healthy control treated +/- IFN-alpha for 15 min, were analysed by immunoblotting for total for STAT3 and beta-actin.

Having observed a significant difference between STAT1 levels, we next compared the levels of pSTAT1 15 mins after stimulation with 1,000 units of IFN in 8 of the patients. Due to a lack of a sufficient number of PBMCs we were unable to perform this analysis in 3 of the patients. These results were compared to healthy volunteers. Again, while the mean pSTAT1 levels of the HIV/HCV patients was less than the healthy control levels, differences between the two groups were not significant.

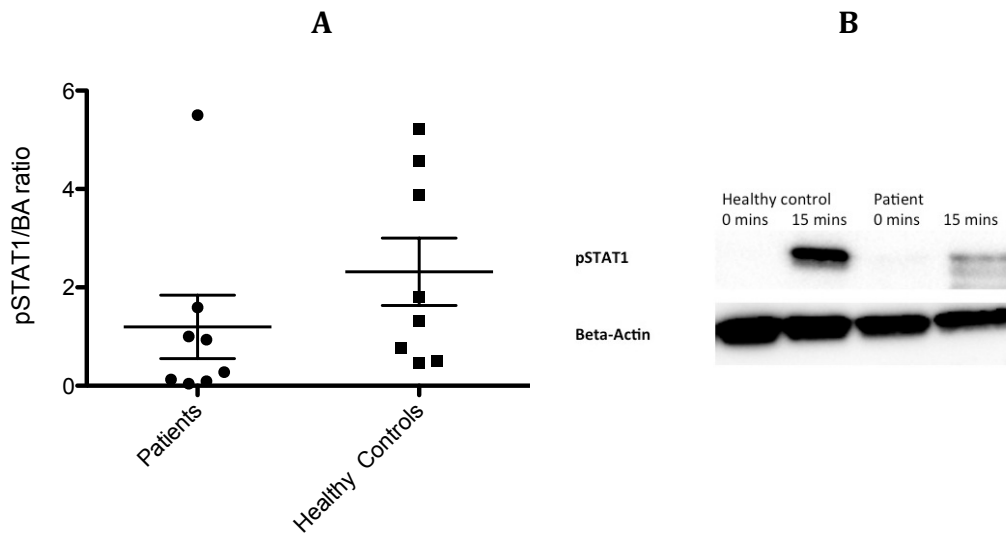


Figure 4-4 Comparison of pSTAT1 protein levels from PBMCs from HIV/HCV co-infected patients and healthy controls

(A) Ratio of pSTAT1: house-keeping protein optical density (OD) measured by densitometric analysis of immunoblots of pSTAT1 and beta-actin (BA) protein levels in PBMCs from HIV/HCV+ patients (n = 8) and healthy (n = 8) individuals. (B) Lysates from PBMCs of an individual HIV/HCV+ patient and healthy control treated +/- IFN-alpha for 15 min, were analysed by immunoblotting for total for pSTAT1 and beta-actin.

In order to track the IFN- α pathway during HCV treatment STAT1 and STAT3 protein levels in PBMCs were monitored by immunoblotting and densitometric analysis at week 4 and 12 of treatment. We found an increase of STAT1 levels over the course of the treatment with a significant difference between week 0 and week 12 ($p < 0.05$).

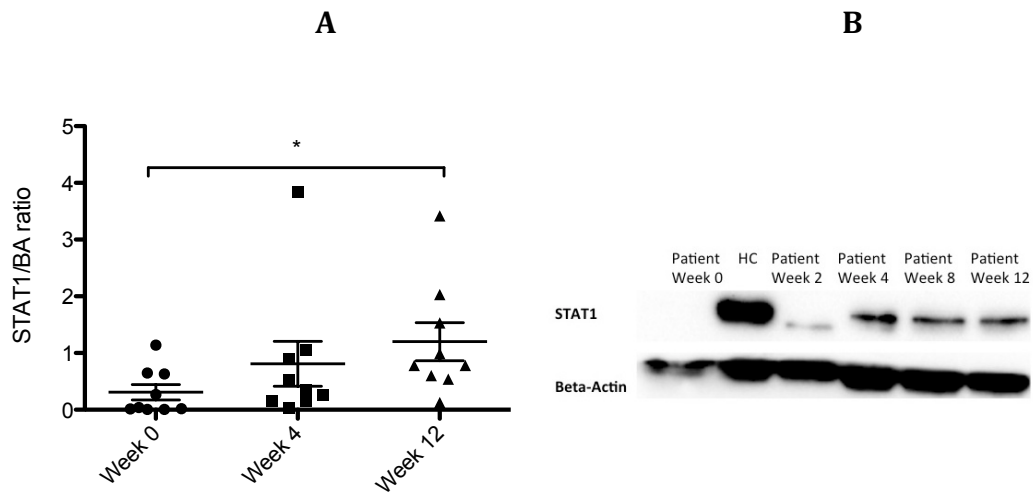


Figure 4-5 STAT1 protein levels from PBMCs from HIV/HCV co-infected patients are increased during HCV treatment

(A) Ratio of STAT1: house-keeping protein optical density (OD) measured by densitometric analysis of immunoblots of STAT1 and beta-actin (BA) protein levels in PBMCs from HIV/HCV+ patients (n = 9) at week 0, and 12 of HCV treatment. (B) Lysates from PBMCs of an individual HIV/HCV+ patient during HCV treatment and a healthy control (HC) were analysed by immunoblotting for total STAT1 and beta-actin. There was a significant difference between week 0 and week 12 (p<0.05 Student t test)

When we analysed STAT3 protein levels from the same patient immune cell samples, we observed no significant differences in STAT3 levels in patients undergoing treatment over this 12 week period (Figure 4-6).

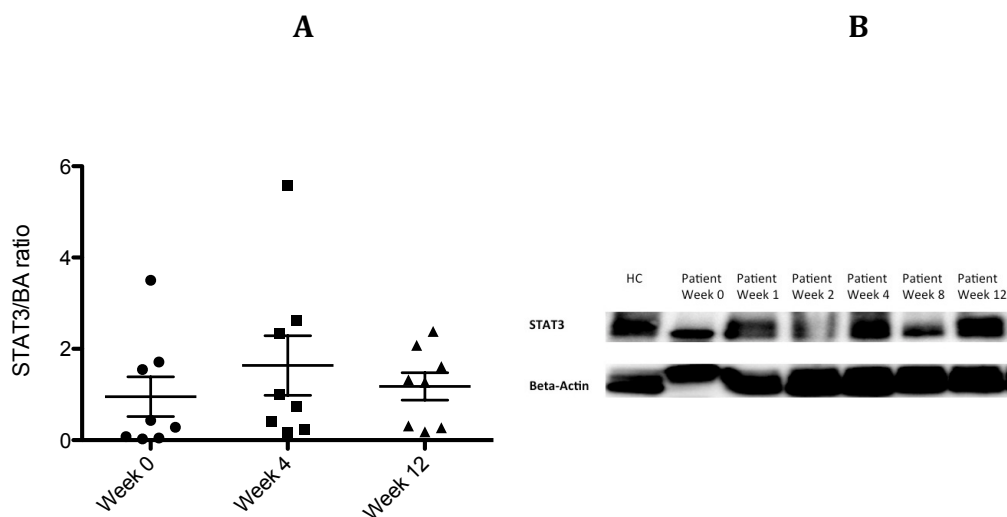


Figure 4-6 STAT3 protein levels from PBMCs from HIV/HCV co-infected patients are not significantly altered during HCV treatment

(A) Ratio of STAT3: house-keeping protein optical density (OD) measured by densitometric analysis of immunoblots of STAT3 and beta-actin (BA) protein levels in PBMCs from HIV/HCV+ patients (n = 8) at week 0, 4 and 12 of HCV treatment. (B) Lysates from PBMCs of an individual HIV/HCV+ patient during HCV treatment and a healthy control (HC) were analysed by immunoblotting for total STAT3 and beta-actin.

4.7 Discussion

These results identify a reduced level of STAT1 (but not STAT3) protein from immune cells from individuals affected with HIV/HCV co-infection, compared to healthy controls. This loss of STAT1 from PBMCs may explain a mechanism as to why patients fail to clear HCV spontaneously following exposure.

In vitro work has sought to examine the effects of IFN- α on HCV replication and antiviral gene expression by targeting STAT [159]. Inhibition of the STAT pathway by a JAK inhibitor significantly enhanced HCV RNA replication and viral protein expression in Huh7.5.1 cells. This work demonstrates that activation of the STAT pathway is important for the anti-HCV activity of IFN- α .

Following 12 weeks of treatment with telaprevir-based therapy for Hepatitis C we observed a significant increase in STAT1 proteins, suggesting restoration of IFN- α signaling pathways in these patients' immune cells. We have previously described reduction of STAT1 and STAT3 proteins from immune cells and hepatocytes [52] of HCV mono-infected individuals. This demonstrated widespread disruption of IFN- α pathway, which identifies the broad immune evasion strategy of HCV and additionally helped to widen our understanding of this virus's spectrum of target cells.

Wanderer et al have previously analysed STAT1 expression and activation in HCV patients who either obtained or did not obtain a SVR post treatment with PEG-IFN and ribavirin [160]. PBMCs from patients who obtained a SVR showed an almost 3-fold stronger increase in IFN- α induced phosphorylation and activation of STAT1 after IFN treatment, compared with cells from patients without SVR. These findings add further evidence to the disruption of IFN- α signaling in patients with chronic HCV infection.

One limitation of this work was that we were unable to analyse the relative contributive effect of both HIV and HCV individually on STAT proteins by recruiting patients with HIV mono-infection and HCV mono-infection.

Fernandez-Botran et al have looked at this issue through investigating systemic cytokine and IFN responsiveness patterns in HIV and HCV mono and co-infections [161]. The mean levels of STAT1 phosphorylation were only significantly reduced in patients with HCV mono-infection. While reduced levels of STAT1 phosphorylation were seen in the 2 HIV-infected groups (HIV/HCV co-infection and HIV mono-infection), these were not significantly different from the control group. These results suggest type I IFN-mediated signaling is especially compromised in HCV mono-infected patients. However, higher levels of pro-inflammatory cytokines were seen in the HIV/HCV co-infected patients which may contribute towards more rapid progression of liver disease. We similarly noticed a reduced mean pSTAT1 level in HIV/HCV patients compared to healthy controls after PBMCs were treated with IFN- α , but this difference was also not significant. However, we were only able to perform this analysis in 8 of the patients.

The HCV NS viral protein NS3/4A has also been shown to interfere with STAT1 phosphorylation [162], supporting the hypothesis that PIs mediate their action, in part, by restoring the JAK-STAT pathway, explaining their synergistic effect with PEG-IFN/ribavirin. Given that HCV NS5A protein has also been shown to disrupt STAT1 phosphorylation and suppress type I IFN signaling [163], it is possible that the mechanism of action of the next generation of DAA agents, that include NS5A inhibitors, may include restoration of type I IFN signaling in order to achieve viral clearance.

While these findings provide a previously undescribed phenomenon in HIV/HCV co-infected patients we experienced a number of limitations which future work would aim to address. For example, our patient recruitment was low due to the short duration Telaprevir was available on the market; significant side effects associated with Telaprevir and the imminent availability of IFN-free DAAs with superior efficacy and tolerability.

Additionally, because of high response rates to DAA based treatment during our trial (SVR rate of 82%), we were unable to determine whether baseline or on-

treatment STAT1, pSTAT1 or STAT3 levels were predictive of response. Sultanik et al have looked at baseline pSTAT1 levels in PBMCs from patients with HCV monoinfection prior to therapy with PEG-IFN, RBV and a first-generation protease inhibitor [164]. They found that pSTAT1 levels, in T cells post IFN- α stimulation, were significantly higher in responders compared to non-responders. However, they acknowledge their results were derived from a small number of heterogeneous patients and would need to be validated in larger cohorts. Similarly, Aceti et al assessed PBMC levels of STAT1 and pSTAT1 from PBMCs of responders and non-responders to IFN-based therapy [165]. They found pSTAT1 was undetectable in 70% of patients with chronic HCV who do not respond to IFN therapy.

Baseline and on-treatment levels of STAT3 were not significantly affected, however a “trend” towards lower STAT3 levels in patients compared to healthy controls indicates that increased recruitment of patient numbers might help determine the true baseline and “on-treatment” levels of STAT3 in co-infected patients undergoing HCV treatment.

Another limitation of this work is that I was unable to assess hepatic protein expression of proteins of interest in IFN signaling prior to treatment initiation. In recent years, the role of liver biopsy in staging severity of liver disease has been replaced by non-invasive methods including biomarkers of fibrosis and liver stiffness assessment. As a result, liver biopsy specimens would only have been available on a minority of the patients studied. Kim et al have examined the hepatic protein expression of STAT1 and suppressor of cytokine signaling 3 (SOCS3) in patients with HCV mono-infection and HIV/HCV co-infection prior to treatment initiation with PEG-IFN and RBV [166]. SOCS3 is a member of the suppression of cytokine signaling family and is thought to interfere with intracellular IFN signaling by directly inhibiting JAK tyrosine kinase activity. They found that non-response to therapy in both patient groups was associated with significantly higher hepatic SOCS3 expression prior to commencing treatment. Interestingly, higher SOCS3 levels were seen in patients with

Genotype 1 disease, a group that traditionally responds less well to IFN-based treatment.

Future recruitment will be easier from the cohort of patients treated with recently approved IFN-free DAA therapy consisting of all-oral combination regimens that are highly potent and well tolerated, [120, 121] with SVR rates typically greater than 90% with few side effects.

Future studies should look at evaluating on-treatment immune responses in patients in an effort to further understand host predictors of treatment response and treatment failure and potentially select patients who would be eligible for either short course or prolonged treatment.

In conclusion, this work has shown that STAT1 protein levels are reduced from primary immune cells in patients with HIV/HCV co-infection. Telaprevir-based regimens result in on-treatment restoration of STAT1 levels in these patients.

Having observed these changes in the JAK/STAT signaling pathway, I now looked to investigate the potential of a preventative HCV vaccine strategy that would look to harness valuable information gained from the study of the host response to HCV infection.

Chapter 5 Hepatitis C Vaccination Strategy

5.1 Introduction – The rationale behind Hepatitis C vaccination

The vaccination work outlined below was carried out as a goal of the PEACHI (Prevention of Hepatitis C Virus (HCV) and HIV-1 Co-Infections) project. The PEACHI project is a collaborative project financed by the EC Framework Program. The goal of the PEACHI project is to develop simple, affordable and effective vaccine strategies that can be given alone or in combination to prevent HCV, HIV-1 and co-infection. The vaccines are based on novel and powerful viral vectors for *in vivo* delivery of antigens. The PEACHI consortium comprises four partners from Italy, Ireland, Switzerland and the UK.

The PEACHI project has 3 major goals: to assess the safety and immunogenicity of adenovirus prime / Modified Vaccinia Ankara (MVA) boost HCV vaccines in HIV-1+ people who are treated with combination antiretroviral therapy (cART); conduct phase I clinical studies using two distinct simian adenoviral vectors simultaneously (aiming to prime responses against both HCV and HIV-1 antigenic targets concurrently) and development and evaluation of a vaccine strategy using adenoviral and MVA vectors when the encoded HCV immunogen is fused to MHC class II invariant chain (Ii).

HCV infection may be particularly amenable to T cell vaccination strategies since effective cellular immunity is known to play a crucial role in spontaneous viral eradication after primary infection, which occurs in up to 40% of humans and also in chimpanzees.

As mentioned in Chapter 1, the hallmark of spontaneously resolved HCV infection, indicated by an undetectable viral load, is a broad and robust HCV specific T cell response. By contrast, a weak and narrowly focused T cell response is observed in chronically infected individuals [53-55]. Spontaneous clearance of HCV has been associated with human leukocyte antigen (HLA) type and the magnitude and quality of CD4+ and CD8+ T cell responses [54, 56, 57]. In

resolved HCV infection, functional HCV-specific CD4⁺ and CD8⁺ T cells are maintained for decades [58] and substantially reduce the risk of persistent infection upon re-exposure [59], whereas in persistent HCV when viral loads are high, these responses are rarely detectable at all in blood [54]. This may be due to a number of factors including T cell exhaustion, downregulation of effector responses by regulatory T cells or T cell priming in the tolerogenic liver environment and viral antigenic variation leading to escape from T cell responses (Reviewed in [60]).

T cell depletion experiments in chimpanzees have confirmed the essential role of cellular immunity in mediating viral control [167, 168]. Secondary exposure to HCV in PWID is associated with the generation of robust T cell immunity that corresponds with protection from chronic HCV infection upon re-exposure to HCV [169].

Proof of principle that a T cell vaccination strategy could be effective for HCV was first shown by Okairos Srl (Rome, Italy): heterologous prime-boost vaccination of chimpanzees with adenoviral and DNA vectors encoding HCV non-structural (NS) proteins elicited potent, broad and long-lived T cell responses that conferred protection against HCV, a 100-fold reduction in peak viraemia post-challenge and subsequent viral clearance [170]. Okairos Srl is now known as Reithera Srl following acquisition by GlaxoSmithKline.

An alternative vaccine approach might be to generate anti-HCV antibodies. However, HCV displays a very high inter- and intra-individual genetic variability [6], especially in the E1 and E2 envelope glycoproteins which are the major targets of anti-HCV antibodies. Neutralising antibody responses are effective, at best, against only a handful of viral variants and their role in viral clearance is still uncertain. Furthermore, development of vaccines that elicit neutralising antibodies conferring sterilising immunity has proved challenging, especially against heterologous viral inocula [171].

These observations provide the rationale for the development of a T cell vaccine and suggest that a HCV vaccine will be effective if it is capable of eliciting a T cell response equivalent or superior to that observed in acute, resolving infection.

5.2 Description of the HCV vaccines used

Two vaccines were used in this study. The vaccines consisted of a replication-defective chimpanzee adenoviral vector (AdCh3) and an MVA vector, both of which encode a 1,985-amino acid sequence encompassing NS3 to NS5B of the non-structural region of HCV (NSmut). The vaccines were administered in a heterologous prime/boost regimen by intramuscular injection.

The extensive genetic variability of HCV is a major challenge for vaccine development. The HCV vaccine is based on NS amino acid sequences from the genotype 1, subtype b, BK strain isolate. The BK strain amino acid sequence is relatively conserved across several genomes ($\geq 80\%$ homology) covering 14 different subtypes belonging to the 6 major genotypes. The NS region encompasses approximately two thirds of the HCV genome and encodes five different proteins (NS3, NS4A, NS4B, NS5A and NS5B). Proteolytic cleavage of the HCV polyprotein is mediated by the encoded NS3 protease (See Figure 5-1). This process recapitulates the same cascade of events occurring *in vivo* upon viral infection and therefore provides a physiological way of presenting the viral antigens to the host immune system.

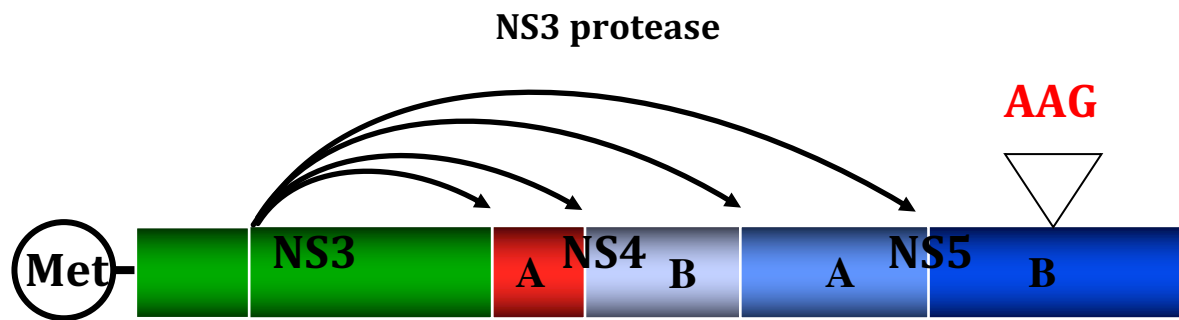


Figure 5-1 The non-structural region of the Hepatitis C virus, by protein schematic, encoded by the vaccines.

The NS region encompasses approximately two thirds of the HCV genome and encodes five different proteins (NS3, NS4A, NS4B, NS5A and NS5B). Proteolytic cleavage of the HCV polyprotein is mediated by the encoded NS3 protease. A mutation (AAG (Alanine, Alanine, Glycine)) inactivating the enzymatic activity of the encoded polymerase gene (NS5B) has been introduced (GDD (Glycine Aspartate Aspartate) \Rightarrow AAG at positions 1711 to 1713).

It is expected that an effective HCV vaccine will need to induce a broad cellular immune response due to the genetic diversity of human MHC alleles and of the virus. The HCV database group has mapped a large number of T cell epitopes in the NS region (more than 90 T helper and more than 70 CTL (cytotoxic T lymphocyte) epitopes [172], and most of the known CD4 and CD8 T cell epitopes are conserved in the BK viral isolate. Recent published data in chronically and acutely infected patients suggest a predominant role for epitopes mapped in the NS region in inducing T cell immunity [60, 173, 174]. Data from the study HCV001 shows that multiple epitopes are targeted within the NS insert in most vaccinated individuals [175].

For safety reasons, a mutation has been introduced to inactivate the enzymatic activity of the encoded polymerase gene and eliminate any potential replication capability of the vaccine. This replaced the original amino acid sequence GlyAspAsp at positions 1711 to 1713 in the catalytic site of the NS5B with the inactive AlaAlaGly [176]. Genetic inactivation of NS5B does not reduce gene expression and polyprotein processing by the NS3 protease present in the encoded HCV fragment [177].

5.3 Development of highly immunogenic replication defective Chimpanzee Adenoviral Vectors

Replication-defective adenoviruses are currently the most potent vectors for induction of human T cell responses to encoded antigens. Adenoviral vectors are genetically stable, therefore, inserted foreign genes are not deleted but remain epichromosomal in the host cell upon infection. The risk of insertional mutagenesis is thus avoided. In addition, transgene expression is limited in time. Adenoviruses are easily propagated in cell culture to high titres, are relatively inexpensive to manufacture and have an excellent safety profile (reviewed in [178]). These characteristics make them ideal for immunogen delivery.

Replication-defective adenovirus vectors based on Ad5 have been extensively used for HIV-1 vaccine development to Phase II clinical trials [179-183]. PEACHI consortium members have previously constructed Ad5-based replication-defective vectors encoding different HCV antigens and have shown that, similarly to the Ad5-vectored HIV vaccine, they are extremely potent vectors for the induction of HCV-specific T cell responses in rodents and non-human primates over a wide range of doses [176].

Collectively, these studies, in addition to recent studies at the University of Oxford into HCV [175], HIV-1 [184] and malaria [185], using adenoviral vectors derived from rare human serotypes (Ad6) and chimpanzee adenoviruses (AdCh3 and AdCh63) (discussed in detail below), show that adenoviral vectored vaccines induce the highest level of T cells targeting the immunogen afforded by any vaccine approach to date.

However, the use of human adenovirus vectors is limited by pre-existing humoral immunity since adenoviruses are ubiquitous infectious agents. For example, between 45-90% of healthy adults carry Ad5 neutralising antibodies [186]. Studies have shown that immunisation with adenovirus vectors in animal models in the context of pre-exposure to heterologous adenoviruses attenuates vaccine responses, probably due to the removal of virus particles by pre-existing

antibodies [187-189]. Vaccines developed using chimpanzee adenoviruses look to address this hurdle in our trial.

To identify novel vectors suitable for vaccine delivery in humans, Okairos isolated and sequenced over a thousand chimpanzee Adenovirus strains (AdChs) [190]. Replication-defective vectors were generated and screened for neutralisation by human sera, and for their ability to grow in human cell lines that were approved for clinical studies. The vectors studied varied by up to a thousand-fold in potency for CD8+ T cell induction in mice. The most potent AdCh vectors, AdCh3 and AdCh63, were selected for Phase I clinical studies at the University of Oxford, where it was shown that AdCh-vectored vaccines for malaria, HCV and HIV-1 infections are safe and highly immunogenic [175, 184, 185, 191].

These viruses were chosen not only on the basis of their potency as vectors but also for their low seroprevalence and lack of pathogenicity in humans [192]. Furthermore, despite the high degree of homology between AdCh3 and human Ad5 and Ad6 in hexon (the major immunogenic adenoviral capsid protein [193]) sequence overall, these viruses are highly divergent in the hypervariable region of hexons that are the target of neutralising antibodies. Indeed, sera with neutralising activity against Ad5 and Ad6 were not able to neutralise AdCh3 virus. Furthermore, pre-immunization with Ad5 or Ad6 did not impair the induction of T cell responses by AdCh3 (S. Colloca, unpublished).

5.3.1 Summary of pre-clinical data with AdCh3NSmut1 HCV Vaccine Vector Candidate

Pre-clinical studies with AdCh3NSmut / AdCh3NSmut1 were performed at the Research Toxicology Centre (RTC), Pomezia, Italy, with analyses for the immunogenicity and bio-distribution being performed at the Okairos laboratories. Each study involved intra-muscular injection of 6.0×10^9 vp/dose of AdCh3NSmut.

In RTC study 57710, tissue distribution after AdCh3NSmut immunisation in mice was assessed in two ways: (i) inoculation of human embryonic kidney (HEK) 293 cells with mouse organ homogenates followed by culture and real-time PCR to detect infectious virus; (ii) quantification of adenoviral genomes in DNA extracted from organ homogenates by real-time PCR. This study showed that one week after intramuscular injection, AdCh3NSmut was only barely detected in the regional lymph nodes while muscles were negative in all mice. Thus, in both compartments there is evidence of a loss of infectious virus. Similar results were obtained by qPCR of adenovirus DNA. These data indicate that AdCh3NSmut was not able to replicate within the mouse tissue.

Lack of replicative capacity of AdCh3NSmut1 was also confirmed by a replication competence assay. Because human adenoviral infection is transient and because the vaccination vector AdCh3 is replication-defective, detectable only at the site of injection (skeletal muscle is the route for vaccine, while wild type adenoviruses may be found at mucosal sites) and does not disseminate or persist, the theoretical risk of recombination of the vector with human adenoviruses *in vivo* is very low.

Several repeated dose toxicity studies were performed using AdCh3NSmut in mice (RTC studies 57710, 70450 and 78130 for AdCh3NSmut, and 82930 for AdCh3NSmut1); these were designed to investigate potential toxicity arising from repeated administration of the vaccine in heterologous prime/boost regimens similar to those proposed for the clinical trials. No toxic effects were noted in mice receiving Ad6NSmut, AdCh3NSmut, AdCh3NSmut1 and MVA-NSmut when given by the intramuscular route.

Immunogenicity was also assessed during the studies outlined above. It was found that all vaccinated mice developed very strong T cell-mediated immune responses directed against multiple epitopes. Similarly, in preclinical studies performed at Okairos laboratories using Rhesus macaques, potent responses targeting different NS antigens were induced in all immunised macaques.

Prior to proceeding to human trials it is important to understand the concept of extrapolation of dose between species [194]. Various factors need to be considered when determining the correct dose when proceeding from animal to human studies including body surface area, pharmacokinetics, physiological clearance and available comparative data with similar products. Larger animals often have slower metabolic rates and require a lower drug dose on the basis of weight alone. The safety to individuals of any new investigational product is the primary priority and the above factors should be taken into account in determining the initial dose for safety assessment in humans with careful titration to determine the safest and most efficacious or immunogenic dose.

5.3.2 Clinical data summary of AdCh3NSmut1 HCV Vaccine Vector Candidate

Completed Phase I trials in Oxford included those using the AdCh3 and Ad6 vectors encoding the NS antigen (AdCh3NSmut and Ad6NSmut) in healthy volunteers (study HCV001), in HCV infected patients (study HCV002TV), and study HCV003 which also assessed the safety and immunogenicity of AdCh3NSmut1 – identical to the adenoviral vaccine to be used in this study – and MVA-NSmut in healthy volunteers.

The phase I clinical trial HCV001 assessed the safety and immunogenicity of AdCh3NSmut in healthy volunteers. This was an open label study where 38 volunteers received AdCh3NSmut at least once during the trial. Priming vaccinations were administered to 22 volunteers at doses ranging from 5×10^8 vp to 7.5×10^{10} vp. A further 16 individuals received a single boosting vaccination of AdCh3NSmut at 2.5×10^{10} vp following priming vaccinations with Ad6NSmut at varying doses [175].

In the HCV003 trial the dose of AdCh3NSmut was 2.5×10^{10} vp, which was determined by complete safety analysis of the HCV001 trial data. This trial also introduced the use of the new improved batch of AdCh3NSmut (AdCh3NSmut1), described below, at the dose of 2.5×10^{10} vp [195]. This new batch AdCh3NSmut1

is also being used in the staged phase I/II trial in progress in the USA (FDA approval, <http://clinicaltrials.gov/ct2/show/NCT01436357>).

The results of the HCV001 trial demonstrate that AdCh3NSmut is well tolerated and there were no reported suspected unexpected serious adverse reactions (SUSARs). 22 volunteers received AdCh3NSmut as a priming vaccine. The observed frequency of local reactions to AdCh3NSmut was 66% overall and appeared to increase with dose. Local pain, erythema, warmth and swelling were the most commonly reported local reactions. 93% of the observed local reactions were mild in severity. Systemic adverse events were observed in 74% of recipients. Fatigue, malaise and headache were the commonly reported adverse events. Of the observed systemic reactions, 96% were mild in severity. In the HCV003 trial local reactions occurred in 94% of healthy volunteers (of which 91% were mild and only 2.2% classified as severe). Local pain and warmth at the vaccination site were the most common local adverse events. Systemic reactions occurred in 81% of participants, headache being the most common followed by fatigue; the majority (74%) of systemic reactions were classified as mild. The bulk of adverse events lasted 1-3 days.

A summary of the numbers of people vaccinated with each vaccine construct is given in Table 5.1 below:

Table 5.1 Number of individuals receiving AdCh3 and MVA HCV vaccines

Study name	AdCh3NSmut	AdCh3NSmut1	MVA-NSmut
HCV001 (healthy volunteers)	38	0	0
HCV002 (HCV infected)	32	0	0
HCV003 (healthy volunteers)	13	0	13
HCV003 (HCV infected)	16	15	34
HCV004 (HCV infected)	0	0	9
HCV005 (Active IVDUs)	0	488	393
PEACHI-04 (healthy volunteers)	0	25	24

Adenoviral vector shedding was explored in urine and throat swabs in the study HCV001. There was no detectable virus in any clinical sample after intramuscular AdCh or AdHu immunisation. These findings are consistent with the pre-clinical studies demonstrating no vector dissemination beyond regional lymph nodes and no vector persistence.

AdCh3NSmut has been shown to be highly immunogenic, priming polyfunctional HCV specific CD4+ and CD8+ T cell responses (expressing IFN- γ , IL-2 and TNF- α). T cell responses were broad, targeting multiple transgene proteins and were capable of recognising the heterologous HCV genotypes 1a and 3a. In exploratory analyses the induced T cell populations consisted of a long-lived central and memory effector T cell pool that retained polyfunctionality and proliferative capacity [175]. These characteristics have been associated with spontaneous clearance of HCV in humans and are therefore under investigation as possible surrogates for vaccine candidate efficacy.

Vaccine trials evaluating another chimpanzee adenovirus vector, AdCh63, in healthy volunteers have also been conducted at the University of Oxford.

Vaccines for malaria incorporated ME-TRAP (a multi-epitope string and the thrombospondin related adhesion protein) inserts in trials VAC033 and MAL034 [196, 197], and an MSP1 (Merozoite surface protein-1) insert in trial VAC037 [185, 198].

Local and systemic adverse events are comparable in incidence and severity to the manifestations observed in HCV001 and HCV003. An HIV vaccine incorporating a conserved region immunogen, HIVconsv, was evaluated in the phase I trial HIV-CORE 002 [184]. In all these trials, there were no vaccine-related serious adverse events or SUSARs.

5.3.3 Manufacture of replication-defective Chimpanzee Adenoviral Vectors

The AdCh3 vector was generated by cloning into bacterial plasmids followed by transformation in *E. coli*. Bacterial clones were then used to prepare the plasmid DNA required for the subsequent construction of replication-defective viral vectors. The genetic integrity of this adenovirus vector and the absence of foreign genetic material therein were assessed by fully sequencing the vector DNA extracted from individual bacterial clones. The molecular cloning procedure along with the sequencing results ensured exclusion of any risk of contamination with adventitious agents.

Chimpanzee Adenoviruses are rendered replication-defective by deletion of genes from the E1 locus. The absence of E1 results in only minimal vector gene expression *in vivo*. However, due to sequence similarity to human adenoviruses, AdChs may be grown in complementing human cell lines. For AdCh3 vector construction the E3 genes were also deleted in order to decrease the fitness of the vector further, because the E3 region is essential for counteracting *in vivo* eradication. Double deletion also minimises the risk of generating replication-competent adenovirus (RCA) recombination *in vivo* [199].

E1-deleted AdCh vectors can be complemented by Ad5 E1 genomic region stably expressed in human cell lines previously used for production of clinical material such as human embryonic kidney (HEK) cells 293, HEK 293 and PER.C6® (Crucell) [200, 201]. The PER.C6 human cell line is considered the most characterised commercial platform for the production of recombinant adenoviruses, in addition to proteins and antibodies. The PER.C6 human cell line is derived from a single, human retina-derived cell, which was purposely immortalised using an expression vector for the E1 region of Ad5. The genetic structure of the E1 expression cassette inserted in the PER.C6® cell genome was designed to avoid regions of homology between the E1-deleted Ad vectors and therefore eliminate the risk of RCA generation during vector manufacture. A diagram of the AdCh3 vector vaccine is shown in Figure 5-2.

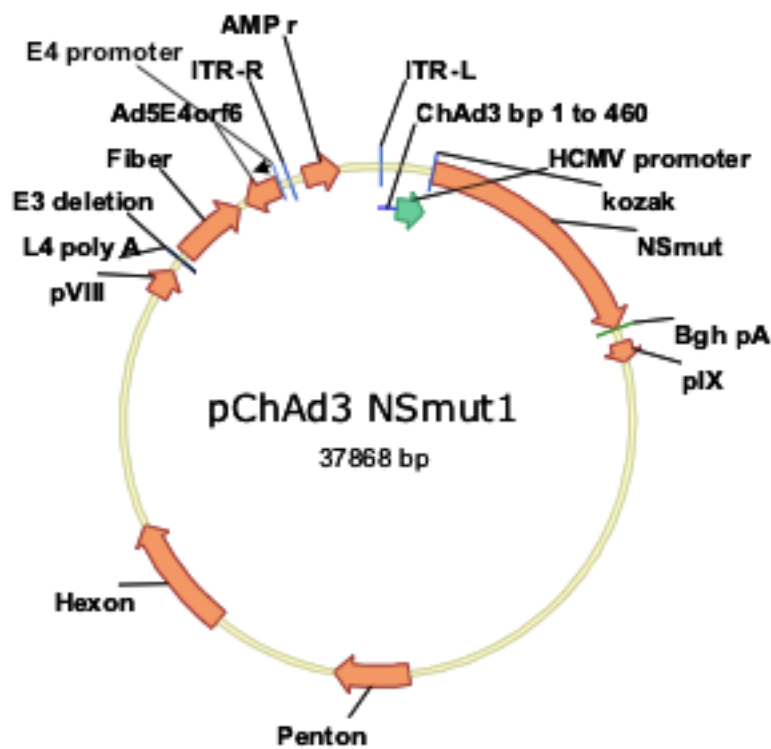


Figure 5-2 Diagram of the novel AdCh3 vector containing the NSmut antigen.

The structure of the modified E4 region is shown in the diagram. The Ad5 E4orf6 was fused directly to the native E4 promoter and the entire ChAd3 E4 region was deleted. (Figure from IMPD for AdCh3NSmut1 v7.0)

A first generation AdCh3NSmut vaccine, with the vector characteristics described above, was used in the HCV001 and HCV003 trials. In order to improve vector production, the following minor changes were introduced in the vector backbone leading to production of a new batch AdCh3NSmut1 that will be used in future clinical development of the HCV vaccines. The changes in the AdCh3 backbone, described in detail in the IMPD of AdCh3NSmut1, are:

1. Deletion of the E4 region of AdCh3. We have experimental evidence that propagation of E4-deleted AdCh3NSmut1 is more productive in PER.C6 cells, and that the two vector backbones have equivalent infectivity and immunogenicity;
2. The purification process has been changed to allow for scale-up of the process, whereas the release criteria will remain the same.

The new batch AdCh3NSmut (AdCh3NSmut1) is currently in use at the same dosage (2.5×10^{10} vp) in the Phase I/II blinded clinical trial in healthy HCV-uninfected PWID in the USA (ClinicalTrials.gov ID: NCT01436357). AdCh3NSmut1 was also used in our vaccine study.

Besides the advantage of this new batch in terms of productivity, vaccine safety has potentially been improved because the detergent Kathon has been removed from manufacturing. This eliminates the risk of allergic reactions to Kathon.

5.4 Modified Vaccinia Virus Ankara (MVA) as Viral Vaccine Candidates

Modified Vaccinia Ankara (MVA) is an attractive candidate orthopoxvirus vaccine vector for safety and immunogenicity reasons. The successful worldwide eradication of smallpox via vaccination with wild-type vaccinia virus is proof of its safety and immunogenicity. However, despite safe administration of replication-competent vaccinia virus to millions of people its small but definite risk of dissemination in immunocompromised individuals led to the development of several attenuated strains of vaccinia during the smallpox

eradication campaign. These attenuated strains have been used recently for vector development for a range of diseases.

MVA was originally derived from the vaccinia strain Ankara by over 500 serial passages in primary chicken embryo fibroblasts (CEF). MVA has six major genomic deletions compared to the parental Ankara genome, including cytokine receptor genes. It replicates well in CEFs and baby hamster cells but is severely compromised in its ability to replicate in mammalian cells [202, 203]. No replication has been documented in non-transformed mammalian cells. The viral genome has been proven to be stable through a large series of passages in CEFs; using restriction enzyme analysis virtually no difference was observed between passage 500 – 572 [203], and MVA also showed no cytopathic effect or plaque formation in cells of human origin. Viral replication is blocked late during infection of cells, but importantly viral and recombinant protein synthesis is unimpaired even during this abortive infection. Because the MVA vector is located exclusively in the cytoplasmic compartment of the host cell, integration into the host genome has not been observed [204].

Towards the end of the smallpox eradication campaign MVA was licensed in Germany [205] and over 120,000 people were vaccinated with MVA, without significant adverse effects. Only a small proportion showed any systemic side effects such as fever over 38.5°C [203] and MVA proved to be non-contagious and non-virulent. MVA did not elicit any morbidity or mortality in irradiated mice even when administered at high doses intra-cerebrally, or simian immunodeficiency virus (SIV)-infected macaques, indicating its safety even in immunocompromised organisms [203]. Recombinant MVA vaccines have been safely administered to HIV-infected individuals in several clinical trials (summarised in Table 5.2).

Recombinant MVA is capable of inducing potent CD8⁺ T cell responses to passenger proteins, particularly when used as a booster in prime-boost regimens. The immunogenicity of MVA has been attributed in part to loss of cytokine and chemokine receptor genes [206]. MVA-vectored vaccines

expressing SIV genes have been evaluated in the macaque model, either as a single vaccine modality or in prime-boost regimens. Vaccination followed by a pathogenic SIV challenge rarely gave complete protection against infection but strong virus-specific CD8⁺ T cell responses were observed [207]. In some monkeys, containment of infection, indicated by significantly lower virus set points compared with sham-vaccinated animals, were observed after infection with the challenge virus [208, 209].

5.4.1 Pre-Clinical Data Summary of MVA NSmut HCV Vaccine Vector Candidate

The safety of MVA-NSmut as part of a prime-boost vaccination regimen has been demonstrated in pre-clinical good laboratory practice (GLP) toxicology studies in mice and macaques (documented in the Investigator's Brochure for MVA-NSmut). Prime-boost vaccinations with AdCh3-NSmut followed by MVA-NSmut were performed at the Research Toxicology Centre (RTC), Pomezia, Italy, with analyses for the immunogenicity and bio-distribution being performed at the Okairos laboratories. Repeated dose toxicity studies with a heterologous prime/boost vaccination schedule (Ad6 and AdCh3NSmut or AdCh3NSmut1 followed by MVA NSmut) given via the intramuscular route were performed in Balb/c mice (RTC study 78130 for AdCh3NSmut and study 82930 for AdCh3NSmut1). No toxic effects were observed in any organ. Immunogenicity was also assessed in this study and in macaques. All vaccinated animals developed strong T cell responses to multiple epitopes in NSmut.

Although formal biodistribution studies were not performed with MVA-NSmut, the biodistribution and persistence of another MVA-vectored HIV vaccine, MVA.HIVA, was assessed in a GLP study of SIV-infected macaques and severe combined immunodeficient (SCID) mice. In SIV-infected macaques, MVA.HIVA was undetectable by nested PCR 6 weeks after dosing. In SCID mice, the MVA.HIVA vaccine was only detected by PCR at the site of injection 49 days after dosing in 4/6 mice, and these sites were negative by day 81 post-injection [210].

These data supported regulatory applications for clinical evaluation of MVA vaccines in HIV-positive human subjects.

5.4.2 Clinical Data Summary of MVA-NSmut HCV Vaccine Vector Candidate

MVA-NSmut was first evaluated in humans in the HCV003 trial in the UK. This was followed by the HCV004 phase I trial in Italy and the phase I/II trial (ClinicalTrials.gov ID: NCT01436357), in the USA. Data from HCV003 and HCV004 confirm the excellent safety profile of MVA-NSmut, consistent with other trials of recombinant MVA vaccines that have been conducted at University of Oxford.

In HCV003, healthy volunteers received MVA-NSmut at a dose of 2×10^8 pfu. Local reactions occurred in 100% of healthy volunteers, local pain being the most frequent event followed by warmth of the vaccination site and erythema. Most local adverse events were mild (74%) and self-limited (median duration 3 days). Systemic reactions occurred in 89% of healthy volunteers, of which 10.4% were severe. They were, however, generally transient (< 2 days).

These data add to the existing safety profile of MVA as a vector for a range of antigens, including the malaria antigens ME-TRAP (study codes VAC033 and Mal034), MSP1 (study code VAC037), CS (study code VAC038), AMA-1 (Study code VAC039), and L3SEPT; antigen 85A from *Mycobacterium tuberculosis*; and influenza antigens NP+M1. To date there have been no vaccine-related serious adverse events in these trials. There are also extensive safety data from trials of MVA HIV vaccines in healthy HIV-uninfected and HIV-positive ART-treated subjects [211-214].

MVA vector shedding has been assessed in a clinical trial evaluating MVA expressing human MUC1 as an antigen-specific anti-tumour immunotherapy [215]. Urine samples collected after 4 hours and 8 days were negative for MVA, confirming that the vaccine vector is not excreted due to the lack of systemic

distribution. In a separate study, shedding was investigated after intradermal immunisation by PCR analysis of blister fluid collected from the vaccination site: no MVA was detected in any sample (Sarah Gilbert, personal communication).

5.4.3 MVA-NSmut Manufacture

The NSmut coding region was subcloned into the MVA shuttle vector pMVA-GFP-TD, flanked by TKL (thymidine kinase gene, left region) and TKR (thymidine kinase gene, right region) and generating the transfer vector pMVA-GFP-TD-NSmut. pMVA-GFP-TD-NSmut, drives the antigen expression using the vaccinia P7.5 early/late promoter, and expression of Green Fluorescent Protein (GFP) using the fowlpox late promoter, FP4b.

The recombinant vaccine MVA-NSmut virus was generated by in vivo recombination between the MVA- RFP vector genome and homologous sequences (TKL, thymidine kinase gene, left region and TKR, thymidine kinase gene, right region) within the transfer vector pMVA-GFP-TD-NSmut [216].

The MVA-NSmut is manufactured under Good Manufacturing Practice (GMP) conditions by the contract manufacturer IDT (Rosslau, Germany). A diagram of the MVA-NSmut structure is shown in Figure 5-3.

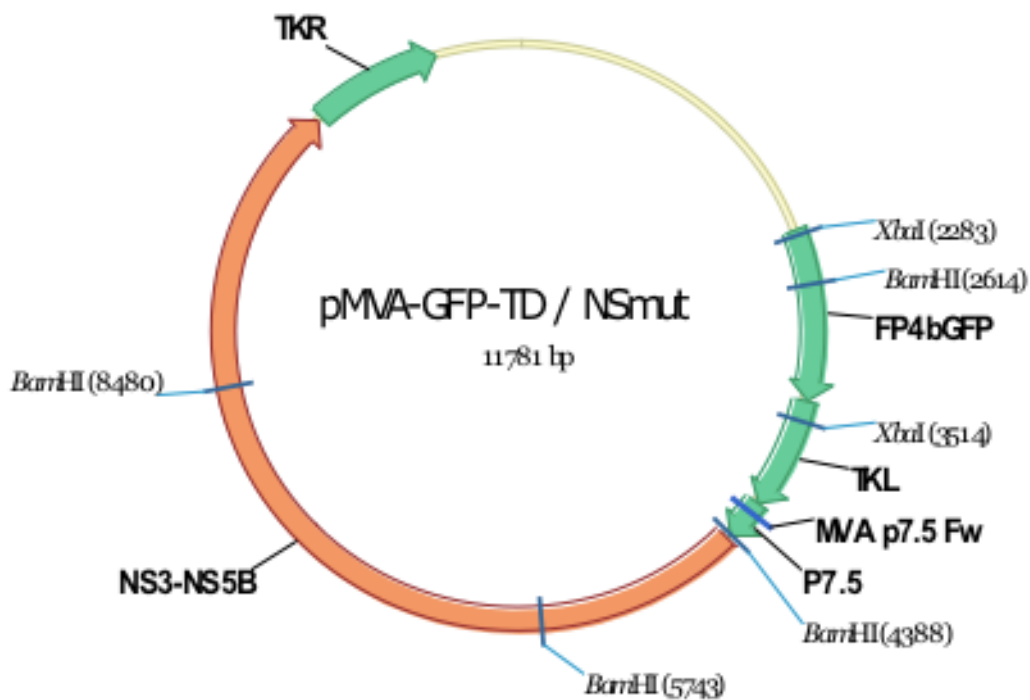


Figure 5-3 Schematic diagram of the MVA-GFP-TD-NSmut structure.

The MVA-NSmut transfer vector encodes for 1985 amino acids of HCV NS region, with genetically-inactivated RNA- dependent RNA polymerase activity. The transgene cassette is located under the control of P7.5 MVA early promoter (277 bp) and flanked by TKL (Thymidine Kinase gene, left, 735 bp) and TKR (Thymidine Kinase gene, right, 835 bp), non-essential region of the MVA genome. (Figure from Investigator Brochure for MVA-NSmut)

5.5 Rationale for heterologous prime boost vaccination

Previous studies have shown that heterologous prime-boost regimens employing different genetic vaccine vectors are an effective means to induce potent T cell responses in both non-human primates and humans [217-219]. Homologous regimens may be less effective due to the development of immune responses to the vector; for example, the neutralizing antibody response against adenoviral capsid proteins following multiple administrations of adenovirus vectors dampens the response to the transgene.

To address this issue, MVA-NSmut was developed as a heterologous boosting vaccine candidate following prime with AdCh3NSmut. The high magnitude of NSmut-specific T cell responses 7 days after the MVA-NSmut boost is illustrated

in Figure 5-4 below. This regimen is more immunogenic than prime-boost vaccinations with heterologous adenoviruses [195].

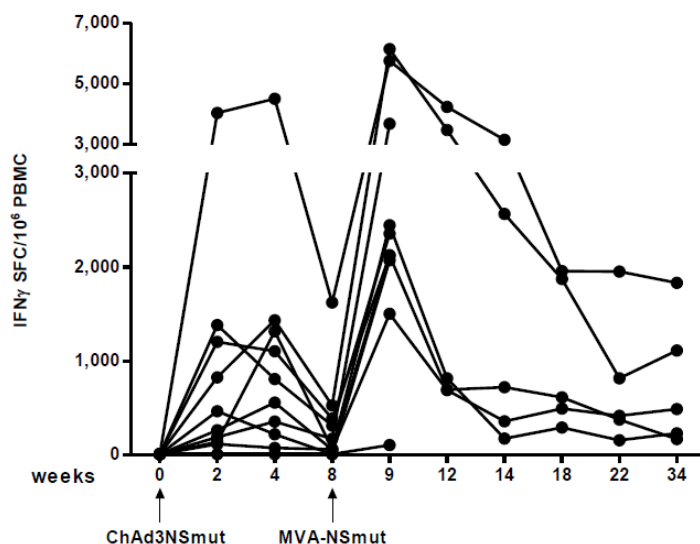


Figure 5-4 AdCh3NSmut prime followed by MVA-NSmut boost vaccination induced sustained and broadly directed T cell responses.

10 healthy volunteers were primed at week 0 with AdCh3NSmut (2.5×10^{10} vp) and boosted at week 8 with MVA-NSmut (2×10^8 pfu). ELISpot was performed on freshly isolated PBMC to measure HCV-specific IFN- γ secreting cells using 6 pools of peptides encompassing the HCV genotype 1b NS3-NS5b sequence (data is expressed as IFN- γ Spot Forming Cells (SFC)/million PBMC) (data from study HCV003 – Source E Barnes).

5.6 Route of injection and dosage

There is a large body of data from clinical trials of replication defective Ad5- and Ad6-vectored HIV vaccines showing an excellent safety profile when injected intramuscularly, with no viral shedding, and high levels of immunogenicity.

MVA-NSmut was also given by the intramuscular route following studies demonstrating comparable immunogenicity and less local reactogenicity when MVA vectors were administered by the intramuscular as opposed to subcutaneous or intradermal routes [220, 221].

In our study, AdCh3NSmut1 vaccine was administered at 2.5×10^{10} vp, and MVA-NSmut at 2×10^8 pfu. This takes into consideration the balance between safety,

reactogenicity and maximal immunogenicity, which is generally observed at higher doses of both vectors.

AdCh3NSmut1: Immunisation studies in non-human primates showed that adenovirus vector doses of 10^{10} - 10^{11} vp elicited T cell responses to NSmut that were comparable or even better than those observed in spontaneously resolved HCV infection in humans. Additionally, these doses consistently induced responses in 100% of animals, while lower doses achieved lower response rates [176]. The “necessary and sufficient dose” capable of eliciting the desired T cell response, was also obtained from extensive studies in humans using similar Ad5- and Ad6-based vaccine vectors (albeit with different antigens). In phase I studies evaluating Ad5- and Ad6-based HIV vaccine candidates at vector doses of 10^9 - 10^{11} vp, peak magnitude and durability of immune responses were dose-related [222]. These adenoviral vectors were safe and well tolerated up to 10^{11} vp/dose. However, increased reactogenicity was observed with the highest dose [179].

The study HCV001 has shown AdCh3NSmut was safe and well tolerated in healthy volunteers at doses ranging from 5×10^8 to 7.5×10^{10} vp . The new vaccine AdCh3NSmut1 was expected to have equivalent safety and immunogenicity to the previous AdCh3NSmut. This was confirmed in the vaccine study HCV003 [195]. This vaccine is also being used in the phase I/II blinded trial, which is ongoing in the USA under the sponsorship of National Institutes of Health. No serious safety concerns have been identified to date.

MVA-NSmut: In our study, MVA-NSmut was administered at 2×10^8 pfu. This dose had been selected based on the use of MVA vectors in primate and human studies. In the University of Oxford, MVA-vectored malaria and HIV vaccines have been safely administered in phase I and phase II studies at a dose of 2×10^8 pfu. In addition, MVA ME-TRAP and MVA-vectored *P. falciparum* CS protein were safe and immunogenic at a dose of 5×10^8 pfu, although MVA MSP1 was associated with more local and systemic reactogenicity at this dose.

Participants in our study were vaccinated with a single dose of AdCh3NSmut1 (2.5×10^{10} vp) on day 0 followed by a single injection of MVA-NSmut (2×10^8 pfu) 8 weeks later.

5.7 Special consideration of the vaccine candidates in HIV-1 seropositive individuals

Vaccination is one of several important measures to prevent serious infections in immunocompromised individuals, including people living with HIV-1 infection. The European Clinical AIDS Society (EACS – www.eacsociety.org) and the British HIV Association (BHIVA) [223] have published recommendations for immunisation in HIV-positive patients.

These recommendations state that live attenuated vaccines (e.g. Measles, Mumps, Rubella (MMR), Varicella, Yellow Fever) can be given safely in individuals with a CD4 cell count >200 cells/ μ l. Subunit, toxoid or killed split-virion vaccines (e.g. HAV, HBV, diphtheria / tetanus / pertussis combination, parenteral seasonal influenza vaccines, pneumococcal vaccines) may be administered irrespective of CD4 cell count, if clinically indicated [223, 224].

In this study, we tested replication-deficient viral vectored vaccine candidates. To date there is only limited experience with AdCh vectored vaccines in HIV-1 seropositive individuals: the HIV vaccine candidates AdChV63.HIVconsv and MVA.HIVconsv are currently being evaluated in a therapeutic study in HIV-positive individuals initiating cART during primary HIV infection (ClinicalTrials.gov No: NCT01712425). To date, a comparable safety and tolerability profile to HIV-1 seronegative individuals has been observed. Furthermore, from recent studies of Ad5-vectored HIV vaccines in HIV-1 seropositive individuals it can be inferred that replication-defective adenoviral vectors have a safety and tolerability profile in HIV-1 seropositive individuals on antiretroviral therapy that is comparable with healthy adults [225, 226].

Extensive data indicate that poxvirus-vectored vaccines are safe in HIV-1 seropositive individuals, particularly those receiving antiretroviral therapy. A summary of trials of attenuated vaccinia (MVA, NYVAC), fowlpox and canarypox vaccines in HIV-1 seropositive individuals is shown in Table 5.2. There have been no reports of SUSARs in these studies and safety and tolerability have been comparable with healthy HIV-uninfected adults. None of the serious adverse events that occurred in these studies were considered possibly, probably or definitely related to the respective vaccine.

Table 5.2 Overview of trials evaluating poxvirus-vectored vaccines in HIV-1 seropositive individuals

Vaccine	Dose/Schedule	Participants	Adverse Events	Reference
MVA.HIVconsv	5x 10 ⁷ pfu im. / 0,4,12 weeks 2x 10 ⁸ pfu im. / 0,4,12 weeks	20 ART-treated adults	3 SAE, unrelated or unlikely related	Dorrell L, 2013, in preparation
MVA.gag/epitopes	5x 10 ⁷ pfu id. / 0,4, weeks	16 ART-treated adults	No SAE	[211]
MVA-HIV/FP-HIV MVA	5x 10 ⁷ pfu im. / 0, 4 weeks FP 5x 10 ⁸ pfu im. / 8, 24 weeks	20 ART treated adolescents	2 SAE, one unlikely related	[227]
MVA-nef	5x 10 ⁸ pfu sc. / 0,4,16 weeks	14 ART-treated adults	No SAE	[228]
FP-HIV	5x10 ⁷ pfu im. / 0, 4, 12 weeks	24 adults, ART initiated during PHI	3 SAEs – not related	[229]
NYVAC-gag/pol/nef/env	im. / 0, 4 weeks	10 ART-treated adults	No SAEs	[230]
MVA85A	5x10 ⁷ pfu id. / 0 weeks	36 adults: 12 on ART, 24 ART-naïve	2 SAEs – not related	[231]
MVA/FP ME-TRAP	1.5x10 ⁸ /1x10 ⁸ pfu im. / 0 weeks	7 ART- naïve adults	1 SAE – unlikely related	[232]
ALVAC vCP1452	10 ⁷ TCID ₅₀ / 0, 30, 90, 180 days	15 ART-treated adults	No SAEs	[233]
ALVAC vCP1433	10 ⁷ TCID ₅₀ / 0, 4, 8, 12 weeks	48 ART-treated adults	No SAEs	[234]
ALVAC vCP1452	10 ⁷ TCID ₅₀ / 0, 4, 8, 20 weeks or 4, 8, 20 weeks	43 ART-treated adults	1 severe local reaction; no vaccine-related SAEs	[235]

In this trial, we enrolled HIV-1 seropositive individuals on an effective ART regimen (with fully suppressed viraemia) with a CD4 cell count >350 cells/ μ L. These criteria are based on: 1) evidence of safety of the AdCh and MVA vectors in previous trials (described in detail above); 2) consideration of current

recommendations with respect to licensed live attenuated vaccines (CD4 count >200 cells/ μ L); 3) anticipation of good responses to the vaccine candidates, given that optimal immune responses to licensed vaccines are more likely to be achieved in individuals with CD4 cell counts above 350 cells/ μ L [224].

5.8 Regulatory Approval

This was the first Phase I vaccine study to be carried out in HIV-1 seropositive patients in Ireland. Approval for conduct of the study in Ireland fell under the remit of a number of regulatory bodies outlined below. Our colleagues in Switzerland made concurrent submissions to Swiss regulatory bodies including Swiss Medic. The Clinical Trials and Research Governance team at the University of Oxford reviewed the relevant submissions.

5.8.1 Ethical Approval

Submission for ethical approval to conduct the vaccine study was made to the local Tallaght Hospital / St James's Hospital Joint Research Ethics Committee in April 2014. Following initial submission, the ethics committee requested some additional information related to the study and for some changes to the patient information leaflet and consent forms. This information was provided, and the changes requested were made and resubmitted to the committee in June 2014. This study received ethical approval (REC Reference 2014/07/List 27 (6)) on 8th July 2014.

5.8.2 Health Products Regulatory Authority (formerly Irish Medicines Board)

An informal meeting was conducted with the Health Products Regulatory Agency on Friday 28th June 2013, outlining the aims of the vaccine study.

The application to conduct a clinical trial was submitted to the Health Products Regulatory Authority on Friday 7th April 2014 (CT Number: CT/900/552/1 – AdCh3NSmut1/MVA-NSmut). Following submission, a detailed letter was received from the HPRA on 9th June 2014 outlining a number of queries related to pharmaceutical, pre-clinical and clinical aspects of the trial. All queries were addressed, and changes made to the clinical trial protocol as requested by the HPRA.

Conditional approval was granted on the 11th July 2014. The conditional approval required confirmation of the genomic identity of the MVA vector by PCR testing of the major genomic deletions that are characteristic of MVA. This test was conducted and submitted to the Health Products Regulatory Authority for review. Full approval was granted on the 3rd December 2014.

5.8.3 Environmental Protection Agency

I had an informal meeting with the Environmental Protection Agency (EPA) in Johnstown Castle Estate, Wexford in June 2013 and outlined details of the proposed vaccine work.

The application to the EPA (GMO Register No: G0536-01, Reference B/IE/14/01 and B/IE/14/02) regarding deliberate release of a GMO was submitted on the 14th June 2014. Multiple documents including Environmental Risk Assessments and Summary Notification Release Formats for both GMO vaccines were prepared and submitted with the application. A newspaper notice was placed regarding the proposed deliberate release on the 16th April 2014.

Consent to a deliberate release of a GMO into the environment for purposes other than placing on the market was granted on 22nd July 2014. Given unforeseen delays in the commencement of the clinical trial, a request to extend the duration of approval above was submitted to the EPA and was granted until

31st December 2016. Full details of the submissions are available for view on the EPA website.

5.8.4 Health and Safety Authority

The Health and Safety Authority were notified regarding the proposed use of GMOs at the trial site on 9th December 2014.

5.8.5 Other Approvals

Details regarding the clinical trial were submitted to the relevant bodies, including biosafety committees and medical legal departments at Trinity College Dublin and St James's Hospital.

An application was made to the Wellcome Trust-HRB Clinical Research Facility at St James's Hospital for conduct of the clinical trial at this site on 10th April 2014. This request was reviewed and approved in July 2014.

5.9 Patient Recruitment

Patients were recruited from the outpatients of the Genito Urinary Medicine and Infectious Diseases (GUIDE) Department at St. James's Hospital.

Prior to patient recruitment we conducted a questionnaire in our outpatient department to evaluate the acceptability and interest in partaking in a HCV vaccine trial. 152 patients completed the questionnaire. Patients, of whom the majority were MSM, were asked whether they considered themselves at risk of HCV, whether they would be interested in a vaccine for HCV if commercially available and whether they would be interested in taking part in a clinical trial for a HCV vaccine. 44% of patients considered themselves at risk of acquiring

HCV, 92% were interested in a HCV vaccine if commercially available and 33% stated that they would be interested partaking in a study of a HCV vaccine.

Potentially eligible patients were approached at the time of their regular clinic follow-up visits and were contacted at home to invite them to participate. Interested patients received a copy of the Patient Information Leaflet and Consent Form for consideration prior to any screening visit. There was no public advertisement. Patient recruitment and enrolment took place at both trial sites between June 2015 and May 2016.

Inclusion Criteria

HIV-1 seropositive adults needed to satisfy all the following inclusion criteria to be eligible for the study:

- Aged 18 to 60 years (inclusive)
- Resident in or near the trial sites for the duration of the vaccination study for the participant
- Able and willing (in the Investigator's opinion) to comply with all study requirements
 - HIV Viral Load <50 copies/mL at the last routine HIV follow-up visit within the last 9 months prior to inclusion whilst on treatment with an effective ART regimen
 - Willingness to remain on ART for the study duration
 - CD4 cell count above 350 cells/ μ l
 - Negative HCV serology and negative HCV RNA PCR testing
- For women of child bearing potential, a willingness to practise continuous effective contraception during the study and a documented negative pregnancy test on the day(s) of vaccination. Effective contraception was defined as a contraceptive method with failure rate of less than 1% per year when used consistently and correctly and, when applicable, in accordance with the product label. In subjects on ART, these were:
 - Injectable progestogen

- Male partner sterilisation prior to the female subject's entry into the study, and this male was the sole partner for that subject
- Male condom combined with a vaginal spermicide (foam, gel, film, cream or suppository)
- Intrauterine device or intrauterine system
- In addition, male partners should use condoms until 3 months after the last vaccination
- Male trial participants with a female partner of child bearing potential should use condoms until 3 months after the last vaccination. In addition, the female partner should use one of the following contraceptive methods: Oral or injectable hormonal contraception; sterilisation; intrauterine device or intrauterine system
- Male trial participants with pregnant partners should use condoms until 3 months after the last vaccination
- Written informed consent

Exclusion Criteria

HIV-1 seropositive adults were unable to enter the study if any of the following exclusion criteria applied:

- Participation in another research study involving an investigational product in the 30 days preceding enrolment, or planned use during the study period
- Prior receipt of a recombinant simian or human adenoviral vaccine
- Clinical, biochemical (abnormal liver synthetic dysfunction defined by an elevated blood prothrombin time or a low blood albumin level), ultrasonographic, Fibroscan™, or liver biopsy (histology) evidence of cirrhosis or portal hypertension
- Ongoing or recent (<12 months) AIDS defining illness (US CDC definition)
- History of allergic disease or reactions likely to be exacerbated by any component of the vaccine, including egg products or gentamicin.
- History of clinically significant contact dermatitis
- Any history of anaphylaxis or serious reaction in relation to vaccination

- Pregnancy, lactation or willingness/intention to become pregnant during the study
- Known active malignant disease (except basal cell carcinoma of the skin and cervical carcinoma in situ)
- Current suspected or known injecting drug use (except individuals participating in a heroin substitution program without known or suspected concomitant drug abuse). Participants were counselled regarding the risk of HCV acquisition during the trial.
- Seropositive for hepatitis B surface antigen (HBsAg)
- Positive test for hepatitis C antibody and/or PCR
- Moderate neutropenia (Absolute neutrophil count of $<1,000$ cells/ μ l)
- Moderate thrombocytopenia (Platelet count $<80,000$ cells/ μ l)
- Anaemia (Haemoglobin <10 g/dL)
- History of pericarditis and/or myocarditis
- Heart failure (left ventricular ejection fraction $<20\%$) in the patient history or current medical treatment for heart failure.
- History of immunologically mediated disease (e.g. inflammatory bowel disease, idiopathic thrombocytopenic purpura, lupus erythematosus, autoimmune hemolytic anemia, scleroderma, rheumatoid arthritis requiring more than intermittent nonsteroidal anti-inflammatory medications for management)
- History of organ transplantation
- History of severe psychiatric disease, including psychosis and/or depression, characterised by a suicide attempt, hospitalisation for psychiatric disease, or a period of disability as a result of psychiatric disease.
- History of a significant coagulopathy (i.e. INR > 1.3 and/or APTT > 1.5 upper limits of normal) or anticoagulant therapy at time of vaccination
- Receipt of any oral or systemic antineoplastic or immunomodulatory (e.g. oral systemic corticosteroids) treatment or radiation within 24 weeks before Day 0 or the expectation that such treatment will be needed at any time during the study. Topical or inhaled corticosteroid use is allowed.

- Any other significant disease, disorder or finding, which, in the opinion of the Investigator, may either put the patient at risk because of participation in the study, or may influence the result of the study, or the patient's ability to participate in the study

5.10 Patient Baseline Characteristics

Baseline characteristics of the patients enrolled in the vaccine study are listed in Table 5.3 below. 12 patients were recruited from St James's Hospital, Dublin and 8 patients from Kantonsspital, St Gallen, Switzerland.

Table 5.3 Baseline characteristics of patients enrolled in the HCV vaccine study (n=20)

Characteristic	
Median age (IQR) - yr	42 (36-52)
Male sex - no. (%)	20 (100%)
Median weight (IQR) - kg	77 (71-83)
Median height (IQR) - m	1.8 (1.79-1.81)
Median BMI (IQR) - kg/m ²	24 (22.4-25.5)
Race or ethnic group - no. (%)	
White	19 (95%)
Latino or Hispanic	1 (5%)
Median CD4+ count (IQR) ^a - cells/mm ³	718 (560-856)
Median nadir CD4+ count (IQR) - cells/mm ³	306 (270-373)
Viral Load <50 copies/mL - no. (%)	20 (100%)
Median duration from initiation of ART to study entry (IQR) - yr	5 (3-8)
Median duration of viral suppression prior to study entry (IQR) - yr	3 (2-6)
ARVs - no. (%)	
Emtricitabine-tenofovir-efavirenz	5 (25%)
Emtricitabine-tenofovir-ritonavir-boosted atazanavir	1 (5%)
Emtricitabine-tenofovir-ritonavir-boosted darunavir	2 (10%)
Lamivudine-abacavir-efavirenz	1 (5%)
Lamivudine-abacavir-nevirapine	5 (25%)
Emtricitabine-tenofovir-rilpivirine	3 (15%)
Dolutegravir-ritonavir-boosted darunavir	1 (5%)
Lamivudine-abacavir-dolutegravir	1 (5%)
Dolutegravir	1 (5%)
History of current or previous smoking - no. (%)	7 (35%)
Median weekly alcohol consumption (IQR) - units	4 (1-8)
History of Injecting Drug Use	0 (0%)
Acquisition Risk	
MSM	20 (100%)

^a Normal CD4 count 502-1749 cells/mm³

Chapter 6 Safety and Immunogenicity of AdCh3NSmut1 and MVA-NSmut

6.1 Safety

The primary outcome measure of this Phase I vaccine study was to assess the safety of these new HCV vaccine candidates, AdCh3NSmut1 and MVA-NSmut, when administered to HIV-1 seropositive HCV-unaffected adults on cART.

Safety was assessed by the frequency, incidence and nature of adverse events and serious adverse events including clinical laboratory abnormalities arising during the study. Other studies using AdCh / MVA vectors have shown that local and systemic side effects arise in the first few days after vaccination.

Local Reaction Events

The presence of local reaction events was assessed at the time points specified in the Schedule of Procedures (See Appendix 3).

- Local reaction events were collected by structured interviews at the vaccination visits and post-vaccination follow-up visits.
- Local reaction events (pain, tenderness, erythema or skin discoloration, skin damage e.g. vesiculation or ulceration, induration i.e. formation or crust or scab, were assessed and graded according to the Adverse Event Grading Toxicity Table.

Systemic Reaction Events

The presence of the systemic reactogenicity events was assessed at the time points specified in the Schedule of Procedures.

- Volunteers recorded their temperature between days 0-3 post-vaccination in a study-specific diary card.
- Systemic reactogenicity events were collected by inspection of the diary cards as well as structured interviews at the vaccination visits and post-vaccination follow-up visits.

- Vital signs (pulse, blood pressure and temperature) were measured by site personnel prior to vaccination, 30 minutes and 1 hour post-vaccination.
- Adverse events such as feverishness, chills, headache, nausea, vomiting, malaise and myalgia were graded according to the Adverse Event Grading Toxicity Table.

All patients were given contact numbers for the clinical trial team to get in touch at any time if they had any concerns.

6.2 Adverse Events Reporting

The following definitions listed in Table 6.1 were used for the purpose of the clinical trial.

Table 6.1 Definitions for safety reporting

Adverse Event (AE)	Any untoward medical occurrence in a participant to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product.
Adverse Reaction (AR)	<p>An untoward and unintended response in a participant to an investigational medicinal product which is related to any dose administered to that participant.</p> <p>The phrase "response to an investigational medicinal product" means that a causal relationship between a trial medication and an AE is at least a reasonable possibility, i.e. the relationship cannot be ruled out.</p> <p>All cases judged by either the reporting medically qualified professional or the Sponsor as having a reasonable suspected causal relationship to the trial medication qualify as adverse reactions.</p>
Serious Adverse Event (SAE)	<p>A serious adverse event is any untoward medical occurrence that:</p> <ul style="list-style-type: none"> • results in death • is life-threatening • requires inpatient hospitalisation or prolongation of existing hospitalisation • results in persistent or significant disability/incapacity • consists of a congenital anomaly or birth defect. <p>Other 'important medical events' may also be considered serious if they jeopardise the participant or require an intervention to prevent one of the above consequences.</p> <p>NOTE: The term "life-threatening" in the definition of "serious" refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.</p>
Serious Adverse Reaction (SAR)	An adverse event that is both serious and, in the opinion of the reporting Investigator, believed with reasonable probability to be due to one of the trial treatments, based on the information provided.
Suspected Unexpected Serious Adverse Reaction (SUSAR)	<p>A serious adverse reaction, the nature and severity of which is not consistent with the information about the medicinal product in question set out:</p> <ul style="list-style-type: none"> • in the case of a product with a marketing authorisation, in the summary of product characteristics (SmPC) for that product

- in the case of any other investigational medicinal product, in the investigator’s brochure (IB) relating to the trial in question.

To avoid confusion or misunderstanding of the difference between the terms “serious” and “severe”, the following note of clarification is provided: “Severe” is often used to describe intensity of a specific event, which may be of relatively minor medical significance. “Seriousness” is the regulatory definition supplied above.

Severity grading of all AEs was assessed and determined by the Principal Investigator at the trial site or designee. Criteria for grading the severity of adverse events (as mild, moderate, severe and very severe) are listed in the Adverse Event Grading Toxicity Table in the clinical trial protocol. The principle used to develop the grading criteria is shown in Table 6.2 below and the same principle was used in grading any events that have not been anticipated in the Adverse Event Grading Toxicity Table.

Table 6.2 Severity Grading Criteria for Adverse Events

Severity Grade	Definition
1	Transient or mild discomfort that does not interfere with daily activities. Occasional over the counter medication (OTC) may be required. Prescribed therapy not needed.
2	Repeated OTC or prescribed medication required and/or normal activity reduced by ≤ 50%.
3	Significant/prolonged medical intervention or repeated prescribed medication required. Inability to perform daily activities. Bed rest needed or normal activity reduced by > 50%.
4	Hospitalisation and/or life-threatening.

Causality Assessment

For every AE, the Investigator at the trial site undertook an assessment of the relationship of the event to the administration of the vaccine. An intervention-related AE referred to an AE for which there is a possible, probable or definite

relationship to the administration of a vaccine. An interpretation of the causal relationship of each AE to the trial medication was determined by a medically qualified individual based on the type of event, the relationship of the event to the time of vaccine administration, and the known biological effects of the vaccines, according to the definitions outlined in Table 6.3 below.

Table 6.3 Guidelines for assessing the relationship of vaccine administration to an AE

Scale	Description	Definition
0	No Relationship	No temporal relationship to study product <i>and</i> Alternative aetiology (clinical state, environmental or other interventions); <i>and</i> Does not follow known pattern of response to study product
1	Possible	Reasonable temporal relationship to study product; <i>or</i> Event not readily produced by clinical state, environmental or other interventions; <i>or</i> Similar pattern of response to that seen with other vaccines
2	Probable	Reasonable temporal relationship to study product; <i>and</i> Event not readily produced by clinical state, environment, or other interventions <i>or</i> Known pattern of response seen with other vaccines
3	Definite	Clear evidence to suggest a causal relationship; <i>and</i> Event not readily produced by clinical state, environment, or other interventions; <i>and</i> Known pattern of response seen with other vaccines

All reporting duties were performed in accordance with the protocol and with local and national law, as appropriate to the trial site. Data were collected and stored in a secure fashion, including encrypted data format requiring password access for electronic records. Data was entered into the patients' Case Report Forms (CRFs) in an electronic format using an OpenClinica™ (Waltham, MA, USA) database stored on a secure University of Oxford server. Data were entered by both the local research nurse and I who had been appropriately trained in the use of OpenClinica™. I had also been involved in the set-up and validation of the database prior to trial commencement as part of the PEACHI consortium. Data were transferred to the OpenClinica™ Database by encrypted (HTTP Secure) transfer. OpenClinica™ has been fully validated for use in clinical trials at the

University of Oxford and is compliant with the requirements of Good Clinical Practice (GCP). Data captured on OpenClinica™ included safety data, clinical laboratory data and outcome data.

Reporting procedures for SAEs and SUSARs were listed in the clinical trial protocol and were consistent with the requirements of GCP.

The first two patients to receive each vaccine at St. James's Hospital stayed under observation for three hours, in case of immediate adverse events. All subsequent patients stayed under observation for one-hour post vaccination. Observations were taken 30 minutes and one hour after vaccination, after which time the sterile dressing was removed and the injection site inspected. An oral thermometer and diary card was given to each patient (for the recording of temperature and any symptoms experienced between 0-3 days post-vaccination) and instructions provided on the recording of information. Participants were advised that the use of paracetamol and ibuprofen was permitted for post-vaccination symptom relief, and they were directed to record usage of these or any other products on the diary card between days 0-3. Subjects were reviewed in the clinic 1, 7 and 28 days, and by telephone 3 days, after both prime and boost vaccinations for primary endpoint safety assessments.

6.3 Adverse Events

The following are the Solicited Adverse Events deemed related to vaccination with AdCh3NSmut1 at Day 0 and MVA-NSmut at Day 56.

6.3.1 Solicited Adverse Events – AdCh3NSmut1

Local and systemic adverse events deemed definitely, probably or possibly related to AdCh3NSmut1 are listed in Table 6.4 and Table 6.5 below. A figurative description of the maximum severity of each solicited AE is presented in Figure 6-1.

Table 6.4 Local adverse events definitely, probably or possible related to vaccination with AdCh3NSmut1 (n=20)

Reaction	Mild N(%)	Moderate N(%)	Severe N(%)	Mean duration (days)
Pain	11 (55%)	0	0	1.18
Itch	1 (5%)	0	0	1
Warmth	2 (10%)	0	0	1
Swelling	1 (5%)	0	0	1
Redness	1 (5%)	0	0	1

Table 6.5 Systemic adverse events definitely, probably or possible related to vaccination with AdCh3NSmut1 (n=20)

Reaction	Mild N(%)	Moderate N(%)	Severe N(%)	Mean duration (days)
Fever	0	0	0	0
Feverishness	3 (15%)	1 (5%)	0	2
Shivering	0	0	0	0
Muscle aches	2 (10%)	4 (20%)	0	1.83
Joint aches	1 (5%)	2 (10%)	0	2
Headache	6 (30%)	2 (10%)	0	1.75
Fatigue	7 (35%)	1 (5%)	2 (10%)	2.4
Nausea	1 (5%)	1 (5%)	0	1.5
Malaise	2 (10%)	2 (10%)	1 (5%)	2.4
Dizziness	1 (5%)	0	0	1
Local feeling of a hot head	1 (5%)	0	0	1
Night sweats	1 (5%)	0	0	1

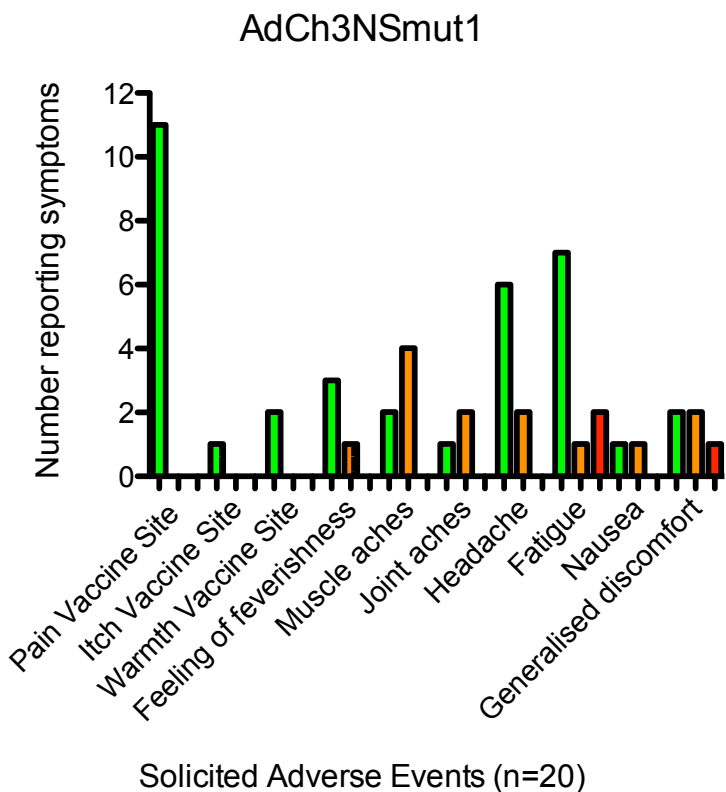


Figure 6-1 Maximum severity of solicited AEs for AdCh3NSmut1 (Green = mild, Orange = moderate, Red = Severe, n=20)

In summary, the AdCh3NSmut1 vaccine was well tolerated. Adverse events occurred in 95% of the participants. The majority of these AEs were mild or moderate, with 3 severe adverse reactions noted. The most common AEs (in order from most to least common) were pain at the vaccination site, headache, fatigue and myalgia. The majority of the AEs had resolved with 24-48 hours of vaccination (range 1-7 days).

6.3.2 Solicited Adverse Events – MVA-NSmut

Local and systemic adverse events deemed definitely, probably or possibly related to MVA-NSmut are listed in Table 6.6 and Table 6.7 below. A figurative description of the maximum severity of each solicited AE is presented in Figure 6-2.

Table 6.6 Local adverse events definitely, probably or possible related to vaccination with MVA-NSmut (n=20)

Reaction	Mild N(%)	Moderate N(%)	Severe N(%)	Mean duration (days)
Pain	6 (30%)	10 (50%)	4 (20%)	3.05
Itch	1 (5%)	0	0	1
Warmth	4 (20%)	1 (5%)	1 (5%)	1.5
Redness at injection site	2 (10%)	0	0	1

Table 6.7 Systemic adverse events definitely, probably or possible related to vaccination with MVA-NSmut (n=20)

Reaction	Mild N(%)	Moderate N(%)	Severe N(%)	Mean duration (days)
Fever	3 (15%)	1 (5%)	0	1
Feverishness	9 (45%)	6 (30%)	1 (5%)	1.375
Shivering	4 (20%)	2 (10%)	0	1
Muscle aches	3 (15%)	4 (20%)	6 (30%)	2.3
Joint aches	2 (10%)	4 (20%)	4 (20%)	2
Headache	6 (30%)	3 (15%)	2 (10%)	1.82
Fatigue	7 (35%)	5 (25%)	5 (25%)	2.29
Nausea	3 (15%)	1 (5%)	1 (5%)	1
Malaise	8 (40%)	5 (25%)	4 (20%)	1.47
Dizziness	1 (5%)	0	0	1
Stomach Pain / Cramps	0	1 (5%)	0	1

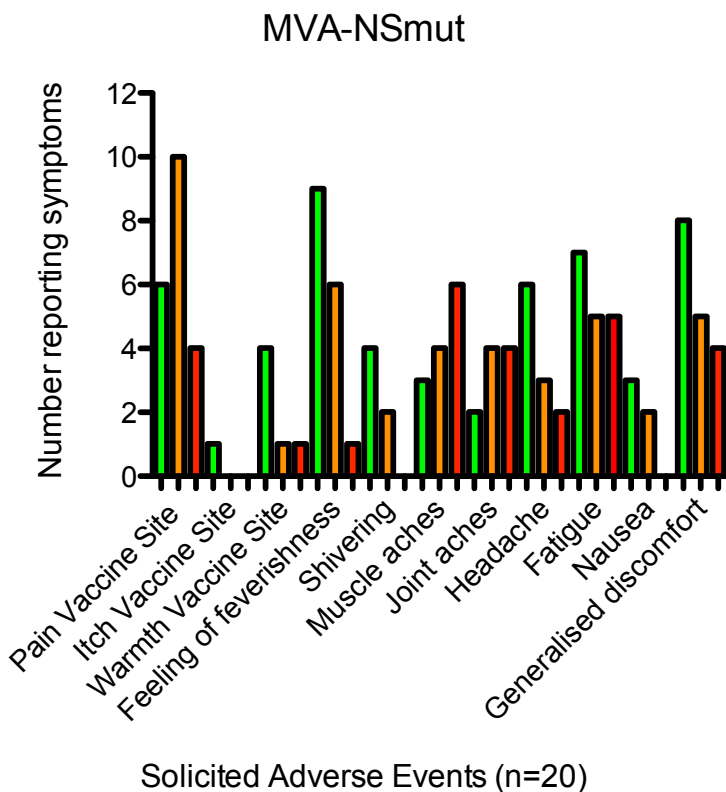


Figure 6-2 Maximum severity of solicited AEs for MVA-NSmut (Green = mild, Orange = moderate, Red = Severe, n=20)

Fever (temperature $>37.7^{\circ}\text{C}$) occurred in 4 (20%) of the patients who received MVA-NSmut. No fevers were recorded with AdCh3NSmut1. All fevers were temporary and occurred within 24 hours after vaccination. Plots of the temperature readings for the 4 patients who developed fever are shown below in Figure 6-3.

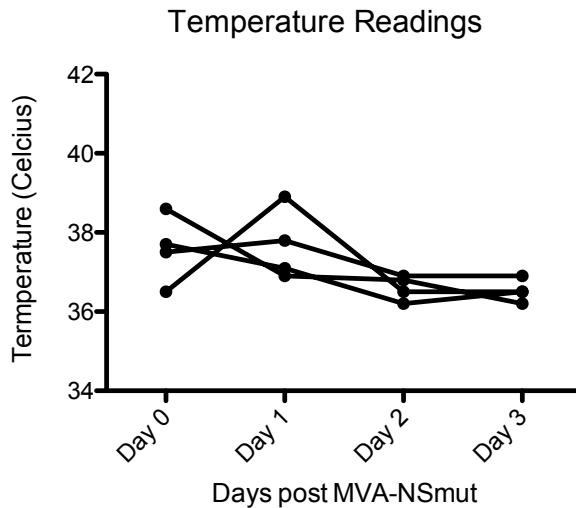


Figure 6-3 Temperature readings by day, post MVA-NSmut, for the 4 patients experiencing fever

In summary, the MVA-NSmut vaccine was generally well tolerated. Adverse events occurred in 100% of the participants. The majority of AEs were either mild or moderate, however AEs rated as severe did occur in a significant number of participants. These AEs were typically short-lived with the peak severity generally occurring within 24 hours post vaccination. As typically higher grades of local and systemic AEs have previously been reported with MVA vaccines over AdCh vaccines, all patients were counseled regarding this and advised to take over-the-counter medications for any AEs as required. The most common AEs (in order from most to least common) were pain at the vaccination site, fatigue, general malaise and a feeling of feverishness. The majority of the AEs had resolved within 48-72 hours of vaccination (range 1-5 days).

6.4 Laboratory and ECG monitoring

Haematological and Biochemical Monitoring were performed as indicated at the time points in the Schedule of Procedures (See Appendix 3).

6.4.1 Haematological monitoring

Laboratory abnormalities were graded according to the clinical trial protocol and local laboratory reference ranges.

We assessed the frequency, severity and duration of possible transient reductions in platelet count as a result of vaccination with an AdCh3 vectored vaccine. This was expected to be within the lab-defined normal range. The reason for this assessment was that a previous vaccine trial using an AdCh vector had suggested a transient reduction in platelets 1 day after vaccination. The results for all 20 participants are shown in Figure 6-4. No platelet count outside the lab-defined normal range was recorded at any time-point for any patient throughout the clinical trial.

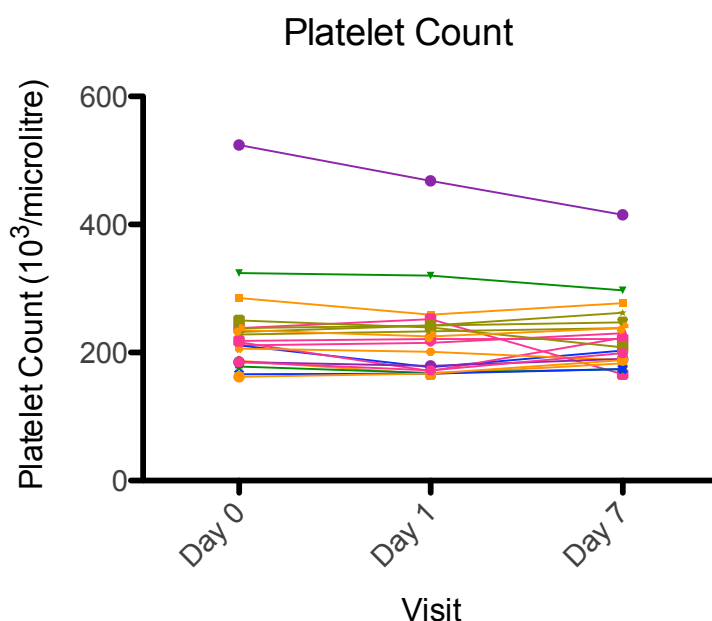


Figure 6-4 Platelet count at Day 0, 1 and 7 following AdCh3NSmut1 vaccination

No significant differences were seen in platelet count at Day 0,1 and 7.

We also assessed white cell count and lymphocyte cell count at the relative time-points in the Schedule of Procedures. Figure 6-5 and Figure 6-6 show lymphocyte count and white cell count pre and post both AdCh3NSmut1.

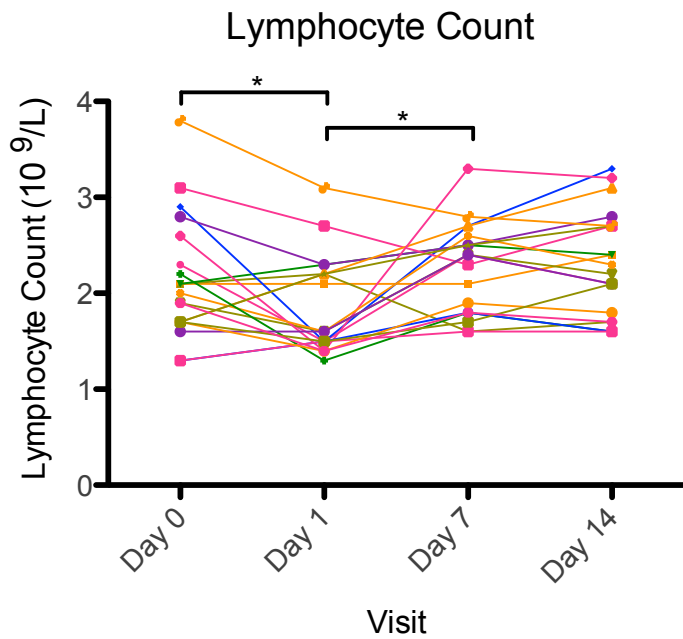


Figure 6-5 Lymphocyte count at Day 0, 1, 7, and 14 post AdCh3NSmut1 vaccination

A transient drop in lymphocyte count was observed at Day 1 post vaccination with a significant reduction in mean lymphocyte count seen between Day 0 and Day 1, that had recovered by Day 7 ($p < 0.05$ for Student t test for both Day 0 and Day 1 analysis and Day 1 and Day 7 analysis).

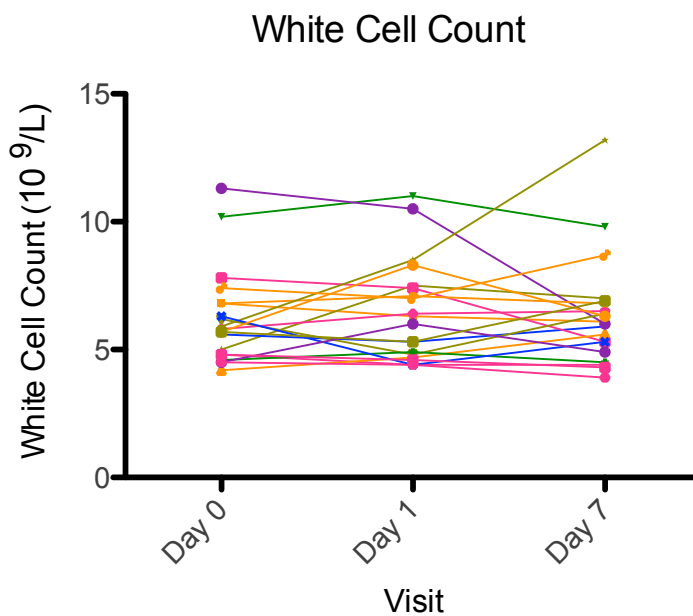


Figure 6-6 White cell count at Day 0, 1 and 7 post AdCh3NSmut1 vaccination

Neutrophil count monitoring is shown in Figure 6-7 and lymphocyte monitoring is shown in Figure 6-8 below for MVA-NSmut.

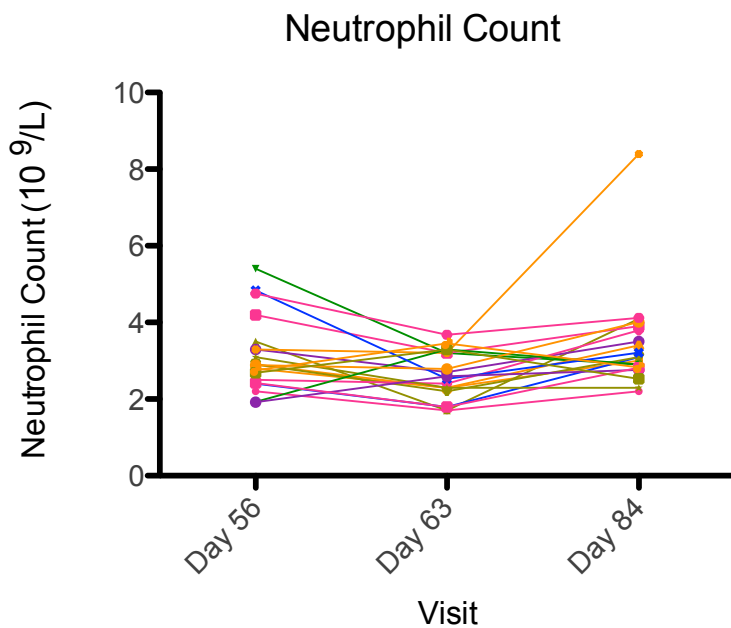


Figure 6-7 Neutrophil counts at MVA-NSmut vaccination (Day56) and post vaccination

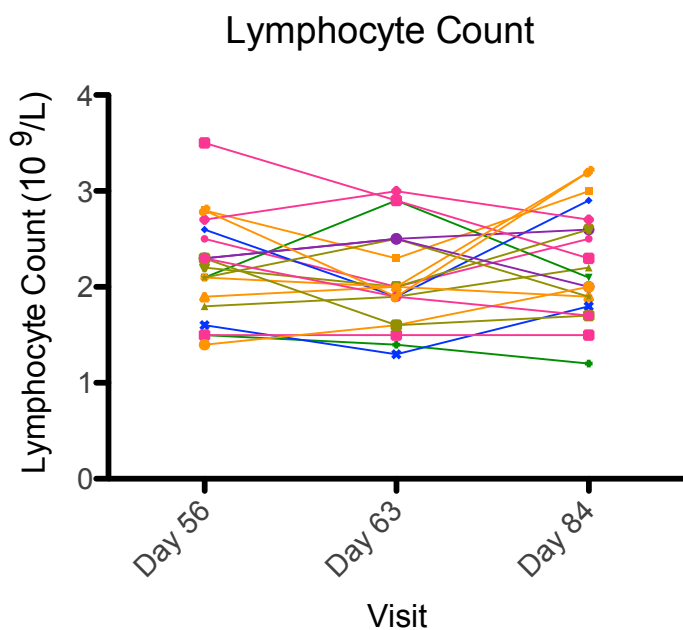


Figure 6-8 Lymphocyte counts at MVA-NSmut vaccination (Day 56) and post vaccination

As demonstrated above, no adverse events resulting from significant alterations in white cell count or white cell subsets related to vaccination occurred during the trial. As evidenced from Figure 6-5 above, it was noted that a number of patients had transient drops in lymphocyte count post vaccination with AdCh3NSmut1. This resulted in a significant mean decline in lymphocytes at Day 1 post vaccination that had recovered by Day 7. This was felt to be secondary to

migration of lymphocytes from peripheral blood to the site of vaccination. A full blood count was not obtained at Day 57 (Day 1 post MVA-NSmut vaccination) so we were unable to assess whether a similar phenomenon would have occurred at this time point.

Given the cumulative volume of blood drawn for the clinical trial was approximately 690mL over 8 months, patients were also monitored for anaemia. This volume was less than the volume allowed for both male and female blood donors over a 9-month period; therefore, we did not anticipate AEs as a consequence of blood sampling. No episodes of anaemia of any grade occurred during the trial.

6.4.2 Biochemical monitoring

Biochemical laboratory abnormalities were graded according to the clinical trial protocol and local laboratory reference ranges. No grade 3 or 4 laboratory abnormalities were observed throughout the study in HIV-infected patients. A number of grade 1 elevations in ALT, GGT, and Creatinine and 1 transient grade 2 elevation in Creatinine were observed in a minority of patients. These abnormalities either were chronic and preceded clinical trial enrollment or were transient and not deemed by the investigators to be related to the vaccines. A grade 1 elevation in bilirubin was also seen in 1 patient and was attributed to his antiretroviral therapy that included the protease inhibitor atazanavir. Atazanavir is known to induce concentration dependent increases in bilirubin plasma levels [236].

6.4.3 ECG and Troponin Monitoring

An ECG was performed at screening visit, week 8 (date of MVA-NSmut booster-vaccination) and week 9 respectively. As mentioned in Chapter 5, patients were excluded if there was a history of heart failure, myocarditis or pericarditis. No ECG findings concerning for myopericarditis were recorded and all troponin

values assessed at these time points were within the normal range (i.e. <14ng/L).

6.4.4 HIV Virological Control

2 episodes of HIV viral load escape were recorded at the St. James's Hospital site. An episode of HIV viral load escape was defined as any HIV viral load recorded that was >40 copies/mL at any time point during the trial.

One was recorded at week 8 – at the time of the MVA-NSmut vaccine administration. The HIV viral load recorded was 2156 copies/mL and felt to be associated with a syphilis re-infection; the patient had a 4-fold rise in syphilis rapid plasma reagin (RPR). Syphilis was promptly treated with a single dose of intramuscular benzathine penicillin and on repeat viral load testing a week later the patient's HIV viral load had suppressed. An appropriate RPR decline was seen following treatment. The patient's viral load remained suppressed for the remainder of the trial.

The second case was noted at Day 0 when the patient was a week post respiratory tract infection. HIV viral load at this visit was 49 copies/mL. Vaccination had been deferred for 1 week because of the patient's respiratory tract infection. Upon retesting at week 2 and week 8, the patient's viral load had suppressed, and remained suppressed for the remainder of the trial.

6.4.5 Serological Monitoring

Patients were monitored for hepatitis B, hepatitis C and syphilis seroconversion throughout the trial. As mentioned above, there was one case of syphilis re-infection at St. James's Hospital with an associated HIV viral blip. The patient's RPR titre fell appropriately following treatment. No hepatitis B infections were documented during the period of the clinical trial.

At the end of the trial, 2 patients had become hepatitis C antibody positive. Both patients had an undetectable HCV viral load on PCR testing. The positive antibody response was felt to be related to the vaccination and has been seen in a small number of volunteers vaccinated in the HCV trials at the University of Oxford. Both patients were counseled appropriately regarding interpretation of future HCV testing results. This antibody response seen in the two patients is not felt to represent the development of potentially protective neutralising antibodies as the HCV envelope glycoprotein is the natural target for neutralising antibodies [237] and the HCV immunogen used in the vaccine study was derived from the NS region.

6.4.5 Pregnancy

There were no pregnancies during the clinical trial. All participants enrolled were male.

6.4.6 Sexually Transmitted Infections

3 patients at the St James's Hospital site tested positive for sexually transmitted infections (STIs) during the clinical trial. As outlined above, one patient tested positive for syphilis re-infection. Another patient tested positive for rectal chlamydia as part of a routine asymptomatic screen and was treated with a week of doxycycline therapy. He had a test-of-cure that showed negative testing for chlamydia. Finally, a third patient tested positive on two occasions during the clinical trial. Initially, he tested positive as part of an asymptomatic screen and was diagnosed with urethral and rectal chlamydia and treated appropriately. At the final trial visit, he complained of a 1-day history of urethral discharge and diagnosed and treated for urethral gonorrhoea.

In Switzerland one patient was treated for both chlamydia and *Trichomonas vaginalis*. A second patient received empiric benzathine penicillin as he was a named contact of an individual with a recent syphilis diagnosis.

6.5 Serious Adverse Events

There were no SAEs or SUSARs during the clinical trial.

6.6 Data Safety Monitoring Committee (DSMC) and Interim Safety Reviews

An independent DSMC was convened to enable review of the safety data from the study and ensure the ethical conduct of the trial. The DSMC would have reviewed the relationship of any SAE to the study vaccines. The DSMC would also have been notified immediately or within 24 hours of the Principal Investigator at the trial site being aware of the occurrence of an SAE. The Principal Investigator at the trial site would also have notified the Chief Investigator in the same correspondence. The DSMC had the power to terminate the study if deemed necessary following a vaccine-related SAE.

The DSMC was chaired by Prof Brian Angus, a Clinical Tutor in Medicine, Honorary Consultant Physician and Director of the Centre for Tropical Medicine at the University of Oxford. The other members on the panel were PD Dr. Andri Rauch, Consultant Physician in Infectious Diseases and Swiss HIV Scientific Board Chairman, University of Bern, Inselspital Bern, Switzerland, and Prof. Dr. Heiner Wedemeyer, Hannover Medical School, Germany, all of whom had no other role in this study.

DSMC meetings were held by teleconference at study initiation, for interim safety reviews (described below), at the end of the study after collation of all safety data and at other times during the study as deemed necessary by DSMC members. The Chief Investigator coordinated DSMC meetings in order to ensure central oversight of the study.

Independent external site-specific safety reviews were performed after the first two participants per site had received the AdCh3NSmut1 priming vaccination

based on the assessment of adverse events at week 1 and before these participants receive a boosting vaccination.

A further two site-specific interim safety reviews were performed after the same two participants per trial site had received the MVA-NSmut boosting vaccination.

No further participants at the relevant trial site were vaccinated until the DSMC had reviewed all safety data and adverse events in these two participants per trial site. The DSMC's opinion, after reviewing the data, was communicated to all sites and investigators involved in the study.

6.6.1 Developmental Safety Update Reports

In addition to the reporting above, the Chief Investigator in the University of Oxford submitted Development Safety Update Reports (DSURs) once a year throughout the clinical trial, or on request, to the Medicines and Healthcare Products Regulatory Agency (MHRA), Health Products Regulatory Authority (HPRA), Swissmedic, relevant Research Ethics Committees and Sponsor, in accordance with local national regulations.

6.6.2 Conclusion

In summary, both vaccines were well tolerated and AEs were similar to what has been seen in clinical trials involving healthy volunteers. AEs occurred in 100% of the participants. The majority of AEs related to vaccination were either mild or moderate, however AEs rated as severe did occur in a significant number of participants following MVA-NSmut. However, all AEs associated with vaccination were typically short-lived with the peak severity generally occurring within 24 hours post vaccination. The majority of the AEs had resolved within 48-72 hours of vaccination (range 1-7 days).

6.7 Immunogenicity

Given this was a Phase I study, the primary endpoint for the clinical trial was the safety of AdCh3NSmut1 and MVA-NSmut when administered to patients with HIV infection. We were not seeking to establish efficacy, however we explored immunogenicity of this prime-boost vaccine regimen as a secondary endpoint for the clinical trial.

Immunogenicity assessments included:

- The proportion of volunteers who develop T cell responses to one or more HCV epitopes, as determined by IFN- γ ELISpot
- The proportion of volunteers who develop an IP-10 (CXCL-10) response to one or more HCV epitopes as determined in ELISA assays

Ex vivo ELISpot assays for IFN- γ were performed following laboratory SOPs at the time points specified in the Schedule of Procedures (See Appendix 3) at the research laboratory affiliated to St James's Hospital site at the Trinity Translational Medicine Institute.

6.8 ELISpot Technique

As outlined in Chapter 2, the activation and expansion of antigen-specific T lymphocytes is a characteristic of primary viral infections. Upon challenge, CD8+ cytotoxic T lymphocytes (CTL) recognise antigenic peptides in conjunction with MHC class I molecules, leading to the lysis of viral-infected cells as well as the secretion of interferon- γ . In addition, CD4+ T helper lymphocytes recognise antigenic peptides in conjunction with MHC class II molecules, also leading to the secretion of interferon- γ , which in turn affects other aspects of the immune system. The number of antigenic-specific precursor T cells available at the time

of challenge determines the magnitude of the immune response and may ultimately affect the course of infection.

An ELISpot assay is capable of detecting IFN- γ -producing T cells in a sample of peripheral blood mononuclear cells (PBMCs) and can be utilised to estimate precursor frequency. PBMCs were serially diluted and placed in microplate wells coated with anti-human IFN- γ antibody. They were cultured with antigens of interest overnight, resulting in re-stimulation of the precursor cells and secretion of IFN- γ . The peptides used were 15 amino acids long and overlapping by 11 amino acids; they span the whole HCV NS3-5B region and were arranged in six pools (Pools F, G, H, I, L and M). The peptides were derived from HCV genotype 1b strain BK (matching the vaccine NSmut immunogen) and were obtained from BEI resources (Manassas, VA, USA).

Following overnight stimulation, the cells were washed away, leaving the secreted IFN- γ bound to the antibody-coated wells in concentrated areas corresponding to the location of the displaced cells. The captured IFN- γ was detected with biotinylated anti-human IFN- γ antibody followed by an alkaline phosphatase-conjugated anti-biotin antibody. The addition of insoluble alkaline phosphatase substrate resulted in dark spots in the wells at the sites where the cells are located, leaving one spot for each T cell that secreted IFN- γ . The number of spots per well is directly related to the precursor frequency of antigen-specific T cells. An example showing results from an ELISpot procedure is shown below in Figure 6-9.

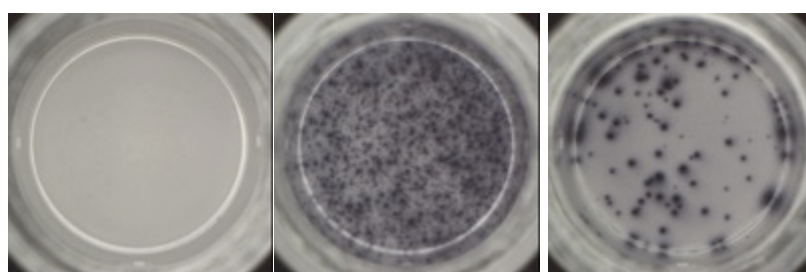


Figure 6-9 Examples of negative control (DMSO), positive control (Phytohaemagglutinin) and an investigational peptide (tuberculin) on ELISpot testing in a healthy volunteer.

6.9 Results

The geometric mean ELISpot results for all 12 patients recruited at St James's Hospital is shown below in Figure 6-10. As mentioned previously, MVA-NSmut was developed as a heterologous boosting vaccine candidate following prime with AdCh3NSmut. The high magnitude of NSmut-specific T cell responses 7 days after the MVA-NSmut boost is illustrated. As previously demonstrated in healthy volunteers, this regimen is more immunogenic than prime-boost vaccinations with heterologous adenoviruses.

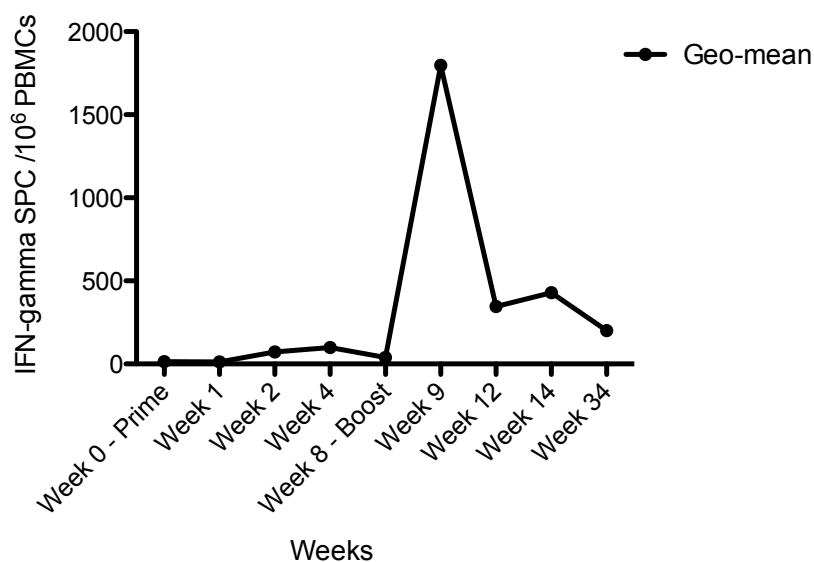


Figure 6-10 AdCh3NSmut prime followed by MVA-NSmut boost vaccination induced sustained and broadly directed T cell responses. The geometric mean responses for all patients at St James's Hospital (n=12) are presented.

20 HIV seropositive patients in total were primed at week 0 with AdCh3NSmut (2.5×10^{10} vp) and boosted at week 8 with MVA-NSmut (2×10^8 pfu). ELISpot was performed on freshly isolated PBMC to measure HCV-specific IFN- γ secreting cells using 6 pools of peptides encompassing the HCV genotype 1b NS3-NS5B sequence (data is expressed as IFN- γ Spot Forming Cells (SFC)/million PBMC).

Individual responses to vaccination for the 12 patients vaccinated at St James's site are shown below in Figure 6-11:

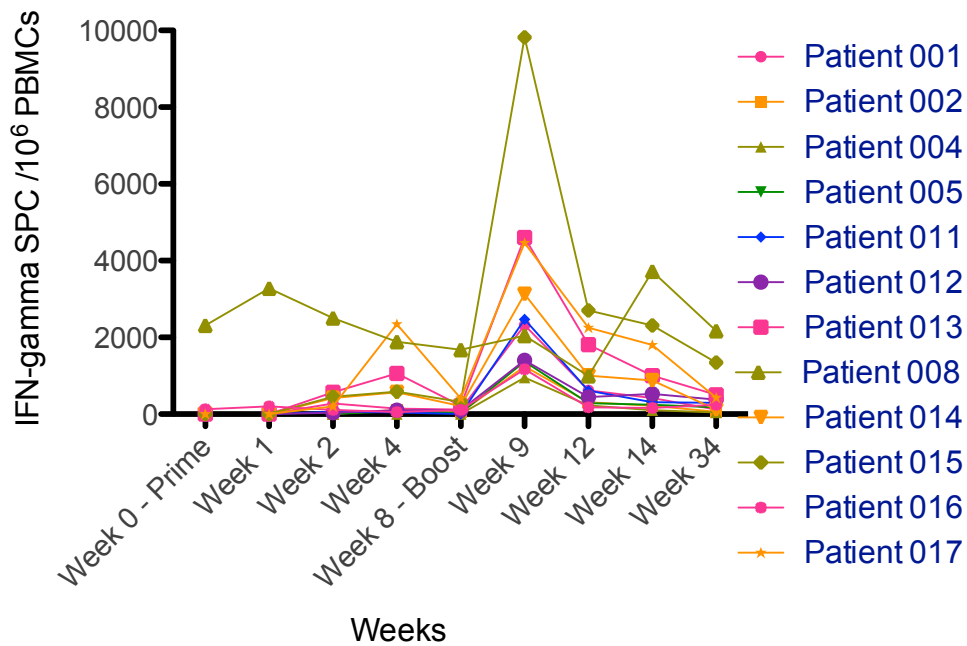


Figure 6-11 AdCh3NSmut prime followed by MVA-NSmut boost vaccination induced sustained and broadly directed T cell responses.

The total individual responses for all patients at St James's Hospital patients (n=12) are presented. 20 HIV seropositive patients in total were primed at week 0 with AdCh3NSmut (2.5×10^{10} vp) and boosted at week 8 with MVA-NSmut (2×10^8 pfu). ELISpot was performed on freshly isolated PBMC to measure HCV-specific IFN- γ secreting cells using 6 pools of peptides encompassing the HCV genotype 1b NS3-NS5B sequence (data is expressed as IFN- γ Spot Forming Cells (SFC)/million PBMC).

To analyse the results further, peak responses recorded post prime and post boost vaccination for all 20 patients are shown below in Figure 6-12 and Figure 6-13.

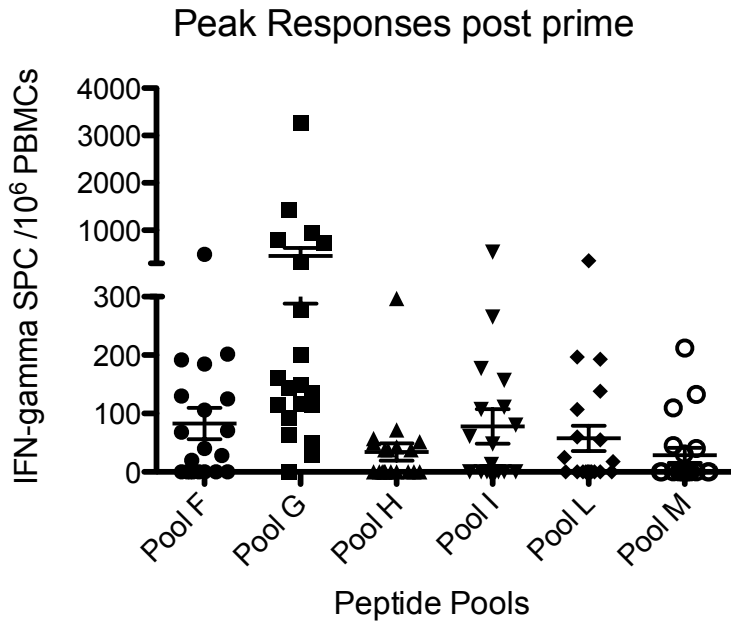


Figure 6-12 Peak Responses by peptide pool measured by fresh ELISpot analysis post MVA-NSmut boosting (n=20)

20 HIV seropositive patients in total were primed at week 0 with AdCh3NSmut (2.5×10^{10} vp) and boosted at week 8 with MVA-NSmut (2×10^8 pfu). ELISpot was performed on freshly isolated PBMC to measure HCV-specific IFN- γ secreting cells using 6 pools of peptides encompassing the HCV genotype 1b NS3-NS5B sequence (data is expressed as IFN- γ Spot Forming Cells (SFC)/million PBMC).

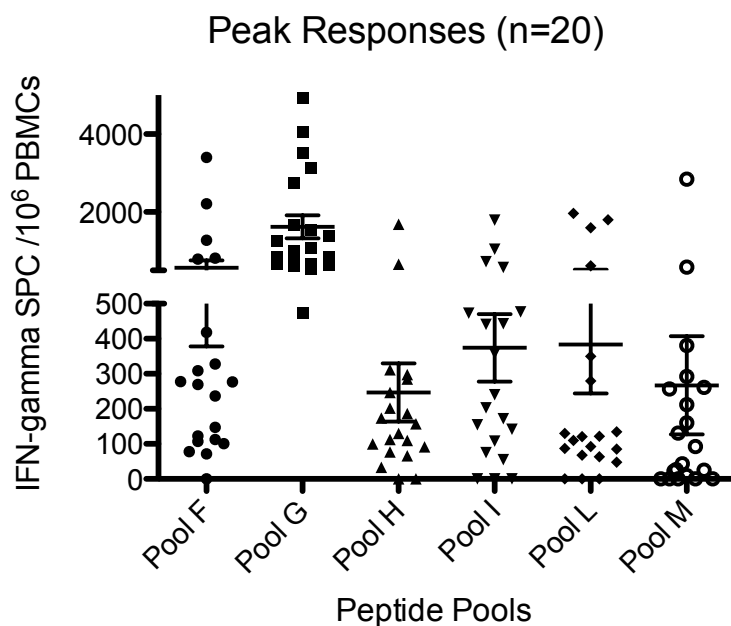


Figure 6-13 Peak Responses by peptide pool measured by fresh ELISpot analysis post MVA-NSmut boosting (n=20)

20 HIV seropositive patients in total were primed at week 0 with AdCh3NSmut (2.5×10^{10} vp) and boosted at week 8 with MVA-NSmut (2×10^8 pfu). ELISpot was performed on freshly isolated PBMC to measure HCV-specific IFN- γ secreting cells using 6 pools of peptides encompassing the HCV genotype 1b NS3-NS5B sequence (data is expressed as IFN- γ Spot Forming Cells (SFC)/million PBMC).

From the results above, it is evident that the vaccine candidates induced sustained and broadly directed T cell responses when delivered to patients who were HIV seropositive.

The majority of patients responded to AdCh3NSmut1 prime with peak responses typically occurring 2-4 weeks after vaccination (median 477, range 0-3275 SFC/10⁶ PBMC). HCV specific T cell responses were significantly enhanced by MVA-NSmut in all patients, typically peaking 1 week post vaccination (median 2381, range 952-9822 SFC/10⁶ PBMC).

At the end of the study (week 34), 6 months after vaccination, T cell responses remained detectable in 19 of the 20 patients.

Analysis of the breadth of T-cell responses induced by the vaccines, using the 6 peptide pools corresponding to the entire immunogen, showed that at the peak

response following MVA-boost most individuals responded to either 5 or 6 peptide pools. The immunodominant T cell response was elicited to the NS3 region.

6.10 Pool G reactivity at baseline

We observed significant reactivity in Pool G in 2 of the 20 patients at baseline prior to administration of the vaccines. This occurred in one patient at St James's Hospital (2533 SFC/10⁶ PBMC in Pool G at Screening/Day 0) and in one patient at St Gallen (1028 SFC/10⁶ PBMC). The reasons for this are unclear. It may represent cross reactivity with a specific antigen in Pool G or possible exposure to HCV in the past with no evidence of sero-conversion. We plan to evaluate these unexpected findings further by performing 'mini-pool' analysis for Pool G peptides on frozen PBMCs from the Screening /Day 0 samples performed on these patients.

6.11 Comparison with Healthy Volunteer Studies

I compared the peak vaccine responses from the HIV-positive patients in this trial with healthy volunteers from the HCV003 who received the same vaccine strategy in Oxford. Results are shown below in Figure 6-14.

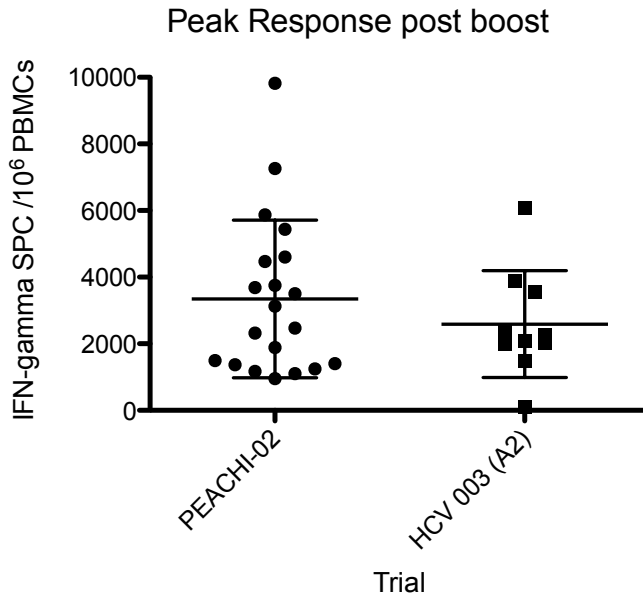


Figure 6-14 Comparison of mean peak ELISpot responses post MVA-boost in PEACHI-02 study (n=20) and healthy volunteers (n=10) in HCV003 - Arm A2 of the trial.

No significant difference was observed between the two groups. Day 63 data are shown for both groups. Data are expressed as IFN- γ Spot Forming Cells (SFC)/million PBMC).

6.12 Whole Blood Assay for IP-10

IFN- γ induced protein 10 (IP-10; CXCL10) is a chemokine produced by innate antigen presenting immune cells – mainly DCs – upon stimulation with IFN- γ . IP-10 is the key driver of a IFN- γ dependent amplification loop supporting a sustained adaptive immune response. The IP-10 receptor CXCR-3 (CD183) is up regulated on pathogen specific effector Cytotoxic T Lymphocytes (CTLs) and Th1-polarised CD4⁺ T cells. CXCR-3 mediated signalling facilitates the CTL trafficking to the lymphoid compartment.

In comparison to IFN- γ , IP-10 secretion upon pathogen specific stimulation is increased significantly [238, 239] rendering IP-10 a valid alternative for the detection of pathogen specific T cell responses *ex vivo* with increased sensitivity. HCV specific CTL responses tend to be weak in peripheral blood due to the compartmentalisation of the CTLs into the liver parenchyma. Although this compartmentalisation is less likely to happen in HCV vaccine recipients without

HCV infection a robust and sensitive assay to detect CTL responses upon vaccination is desirable.

I performed stimulation of whole blood from all patients enrolled at St James's Hospital at baseline, week 2, week 8, week 9 and week 34. Supernatants and homogenised cell pellet samples in DNA/RNA shield were stored at -80°C. Samples were shipped to St Gallen in Switzerland in early 2017 for further analysis on all 20 patients enrolled in the trial. Initial evaluation of this novel whole blood stimulation assay with IP-10 detections by ELISA in the supernatants at each time point is shown in Figure 6-15.

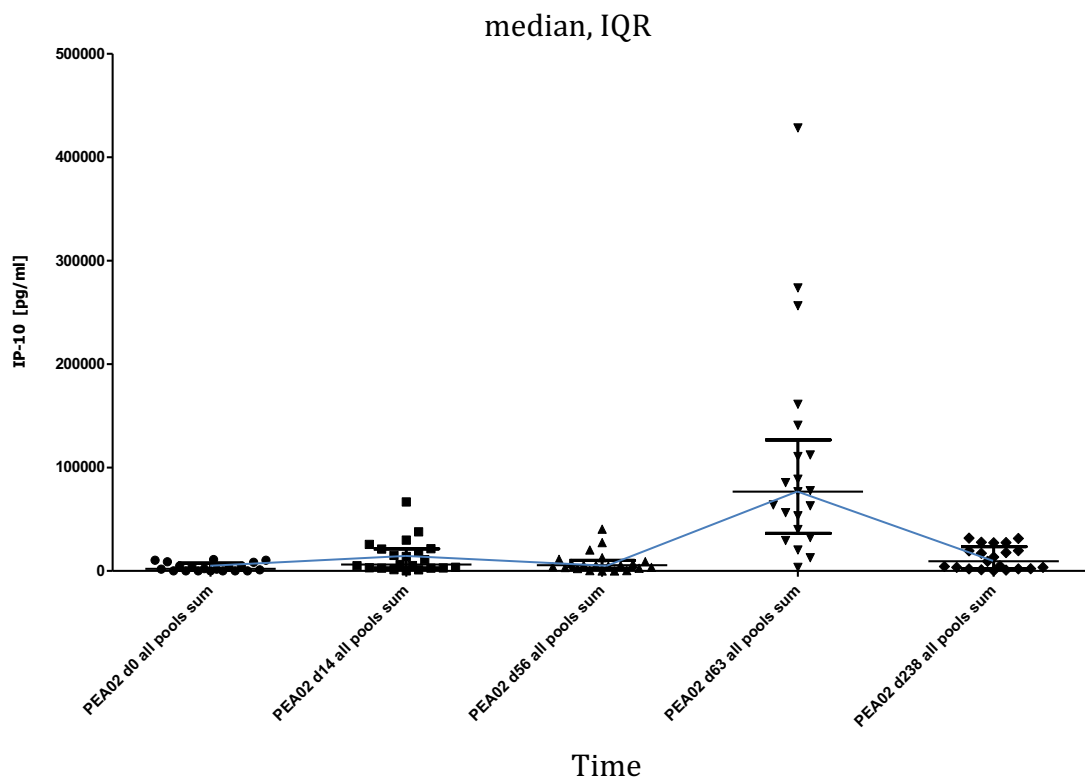


Figure 6-15 IP-10 responses with AdCh3NSmut prime followed by MVA-NSmut boost vaccination

20 HIV seropositive patients in total were primed at week 0 with AdCh3NSmut (2.5×10^{10} vp) and boosted at week 8 with MVA-NSmut (2×10^8 pfu). IP-10 detection by ELISA following overnight stimulation of whole blood with 6 pools of peptides encompassing the HCV genotype 1b NS3-NS5B sequence (data is expressed as pg/ml). Figure courtesy of M Hoffmann.

The objective of this work on the whole blood assay was to develop and assess a robust, simple and affordable assay to measure vaccine-induced T cell immunogenicity. A positive signal is seen both 2 weeks post prime vaccination and 1 week post boost vaccination. Further work is in progress to correlate the

IP-10 ELISA results with the ELISpot results and look to obtain a cut-off definition for the IP-10 ELISA.

6.13 Discussion

The ability to produce a vaccine for HCV is hampered by the large diversity of the virus, its capacity to evade the immune system and to downregulate T cell immunity. The fact that a significant proportion of those infected are able to control the virus spontaneously makes the quest for a vaccine against HCV distinct from HIV vaccines. What a vaccine should ideally achieve is to accelerate generation of immunity (which could involve neutralising antibodies) after exposure and enhance the chances of clearance.

Although, the correlates of protection in HCV are poorly defined, studies of T cell immunity in natural infection, discussed in Chapter 5, suggest targeting of multiple HCV antigens and the generation of a robust T cell response are some of the key parameters.

This study shows that AdCh3/MVA vaccination delivered in a prime-boost regimen to HIV-infected patients induces T-cell responses in the majority of NS antigenic pools in most individuals. This work shows that it is possible to develop broad, long lasting T cell responses against HCV in HIV seropositive patients.

The next step will be to test whether these vaccines can be protective in a setting where HCV exposure is common and the results of the ongoing Phase II study to assess this effect are eagerly awaited.

Chapter 7 Final Discussion

7.1 Final Discussion

As outlined in Chapter 1, the aims of this thesis were threefold. Firstly, I looked to examine therapeutic outcomes for patients with HIV/HCV co-infection treated for HCV from 2001 to 2016. I looked to examine outcomes for a variety of evolving treatment regimens across a number of patient groups. I also looked to report liver-related morbidity and mortality data in these patient groups.

Further understanding of the complex interaction between HCV and the immune system is likely to yield benefits in terms of therapeutic and preventative strategies. Secondly, we looked to examine alterations in the IFN- α JAK-STAT signaling pathway in primary immune cells of co-infected patients as a result of HCV treatment with a Telaprevir-based regimen.

Finally, as significant barriers exist towards eradication of HCV, I looked to examine the safety and immunogenicity of novel vaccine candidates (AdCh3NSmut1 and MVA-NSmut) for the prevention of Hepatitis C in HIV seropositive individuals.

Therapeutic Outcomes for HCV

This work has shown the evolution of therapeutic responses to HCV treatment in co-infected patients with current SVR rates of >90% for all genotypes at our facility. These response rates mirror the results seen in clinical trials and real-life settings in co-infected patients [122]. Overall, these new regimens offer hope towards eradication of HCV in specific patient groups including haemophiliacs, liver transplant patients, patients on haemodialysis and patients with HIV/HCV co-infection [240]. Significant reductions in mortality and liver-related morbidity for patients treated with IFN-based regimens have been demonstrated, mirroring results from other HCV-infected cohorts [32, 33]. We have also shown that in a large urban cohort, outcomes for people who inject

drugs treated with PEG-IFN and ribavirin, a group historically viewed as 'difficult to treat' are comparable to non-drug users [241]. These data support a public health strategy of HCV treatment and eradication in this population in the DAA era.

Patients with decompensated cirrhosis, however, remain a significant challenge. As discussed in Chapter 3, at 2 years of follow up, for HIV/HCV co-infected patients with decompensated liver disease treated as part of the national EAP, the overall mortality rate was 54%. This reflects the considerable challenges in treating this patient group, given the poor overall prognosis for patients with decompensated liver disease. 2 of these patients died while on treatment with DAA therapy. High mortality during therapy has been reported on a national level for the Irish EAP [146], especially amongst patients with Child-Pugh C cirrhosis. These findings were mirrored in patients with Child-Pugh C cirrhosis treated with antiviral therapy in the United Kingdom as part of the National Health Service EAP [147]. It is paramount clinicians should reflect very carefully on the risk-benefit ratio of therapy. Rapid referral for liver transplantation evaluation should be considered in such patients. However, the shortage of available donor livers needs to be addressed also. Predictors of response and subsequent improvement in liver function and mortality for patients with decompensated cirrhosis who are ineligible for liver transplantation are urgently required. It is planned to analyse this on a national level for all patients enrolled in the EAP.

A limitation of this work is that it was predominantly retrospective, and while data were available for follow-up on the majority of patients who continued to engage in care for ongoing HIV management, we would have been unable to account for patients who transferred care or who were lost to follow up. The establishment of a national HIV registry in Ireland, coupled with the introduction of a national unique Individual Patient Identifier, would be a significant step forward towards overcoming this limitation in future studies.

While response rates with DAA agents often show SVR rates >95%, they are not 100% effective. Patients failing treatment risk HCV viral resistance [126], and resistant variants that emerge during therapy, particularly resistance to NS5A inhibitors, can persist long term in the absence of the drug [242]. Successful therapy with DAA regimens also does not protect against re-infection. We have documented 10 cases of re-infection to date following initial successful treatment for HCV.

The recent establishment of a national registry (ICORN - The Irish Hepatitis C Outcomes Research Network) for Hepatitis C treatment outcomes should allow for prospective assessment of treatment outcomes for emerging HCV therapeutics and enable further research into this field. Additionally, data obtained from studies in real world clinical practice provide valuable and robust information fundamental for input into future economic evaluations for therapeutic agents used for the treatment of HCV infection.

Evaluation of the JAK-STAT Signaling Pathway in Patients undergoing Treatment for HCV with Telaprevir-based regimens

We have previously described reduction of STAT1 and STAT3 proteins from immune cells and hepatocytes [52] of HCV mono-infected individuals. This helped to widen our understanding of HCV's spectrum of target cells and demonstrate widespread disruption of IFN- α pathway, and further outline the broad immune evasion strategy of HCV.

Given the IFN- α pathway and induction of anti-viral ISGs are essential for successful viral clearance, we hypothesised that in suppressing viral replication directly DAAs may indirectly enable restoration of the endogenous IFN- α signaling pathway and that indeed this may be crucial in achieving full clearance of HCV.

Our results identify a reduced level of STAT1 (but not STAT3) protein from immune cells from individuals affected with HIV/HCV co-infection, compared to

healthy controls. After 12 weeks of treatment with telaprevir-based therapy for HCV we observed a significant increase in STAT1 proteins, suggesting restoration of IFN- α signaling pathways in these patients' immune cells. It remains unclear as to whether the rise in STAT1 is related to PEG-IFN therapy, HCV viral decline or a combination of both of these factors. This issue could be addressed by looking at earlier time points e.g. Day 1 of therapy and evaluating responses in IFN-free therapies.

Despite the high SVR rates now seen with IFN-free DAA treatments, it is important to obtain further information relating to immunological interactions between the host and the virus. For example, recent reports that CD4+ regulatory T cells, which have been linked to inhibition of antiviral immunity and promotion of fibrosis, remain active post elimination of HCV after DAA treatment in mono-infected patients [243] and HIV/HCV co-infected patients [244], highlight that on-going work is required to tease out the complexities of immunological consequences of DAA treatment. The long term clinical impact of this persistence remains unknown.

Future work should look to enable identification of patients likely to fail standard therapy, and patients who would require only short course therapy. The personalisation of DAA regimens could have implications for significant cost savings as evaluation and access to DAA treatment is being rolled out to all patients.

It is unknown whether on-treatment changes in STAT proteins from immune cells may serve as a biomarker for evaluating patients for whom short course or long course DAA treatment may be appropriate, but this should be explored further through assessment of IFN signaling with IFN-free regimens.

As mentioned previously, despite advances in HCV therapeutics outlined above, DAA therapy will have a limited impact on the burden of HCV-related disease on a population level unless barriers to HCV education, screening, evaluation and treatment are addressed and treatment uptake improves [128].

Accurate HCV prevalence and incidence data are required to assess the magnitude of the pandemic and to implement appropriate public health interventions. The Centers for Disease Control and Prevention (CDC) in America recommends 1-time HCV testing in the 1945-1965 birth cohort, in addition to targeted risk-based testing. However, research demonstrates that one-quarter of infections would remain undiagnosed if current CDC birth cohort recommendations were employed, suggesting that in high-risk urban Emergency Department (ED) settings a practice of universal 1-time testing might be more effective [245]. Programs such as opt-out testing for HIV, HBV and HCV at the Emergency Department at St James's Hospital have been found to be feasible and acceptable. Blood borne viral infections were prevalent in our population and newly diagnosed cases were diagnosed and linked to care [246]. Similarly, we have shown that large-scale point-of-care testing for HIV in an Irish prison setting is acceptable and achievable [247]. Current Irish HCV screening guidelines recommend screening for HCV should be offered to all prisoners on entry to prison. The development of an affordable, rapid, commercially available point-of-care diagnostic to detect HCV RNA would contribute towards efforts to eliminate HCV [248].

The Extension for Community Healthcare Outcomes (ECHO) model was developed at the University of New Mexico to improve access to care for underserved populations with complex health problems such as HCV infection [249]. Through the use of video-conferencing technology, the ECHO program trains primary care providers to treat complex diseases. Similar programs, worldwide, have enabled delivery of HCV care to remote communities [250]. We are currently exploring such a model to enable delivery of HCV care to vulnerable patient groups.

Therefore, as part of a multidisciplinary approach in working towards HCV eradication, the development of a vaccine that prevents HCV infection would have a substantial impact on morbidity, quality of life and treatment costs.

Safety and Immunogenicity of AdCh3NSmut1 and MVA-NSmut

The final work of my thesis evaluated the safety and immunogenicity of a novel vaccine strategy in HIV-positive patients. The vaccine strategy has been shown to be safe and highly immunogenic. Adverse events associated with the vaccine were consistent with previous trials of AdCh and MVA vectors. The majority of AEs lasted 24-48 hours and typically peaked in severity within the first 24 hours after vaccination. Local and systemic AEs were increased in frequency and severity after MVA-NSmut compared to AdCh3NSmut1, consistent with previous published literature for MVA. However, the overall safety profile was acceptable. No SAEs or SUSARs were recorded.

The challenge to produce a vaccine for HCV is hampered by the large genetic diversity of the virus, its ability to evade the immune system and to downregulate T cell immunity. The fact that a significant proportion of individuals infected are able to control the virus spontaneously, leading to viral eradication and effectively representing long-term clinical cure makes the quest for a vaccine against HCV distinct from HIV vaccines. An effective vaccine against HCV would not need to provide sterilising immunity. What a vaccine should ideally achieve is to accelerate generation of immunity (which could involve neutralising antibodies) after exposure and enhance the chances of clearance.

It was shown in our study that the vaccines induce T-cell responses in the majority of NS antigenic pools in most patients. This work shows that it is possible to develop broad, long lasting T cell responses against HCV in HIV seropositive patients. Although, the correlates of protection in HCV are poorly defined, it is suggested targeting of multiple HCV antigens and the generation of a robust T cell response are some of the key parameters.

Ideally a vaccine strategy should result in the maintenance of a memory pool over time with the capacity to proliferate with a population of T cells with immediate effector function. A critical threshold for the magnitude of the T cell

response required has yet to be defined, although that it is likely that in the context of a prophylactic vaccine, “more is better.”

In our study, we selected patients with a robust CD4 cell count (>350 cells/ μ L) and all patients were on antiretroviral therapy with an undetectable viral load. How patients with a CD4 count between 200-350 cells/ μ L and patients with a low nadir CD4 count would respond to such a vaccine strategy remains unknown. A potential future venture would be to look at vaccinating patients post treatment with IFN-free DAA agents to assess immunogenicity responses and conceivably later assess for efficacy against HCV reinfection in high-risk groups.

We eagerly await the results of the on-going Phase 2 study evaluating the efficacy of this regimen.

7.2 Future work arising from this work

We now propose to consolidate the work completed in the initial phase of this project, reviewed in this thesis, by extending it out to other research projects including:

We plan to undertake transcriptomic evaluation of PAXgene samples frozen at the Screening Visit/Days 0, 1, 7, 14, 28, 56, 57, and 63.

As HCV exists as distinct genotypes that are broadly segregated geographically, previous work in healthy volunteers has assessed the capacity of T cells generated by MVA boost encoding a subtype 1b immunogen to target genotypes 1a, 3a, and 4a. It was found that cross-reactive T cells responses between heterologous viral genotypes are readily generated but at a reduced magnitude [195]. We plan to assess these responses in the vaccinated HIV seropositive patients. Whether these responses observed in healthy volunteers are sufficient to provide protection remains unknown and will require efficacy studies in

mixed genotype populations. Further assessment of the significant T cell responses in Pool G in 2 of the 20 patients prior to administration of the vaccines will also be evaluated. This will be achieved by examining 'mini-pools' of the Pool G peptides in an effort to determine which peptide(s) are reactive.

HLA typing has been performed on all patients in the vaccine study. In the HCV003 study, the AdCh3NSmut / MVA-NSmut vaccine approach generated very high numbers of both CD4+ and CD8+ T cells, targeting multiple HCV antigens irrespective of host HLA background [195]. We plan to look at the magnitude and phenotype of HCV-specific CD8+ T cell populations identified by tetramer staining, before and after vaccination, in patients with the appropriate HLA class I alleles.

Several markers of T cell functionality (e.g. perforin and granzyme B expression) and the T cell phenotype (e.g. effector memory T cells) have been associated with HCV infection control. Therefore, we plan to use frozen PBMCs to evaluate exploratory immunology assays (including intracellular cytokine staining (ICS) phenotyping, viral suppression in vitro and proliferation). Included in this work, we plan to evaluate whether CD4+ or CD8+ T cells were the predominant producers of IFN- γ . We also plan to evaluate for rates of multifunctional T cells by assessing production of IFN- γ , IL-2 and TNF- α . Previous work in the HCV003 trial demonstrated the polyfunctionality of CD4+ and CD8+ cells peaked at week 18 and 22 of the trial respectively, following MVA vaccination [195]. We plan to compare our results with the healthy volunteers who received the same vaccine strategy in the PEACHI-04 study at the University of Oxford.

Similarly using frozen PBMCs and serum for innate immunological assays we plan to measure natural killer cell (NK) function, at specific time points including before, within 24 hours and 7 days after vaccination. The relationship between innate and adaptive immune responses to HCV vaccination will be explored.

One of the host factors that may determine the immunogenicity of AdCh3NSmut1 in our study population is the potential for neutralisation of the vaccine by pre-

existing adenovirus-specific antibodies. This hypothesis is based on the observed lower magnitude responses to encoded HIV proteins among recipients of Merck's trivalent Ad5-vectored HIV-1 vaccine with pre-existing humoral immunity to Ad5 [251, 252]. We therefore considered the following possibilities in the design of the vaccine trial: 1) neutralisation of the AdCh3 vector by pre-existing AdCh3-specific antibodies; 2) neutralisation of the AdCh3 vector by pre-existing antibodies to human adenoviruses that could cross-react with AdCh3.

PEACHI consortium members have screened sera from 193 human subjects from Europe and the US for neutralising antibodies against a panel of human and chimpanzee adenoviruses: AdCh3-specific antibody positivity (titre >200) was observed in just over 10%, while 40% had Ad5-specific antibody titres >200 [190]. Furthermore, in another study, AdCh-specific neutralising antibodies were rarely detected among human subjects from the US and Thailand and prevalence was $\leq 10\%$ in subjects from sub-Saharan Africa [253]. Colloca *et al* also found that pre-existing Ad5-specific antibodies did not cross-neutralise AdCh3 in mice that were vaccinated with a AdCh3-vectored HIV-1 immunogen: T cell responses to the encoded HIV-1 proteins were similar in Ad5-immune and control mice [190].

Taken together with data from phase I clinical trials showing strong T cell responses, following vaccination of healthy subjects with AdCh3NSmut, the risk of pre-existing humoral immunity to adenoviruses impairing vaccine responses in our study is considered to be low. However, to address this question definitely, serum samples from study participants were stored for analysis of neutralising activity against a panel of human and chimpanzee adenoviruses including AdCh3. These samples have now been shipped to our colleagues at ReiThera in Rome for further analysis.

We plan to further evaluate the whole blood stimulation assay with further analysis of IP-10, IFN- γ and interleukin 2 (IL-2) detections by ELISA in supernatants and the correlation of chemokine / cytokine secretion and respective mRNA cell levels.

Given, 2 of the patients who participated in the vaccine trial became HCV antibody positive at the last clinic visit, we plan to analyse Day 0 and Day 238 serum samples from patients with a spectrum of Hepatitis C antibody assays, to further understand the antibody response in these patients. As mentioned before, no patient had evidence of detectable HCV by RNA testing at any point in the study.

PEACHI investigators have also developed further vaccines using adenoviral and MVA vectors when the encoded HCV immunogen (NSmut) is fused to MHC class II invariant chain (Ii). We plan an evaluation of a vaccine strategy in healthy volunteers in Oxford initially. If safety data are favourable, we plan to expand this vaccine strategy to further populations including patients who have been successfully treated with IFN-free DAA regimens and patients with HIV infection.

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Appendix 1

Publications and Presentations related to this work

Outcomes of a HCV treatment programme in PWID over 10 years support a disease eradication strategy for all patients in the DAA era
Omar El-Sherif, **Ciaran Bannan**, Shay Keating, Susan McKiernan, Colm Bergin, Suzanne Norris
Poster at AASLD Annual Meeting, 2014

Outcomes from a large 10 year hepatitis C treatment programme in people who inject drugs: No effect of recent or former injecting drug use on treatment adherence or therapeutic response
Omar El-Sherif, **Ciaran Bannan**, Shay Keating, Susan McKiernan, Colm Bergin, Suzanne Norris
PLoS One. 2017 Jun 21;12(6):e0178398. doi: 10.1371/journal.pone.0178398. eCollection 2017.

Hepatitis C Virus (HCV) Immune Evasion Chapter
Silvia Napoletano, **Ciaran Bannan**, Colm Bergin and Nigel J Stevenson
Hepatitis C Virus: Molecular Pathways and Treatment (Omnicis Group International)

Outcomes of HCV treatment in HIV co-infected patients in the pre-DAA era: 2001-2012
CL Bannan, M Coghlan, G Farrell, CJ Bergin
Presentation at IDSI Annual Scientific Meeting 8th May 2015

From Clinical Trial to Real World: Treatment outcomes for DAA based Hepatitis C triple therapy a HIV co-infected and Methadone Maintenance Therapy population
M Coghlan, S Kelly, **C Bannan**, G Farrell, M Broderick, C Murray, S O'Dea, C Bergin
Poster at IDSI Annual Scientific Meeting, May 2015

Interferon Free Hepatitis C Treatments: Experience to date in the co-infected setting
M Coghlan, **C Bannan**, G Farrell, M Broderick, C Murray, S O'Dea, C Bergin
Poster at IDSI Annual Scientific Meeting, May 2015

Point-of-care testing for HIV in an Irish Prison Setting: results from three major Irish prisons
Bannan CL, Lynch PA, Conroy EP, O'Dea S, Surah S, Betts-Symonds G, Lyons FE
Int J STD AIDS 2016 Oct ;27(11)950-4

Hepatitis C in MSMs: A review of testing practices in the GUIDE clinic & a description of recent cases
N Lynn, J Dean, E Quinn, G Farrell, C Murray, F Lyons, **C Bannan**, C DeGascun, C Bergin
Oral Presentation at SSSTDI Autumn Conference November 2016

NS5A resistance leading to failure of 24-week therapy with sofosbuvir/ledipasvir and ribavirin for the treatment of hepatitis C genotype 1a infection in a HIV-1 co-infected patient.

Sevastianova K, Dean J, **Bannan C**, Coghlan M, Murray C, Farrell G, De Gascun CF, Bergin C
J Clin Virol. 2016 Sep;82:66-9

Assessment of the safety and immunogenicity of a heterologous prime-boost hepatitis C vaccine strategy in HIV-1 seropositive adults on antiretroviral therapy

Ciaran Bannan, Stefania Capone, Anthony Brown, Felicity Hartnell, Antonella Folgori, Pietro L. Vernazza, Colm Bergin, Bethany Turner, Ellie Barnes, Lucy Dorrell, Matthias Hoffmann
Oral Presentation at EACS, 27 October 2017

Control of HIV infection by IFN- α : implications for latency and a cure

N Bourke, S Napoletano, **C Bannan**, S Ahmed, C Bergin, A McKnight, N Stevenson
Cellular and Molecular Life Sciences
DOI 10.1007/s00018-017-2652-4 – Accepted 5 September 2017

Appendix 2

Reagent Preparation

Preparation of R0 Medium:

R0 was prepared by mixing 500 mL of RPMI medium 1640, 5mL of 200mM L-glutamine (2mM final concentration) and 5 mL of 100X penicillin-streptomycin solution (1X final concentration). R0 medium was stored at 2-8°C

Preparation of Complete RPMI Medium (R10):

R10 was prepared by mixing 500 mL of RPMI medium 1640, 50 mL FBS (with heat inactivation at 56°C for 60 min) or FCS (10% final concentration), 5mL of 200mM L-glutamine (2mM final concentration) and 5 mL of 100X penicillin-streptomycin solution (1X final concentration). R10 medium was stored at 2-8°C for up to 2 months.

Preparation of Thawing Medium (TM):

TM was prepared when 10 µl DNase I (Type IV) (final concentration of 60 µg/ml) was added to 10 ml of R10. TM was prepared on day of use and pre-warmed in a water bath at 37°C for at least 10 minutes

Preparation of PBS/0.05% TWEEN 20® Wash Buffer (WB):

0.5 ml TWEEN 20 was added to 1 x 1000 ml bottle of D-PBS, mixed by inversion and stored at room temperature (18-22°C) for up to one year from the date of preparation.

Preparation of Assay Diluent (AD):

5g of BSA was added to a 1000 ml bottle of D-PBS and gently mixed by inversion. The bottle was left at room temperature until the BSA is completely dissolved and stored at room temperature for up to three months from the date of preparation.

Running buffer (for Western Blotting):

14g Glycine

3 g Trizma Base

1g SDS

1L distilled H₂O

Transfer buffer (for Western Blotting):

3.03g TRIS

14.4g glycine

200mL methanol

Final solution made up to 1L with 800mL dH₂O

TBST - 1L (for Western Blotting):

1.21g 10mM Tris

5.84g 100mM NaCl

1ml Tween 10

Adjust with HCl to a pH of 7.4.

Appendix 3

Schedule of Procedures and Copies of Publications related to this work

Table 3 – Schedule of Procedures

Procedures	Screen (≥ day -42)	Day 0	Day 1 (18-24 hrs post-vacc.)	Day 7	Day 14	Day 28	Day 56	Day 57 (18-24 hrs post-vacc.)	Day 63	Day 84	Day 98	Day 238 (Final Visit / Early Termination)
Visit number	1	2	3	4	5	6	7	8	9	10	11	12
Visit windows (days)		0	0	±3	±3	±7	±7	0	±3	±7	±7	±7
Week		0	0 + 1 day	1	2	4	8	8 + 1 day	9	12	14	34
Informed consent	X											
Confirm eligibility	X	X					X					
Demographics & medical history	X											
Concomitant medications and directed physical examination	X	X		X		X	X		X	X	X	X
Vital signs	X	X		X	X	X	X		X	X	X	X
ECG	X						X		X			
Vaccination – prime (AdCh3NSmut1)		X										
Vaccination - boost (MVA-NSmut)							X					
Vaccine reactions – local/systemic		X	X	X			X	X	X			
Review medical history/adverse events		X	X	X	X	X	X	X	X	X	X	X
Urinalysis (protein, glucose and multistubstance drug screening)	X											
Pregnancy test	X	X					X					
Serology and Virology - HBsAg, HCV antibodies, Syphilis, HCV and HIV-1 RNA	X	X					X					X
Haematology & clinical biochemistry	X	X	X ⁶	X	X	X	X		X	X	X	X
Cellular immunogenicity assays including IP-10 assay (Li/Heparin tube)	X	X	X	X	X	X	X	X	X ^{**}	X	X	X
Transcriptomics (PAXgene tube)	X	X	X	X	X	X	X	X	X			
CD4 cell count	X						X					X
Anti-adenovirus antibodies and innate immune studies (STT/Clotted tube)		X [#]	X	X	X	X [#]	X	X	X			X [#]
HLA typing		X										
Diary cards provided		X					X					
Blood volume per visit (mL)*	60	60	25	45	60	70	70	25	90	70	45	70
Cumulative blood volume (mL)		120					390					690

Table 3 – Schedule of Procedures

* 15-60 mL blood per visit for exploratory immunology (approximately 80 mL for visits shown above as **), 2.5 mL per visit for transcriptomics, 5 mL per visit for anti-adenovirus antibodies (visits shown above as # only), 5 mL per visit for innate immune studies, 10 mL per visit for HIV/HCV PCR, 2 mL per visit for haematology, 4 mL per visit for biochemistry, 2 mL per visit for glucose, 4 mL per visit for HLA typing, haematology only at this time point.

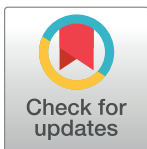
RESEARCH ARTICLE

Outcomes from a large 10 year hepatitis C treatment programme in people who inject drugs: No effect of recent or former injecting drug use on treatment adherence or therapeutic response

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Abstract

Background and aims

People who inject drugs (PWID) are historically viewed as having “difficult to treat” hepatitis C disease, with perceived inferior treatment adherence and outcomes, and concerns regarding reinfection risk. We evaluated for differences in treatment adherence and response to Peginterferon-alfa-2a/Ribavirin (Peg-IFN α /RBV) in a large urban cohort with and without a history of remote or recent injection drug use.

Methods

Patient data was retrospectively reviewed for 1000 consecutive patients—608 former (no injecting drug use for 6 months of therapy), 85 recent (injecting drug use within 6 months) PWID, and 307 non-drug users who were treated for chronic hepatitis C with Peg-IFN α /RBV. The groups were compared for baseline characteristics, treatment adherence, and outcome.

Results

There was no significant difference in treatment non-adherence between the groups (8.4% in PWID vs 6.8% in non-PWIDs; RR = 1.23, CI 0.76–1.99). The overall SVR rate in PWID (64.2%) was not different from non-PWIDs (60.9%) [RR = 1.05, 95% CI 0.95–1.17]. There was no significant difference in SVR rates between the groups controlling for genotype (48.4% vs 48.4% for genotype 1; 74.9 vs 73.3% for genotype 3). Former and recent PWID had similar adherence rates.

Conclusions

PWID have comparable treatment adherence and SVR rates when compared to non-drug users treated with Peg-IFN α /RBV. These data support a public health strategy of HCV treatment and eradication in PWID in the DAA era.

Abbreviations: PWID, People who inject drugs; Peg-IFN α , Pegylated Interferon- α ; RBV, Ribavirin; HIV, Human Immunodeficiency virus; SVR, Sustained viral response; DAA, Direct acting antivira; HCV, Hepatitis C virus.

Introduction

People who inject drugs (PWID) represent the majority of the hepatitis C virus (HCV) epidemic in the developed world [1]. The majority of new infections develop in active PWID, with this group accounting for more than 80% of new infections in high-income countries [2]. Furthermore, an additional large reservoir of infection exists amongst former PWID who remain undiagnosed. Public health initiatives which aim to reduce the burden of HCV and its attendant complications, or more ambitious strategies to target HCV elimination will therefore be unsuccessful unless they include strategies for engagement and retention of PWID in treatment and follow-up.

PWID are a highly marginalised group and HCV treatment uptake remains poor in this group with studies reporting annual treatment rates of less than 1.6 per 100 person-years in a community-based inner city cohort in Canada, with similar rates reported in Australia [3–4]. Data from the UK demonstrates a geographical variation in PWID treatment rates, ranging from 5 to >25 per 1000 PWID, and highlights the opportunity for upscaling treatment in areas of low treatment uptake. [5] Barriers to treatment of PWID relate to both system and patient factors, but also to reluctance among providers who perceive this group as “difficult to treat”.

Historically, HCV treatment guidelines have excluded PWID from consideration for treatment because of concerns regarding compliance and re-infection. These concerns have not been realised in several small studies assessing treatment for PWID, and outcomes have been comparable to large randomised trials of dual therapy in non-drug users [6–9]. The American Association for the Study of the Liver (AASLD) guidelines recommend that HCV therapy be considered in persons actively using illicit drugs or on opiate substitution therapy provided they wish to be treated and are willing to maintain frequent monitoring [10]. The recently updated European Association for the Study of the Liver (EASL) recommendations on treatment of HCV advocate treatment for individuals with ongoing risk of infection including active PWID, provided they wish to receive treatment and are willing to maintain regular appointments [11].

Important factors that have limited treatment uptake in PWID are the contraindications and adverse effects of Interferon (IFN) based therapy. A study of PWID in a UK prison outreach clinic found only 15% of HCV RNA positive inmates to be eligible for treatment, with only 50% of eligible patients receiving treatment [12]. The development and availability of IFN-free direct acting antiviral (DAA) regimes with high efficacy, improved tolerability and a limited side effect profile will significantly increase the proportion of patients who can be offered HCV therapy [13–15]. However, adherence to therapy in “real world” population groups will remain paramount in the DAA era to realise the SVR rates observed in clinic trials, as well as limiting the emergence of antiviral resistance. In a recent IFN-free DAA trial in patients with cirrhosis, those with a history of injecting drug use were found to have reduced likelihood of sustained viral response (SVR) [16]. We investigated for differences in HCV treatment adherence and outcome between former PWID, recent PWID and non-drug users treated with IFN and ribavirin.

Methods

Differences between PWID and non-drug users were analysed for adherence to treatment and outcome in all patients treated for chronic HCV infection in a university teaching hospital from 2002–2012. Anonymised patient data was retrospectively reviewed for the treatment period and monitored for at least 6 months follow-up after treatment. The PWID group also included former and recent drug users who were treated in a community based drug treatment centre. Former PWID was defined as having stopped drug use for 6 months prior to treatment,

whereas recent PWID was defined as drug use in the 6 months leading up to treatment. Former PWID did not have routine urinary drug testing during treatment, and abstinence was assessed from patient self-reporting alone. Recent PWID provided weekly urine samples for drug testing on treatment.

Patients

All patients who received treatment had compensated chronic HCV infection and detectable HCV RNA pre-treatment. Patients with cirrhosis were screened for hepatocellular carcinoma prior to treatment with combined liver ultrasonography and alfafetoprotein. All patients treated over the 10 year period were included in the final analysis. This retrospective audit of clinical outcomes was performed in accordance with the Royal College of Physicians of Ireland guidance on clinical audit. As data controller, the St. James's Hospital review board approved this retrospective audit of anonymised patient data.

Patients co-infected with HIV were considered for HCV treatment once they were established on an effective antiretroviral regimen or they had evidence of a satisfactory CD4 count (>350 cells/mm³) prior to initiation of treatment.

Exclusion criteria for HCV treatment included active alcohol abuse at the time of screening, decompensated cirrhosis, untreated psychiatric conditions, a Haemoglobin of less than 12g/dl at baseline. Relative contraindications to treatment were a neutrophil count of less than 1500/mm³, and a platelet count of less than 50×10^9 /mm³, although a small number of patients ($n = 10$) with HIV co-infection with moderate neutropenia and/or a platelet count of 25-50x10⁹ were treated at the discretion of the treating physician. Risk factors for HCV transmission were recorded from the patient history and referral source at the screening assessment visit. Information on illicit or non-prescription drug use was collected directly from patients. The non-PWID group's risk factors included receipt of infected blood products, sexual transmission, or birth in an area of high HCV prevalence. Alcohol abuse was defined as the consumption of more than 21 standard units per week for men and 14 units per week for women.

Treatment protocol

All patients received comprehensive psychological assessment by specialised treatment nurses prior to therapy. Patients received standard dose weekly Peginterferon- α subcutaneous injections and weight based Ribavirin for 24 or 48 weeks based on genotype and the presence of pre-existing cirrhosis. Individuals who were HCV RNA positive by polymerase chain reaction (PCR) at week 24 were defined as non-responders and treatment was discontinued. Anaemia was managed by Ribavirin dose reduction and/or the use of Erythropoietin at the discretion of the treating physician. HCV RNA was measured using a highly sensitive serum PCR test (HCV Versant 3.0 Assay, Roche COBAS Taqman or Abbott Real Time II HCV assay). Patients treated after 2006 were routinely assessed for rapid virologic response (undetectable HCV RNA after 4 weeks of therapy).

Outcome measures

A sustained viral response (SVR) was defined as undetectable HCV RNA in serum at 24 weeks follow-up after completion of therapy. Treatment adherence was determined as patients who attended up to the pre-defined treatment completion date, and/or met virological stopping rules for non-response. Non-response was defined as ≤ 2 log decline in HCV RNA by treatment week 12, and/or detectable HCV RNA in serum after 24 weeks of therapy. Relapse was defined as patients with an end of treatment response (HCV PCR undetectable) who did not achieve an SVR. Re-infection was defined as any patient who achieved SVR₂₄, but had

detectable HCV RNA during longer term follow-up. The classification of reinfection included patients who achieved an end of treatment response, but were diagnosed with a different HCV genotype during 24 weeks of follow-up.

Analysis

SPSS (Version 21, IBM, Chicago, Illinois, USA) was used for calculations. Chi-squared test for independence and the independent sample t-Test were used to compare baseline characteristics between the PWID and non-PWID groups. The proportions of non-adherence and treatment response between groups were compared and expressed as relative risks (RRs) with a calculated 95% confidence interval. The available number of patients was sufficient to significantly identify a RR of 1.5 between PWID and non-PWID groups with a power of 99% ($\alpha = 0.05$).

Results

From January 2002 to December 2012, one thousand patients were treated for chronic HCV infection. Of these, 608 were former PWID, and 85 were recent PWID. These groups were compared with 307 non-drug users who had other defined risk factors for HCV. These risk factors included receipt of infected blood or blood products (clotting factors, anti-D), sexual transmission and birth in an area of high HCV prevalence.

Baseline patient characteristics

Baseline characteristic for both groups are outlined in Table 1. The majority of former PWID and recent PWID were male, and were significantly younger than the non-PWID group. More than half of former PWID were infected with genotype 3 (52.5%), with genotype 1 infection being the second most common (41.8%). Only 5.6% had a genotype other than 1 and 3. In all, 12% of former PWID had underlying cirrhosis, and 114 (16.5%) were co-infected with HIV.

Of the 85 recent PWID treated for HCV in a community based drug treatment centre during the study period, all received opiate substitution therapy. Genotype 1 infection was more common with lower degrees of fibrosis in the recent PWID group. None of the patients had established cirrhosis.

In the non-PWID group, there were more women (36.2%), and the mean age was significantly higher at 43.0 years. Genotype 1 infection was the most common genotype (46.3%) followed by genotype 3 (42.7%). The proportion of patients with cirrhosis was similar to the former PWID group (14%), as was the percentage with HIV co-infection (14%).

Table 1. Baseline patient characteristics of former and recent PWID and non-PWIDs treated for chronic HCV infection.

	Former PWID (n = 608)	Recent PWID (n = 85)	non-PWID (n = 307)
Age (years) [mean ± SD]	36.2 ± 7.7	35.9 ± 6.6	43.0 ± 11.6
Sex (male) [n (%)]	547 (78.9)	67 (78.8)	196 (63.8)
Genotype [n (%)]			
1	290 (41.8)	42 (49.4)	142 (46.3)
2	29 (4.2)	5 (5.9)	15 (4.9)
3	364 (52.5)	38 (44.7)	131 (42.7)
4–6	10 (1.4)	0 (0)	19 (6.3)
Cirrhosis [n (%)]	83 (12)	0 (0)	43 (14)
HIV Co-infection	114 (16.5)	0 (0)	43 (14)
Log ₁₀ Viral Load—mean (range)	6.53 (2.10–7.84)	6.63 (4.30–7.33)	6.49 (4.06–7.64)

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Pre-treatment HCV viral load was available in 557 (91.6%) of former PWID, 85 (100%) of recent PWID and 253 (82.4%) of non-PWID, and a higher percentage of PWID had a viral load of greater than 10⁶ IU/ml.

Treatment completion/compliance

Six hundred and eight (608) former PWID, 85 recent PWID, and 307 non-PWIDs were commenced on HCV therapy. Treatment was discontinued in 71 former PWID (11.5%), 8 recent PWID (9.4%), and 46 non-PWIDs (15%) for virologic failure at week 24 of therapy. There was no significant difference in treatment non-completion (for reasons other than virologic non-response) between PWID and non-PWIDs [8.4% vs 6.8%, RR = 1.23, 95% CI 0.76–1.99]. Additionally, there was no significant difference in treatment non-completion between former and recent PWID [8.7% vs 5.9%, RR = 0.84, 95% CI 0.33–2.10].

Fifteen patients (17.6%) in the recent PWID group tested positive for opiates at least once during treatment, 11 (12.9%) tested positive for benzodiazepines, and 5 (5.8%) tested positive for cocaine. Seven patients tested positive for two of the drug classes, while 5 tested positive for all three classes. No patients reported injecting illicit drugs during treatment or in the 6 month post-treatment follow-up period.

Response to treatment

Treatment response rates are detailed in Table 2. The overall SVR rate in PWID (64.1%) was not different from non-PWIDs (60.9%) [RR = 1.05, 95% CI 0.95–1.17]. There was no significant difference in SVR rates between the groups when comparing genotype 1 and genotype 3 infections (Fig 1). As expected genotype 1 infection was less responsive to Interferon therapy in both PWID and non-PWIDs (47.7% versus 48.4%, *p* = non-significant). PWID were more likely to be lost to follow-up after achieving a viral response at the end of treatment [RR = 2.73, 95% CI 1.16–6.43]. There was no significant difference in SVR between former and recent PWID for genotype 1 [47.2% vs 54.8%, RR = 0.82, 95% CI 0.61–1.10] and genotype 3 infection [73.9% vs 83.8%, RR = 0.91, 95% CI 0.77–1.07].

Table 2. Adherence and treatment responses in people who inject drugs (PWID) and non-PWIDs.

	Former PWID n (%)	Recent PWID n (%)	Non-PWIDs n (%)	RR (95% CI) in Former versus recent PWID	RR (95% CI) in PWID versus non-PWID
Non-responders	71/608 (11.7)	8/85 (9.4)	46/307 (15)	1.24 (0.62–2.49)	0.74 (0.53–1.05)
Genotype 1 patients	52/248 (21)	8/40 (19)	32/142 (22.5)	1.02 (0.53–1.98)	0.92 (0.63–1.35)
Genotype 3 patients	14/326 (4.3)	0 (0)	7/131 (5.3)	-	0.72 (0.30–1.75)
End of treatment response	454/608 (74.7)	68/85 (82.9)	234/307 (76.2)	0.93 (0.83–1.05)	0.99 (0.92–1.07)
Genotype 1 patients	155/248 (62.5)	28/40 (70)	98/142 (69)	0.78 (0.68–0.90)	0.92 (0.80–1.05)
Genotype 3 patients	270/326 (82.8)	35/38 (94.6)	111/131 (84.7)	0.90 (0.81–0.99)	0.99 (0.91–1.08)
Sustained Viral Response	384/608 (63.2)	60/85 (70.6)	187/307 (60.9)	0.89 (0.77–1.04)	1.05 (0.95–1.17)
Genotype 1 patients	117/248 (47.2)	23/40 (54.8)	69/142 (48.6)	0.82 (0.61–1.10)	1.00 (0.81–1.23)
Genotype 3 patients	241/326 (73.9)	31/38 (83.8)	96/131 (73.3)	0.91 (0.77–1.07)	1.02 (0.91–1.15)
Non-adherence	53/608 (8.7)	5/85 (5.9)	21/307 (6.8)	0.84 (0.33–2.10)	1.23 (0.76–1.99)
Lost to Follow up	30/608 (4.9)	1/85 (1.2)	6/307 (2)	4.19 (0.58–30.4)	2.3 (0.97–5.45)

<https://doi.org/10.1371/journal.pone.0178398.t002>

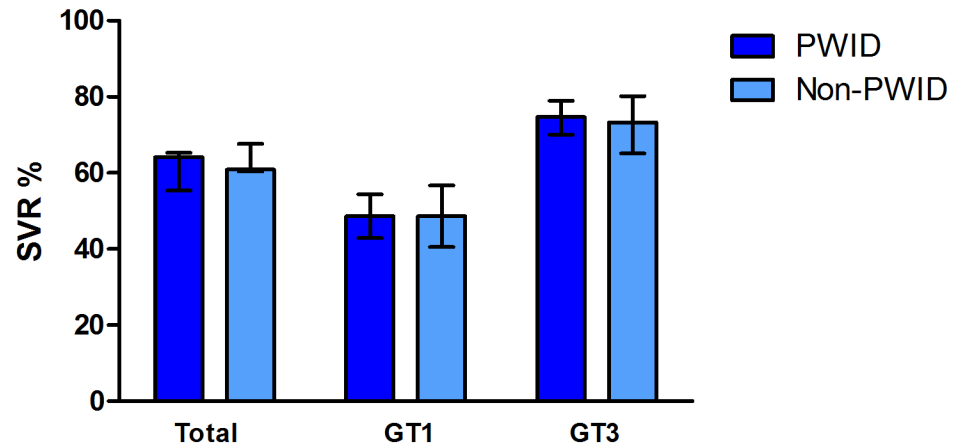


Fig 1. Treatment response. Rates of sustained viral response (95% CI bars) with dual therapy in PWID (people who inject drugs) and non-PWID (a) for all genotypes—64.1% vs 60.9% (b) genotype 1—48.6% vs 48.6% and (c) genotype 3—74.7% vs 73.3%.

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Follow up data on 219 former PWID with who achieved SVR between 2002–2007 for a median of 57 months (range 6–168 months) indicated that 13 patients were re-infected with HCV, a reinfection rate of 10.5/1000 person years of follow-up. All 13 patients had a relapse in injecting drug use. There was no significant difference in re-infection rate between former PWID with and without HIV co-infection.

Discussion

This large retrospective study of a decade of HCV treatment outcomes demonstrates that PWID have similar treatment adherence to Peginterferon and Ribavirin as non-PWID patients with chronic HCV infection. Of the 693 recent or former PWID who commenced treatment, over 90% completed treatment at our centre.

Additionally, PWID patients had a comparable response to treatment with an overall SVR rate of 62.4%. More than half of PWID patients were infected with genotypes 2 and 3, which have favourable response rates to interferon-based therapy. Allowing for this, there was no significant difference in SVR observed between the groups when controlling for genotype. PWID patients were younger but this had no effect on the response to therapy.

These data are in line with previously published cohort studies of treatment adherence and outcome in PWID. The Benelux study group reported no effect of a history of drug use on HCV treatment completion [7]. The SVR rate was approximately 40% and did not differ significantly between PWID and non-drug users having controlled for genotype. In the Benelux study, patients received non-pegylated Interferon, and consequently improved response rates were expected with the use of Peginterferon. Recent systematic reviews of HCV (dual therapy) have also illustrated that active PWID can respond favourably to treatment. A high-pooled SVR rate of 55.5% (95% CI 50.6–60.3%) with a high treatment completion rate of 83.4% was found in one systematic review of 11 studies among active PWID [17–18].

HIV co-infection is associated with accelerated progression of liver disease in patients with HCV resulting in increased morbidity and mortality [19–20]. Co-infected patients with a history of injection drug use have an even higher risk for poorer clinical outcomes and consequently have a greater need for therapy. Our co-infected PWID cohort achieved an SVR rate of 60.5%, indicating that this group at risk for unfavourable outcomes has comparable outcomes with the entire cohort.

The accepted consensus just over a decade ago was that PWID should not be offered therapy until they have been drug free for a period of six months [22–23]. Several small studies have since demonstrated that treatment outcomes even in active PWID are non-inferior, which resulted in updated guidelines [6–9]. However, recommendations that active injection drug use should not preclude access to antiviral therapy have not been translated into enhanced treatment uptake among PWID. A study of physicians' attitudes in Canada found that whilst 90% would consider treating former PWID, only 20% of service providers were likely to treat active or recent drug users [21]. Community based studies of PWID in Australia have shown only a modest improvement in HCV treatment uptake, ranging from 0.5–1.0% in 2004–05 (5–10 per 1000 infected) to 1.5–2.0% in 2009–10 (15–20 per 1000 infected) [24]. The biggest improvements in HCV treatment uptake have been observed in countries that have employed a comprehensive national strategy such as Scotland's HCV Action Plan [25–26]. Early results have been encouraging in terms of patient attendance at specialist centres following diagnosis and treatment uptake thereafter [27].

Another provider factor that may limit treatment access for PWID is the concern regarding re-infection risk in patients who continue to inject drugs. In our low risk population of former PWID with HCV mono-infection, the re-infection rate was low at 10.5/1000 person years of follow-up, with a median follow-up period of nearly 5 years. As viral homology sequencing was not performed in all patients with detectable HCV RNA after SVR, there was a risk of classifying some late relapse cases as re-infection. This potential classification error may be associated with an overestimation of the re-infection rate. All re-infection cases were associated with a relapse in injection drug use. Uncertainty about re-infection rates in published studies remain, due to high dropout rates in long term follow up. Re-infection rates in a number of studies are consistently low between 3–5% [28–30] with a slightly higher re-infection rate of 6.4% in PWID who reported ongoing injecting drug use post-SVR [17]. A meta-analysis of 43 studies which included 7969 patients treated for HCV demonstrated that late-relapse or re-infection is higher in prisoners or those with on-going drug use (22.32/1000 person years of follow-up). The highest risk was in patients with HIV/HCV co-infection (32.02/1000 person years of follow-up). These rates are much higher than we observed in our cohort. Genotyping and viral sequencing was not universally used in classifying late relapse or re-infection in these studies, possibly resulting in an over-estimation of the re-infection rates [31].

A number of factors preclude some HCV infected PWID from successful treatment with existing Interferon-based therapy. Patient related factors which include chaotic lifestyles, psychiatric comorbidity and depression may influence patient adherence during treatment. Patient selection is therefore critical and may be optimised through careful pre-treatment assessment management, with particular attention given to patient and provider factors known to impact on compliance. In our study, a combined clinician and nurse specialist assessment was used to select patients for treatment on a case-by-case basis. Patients with active psychiatric comorbidity were assessed by liaison psychiatry as part of the multi-disciplinary pre-treatment assessment. Whilst this assessment process introduces an element of selection bias, the high observed adherence and SVR rates in PWID result from treatment recruitment decisions made on an individual basis. A multidisciplinary approach to HCV treatment where treatment and counselling services are offered in a “one-stop-shop” has been shown to improve treatment uptake and adherence with therapy [32].

These data support the inclusion of recent and former PWID in any public health HCV treatment strategy aimed at reducing HCV prevalence as a first step towards elimination. The European guidelines advocate early DAA treatment for those with an on-going infection risk including PWID [11]. Interferon free DAA regimens are now the standard of care, with treatment duration as short as 8 weeks, and observed SVR rates of > 95% [13–15]. Co-morbid

psychiatric conditions seen in up to 50% of PWIDs will no longer be a contraindication to therapy [33]. As treatment regimens become simpler and more tolerable, this should result in an expansion of the eligible treatment population. With simplification of therapy, it seems paradoxical that many payers have imposed even greater access restrictions to treatment, with some states in the US requiring 12 months abstinence from drug and alcohol use for re-imburement [34]. These restrictions are difficult to justify in the light of increasing evidence that HCV treatment is effective in PWID. The use of pre-treatment screening tools for illicit drug use do not help in identifying patients more likely to respond to therapy, and only serve as an added barrier to treatment, and are therefore discouraged by treatment guidelines [35]. Some concerns remain regarding the risk of re-infection, but this requires strategies to reduce the re-infection risk as part of treatment strategies rather than excluding PWID altogether. The nature of epidemic control is that re-infection risk will decline by successful treatment scale-up strategies in the populations with the highest prevalence, as the reservoir for re-infection in PWID is reduced. There is a growing body of evidence supported by modelling data of such a “treatment as prevention” paradigm [36]. Additionally, prioritising PWID for treatment appears to be more a cost-effective initiative at reducing long-term health costs than treating non-PWID with comparable degrees of fibrosis [37]. HCV elimination is an ambitious target, but it will not be achieved by excluding PWID from treatment.

Supporting information

S1 File. Anonymised PWID study source data.
(XLSX)

Author Contributions

Conceptualization: SN C. Bergin.

Data curation: OE C. Bannan.

Formal analysis: OE C. Bannan.

Investigation: SN C. Bergin SMK SK.

Methodology: SN C. Bergin.

Supervision: SN C. Bergin SMK.

Writing – original draft: OE C. Bannan.

Writing – review & editing: OE C. Bannan SB SN.

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Case report

NS5A resistance leading to failure of 24-week therapy with sofosbuvir/ledipasvir and ribavirin for the treatment of hepatitis C genotype 1a infection in a HIV-1 co-infected patient



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ABSTRACT

Herein we report a previously undescribed case of treatment-emergent non-structural protein 5A (NS5A) resistance mutations, Q30H and Y93C, leading to a failure of 24-week course of sofosbuvir/ledipasvir+ribavirin therapy for the treatment of hepatitis C virus (HCV) genotype 1a in interferon-experienced, human immunodeficiency virus type 1 (HIV-1) co-infected patient with cirrhosis.

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1. Case importance

Chronic infection with hepatitis C virus (HCV) poses a significant healthcare burden with over 185 million people infected worldwide [1]. HCV is associated with considerable morbidity and mortality, and remains the leading indication for liver transplantation [2]. Recently licensed direct-acting antiviral (DAA) agents significantly improve treatment outcomes for patients with HCV of all genotypes (GT) [3], even in the setting of human immunodeficiency virus type 1 (HIV-1) co-infection [4–8].

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; cART, combination antiretroviral therapy; DAA, direct-acting antiviral; DDI, drug–drug interaction; EOTR, end-of-treatment response; GT, genotype; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; IFN, interferon; INR, international normalized ratio; LDV, ledipasvir; MELD, Model for End-Stage Liver Disease; NS3, non-structural protein 3; NS5A, non-structural protein 5A; NS5B, non-structural protein 5B; PI, protease inhibitor; PPI, proton pump inhibitor; RAM, resistance-associated mutation; RBV, ribavirin; RNA, ribonucleic acid; RT, reverse transcriptase; RT-PCR, reverse transcription polymerase chain reaction; SOF, sofosbuvir; SVR, sustained virologic response.

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HCV is a spherical enveloped virus ca. 50–60 nm in diameter with a positive-polarity, single-stranded, non-segmented ribonucleic acid (RNA) genome of 9.6 kb [9]. As HCV replication generates more than 10^{10} new virus particles each day in the untreated individual and due to the lack of a proofreading mechanism in the virus-encoded RNA-dependent RNA polymerase, mutations occur as part of the natural history of infection [10]. Some of these mutations confer resistance to antiviral agents [11].

To date there are no published reports of cases of resistance-associated mutations (RAMs) leading to failure of a 24-week course of sofosbuvir (SOF)/ledipasvir (LDV) + ribavirin (RBV) used for the treatment of HCV GT1a in cirrhotic, treatment-experienced patients.

2. Case description

Our patient is a 62-year-old white male who was diagnosed with HIV-1 and HCV in the early 1990s. Both infections were sexually-acquired. Other co-morbidities include a history of combination antiretroviral therapy (cART)—associated lipodystrophy, dyslipidemia, and recurrent herpes simplex virus infection. Our patient is infected with multidrug-resistant dual-tropic HIV-1 following extensive exposure to antiretroviral agents in the past. The most

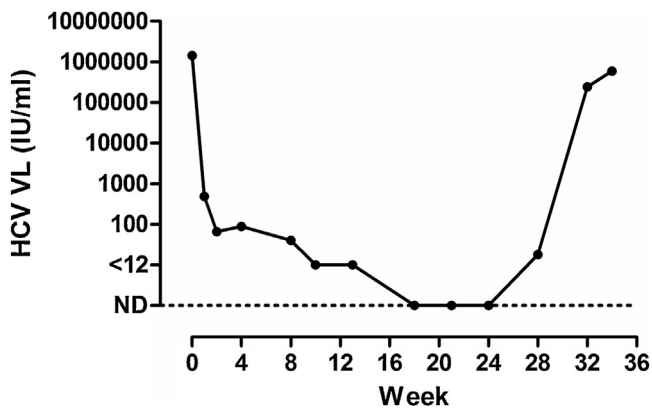


Fig. 1. Hepatitis C viral load (HCV VL) before, during and after treatment with sofosbuvir, ledipasvir and ribavirin. ND, not detected.

recent sequence analysis of HIV-1 demonstrates the reverse transcriptase (RT) mutations M41L, E44D, D67N, T69D, V118I, M184V, Y188L, L210W, T215Y, and the protease inhibitor (PI) mutations L10I, M36I, I54V, L63P, A71V, I84V, L90M. Prior to consideration for SOF/LDV treatment for HCV, our patient's CD4 count was 649 cells/mm³ and HIV RNA was not detected. Antiretroviral regimen consisted of tenofovir 245 mg once daily, efavirenz 200 mg twice daily, raltegravir 400 mg twice daily, darunavir 600 mg twice daily and ritonavir 100 mg twice daily. Other regular medications were valaciclovir 500 mg once daily, pravastatin 40 mg once daily, propranolol 20 mg twice daily, lansoprazole 30 mg as required and tadalafil 10 mg as required.

Our patient is infected with HCV GT1a. His IL28B genotype is CT [12]. Clinically, he has Child-Pugh A disease with Model for End-Stage Liver Disease (MELD) score of 8. Liver biopsy in 2002 demonstrated stage 2 fibrosis and repeat biopsy in 2005 revealed cirrhosis. FibroScan[®] score in 2012 was 18.2 kPa. In 2006, he was a non-responder to a 24-week course of pegylated interferon (IFN) and RBV with detectable HCV RNA at week 24 of therapy, and the treatment was discontinued as futile. In 2013, he was deemed ineligible for treatment with telaprevir through the Early Access Programme due to potential drug–drug interactions (DDIs) with his antiretroviral regimen.

In February 2015, our patient commenced HCV treatment with SOF/LDV as a single fixed-dose combination together with weight-based RBV (daily dose of 1000 mg). Tenofovir was held from his antiretroviral regimen due to the increased risk of tenofovir toxicity when used concomitantly with ledipasvir and a HIV PI. Lansoprazole dose was decreased to 15 mg on an as required basis after consultation with the Drug Interaction Database of the University of Liverpool (www.hep-druginteractions.org). At baseline, HCV RNA was 1422485 IU/mL, bilirubin 25 µmol/L, alanine aminotransferase (ALT) 88 IU/L, aspartate aminotransferase (AST) 97 IU/L and international normalized ratio (INR) 1.1. Our patient was treated with SOF/LDV + RBV for a total of 24 weeks. The treatment was well-tolerated, except for anemia, which was managed with erythropoietin for 4 weeks and with RBV dose reduction to a daily dose of 800 mg at week 4. HIV RNA remained undetectable and CD4 count was stable throughout the treatment. Undetectable HCV RNA was achieved at week 18 and an end-of-treatment response (EOTR) at week 24 (Fig. 1). At week 4 following the completion of treatment, however, virologic relapse occurred (Fig. 1). Plasma samples taken prior to treatment and at the time of relapse were sent to the Irish National Virus Reference Laboratory for HCV sequence analysis to ascertain whether one or more RAMs were present at baseline, or had developed during the course of treatment, to account for the SOF/LDV failure.

3. Methods

Nucleic acid was extracted from 140 µL of serum using the QIAamp Viral RNA minikit (Qiagen, Crawley, UK) as per the manufacturer's instructions. HCV RNA was measured using the Abbott Molecular m2000 RealTime System (Abbott Molecular Diagnostics, Wiesbaden, Germany), and HCV genotyping was performed using the RealTime HCV Genotype II assay (Abbott Molecular Diagnostics, Wiesbaden, Germany), both in accordance with the manufacturer's instructions. HCV RNA was reverse-transcribed and the non-structural protein 3 (NS3), non-structural protein 5A (NS5A) and non-structural protein 5B (NS5B) coding regions were amplified by nested reverse transcription polymerase chain reaction (RT-PCR) using primers and protocols adapted from those previously published [13,14]. The RT-PCR reaction was performed using the Superscript[®] III High Fidelity with Platinum Taq RT-PCR kit (Invitrogen[™] Life Technologies, Paisley, UK), while second-round amplification was performed using the Expand High Fidelity PCR system (Roche Applied Sciences, Mannheim, Germany). Thermal cycling conditions used for the respective RT-PCR and PCR reactions are available from authors upon request. Following nested PCR, unincorporated primers and dNTPs were removed from HCV amplicons using ExoProSTAR One-Step (GE Healthcare, Buckinghamshire, UK) and purified products were sequenced bidirectionally using ABI Big Dye v3.1 kit chemistry on the ABI 3500Dx sequencing platform (Life Technologies, Paisley, UK). Contiguous assembly of nucleotide sequences for the NS3, NS5A and NS5B genomic regions was performed by SeqMan version 11 (DNASTAR, Madison, WI, USA) [15]. Resistance mutation analysis was performed using the Geno2Pheno_[HCV] online algorithm (hcv.geno2pheno.org) operated by the Max Planck Institute.

4. Results

No RAMs were detected in any of the regions sequenced at baseline. Furthermore, no RAMs were detected in either the NS3 or NS5B regions at the time of relapse. Two distinct RAMs, Q30H and Y93C, were detected in the NS5A region sequence at the time of relapse (Fig. 2). In the case of Q30H mutation, the change had arisen as a result of transversion of a single nucleotide position (CAA → CAC) in the corresponding codon (Fig. 2). Likewise, the Y93C mutation had arisen from a single nucleotide transition from A to G at position 2 of codon 93 (TAY → TGC) (Fig. 2). Both RAMs existed as a single population with no evidence of heterogeneity at the relevant nucleotide positions (Fig. 2).

5. Similar and contrasting cases in the literature

The few reported cases of relapse following a SOF/LDV treatment in patients with HCV GT1 disease had, in contrast to our case, all occurred in the setting of a 12-week treatment course without RBV [4,7]. The relapses were associated with patient characteristics such as black ethnicity, TT allele in the gene encoding for IL28B and poor adherence [4,7]. These genetic and ethnic factors, however, are not applicable to our patient. Whilst no therapeutic drug monitoring data are available on our patient, in the setting of the longevity of his attendance at our clinic, the complexity of his HCV/HIV-1 care and his longstanding virologic suppression of HIV-1, there were no concerns regarding his adherence to DAA therapy. Furthermore, as the patient was not engaging in risk activities during the DAA treatment, re-infection was not considered plausible. His therapeutic failure, therefore, was assumed to be resistance-related.

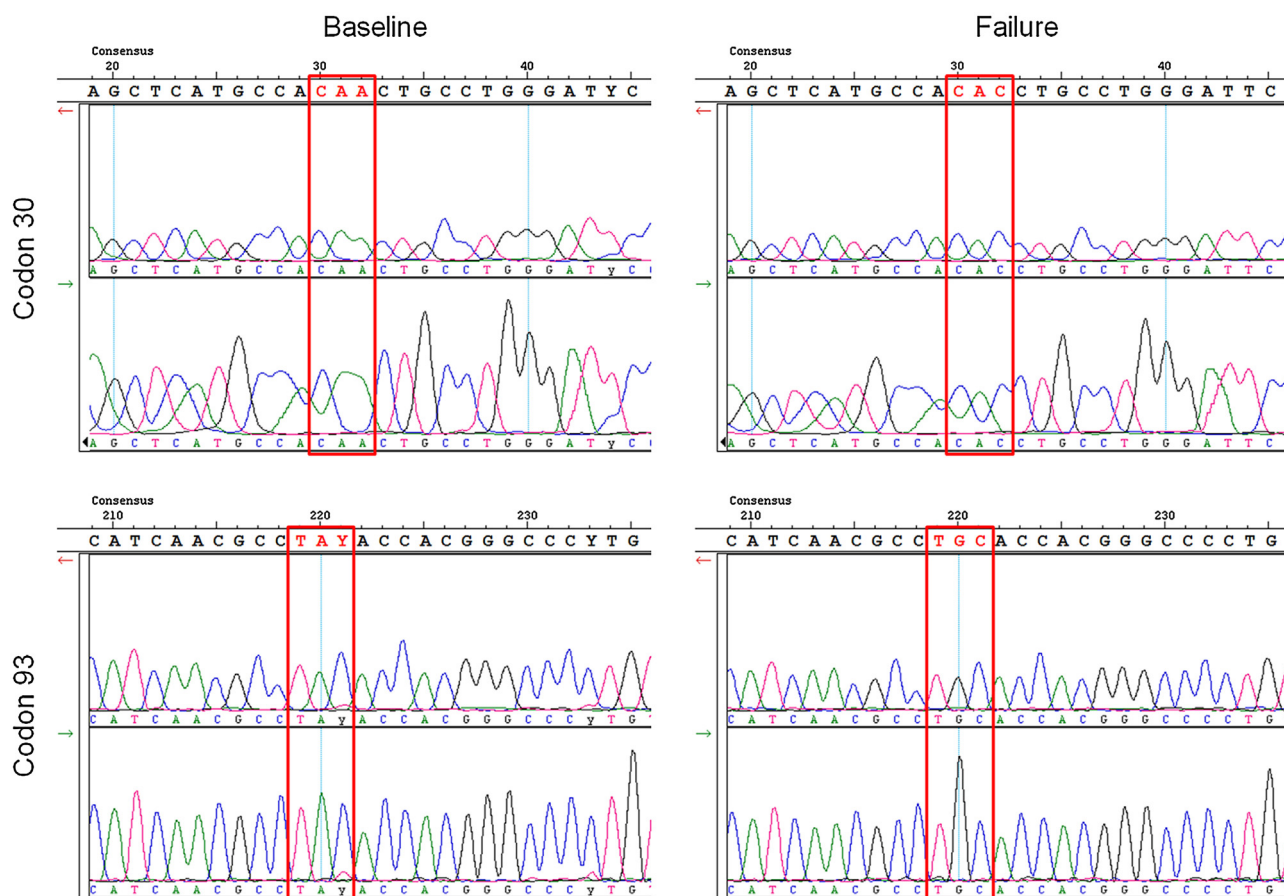


Fig. 2. Aligned sequences of hepatitis C virus (HCV) non-structural protein 5A (NS5A) domain at codons 30 and 93 demonstrating nucleotide sequence at baseline and single nucleotide changes at the time of relapse.

6. Discussion

Herein we report a case of virologic relapse at week 4 following satisfactory EOTR after a 24-week treatment with SOF/LDV + RBV for HCV GT1a infection in an IFN-experienced, HIV-1 co-infected patient with cirrhosis. While no baseline RAMs were identified in the sequenced regions, NS5A was found to harbour two treatment-emergent RAMs, Q30H and Y93C. Both of these RAMs have been reported to become selected under high-dose NS5A inhibitor pressure, and confer high-level resistance to all currently licensed NS5A inhibitors, including LDV [16,17], and have likely led to treatment failure. As HCV RNA was detectable at weeks 4 and 8 of DAA therapy, it is possible that these RAMs emerged early on in the course of therapy leading to a high probability of overall treatment failure. Of note, however, presently there is no established guidance for response-guided interventions to viral kinetic monitoring and for sequential on-treatment resistance profiling. To the best of our knowledge, this is the first reported case of NS5A resistance-associated failure of 24-week SOF/LDV + RBV therapy in HCV GT1a disease.

As in the treatment of HIV-1 mono-infection, DDIs need to be considered as a potential cause of suboptimal treatment response and, equally, as a cause of treatment-emergent RAMs. In our patient's case, the only significant DDI identified by a team of physicians and hospital pharmacists with the support of the Drug Interaction Database of the University of Liverpool (www.hep-druginteractions.org) was with lansoprazole. This was initially assessed prior to commencement of SOF/LDV + RBV therapy in February 2015 and, thereafter, monitored for updates throughout the treatment. Lansoprazole was used by the patient on an

as required basis weekly, but not daily. In accordance with the recommendations, lansoprazole dose was decreased by 50%. It is possible that this may not have been sufficient, however, as novel data suggest that even baseline use of proton pump inhibitors (PPIs) is associated with significantly lower rates of sustained virologic response (SVR) to SOF/LDV [18], and ongoing lansoprazole use could have been an important factor contributing to a virologic failure in our patient. As the evidence base in this area is minimal at present, additional real-world data are necessary on DDIs between DAAs and the commonly-used drugs such as PPIs.

Identification of RAMs at the time of relapse, but not at baseline, and in the absence of other likely confounding factors suggests that it is the emergence of these RAMs that resulted in treatment failure in our patient's case. In contrast to the HIV-1 paradigm of care [19], at present there is no requirement for universal baseline HCV resistance testing as the impact of baseline RAMs on DAA SVR remains uncertain [20,21]. This case highlights the importance of performing resistance testing in patients failing treatment as a minimum, and questions whether baseline samples should be stored if baseline resistance profiling is not routinely undertaken. Further studies on the role of baseline resistance in HCV are required before such a recommendation can be made.

In summary, this is a first report of NS5A resistance-driven virologic relapse post 24-week treatment with SOF/LDV + RBV for HCV GT1a infection in an IFN-experienced, HIV-1 co-infected patient with cirrhosis. By demonstrating a failure of what has been clinically felt to be a very robust regimen, this case highlights a need to identify additional predictors of DAA treatment failure. It also showcases the challenges faced by patients like ours both in terms of available salvage treatment options for HCV and the

DDI concerns related, but not limited, to management of multi-drug resistant HIV-1 infection. Finally, this case illustrates that despite the great advances made in the area of HCV treatment in recent years, the repertoire of treatment regimens remains small. The patient described in the current report has not yet been retreated as due to DDIs and resistance-related considerations all DAAs in the current HCV GT1a treatment armamentarium, particularly simeprevir-based combinations, are incompatible with his antiretroviral regimen which cannot be altered in the setting of extensive antiretroviral resistance. Due to the advanced nature of chronic liver disease in our patient's case, the only option at present was to refer him for consideration for liver transplantation and to defer retreatment of his HCV infection until a new generation of DAAs becomes available.

Conflict of interest

None.

Financial support

None.

Ethical approval

No ethical approval was necessary for this submission.

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Hepatitis C Virus: Molecular Pathways and Treatments

Chapter: HCV Immune Evasion

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HCV Immune Evasion

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Introduction

Infection with Hepatitis C Virus (HCV) results in chronic infection in the majority of infected individuals. Primary (acute) infection is usually asymptomatic, although some patients present with jaundice. Following acute infection approximately 20% of patients will spontaneously clear the virus, while 80% will develop persistent HCV infection. The mechanisms by which some individuals clear the virus and others develop chronic infection are not yet fully understood, but likely reflect a complex interplay between the host and the virus at the level of the immune response [1].

Persistent HCV infection is a significant cause of morbidity and mortality due to progressive liver fibrosis. Eventually 20-50% of patients will develop cirrhosis. Individuals with chronic hepatitis C and cirrhosis are at increased risk of liver failure, liver transplantation and hepatocellular carcinoma development [2].

Following initial infection, HCV RNA can be detected in plasma within days of exposure, often weeks before liver enzyme levels rise. Peak viraemia occurs in the first 8 to 12 weeks of infection, then drops to lower levels and persists. A number of situations may occur at this time:

- Plasma HCV RNA becomes undetectable within the first few months of infection and remains undetectable indefinitely (viral clearance)
- Plasma HCV RNA is inconsistently detected early in infection and a stable pattern of recovery or persistence is not evident for more than 6 months. Intermittent viraemia may represent re-infection (which is observed in intravenous drug users) or escape from an initially successful immune response.

The mechanisms of persistence of HCV remain incompletely understood, but recent studies have revealed important insights into the mechanisms of HCV immune evasion.

The human immune system represents an extraordinary and complex barrier against environmental threats, such as bacteria and viruses, and comprises many effector systems, cells and molecules, which are present in all the organs of the body and defend it from infection

and disease. The innate and the adaptive immune systems, are effective providers of anti-viral immunity. Innate immunity represents the first, non-specific, arm of defence against pathogenic entry, and it is capable of repeatedly eliminating many potential infections through activation of Pattern Recognition Receptors (PRRs). Shortly after the innate immune response begins, the more specific adaptive immune response develops and acts in a more targeted manner, retaining immunological memory. Like other viruses, HCV has evolved immune evasion mechanisms which allow it to overcome the body's natural anti-viral defences, with major consequences, not only for spontaneous viral clearance, but also for response to conventional therapies.

Pattern Recognition Receptors (PRRs)

Dendritic Cells (DCs) are an important cell population involved in innate immunity and represent key mediators of anti-viral immunity. They are classified into conventional DCs (cDCs), which target a wide range of pathogens, and plasmacytoid DCs (pDCs), which, in response to viral infection, produce large amounts of Type I IFNs, and have been implicated in restraining HCV infection [3].

Upon viral infection, PRRs of the innate immune system sense the virus as “non-self”, via the identification of defined conserved patterns within viral RNA, DNA and/or protein called Pathogens Associated Molecular Patterns (PAMPs). PRRs trigger signalling cascades (see Figure 1), leading to transcription of numerous anti-viral genes, including IL-1 β and Type I and III Interferons (IFNs), which are then secreted by the infected cells and activate cytokine-specific signalling pathways [4].

The innate immune response utilises three main classes of PRRs to sense HCV: Toll-Like Receptors (TLRs), the Retinoic Acid-Inducible Gene I (RIG-I)-like Receptors (RLRs), and the Nucleotide oligomerisation domain-Like Receptors (NLRs) [4,5]. PRRs are highly expressed in bone marrow-derived immune cells, such as the liver-based macrophages (Kupffer cells) and hepatic dendritic cells, but also on resident liver cells [hepatocytes, Liver Sinusoidal Endothelial Cells (LSECs), and hepatic stellated cells] [6].

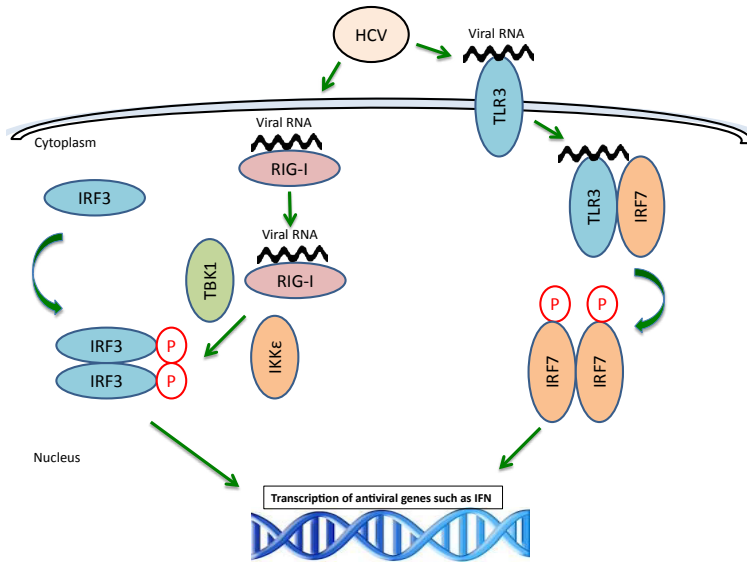


Figure 1: Upon HCV entry and uncoating, viral RNA is detected through pattern recognition receptors (PRRs) such as RIG-I or Toll-like receptors. Upon activation and binding to its adaptor molecule MAVS, RIG-I initiates a signaling cascade leading to phosphorylation and nuclear accumulation of IRF3. In the nucleus IRF3 promotes transcription of antiviral and immunomodulatory genes including IFNs. Similarly, binding of HCV RNA to TLRs results in a signaling cascade leading to production of IFNs and inflammatory cytokines. HCV has been reported to subvert these innate immune defense mechanisms and others at several levels explained in detail in the text.

Toll-Like Receptors (TLRs)

TLRs are highly expressed by innate immune cells, such as DCs, but also by hepatocytes and Kupffer cells. This family of receptors include TLR1, 2, 4, 5 and 6, which are found on the cell membrane, and TLR3, 7, 8 and 9, which reside on endosomal membranes. However, only a few members of the family are currently known to be involved in HCV detection. Cell membrane TLRs signal through five main adaptor molecules containing a Toll/IL-1 Receptor (TIR) domain: the adaptor molecule Myeloid Differentiation primary response gene 88 (MyD88), MyD88-Adapter-Like (MAL), TIR-domain-containing adapter protein inducing IFN- β (TRIF), TRIF-Related Adapter Molecule (TRAM) and the negative signalling regulator Sterile α and Armadillo-Motif-containing protein (SARM). These TLRs activate MyD88 via MAL, and initiate a signalling cascade involving IL-1 Receptor-Associated Kinase (IRAK) and TNF Receptor Associated Factor (TRAF) family members, resulting in activation of the transcription factor NF- κ B, thus promoting induction of pro-inflammatory cytokines. Conversely, endosomal TLRs and TLR4 signal through TRAM, TRIF and the TANK-binding kinase 1/I κ B kinase ϵ (TBK1/IKK ϵ) complex, which in turn triggers Interferon Regulatory Factors 3 (IRF3) translocation to the nucleus and consequently induces Type I IFN production [7-11].

Traditionally, TLR2 and 4 are activated in response to non-viral PAMPs. However, a new role of these receptors in virus recognition has recently emerged: indeed, TLR2 can recognise HCV core and NS3 proteins, and HCV-dependent activation of TLR4 can stimulate Type I IFNs and IL-6 expression [12-14]. Conversely, TLR3 induces IFN- α , β and other inflammatory

cytokine production in response to the presence of replicating HCV double-stranded RNA intermediates during active infection in liver cells [15,16], whereas TLR7 and 9 expressed in pDCs and Kupffer cells produce high amounts of IFN- α in response to HCV infection [3,17].

The HCV non-structural proteins NS3 and NS4A play a major role in immune evasion, as they oligomerise to form a NS3/4A complex with protease activity. Indeed, NS3/4A cleaves and inactivates various innate immune signalling adaptor proteins, including members of the TLR signalling pathway, in particular TRIF, thereby blocking TLR3-dependent and independent signalling [18]. Another HCV protein, NS5A, can also bind to MyD88, inhibiting recruitment of IRAK, resulting in interference with TLR signal transduction [19].

Retinoic Acid-Inducible Gene I (RIG-I)-Like Receptors (RLRs)

Among the RLRs family of cytosolic receptors, RIG-I recognises short 5' triphosphate dsRNA, whereas Melanoma Differentiation-Associated (MDA) protein 5 interacts with longer dsRNA (over 2000 nucleotides). RLRs are characterised by a C-terminal domain, a DExD/H-box RNA helicase domain, and an N-terminal Caspase Activation and Recruitment Domain (CARD). The third member of the family, Laboratory of Genetics and Physiology-2 (LGP2), also known as Probable ATP-dependent RNA helicase DHX58, cannot induce a cellular response alone, as it lacks the CARD domain, but it is essential for effective anti-viral responses mediated by RIG-I and MDA5 [20]. When activated, these receptors signal through the mitochondrial-associated adaptor molecule, IFN Promoter Stimulator-1 (IPS-1) (also known as MAVS/VISA/Cardif), and TBK1/IKK ϵ complex, leading to activation of AP-1, IRF3/7 and NF- κ B to induce pro-inflammatory and anti-viral cytokines, including type I interferon expression [21].

RIG-I detection of HCV is well characterised and involves interaction between the RIG-I helicase domain, a 5'-triphosphate (5'-ppp) and the poly U/UC region of the HCV genome in the 3' non-translated region [22-24]. This is a highly conserved sequence among all HCV genotypes and also essential for viral replication [25-27]. HCV binding to RIG-I promotes a conformational change which triggers TRIM25-dependent ubiquitination of the CARD domains. This step is essential for RIG-I binding to the adaptor protein 14-3-3 ϵ , which facilitates its translocation to the Mitochondrial-Associated ER Membrane (MAM), interaction with IPS-1 and ultimately activation of IRF3 and NF- κ B [28-30].

HCV NS3/4A protein not only blocks RLR signalling by targeting and cleaving the mitochondrial associated IPS-1, thus rendering it incapable of activating the RIG-I signalling pathway [29,31], but it can also block the phosphorylation and activity of IRF-3 [32].

To date, there is no evidence of an involvement of MDA5 in sensing HCV PAMPs. However, as this PRR recognises long dsRNA, the possibility of an interaction with HCV dsRNA replicative intermediates during late infection stages cannot be excluded.

Nucleotide oligomerisation domain-Like Receptors (NLRs)

NLRs are intracellular sensors of PAMPs and Damage-Associated Molecular Pattern Molecules (DAMPs), and contribute to the formation of the inflammasome. This complex comprises a sensor protein, the adaptor protein Apoptosis-associated Speck-like protein Containing a CARD (ASC) and caspase 1 [33]. Once activated by RNA viruses, NALP3 triggers expression of the pro-forms of IL-1 β and IL-18; this is followed by recruitment and activation of caspase 1, which in turn triggers secretion of the active forms of IL-1 β and IL-18 [34]. During chronic HCV infection, this signalling pathway is activated in Kupffer cells, which phagocytose

HCV, triggering TLR7 signalling. Caspase-1-dependent IL-1 β mature form production is, in this case, driven by potassium efflux, although the exact mechanism is yet to be fully understood [35]. Conversely, IL-18 secretion has been linked to acute HCV infection [36]. IL-1 β could also play a direct role in viral clearance during acute HCV infection as it has been reported to affect HCV RNA replication through activation of the Extracellular Regulatory Kinase (ERK) [37].

The Interferon Anti-Viral System

IFN was first identified in 1957, when chick embryos infected with an inactivated influenza virus produced and released an unknown protein into the surrounding fluid that was able to protect non-infected cells against viral infection. This factor was simply named “interferon” as it “interferes” with viral infection [38]. This discovery represented a significant milestone in understanding how the immune system responds to pathogens and, in particular, viruses. Nearly four decades later, the IFN, Janus Kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) signalling pathway has been well characterised and has provided new insights into the anti-viral immune response and the mechanism of many diseases.

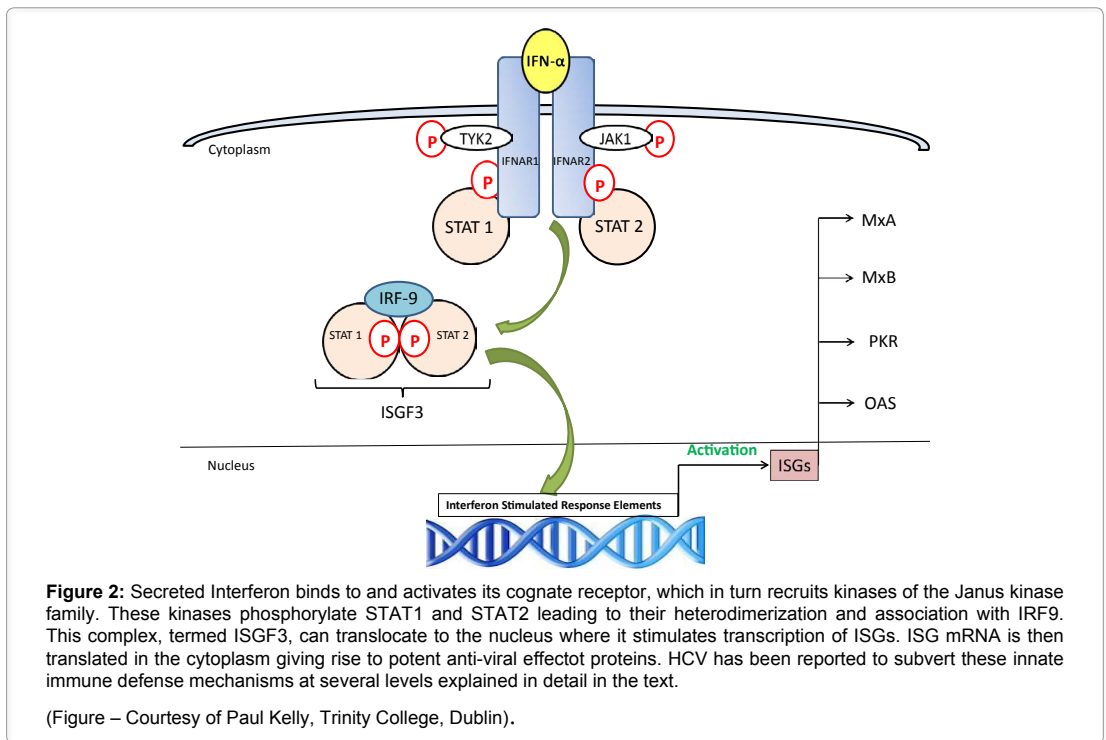
Interferon Family of Cytokines

IFNs are a multi-gene family of inducible cytokines that are best known for their potent anti-viral properties [39-41], although they also play a critical role in cell growth [42] and have immunomodulatory effects [43]. There are three main classes of IFNs: Type I, Type II and Type III. Type I IFNs include IFN- α (subdivided into IFN- α 1, - α 2, - α 4, - α 5, - α 6, - α 7, - α 8, - α 10, - α 13, - α 14, - α 16, - α 17, and - α 21), IFN- β , IFN- ϵ , IFN- κ , and IFN- ω (which are expressed in humans), IFN- δ (which is found in pigs and cattle), IFN- ζ (only expressed in mice), and IFN- τ (only found in cattle) [44-46]. Type II IFN is represented only by IFN- γ and it is mainly involved in bacterial infections and is expressed by activated T cells, macrophages and NK cells. Type III IFNs are classified into four family members: IFN- λ 1, [Interleukin-29 (IL-29)], IFN- λ 2 (IL-28A), IFN- λ 3 (IL-28B), and the newly discovered IFN- λ 4 [47-49]. Until recently, the role of type III IFNs in anti-viral immunity remained largely unknown. However, the discovery of a Single Gene Polymorphism (SNP) upstream of the IFNL3 (IL-28B) gene, which predicts both viral clearance in acute Hepatitis C infection and response to exogenous IFN treatment, has highlighted the importance of this family of cytokines in HCV infection [50].

Each type of IFN activates a specific cognate receptor, and this interaction dictates how a cell responds to infection. All type I IFNs bind to the IFN- α receptor (IFNAR), a two-chained heterodimer, composed of IFNAR1 and IFNAR2 subunits. As opposed to IFNAR1, IFNAR2 can have 3 different spliced variants: a long transmembrane IFNAR2c chain and two truncated forms, a transmembrane IFNAR2b and a soluble sIFNAR2a, which can modulate IFN- α signalling [51,52]. Type II IFN binds to IFN-GR (IFN-GR1 and IFN-GR2 subunits). Type III IFN receptor complexes consist of two chains, IFNLR1 [IL-28 Receptor- α , (IL-28Ra)] and IL10R2 [51]. Upregulation of IFNAR1 and IFNAR2 often occurs on circulating cells upon HCV infection and this expression of IFNAR in the liver of chronic HCV-infected patients is linked to a positive response to IFN- α therapy [53-55]. Type I IFN receptors are ubiquitously expressed in the body, while type III IFN receptors have a more restricted cellular distribution, and, as for IFNARs, have been strongly implicated in response to HCV infection [56-58].

Interferon Signalling Pathway

Once released by infected cells, IFNs establish an anti-viral state via signalling through the Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, resulting in upregulation of over 500 IFN-Stimulated Genes (ISGs). An overview of this process is shown in (Figure 2). Binding of IFNs to their corresponding receptor results in auto-phosphorylation and activation of the receptor-associated JAK kinases, which subsequently phosphorylate the receptor chains and consequently recruit STAT transcription factors, which form homo- or heterodimers and translocate into the nucleus where they bind to specific promoter elements. IFN- α -induced JAK/STAT signalling involves activation of the receptor-associated Tyrosine Kinase (TYK2) and JAK1 and the STAT1-STAT2 heterodimer, which ultimately binds to a third transcription factor, Interferon Regulatory Factor (IRF9) to form the IFN-Stimulated Gene Factor 3 complex (ISGF3). Following translocation to the nucleus, ISGF3 binds to a specific element known as the IFN-Stimulated Response Element (ISRE), which is present in the promoter region of certain ISGs, thereby inducing their transcription. Some ISGs possess only ISRE or, Gamma-Activated Site (GAS) elements in their promoter regions while others contain both elements [48,59].



JAKs and STATs Proteins

In mammals, there are four main JAK proteins [JAK1-3 and TYK2)]. These tyrosine kinase proteins become activated after interaction between the IFN ligand and its receptor. JAK proteins contain seven JAK homology domains (JH1-7) which are widely expressed in

all cell types, except for JAK3, which is found exclusively in cells of myeloid and lymphoid origin. The JH1 domain is important for the JAK enzymatic activity. Phosphorylation of JH1 tyrosine residues leads to JAK conformational changes in the protein to facilitate binding of IFN receptors. JH2 is a pseudokinase domain as it lacks a functional enzymatic activity, but is most likely involved in regulating the activity of JH1. JH3 and the C-terminal portion of JH4 are Src-Homology-2 (SH2)-like domains. The N-terminal end of JH4 up to the JH7 of JAKs form the FERM domain, which is also involved in cytokine receptor binding [60].

In mammals, 7 STAT proteins have been identified (STAT1-4, 5a, 5b and 6). All members of the STAT family share 6 structurally conserved domains: N-terminal (NH2), coiled-coil, DNA Binding (DBD), linker (link), SH2 and the Transcriptional Activation Domains (TAD). The NH2 domain stabilises inactivated STAT proteins, whereas the coiled-coil is involved in nuclear import. The DBD mediates STAT binding to ISRE or GAS elements, while the linker domain acts as a bridge between the DBD and the SH2 domain. The SH2 domain recognises phosphorylated proteins on cell membrane receptors and promotes homo- and/or heterodimer formation. Finally, the TAD domain contains the STAT phosphorylation sites (tyrosine and serine residues) activated during IFN signalling [60,61].

To date, STAT1 and 2 are the best characterised members of the family in relation to IFN- α signalling and are primarily involved in the canonical pathway. STAT3 may also play an important role in IFN- α -mediated anti-viral immunity: recent evidence has shown that HCV evades IFN- α mediated antiviral activity by directly promoting STAT1 and STAT3 protein degradation both in vitro and in immune cells from HCV infected patients, suggesting a fundamental role of the JAK/STAT pathway in mediating viral infection [62].

Once in the nucleus, STAT proteins interact with several transcriptional co-activator proteins, whose recruitment depends on STAT phosphorylation state. These include p300 and cAMP-responsive-element-binding protein Binding Protein (CBP), which possess histone acetyltransferase activity and are important for the regulation of chromatic remodelling that increases IFN- α or IFN- γ -dependent transcription, and Minichromosome Maintenance deficient 5 (MCM5), which is essential for STAT1 target gene transcription [63-67]. HCV NS3/4A and core proteins have been shown to target the JAK/STAT pathway by inhibiting STAT1 phosphorylation at serine 727 and by interacting with the STAT1 SH2 domain, respectively [68,69]. Protein Phosphatase 2A (PP2A)-dependent STAT1 hypomethylation is also induced by HCV, with consequent decreased STAT1 DNA-binding and increased STAT1 association with the negative regulator Protein Inhibitors of Activated STATs (PIAS) [70].

Negative Feedback Controls of IFN Signalling

Negative feedback of inflammatory signalling pathways plays a pivotal role in controlling immune responses and preventing autoimmunity. The JAK/STAT pathway is negatively regulated by PIAS, Suppressor of Cytokine Signalling (SOCS), the Ubiquitin Specific Peptidase 18 (USP18), and the T cell Protein Tyrosine Phosphatase (TcPTP).

SOCS proteins are induced in response to JAK-STAT activation, and prevent further STAT activation by a variety of mechanisms, including binding to the receptors and/or JAKs, thus inhibiting receptor phosphorylation and subsequent STAT recruitment. SOCS proteins also promote proteasomal degradation of JAKs [71,72]. In particular, SOCS1 and SOCS3 are induced in response to Type I IFNs, and are important regulators of the anti-viral response [73]. Interestingly, SOCS3 overexpression has been shown to suppress HCV replication in

in vitro in an mTOR-dependent manner, and, most recently, SOCS1 have been found to promote HCV replication by blocking the anti-viral activity of IFN [74,75], suggesting that both proteins act via alternative mechanisms regarding regulation of IFN activity. However, HCV has been shown to reduce SOCS1 gene expression and upregulate SOCS3 [76-78]. This mechanism employed by HCV might represent a crucial step in HCV infection pathogenesis, including suppressed inflammation and even hepatocellular cancer progression.

PIAS1 and PIAS3 proteins specifically inhibit STAT protein transcriptional activity by binding to activated STAT1 and STAT3, respectively, preventing STAT/DNA binding, thus preventing ISGs expression [79]. Upregulation of PIAS3 has been reported in HCV-infected heroin users [80]. USP18 (also known as UBP43) was initially identified as the specific protease for ISG15 cleavage, and specifically binds to the IFNAR2 receptor subunit of IFN- α , thus inhibiting JAK1/IFNAR2 receptor interaction [81]. Finally, TcPTP dephosphorylates activated STAT1 and STAT3, but a role in JAK inactivation has been also documented [82-84].

Anti-viral Effector Genes of HCV

Activation of IFN signalling can promote the expression of over 500 genes. Type I IFNs upregulate hundreds of ISGs involved in direct anti-viral effects, innate immune signalling, metabolism and apoptosis [85]. Type III IFNs stimulate expression of a similar set of ISGs, despite signalling through distinct receptor types [86]. To date, several ISGs targeting each step of the HCV life cycle have been identified, either preventing or promoting virus replication [87-89]. This was achieved using in vitro hepatic cell lines, due the lack of a suitable small animal model with full HCV permissiveness. The exact role of the majority of these genes in modulating HCV replication and vice versa is still unknown, although some of these ISGs with potent anti-viral activity have been characterised, such as Protein Kinase R (PKR), 2'-5' Oligoadenylate Synthetase (OAS), Myxovirus resistance gene A (MxA), and Interferon Stimulated Gene 15 (ISG15).

PKR

Upon binding to dsRNA, PKR becomes activated by autophosphorylation. Fully functional PKR subsequently deactivates the eukaryotic initiation factor-2- α (eIF2- α) by phosphorylation of the α subunit, blocking protein translation of host mRNA, including ISGs. However, HCV uses this mechanism for its own advantage; as RNA translation occurs via an Internal Ribosomal Entry (IRES), viral protein production is not affected by eIF2- α blockage [90]. HCV E2 and NS5A protein can also bind to PKR protein kinase catalytic domain, rendering this kinase unable to block protein translation and therefore viral replication [91,92].

2'-5'- OAS

As with PKR, the three OAS proteins (OAS1, OAS3, and OASL) are activated by dsRNA and trigger oligoadenylate synthetase/ribonuclease L (RNase L)-dependent cleavage of viral RNA and, interestingly, host RNA in single stranded regions, preferably after UU or UA dinucleotides. These cleaved RNA products can further activate cytosolic PRRs, such as RIG-I and MDA5, thus enhancing Type I IFN signalling response [93-95]. Similar to PKR, the HCV NS5A protein can inhibit OAS anti-viral activity by binding to the N-terminal region of the protein [96].

MxA

MxA is involved in vesicle trafficking and is known to prevent replication of many types

of viruses by binding and wrapping around viral nucleocapsids, rendering them incapable of replication [97]. The exact mechanism involved in HCV replication is not fully understood; however, recent evidence showed that the HCV core protein specifically colocalises with MxA in a granular pattern in the cell cytoplasm, and this is enhanced by treating cells with Ribavirin and IFN- α [98].

ISG15

ISG15 is an ubiquitin-like molecule that targets protein in a mechanism called “ISGylation”, which involves sequential activation of an E1-activating, an E2-conjugating, and an E3 ligation enzyme. ISGylation alters protein property directly by addition of ISG15 and reduces target protein degradation by competing with ubiquitin conjugation. Although ISG15 promotes protection against most viruses, HCV seems to represent the exception to this rule. ISG15 can ISGylate a wide variety of proteins, including PKR and RIG-I, inducing a proviral state within the host cell during very early stages of HCV infection [99,100], and it can also promote HCV virus production in in vitro infection models [101].

Natural Killer Cells

Another component of the innate immune system, Natural Killer (NK) are very important in the primary immune response against viral pathogens [102], eliminating virally infected cells both directly via cytolytic mechanisms and indirectly by secreting cytokines such as IFN- γ [103]. NK cells also demonstrate regulatory and reciprocal interactions with T and B cells, DCs, macrophages and endothelial cells, thus functioning to amplify or attenuate immune responses [104].

In HCV infection NK cells are thought to be necessary for optimal priming and cytolytic function of virus specific T cells due to production of IFN- γ [105]. Cytokine-stimulated NK cell lines and primary NK cells isolated from healthy donors have the ability to lyse HCV replicating cells [106,107].

It has been reported that NK inhibition can occur through binding of HCV viral envelope protein E2 [108]. This binding results in decreased cytotoxicity and IFN- γ production, and can be regarded as a strategy to establish HCV as a chronic infection. It has also been shown that HCV NS5A protein, through monocyte-derived Transforming Growth Factor- β (TGF- β) production, down-regulates expression of NKG2D on NK cells, thus reducing their cytotoxic potential and IFN- γ production [109]. Another mechanism by which HCV avoids recognition by NK cells is through HCV core protein inducing p53-dependent gene expression of TAP1 (Transporter associated with Antigen Processing 1) and consecutive Major Histocompatibility Complex (MHC) class I up-regulation. Elevated MHC class I levels following HCV core protein expression induce negative signals that lead to inhibition of NK cytotoxicity [110].

Adaptive Immune Responses

The adaptive immune system plays a central role in pathogenesis and outcome of disease in patients with HCV infection. The mechanisms involved in this specific immune response, as well as counter measurements taken by the virus to evade this response will be described.

B Cells and Humoral Immunity

The humoral component of the adaptive immune response makes use of viral specific antibodies produced by B cells. Typically anti-HCV antibodies may be detected 50-60 days after HCV infection [111]. However, these antibodies only signify a humoral response to HCV proteins and do not evaluate the ability of these antibodies to neutralise HCV infection. Only a small fraction of antibodies are able to inhibit virus binding, entry or uncoating. These antibodies may potentially block HCV infection and are termed 'neutralising antibodies'. The HCV envelope glycoproteins E1 and E2 are important targets for virus neutralisation as their interaction with host cell factors is required to initiate productive infection [112].

Neutralising antibodies are generally considered an important mechanism for the control of initial viraemia and protection from reinfection in viral infections. In HCV infection, the role of neutralising antibodies in mediating viral clearance remains controversial. The recognition that hypogammaglobulinemic humans can spontaneously eradicate HCV suggests that antibody responses may be dispensible [113].

Studies investigating immune responses in chimpanzees and humans suggest that HCV clearance can occur in the absence of neutralising antibodies or those antibody responses alone are insufficient to eradicate HCV in the majority of cases [114-116]. Additionally HCV infection does not elicit protective immunity against reinfection [117]. However chimpanzees and humans who have cleared HCV spontaneously appear to be less likely to develop chronic infection after re-exposure [118,119]. A study of healthy young women infected with an identical, single source viral inoculum demonstrated that early induction of neutralizing antibodies was seen in individuals with viral clearance. By contrast, in individuals with viral persistence, absent or low-titer neutralizing antibodies were observed in the early phase of infection. Persistence of infection occurred in these individuals despite the induction of cross-neutralizing antibodies in the late phase of infection [120].

In chronic infection, HCV-specific neutralizing antibodies may be detected in most patients. Multiple mechanisms for the failure of the humoral immune response have been suggested. In infected individuals, HCV exists as a quasispecies i.e. a pool of constantly changing, distinct, but related genomic variants. Evolution of the quasispecies within targeted epitopes, such as the Hypervariable Region 1 (HVR1) of the Envelope 2 (E2) protein, may lead to escape from neutralizing antibodies [121]. It has been shown that the interplay of HVR1, High-Density Lipoproteins (HDL) and the Scavenger Receptor (SR-B1) can prevent the effect of neutralizing antibodies, enhancing cell entry of HCV and infection [122]. HVR1 may play a more general role in mutational escape by serving as a decoy for neutralizing antibodies. In this way it may protect less mutable, but functionally important epitopes [123]. HVR1 influences the biophysical properties of released viruses and this domain is particularly important for infectivity of low-density particles. HVR1 obstructs the viral CD81 binding site and protects conserved neutralizing epitopes [124].

The interaction between non-neutralizing and neutralizing antibodies may play a key role in determining the outcomes of HCV infection. Binding of non-neutralizing antibodies to specific epitopes results in protection against neutralizing antibodies [125]. Specific glycans have been identified on E2 responsible for modulating cell entry and protection against neutralizing antibodies [126]. Finally, recent work has demonstrated the ability of HCV to escape neutralization via direct cell-to-cell transfer of the virus [127,128].

T Cell Responses

The majority of acute infections with HCV are asymptomatic and often unrecognised. Therefore, *in vivo* studies of T cell immune responses during acute infection have only been possible in experimental chimpanzee models or individuals with an identifiable exposure for which the time of infection may be documented.

The hallmark of HCV viral clearance is a robust and broad specific T cell response. By contrast a weak and narrowly focussed T cell response is observed in chronically infected HCV individuals [129,130]. An efficient and vigorous T cell response during the acute phase is typically seen in individuals with spontaneous clearance of infection [131]. In resolved HCV infection, circulating HCV specific antibodies decline over a period of decades and may often become undetectable, while functional CD4⁺ and CD8⁺ T cells are maintained for decades [132]. These T cells substantially decrease the risk of persistent infection upon re-exposure to HCV [118]. In individuals who go on to develop chronic infection, a transient or absent primary T cell response is observed [129].

The exact mechanisms underlying the cellular immune evasion capacities of HCV remain incompletely understood. Impairment of the interactions between antigen-presenting DCs and T cells may result in an impaired cellular response. It has been shown that a decrease and dysfunction of DCs correlates with impaired HCV-specific CD4⁺ T-cell proliferation in patients with HCV infection [133]. Individual HCV proteins, core, NS3, NS4, NS5 as well as fused Polyprotein (Core-NS3-NS4) were found to impair functions of both immature DCs and mature DCs on multiple levels including reduced IL-12 secretion, induction of Fas ligand expression to mediate apoptosis and inhibition of TLR signalling [134]. Also, HCV interactions with DC-specific receptors DC-SIGN and DC-SIGNR may contribute to the establishment or persistence of infection both by the capture and delivery of virus to the liver and by modulating DC function [135].

CD8⁺ T cell dysfunction

It has been suggested that dysfunction of CD8⁺ T cells, the primary effector cells that mediate viral clearance through the secretion of antiviral cytokines, is a major determinant of viral persistence [136,137]. T cell exhaustion results in the inability to proliferate in response to antigen and the failure to produce cytokines such as IFN- γ . T cell exhaustion is recognized in other viral infections such as HIV and is characterized by high levels of expression of inhibitory receptors such as protein-Programmed Death 1 (PD-1). It has been shown that large proportions of HCV-specific CD8⁺ T cells express high levels of PD-1 [137] and that these cells are prone to apoptosis [138]. Not all dysfunctional T cells express PD-1, nor are they all rescued by blockade of the PD-1/PD-1 ligand pathway and as a result a number of emerging molecules and pathways have been implicated in mediating the T cell exhaustion characteristic of chronic viral infection, including T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) [139] and the receptor 2B4 (CD244) [140].

Factors potentially leading to T cell exhaustion include chronic antigen stimulation, dysfunction of CD4⁺ T cells or the action of regulatory T cells or cytokines.

CD4⁺ T cells

While CD8⁺ T cells are the major effector cells against viral pathogens, the presence of CD8⁺ T cells that can proliferate, exhibit cytotoxicity, and produce IFN- γ does not ensure recovery. Mouse models have shown that CD4⁺ T cells play an important role in sustaining virus-specific CD8⁺ T cells [141]. A critical determinant of outcome in HCV is whether these

CD8⁺ T cells were primed in the presence or absence of CD4⁺ T-cells [142]. In patients with chronic infection, failure of IL-2 secretion, as opposed to physical deletion or complete functional unresponsiveness, appears to be an important determinant of the status of CD4⁺ T cell populations in chronic HCV infection [143].

Regulatory T Cells

Different T cell subsets with suppressive functions have been described. Among these are regulatory T cells (Tregs), a subpopulation often identified during chronic HCV infection. They are identified by the constant expression of factor Forkhead box P3 (FoxP), CD4⁺ and CD25⁺. The signature of the Tregs is their potent ability to suppress the effector cells. In chronic HCV infection Tregs have been found at a higher frequency when compared with resolved HCV infection [144]. Research into protein-derived peptides from HCV and the antigen specificity of Tregs showed that only a few peptides, with overlapping regions, were able to stimulate Tregs [145]. This indicates that there could be a dominant region of the core of HCV capable of activating Tregs, thereby suppressing the immune response. Evidence against Tregs in promoting the development of chronic infection was recently reported in a prospective study of 27 acutely infected patients. In fact, no significant difference was seen in the proportion of CD4⁺CD25⁺high T cells in the peripheral blood at baseline between the individuals who cleared infection and those who developed chronic infection [142].

Viral Mutation

One method used by many viruses to evade the immune system is that of viral mutation. The estimated replication rate of HCV is 1012 virions per day [146]. The error-prone RNA polymerase that HCV uses for replication results in minor virus variants with the potential for immune evasion. As the replication rate for HCV is so high, this gives rise to quasispecies of HCV in each infected individual [147]. The most conserved region of the HCV genome is the 5' non-coding region demonstrating 90% homology with other viral strains [148]. The most variable part of the genome encodes the envelope proteins, E1 and E2 [149]. Within these proteins lie the hypervariable regions, HVR1 and HVR2, regions that are very 'mutation prone'. Two kinds of mutation can occur during viral replication. Silent or synonymous mutations do not result in a change to the amino acid sequence of a protein, or result in the insertion of an alternative amino acid with similar properties to that of the original amino acid, and in either case there is no significant change in phenotype. Non-synonymous mutations lead to amino acid changes in the virus that may leave it less fit or may even be lethal to the virus.

It has been shown that during chronic infection HCV is subjected to selection pressures from both humoral and cellular immunity, resulting in the continuous generation of escape variants [123]. If a mutation is beneficial to the virus, it will adopt this mutation allowing it to escape the immune system [150]. The altered peptide ligands that arise may even down-regulate the T cell response against original ligands [151].

HCV is in a constant search to optimise its genome, without impairing function, and with a mutation rate of approximately 1.92×10^{-3} base substitutions per genome site per year [152], it makes use of a very capable system to evade the immune response.

Conclusion

To summarise, it appears many factors contribute towards HCV persistence and HCV has

developed a variety of overlapping immune evasion mechanisms, targeting both the innate and adaptive immune response, that contribute to preventing viral clearance in the majority of individuals. Ongoing research will likely identify new mechanisms of immune evasion in the future that will be fundamental to our understanding and development of novel strategies to prevent and control HCV infection.

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Control of HIV infection by IFN- α : implications for latency and a cure

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Abstract Viral infections, including HIV, trigger the production of type I interferons (IFNs), which in turn, activate a signalling cascade that ultimately culminates with the expression of anti-viral proteins. Mounting evidence suggests that type I IFNs, in particular IFN- α , play a pivotal role in limiting acute HIV infection. Highly active anti-retroviral treatment reduces viral load and increases life expectancy in HIV positive patients; however, it fails to fully eliminate latent HIV reservoirs. To revisit HIV as a curable disease, this article reviews a body of literature that highlights type I IFNs as mediators in the control of HIV infection, with particular focus on the anti-HIV restriction factors induced and/or activated by IFN- α . In addition, we discuss the relevance of type I IFN treatment in the context of HIV latency reversal, novel therapeutic intervention strategies and the potential for full HIV clearance.

Keywords HIV · Interferon · Latency · Anti-viral · JAK/STAT · Cure

Introduction

The innate immune response is an effective and advanced system that constitutes the body's first line of defence against pathogenic infection. Upon viral detection, activation of this host defence-mediated mechanism leads to the production of a family of protective cytokines, known as type I IFNs. IFN signalling pathways regulate hundreds of genes involved in anti-viral, pro-inflammatory and anti-proliferative activity, which has led to their use in treating several diseases, including viral infection, cancer and autoimmunity [1, 2]. The type I IFN family members include the IFN- α 's, IFN- β , IFN- ϵ , IFN- κ , IFN- τ and IFN- ω . Type I IFNs are critical controllers of viral infection and it is increasingly evident that in the context of HIV, IFN- α plays a key role in the control of this infection and is elevated in the plasma of HIV-infected individuals [3]. IFN- α 's importance is particularly evident during acute infection, when early production of IFN- α restricts HIV replication [4]. However, for reasons that are not fully elucidated, this innate immune response does not sufficiently halt either chronic HIV progression or integration of HIV DNA into the human genome.

HIV-induced IFN- α production

IFN- α is a rapid and potently induced cytokine. Its production is stimulated upon detection of virally derived products via pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), Retinoic acid inducible gene-like receptors (RLRs) and cytosolic DNA sensors [5]. Following virus detection, signalling pathways are triggered downstream of PRRs, culminating in the activation of transcription factors, such as IFN regulatory factors (IRF)3, IRF7, NF- κ B and activator protein (AP)1, which leads to the upregulation of

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pro-inflammatory and anti-viral cytokines, including type I IFNs [6].

Several PRRs recognise HIV RNA and DNA, leading to IFN- α induction (Fig. 1). Viral RNA detection has been at the forefront of research discoveries over the last decade, such as the recognition of HIV-1 RNA by TLR7, which stimulates production of IFN- α from plasmacytoid dendritic cells (pDCs) [7], although this IFN- α induction has been shown to be relatively weak in comparison to other viruses [8]. However, with the recent discovery of several cytosolic PRRs capable of sensing DNA, there is now a large body of work detailing cellular sensing of HIV DNA, which has also revealed cells other than pDCs, such as T cells, B cells and macrophages, that may be important inducers of IFN- α following HIV detection [3, 9]. IFI16 was identified as a PRR for HIV DNA in macrophages and is implicated in subsequent regulation of HIV replication and HIV-mediated

CD4⁺ T cell death [10, 11]. Furthermore, cells depleted of the nuclease, TREX1, have increased levels of HIV cDNA, which triggers HIV DNA detection and subsequent induction of type 1 IFNs [12]. The cytosolic DNA sensor, cGAS, also plays a crucial role in detecting HIV DNA in fact, knockdown of cGAS has been shown to block cytokine induction by HIV [13]. Interestingly, many of these PRRs are constitutively expressed, but are further upregulated by IFN- α , demonstrating a positive feedback loop that enhances anti-viral detection.

IFN- α intracellular signalling

Following PRR detection of HIV, IFN- α is released, which protects cells in both an autocrine and paracrine fashion. IFN- α binds to the type I IFN receptor (IFNAR) complex,

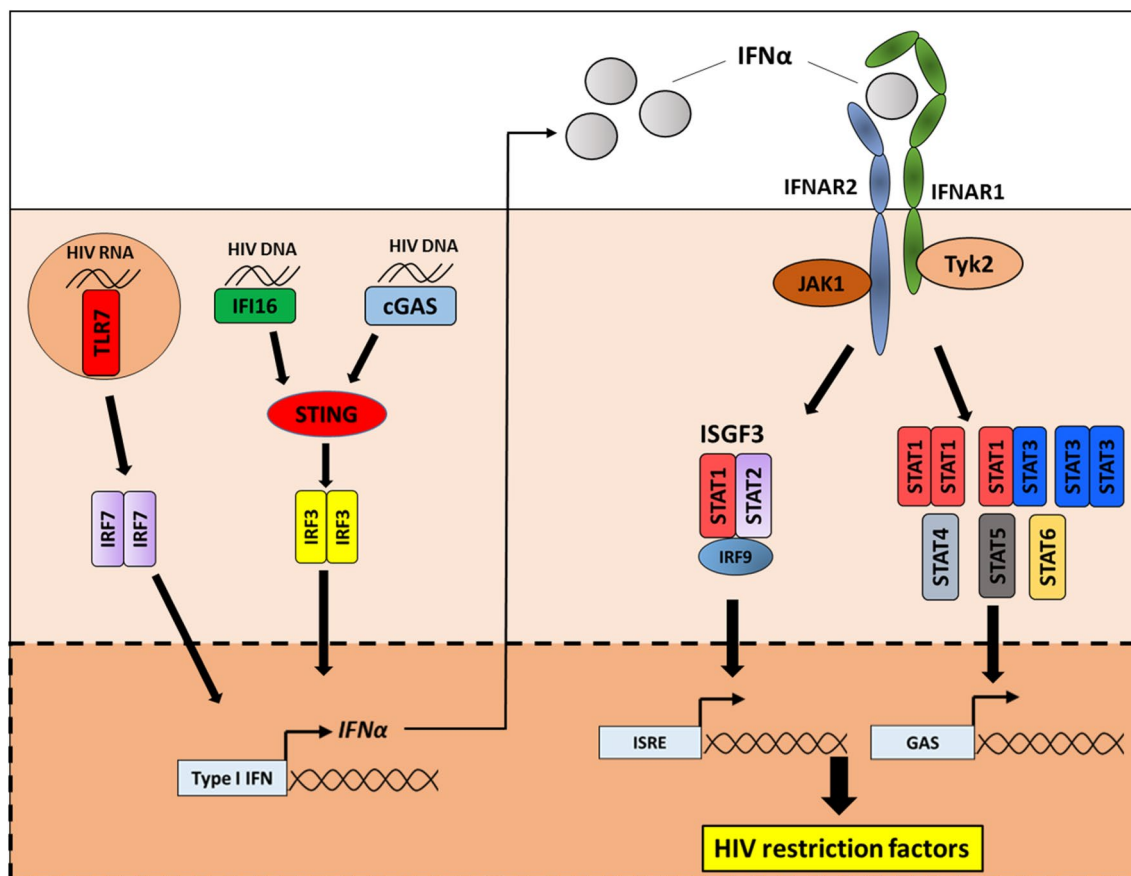


Fig. 1 Innate immune detection of HIV induces anti-viral immunity via upregulation of IFN- α . HIV RNA is detected by TLR7 located in endosomes and leads to the phosphorylation and homodimerization of the transcription factor IRF7, which translocates to the nucleus and induces the expression of *IFN- α* . Cytosolic detection of HIV nucleic acid by the sensors IFI16 and cGAS can also lead to *IFN- α* induction via activation of STING and the subsequent phosphorylation and homodimerization of IRF3. IFN- α binds to its cognate receptor, which is composed of the IFNAR1 and IFNAR2 subunits, which are

ubiquitously expressed on all cell types. Binding of IFN- α results in the activation of receptor-associated kinases, Tyk2 and JAK1, subsequently leading to the activation of the STAT family of transcription factors. Activated STAT1 and STAT2 bind IRF9 and form the transcription factor complex ISGF3, which can bind to the ISRE promoter element. STAT1–6 can also homo- and heterodimerise following activation with IFN- α and can bind to GAS promoter elements. Many genes activated through this response encode for proteins with potent anti-viral functions, including HIV restriction factors

which is composed of the receptor subunits, IFNAR1 and IFNAR2. Unlike IFN- β , which can signal in the absence of IFNAR2 to mediate distinct biological effects, IFN- α absolutely requires the presence of both IFNAR1 and IFNAR2 to signal [14]. IFNAR engagement activates several intracellular signalling cascades, most notably the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathways [15]. The JAK/STAT pathway is stimulated by a spectrum of cytokines, such as IL-6, Granulocyte colony-stimulating Factor (G-CSF) and IFN- α , which initiate a broad range of biological processes, including growth, differentiation and inflammation [16–18]. IFN- α -induced signalling controls the expression of hundreds of IFN-regulated genes (IRGs), many of which encode anti-viral mediators, such as Myxovirus resistance gene A (MxA), Protein Kinase R (PKR) and 2'-5'-Oligoadenylate Synthetase (OAS) [19] (Fig. 1). Indeed, several of these inducible anti-viral IRGs encode proteins that directly restrict HIV replication. A recent increase in our understanding of these IRGs is beginning to provide mechanistic insight into the immune-mediated restriction of HIV infection and is sure to be instrumental in our understanding of how HIV resists elimination and remains latent.

IFN- α -induced anti-HIV restriction factors

Following cell fusion, the HIV capsid (which contains two negative strand RNA genomes and several proteins required for the early life cycle), is released into the cytoplasm. At this point, HIV is vulnerable to constitutively expressed and IFN- α -induced and/or activated anti-viral restriction factors [20]. There are numerous well-defined IRGs that impede HIV's replication cycle, including Tripartite motif 5 α (TRIM5 α), Apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3)G/F, Interferon-induced transmembrane protein (IFITM)1–3, MXB, SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase (SAMHD)1, Schlafen (SLFN) and Tetherin [21] (Fig. 2). Recent literature suggests that many more IRGs than previously thought act to limit HIV replication, with indoleamine 2,3-dioxygenase (IDO)1 and TRIM56 also identified in 2016 as effective restriction factors [22]. This library of increasing evidence highlights the role of IRGs in restricting HIV replication, which could hold the key to a much-needed cure. Since future in-depth analysis of individual IRGs is likely to reveal novel therapeutic targets, the following section of this review provides an up-to-date “snapshot” of IRG actions against HIV infection and replication.

TRIM5 α

Rhesus (rh) macaque TRIM5 α was initially found to be both a cytoplasmic restriction factor and a PRR for the HIV-1 capsid, associating via the polymorphic PRYSPRY domain [23]. TRIM5 α recognition of the HIV capsid stimulates E3 ubiquitin ligase activity, thus promoting its proteasomal degradation, which inhibits HIV replication [24, 25]. Soon after viral cell entry, rhTRIM5 α targets HIV-1 and initiates premature disassembly of the HIV capsid, consequently blocking reverse transcription [23]. Several groups have also implicated TRIM5 α polymorphisms and expression levels with disease severity, highlighting the *in vivo* importance of TRIM5 α [26–29]. While it was previously thought that human TRIM5 α had no specific anti-HIV-1 activity, a recent study demonstrated that silencing TRIM5 α restricts HIV in Langerhans cells, possibly identifying cell specific anti-viral activity [30].

APOBEC

APOBEC3-G and -F strongly inhibit HIV infection [31, 32]. Prior to HIV exiting the cell, APOBEC molecules are loaded onto the virus through interactions with HIV RNA [33]. In addition, when HIV attempts to infect the next cell, the “passenger” APOBEC (using its cytidine deaminase activity), introduces C to U changes in the minus DNA strand of HIV. The result is a guanine to adenine mutation in the genomic sense DNA and overall hypermutation of the replication reverse transcripts, thus inhibiting HIV's ability to replicate [34]. Interestingly, APOBEC3B is also a potent HIV inhibitor; however, as it is not expressed in typical HIV target cells, the HIV protein Vif, which can target other APOBECs for degradation, has not evolved to target this particular APOBEC [35], thus highlighting the co-evolution of HIV to specifically target such restriction factors in HIV target cells.

SAMHD1

Through the reduction of cellular dNTP levels, SAMHD1 impedes efficient reverse transcription and DNA production of HIV and simian immunodeficiency virus (SIV) [36, 37]. Interestingly, re-addition of dNTP pools into myeloid cells is not sufficient to rescue 2-long terminal repeat (LTR) circles or proviral DNA, indicating that SAMHD1 may have other restrictive mechanisms beyond solely depleting the dNTP pool [38]. The anti-viral activity of SAMHD1 is dependent on it being phosphorylated by cyclin A2/Cyclin-dependent kinase (CDK)1 in proliferating cells; an area of research that is highly anticipated to identify new targets for immune restoration against HIV [39, 40].

MX2

Recent studies have identified the IFN-induced GTPase, MX2 (also known as MXB), as a potent inhibitor of HIV-1 infection [41–43]. MX2 targets both the nuclear accumulation and genomic integration of viral DNA. This is dependent on the interaction with the viral capsid (as capsid mutations confer relative resistance to MX2). MX2 protein localises to the nuclear pore [42], suggesting that it may target nuclear transport of the pre-integration complex. As with other GTPases, MX2 requires oligomerisation to suppress HIV infection [44].

SLFN

Both type I IFNs and specific IRGs induce the expression of SLFN genes [45]. The human SLFN proteins were originally

thought to only play a role in T cell development [46]; however, through targeting cellular transfer RNAs, human SLFN11 has been found to inhibit the late stage production of HIV-encoded proteins [47]. Knockdown of SLFN11 in HEK293T cells does not inhibit reverse transcription, integration, production or nuclear export of viral RNA; however, it inhibits the synthesis of virally encoded proteins [46]. Further studies are still needed to address whether HIV-1 counteracts the anti-viral function of SLFN11.

Tetherin

Also known as BST-2, CD317 or HM1.24, Tetherin is a membrane glycoprotein, normally expressed in primary cells such as CD4⁺ T cells and macrophages [48]. Its production is upregulated by cellular activation and maturation, and further induced by IFN- α [49, 50]. Tetherin blocks HIV release

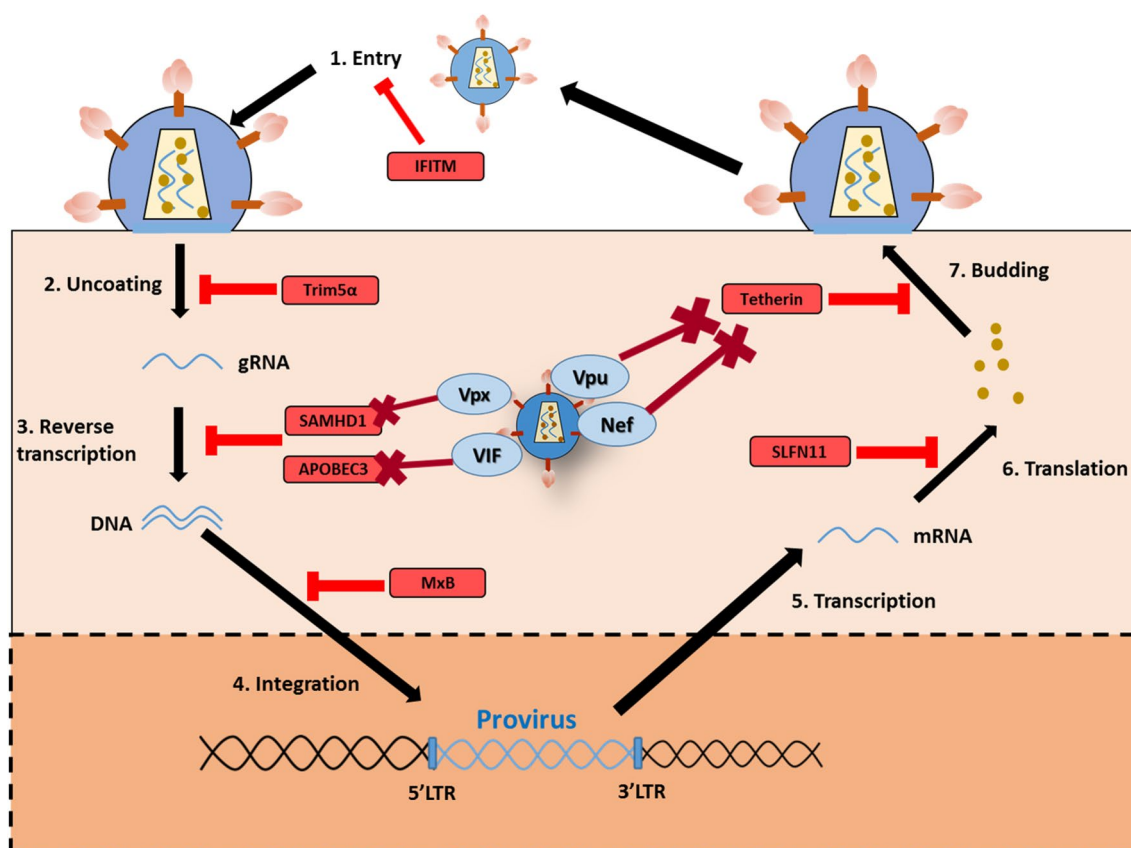


Fig. 2 IFN- α -induced restriction factors block all stages of the HIV life cycle. IFN- α upregulates the expression of many proteins capable of blocking HIV replication at every stage of the viral life cycle. HIV entry into the cell is antagonised by IFITM family members. After uncoating of the viral envelope, TRIM5 α inhibits HIV by directly binding to the capsid and promoting its proteasomal degradation. SAMHD1 and APOBEC3 can both block HIV at the stage of reverse transcription. SAMHD1 decreases the cellular pool of dNTPs available to the virus for cDNA synthesis, whereas APOBEC3 causes hypermutation in viral cDNA during the reverse transcription

process. MX2 inhibits the nuclear import of HIV DNA and thus its integration into the host genome. SLFN11 targets tRNAs for depletion and thus inhibits translation of viral proteins. Finally, Tetherin restricts newly assembled virions from budding from infected cells. Together these restriction factors represent a powerful arsenal to block HIV infection and it is therefore not surprising that the HIV has evolved its own powerful counter mechanisms to antagonise the actions of many of these restriction factors. HIV Vpx targets SAMHD1; HIV Vif targets APOBEC3, and HIV Vpu and Nef target Tetherin

by recruiting and trapping viral particles to the membrane of infected cells. Its broad anti-viral importance is highlighted by its additional role as a PRR, inducing NF- κ B-dependent pro-inflammatory gene expression in virally infected cells [51].

IFITMs

Following screens for HIV-restrictive IFN-inducible genes, IFN-induced transmembrane proteins 1–3 (IFITMs 1–3), have emerged as another class of proteins capable of powerfully suppressing the HIV replication cycle [52, 53]. While early studies discovered that IFITM3 restricted replication of Influenza virus [54], further investigations showed IFITMs block HIV replication, via incorporation into the HIV virion during assembly [55, 56] and by interfering with viral envelope processing [57]. A recent study found that the ability of IFITMs to inhibit HIV replication was dependent on co-receptors used by the virus during cell entry, but did not observe IFITM incorporation into the virion [58]. Interestingly, this study also discovered that transmitted founder viruses are initially resistant to the actions of IFITMs, although sensitivity of HIV to IFITMs increases during the first few months of viral infection, highlighting their anti-viral importance during the course of HIV infection.

IFN- α and acute HIV infection

IFN- α is usually induced early and its expression is often transient during the first weeks of acute HIV-1 infection [59, 60], and IFN- α potently inhibits HIV replication in primary macrophages and CD4 + lymphocytes [61–64]. Indeed, IFN- α treatment of rhesus macaques prior to administered SIV prevents systemic infection, clearly demonstrating the anti-viral strength of IFN- α in vivo [65]. However, if IFNAR is blocked (thus inhibiting type I IFN signalling), SIV infection accelerates, with an increased viral reservoir and progression to AIDS, highlighting the essential requirement of intact type I IFN signalling against SIV infection. Indeed, recent studies in humanised mouse models have yielded greater insight into the intricate and complex responses to type I IFN during HIV infection. Two important studies reported that by blocking type I IFN signalling (using IFNAR antibodies), during HIV infection, restored HIV-specific T cell function and activation [66, 67]. However, using an antibody against IFNAR1 (thus blocking all type I IFN signalling), Cheng et al., reported suppressed HIV viral replication [66], whereas Zhen et al., using an antibody against IFNAR2 (thus only blocking IFN- α signalling and not IFN- β signalling), reported increased HIV viral loads [67]. These

studies therefore suggest that specifically IFN- α is required for efficient suppression of HIV infection in vivo.

Unexpectedly, the beneficial effects of IFN- α observed during early SIV infection seem to be lost during prolonged IFN- α therapy [65], a phenomenon also observed in hepatitis C virus (HCV)-infected humans treated with IFN- α [68]. Interestingly, Fenton-May et al., observed more in vitro resistance to IFN- α of HIV-1 isolates from acutely infected patients compared to viral isolates from the same patients during their chronic phase of infection [69]. Together these important studies emphasise the significant potential of IFN- α in combating HIV infection and while highlighting the current limitations, they emphasise the need to focus our research efforts on further unravelling the viral immune evasion mechanisms that induce this resistance to IFN- α .

Mechanisms of HIV resistance to type I IFN

The antagonism of HIV against type I IFN production and their anti-viral responses further support the hypothesis that type I IFNs have the innate potential to control HIV infection. HIV can block PRR-mediated induction of type I IFNs. In fact, HIV blocks its own recognition by PRRs by recruiting host co-factors, including cleavage and polyadenylation specificity factor subunit 6 (CPSF6) and cyclophilins, to its capsid protein, which enables the virus to evade detection by the PRR cGAS [70]. Following type I IFN production, HIV can also subvert their mechanism of action. Other viruses, such as HCV and respiratory syncytial virus (RSV) [71, 72], block JAK-STAT signalling to inhibit type I IFN mechanism of action. However, to date, HIV has only been shown to directly block the effects of IFN-induced restriction factors, such as APOBEC3G, Tetherin and SAMHD1. The HIV Vif protein counteracts APOBEC activity by recruiting E3 ligase components that promote its proteasomal degradation [31, 73, 74]. The HIV protein Vpu inhibits Tetherin activity by depleting it from the cell surface, through either the proteasome or the endolysosomal system [50, 75], and can trigger its proteasome-dependent degradation [76]. The importance of Vpu is seen during Vpu-deficient HIV infection, when Tetherin expression increases antibody opsonisation of HIV-infected cells and stimulates cytotoxic responses by Natural Killer (NK) cells [77]. The importance of Tetherin in restricting HIV replication is again emphasised by the fact that another HIV protein, Nef, can also target its down-modulation from the cell surface [78, 79]. Another HIV protein, Vpx, present only in the HIV-2/SIVsm/SIVmac group, has been shown to antagonise the restriction factor SAMHD1 [80]. Further research into HIV's inhibition of both the induction and actions of type I IFN-induced genes may reveal molecular mechanisms that could be harnessed to restore innate anti-viral activity against the virus.

Clinical IFN- α treatment for HIV

For many years, the anti-viral properties of IFN- α have been employed as a treatment of specific viral infection, such as HCV [81], and were also trialled for HIV before the advent of highly active anti-retroviral treatment (HAART), although results from these historical trials must be viewed with caution, given the lack of appropriate placebo control groups. The first in vivo studies (given to immune-suppressed HIV-infected patients with Kaposi's sarcoma), involved recombinant exogenous IFN- α administration as a single agent or in combination with the ART Azidothymidine (AZT). These trials observed that IFN- α induced tumour regression and suppressed HIV p24 core antigen levels [82–84]. However, while reductions in viral load were modest, response to IFN- α was most pronounced in patients with high CD4⁺ T-lymphocyte counts (over 200 cells/mm³), and was dose dependent. Furthermore, clinical trials conducted on patients with HIV infection showed that IFN- α , alone or in combination with ART, significantly decreased HIV viral loads and was well-tolerated [85, 86]. However, in contrast, clinical trials of chronically infected patients found that IFN- α therapy failed to inhibit HIV-1 replication and caused severe side-effects, including toxicity, ART failure and progression to AIDS [87]. It is the ability of HIV to acquire resistance to IFN- α after infection and during IFN- α therapy that again highlights HIV's immune evasion mechanism to avoid anti-viral responses to IFN- α [88]. Despite there being evidence of IFN- α 's therapeutic benefit against HIV, problematic issues of toxicity, refractory signalling and viral immune evasion still exist. However, since HIV eradication is not achieved through HAART, there remains a strong rationale to investigate the curative potential of IFN- α , particularly in the context of novel latency reversal agents (LRAs).

HIV latency and IFN- α treatment

To achieve HIV eradication we need to develop novel methods and therapeutic strategies that can overcome viral latency. Latency occurs following the integration of the HIV provirus into the human genome of a small fraction of long-lived memory CD4⁺ T cells [89]. If HAART treatment is interrupted, the virus rapidly re-establishes productive viral replication [90]. Recently, it was found that reactivation of HIV replication can even occur within a week of therapy cessation [91, 92]. It is postulated that this window of viral reactivation may be a key period where the virus is not shielded from the immune system and may therefore be responsive to additional therapeutic intervention. Consequently, there has been a drive to develop novel LRAs to

be used in conjunction with existing HAART, which could stimulate the provirus to become transcriptionally active and overcome this state of viral quiescence.

Several types of LRAs have been assessed, but the class applied clinically to date are the Histone deacetylase (HDCA) inhibitors (HDACi). HDACs remove the acetyl groups from lysine residues of core histones enhancing the formation of a condensed and transcriptionally silenced chromatin. HDACs recruited to the HIV promoter repress transcription of the HIV LTR and HDACi reverse this transcriptional repression. Although studies have demonstrated that patients using HDACi had increased cell-associated HIV RNA, the frequency of latently infected cells did not decrease, for reasons that are still unknown [93]. These trials have revealed that (1) there is a need for better, more targeting LRA development and (2) a LRA in combination with current HAART may benefit from immunotherapy using a "shock and kill" approach [94]. Therefore, a targeted restoration of IFN- α responses during latency reversal may be highly beneficial.

A recent clinical study utilised IFN- α therapy against HIV infection following a staged interruption to their HAART therapy [95]. Patients received therapeutic IFN- α in addition to HAART for 5 weeks, followed by IFN- α alone. HIV-1 replication remained suppressed in 45% of patients after 12 weeks and 30% of patients at week 24 of IFN- α monotherapy. Interestingly, integrated HIV-1 DNA was also significantly reduced in patients who remained virally suppressed, suggesting a crucial reduction in the HIV-1 proviral DNA reservoir. Sun et al., found that IFN- α treatment of HIV/HCV co-infected patients decreased proviral DNA levels in CD4⁺ T cells [96]. In addition, a recent study found that enhanced signalling by the PRR RIG-I (and thus induction of type I IFN responses) led to decreased HIV latency and increased productive HIV infection [97]. Importantly, this viral reactivation preferentially induced apoptosis of HIV-infected cells, responses that were all potentiated when combined with an HDACi. RIG-I is encoded by the gene DDX58, which is strongly upregulated following type I IFN stimulation, thus providing further reasoning to revisit IFN- α therapy in the context of being used alongside LRA. As discussed previously there is a precedence for using therapeutic IFN- α during acute HIV infection IFN- α 's promotion of HIV restriction factor expression could be used to inhibit reactivated viral replication and prevent viral dissemination in addition to blocking re-integration of HIV into the host genome while also inducing infected cell death [21]. However, an outstanding limitation could be the ability of HIV to antagonise IFN- α -induced responses, an area that requires further research exploration.

Conclusion

Despite marked advances in the management of HIV infection, there remains a need for novel therapies specifically designed to obtain a functional cure that eliminates all infectious HIV genomes. The advent of LRAs has presented an exciting and progressive prospect that HIV can ultimately be cleared; however, evidence thus far suggests that use of LRAs, either alone or in combination, will require an additional immune boost. This is a key opportunity for novel strategic thinking regarding HIV treatment. Recent studies are bolstering data from previous findings that show IFN- α has a beneficial effect during acute HIV infection, which strongly suggests that it is now time to re-consider IFN- α therapy, especially in the context of LRAs, towards the ultimate goal in this field: that HIV/AIDS becomes a curable disease.

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Point-of-care testing for HIV in an Irish prison setting: results from three major Irish prisons

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Abstract

HIV is more prevalent in the prison population compared to the general population. Prison inmates are at an increased risk of blood-borne infections. Considerable stigma has been documented amongst inmates with HIV infection. In collaboration with the schools, healthcare facilities, prison authorities and inmate Irish Red Cross groups in Wheatfield, Cloverhill and Mountjoy prisons in Dublin, Ireland, the Department of Genito Urinary Medicine and Infectious Diseases at St James’ Hospital in Dublin developed a campaign for raising awareness of HIV, educating inmates about HIV and tackling HIV stigma. Following this campaign, large-scale point-of-care testing for HIV was offered over a short period. In total, 741 inmates were screened for HIV. One inmate tested positive for HIV. We experienced a large number of invalid test results, requiring formal laboratory serum testing, and a small number of false positive results. Large-scale point-of-care testing in the Irish prison setting is acceptable and achievable.

Keywords

Europe, screening, HIV/AIDS, point-of-care testing, prison, stigma

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Introduction

With early diagnosis and access to effective antiretroviral therapy (ART), morbidity and mortality related to HIV can be reduced, and survival approaches that of the general population.¹ High-risk behavior is substantially reduced after people become aware they are HIV-positive,² and they are substantially less likely to transmit HIV to partners if they are maintained on effective ART.^{3,4}

The Irish Prison Service caters for male offenders who are 17 years or over and female offenders who are 18 years of age or over. There are 14 institutions in the Irish Prison service of which 11 are traditional ‘closed’ institutions, two open centres, operating with minimal internal and perimeter security, and one ‘semi-open’ facility. Delivery of health care in the prison setting can be challenging, where the average inmate weekly turnover rate can be high and the majority of sentences are under three months. In 2012, there were 17,026 committals from 13,860 individuals (84% male) to the Irish Prison Service (Source – www.irishprisons.ie).

Prison inmates are at increased risk of blood-borne virus (BBV) infections compared to the general population. HIV is more prevalent in the prison population as are rates of other BBVs such as hepatitis B and C.^{5,6} In the United States, it was estimated that in 1997, 20–26% of people living with HIV in that year passed through a correctional facility.⁷ Similar data from the Republic of Ireland estimated that between 1987 and 1991, 168 known HIV-infected prisoners had been incarcerated in Dublin’s Mountjoy prison, constituting 16.6% of the total known HIV-infected population.⁸ At present, prisoners are not routinely tested for HIV

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infection at prison entry. The current Irish Prison Healthcare Standards (2011) recommend that all persons who have a background history with risk factors for any infectious disease should be offered any available screening for that condition. Previous work in 1999 by Long et al.⁶ sought to determine the prevalence of hepatitis B, hepatitis C and HIV and risk factors in entrants to Irish prisons. The prevalence of antibodies to HIV infection at that time was estimated to be 2%. Prior to our initial testing in 2010, it was estimated only 10% of the prison population of Wheatfield were aware of their HIV status.

Inmates also tend to have barriers that result in underutilization of ART such as mental illness⁹ and substance use disorders.¹⁰ Considerable stigma regarding HIV has been documented amongst inmates and staff in correctional facilities.^{11,12} The prison system represents an important venue for delivering a variety of HIV services (including testing, treatment and organisation of follow-up care in the community post-release). Collaboration between clinical and prison personnel is an important component of service delivery.¹³ Provision of these services can lead to improvement in the health of inmates, family members and communities into which they return.

Since 2007 a HIV and sexually transmitted infection (STI) consultant-delivered in-reach service has been provided by the Department of Genito Urinary Medicine and Infectious Diseases (GUIDE) at St James's Hospital, Dublin. This service serves Cloverhill Prison (a closed, medium security prison for adult males; daily occupancy – 388 inmates) and Wheatfield Prison (a closed, medium security place of detention for adult males and for sentenced 17-year-old juveniles; daily occupancy – 431 inmates). The in-reach service provides testing for STIs and BBVs, as well as assessment and management of STIs and chronic diseases such as HIV, hepatitis B and hepatitis C. Mountjoy Prison is the main committal prison for Dublin city and county and is also a closed medium security facility with an operation capacity of 554 inmates.

Methods

In collaboration with the prison schools, health care facilities, prison authorities and Irish Red Cross inmate volunteer groups in all three Dublin male prisons, the GUIDE clinic developed a campaign with the objective of raising awareness of HIV, educating prisoners about HIV and tackling HIV stigma. The end of the awareness and education campaign was marked by the offering of HIV point-of-care testing to prison inmates on a large scale over a short period of time.

The point-of-care testing was planned to mark important dates in the HIV calendar: Irish AIDS Day

2010 in Wheatfield prison, Irish AIDS Day 2011 in Cloverhill prison and Irish AIDS Day 2013 in Mountjoy prison. Prior to testing in all three prisons, HIV medical and social work teams from the GUIDE clinic, working with the prison schools and inmate volunteers from the Irish Red Cross, provided education and raised awareness about HIV in an effort to reduce stigma and promote awareness of HIV and encourage HIV testing. This practical application was linked to the classroom learning that the inmate volunteers were receiving as part of their Red Cross Community Based Health and First Aid (CBHFA) course.

In 2009, Ireland became the first country in the world to have prisoners as volunteers of its national Red Cross Society, the Irish Red Cross. These volunteers provide peer-to-peer health awareness and education in all of Ireland's 14 prisons as part of the CBHFA course and were very active in the HIV testing campaigns. The inmate Irish Red Cross volunteers organised group learning sessions and used multimedia materials including DVDs, posters and flyers for educational purposes with their peers within the prison landings. Information regarding limitations of the test and testing dates was disseminated. In all Irish prisons, the inmate Irish Red Cross volunteers are important community advocates in all relevant areas of health and well-being because of the power of peer-to-peer education.

In the run up to rapid HIV testing days, the volunteers did cell leaflet drops to estimate acceptability and uptake of HIV testing. In all three institutions returned leaflets suggested that large-scale rapid HIV testing would be acceptable with anticipated high uptake rates. Multiple planning meetings took place between prison authorities and health care personnel to ensure safe, efficient and confidential HIV testing. Prisoners were grouped and tested by level of security risk. Interview data were collated from discussion with prisoners prior to and on the testing days.

Health care staff involved in testing included members of medical and nursing staff from the GUIDE clinic and the Irish Prison Service. All health care personnel involved in testing received training on HIV, HIV testing, the tests being used (with particular emphasis on the window period for third-generation HIV tests) and the process for giving results, and worked in line with locally developed standard operating procedures. Inmate Irish Red Cross volunteers had no involvement in the clinical aspect of this project or access to any confidential information.

The Hexagon HIV test (Human, Wiesbaden, Germany) was used in testing in Wheatfield and Cloverhill prisons. This is a third-generation immunochromatographic rapid test for the detection of antibodies to HIV 1 and 2. A finger prick sample of whole blood (20 µl) is collected into a pipette and placed on a sample window leading to a

test result in 5–20 min. The test was chosen to minimise waiting times and expedite prisoner flow during the testing process.

The OraQuickADVANCE® Rapid HIV-1/2 Antibody test (OraSure Technologies) was used during the testing in Mountjoy Prison. Testing was performed on oral fluid samples. Volunteers emphasised the need for nil orally (including washing teeth and mouth wash) for 30 min before testing.

Once test results were available, every individual received their test results individually and confidentially.

In the event of a reactive test or a persistent invalid test result, a serum sample was obtained for conventional HIV testing and the results were made available to all individuals within 24 h. Any individuals testing positive on conventional testing were to be linked with the HIV in-reach services within 48 h for appropriate support and medical assessment.

Testing was performed utilising a one-way system to ensure rapid and efficient inmate flow. Following an informal group discussion led by the medical social work team from GUIDE regarding HIV and testing, inmates were led to the testing waiting area. Following

testing, they were led to the results waiting area, where interview data were obtained from inmates in small groups using open-ended questions. Inmates were asked a variety of questions including reasons for testing, anxieties regarding testing, prior and current knowledge of HIV and the stigma surrounding it and asked for general comments on the information dissemination and testing procedures. Once results were available, they were brought into individual rooms to obtain results and discuss any concerns. Inmates were then escorted back to their landing (see Figure 1).

Results

From interview data prior to testing, it was apparent that prisoners had little knowledge regarding HIV testing, treatment and prevention and that the subject had always been somewhat taboo and rarely openly discussed. Following the campaign, it was evident from interviews and informal discussion that knowledge of HIV had disseminated throughout the prison and many simple but important misconceptions regarding HIV had been clarified.

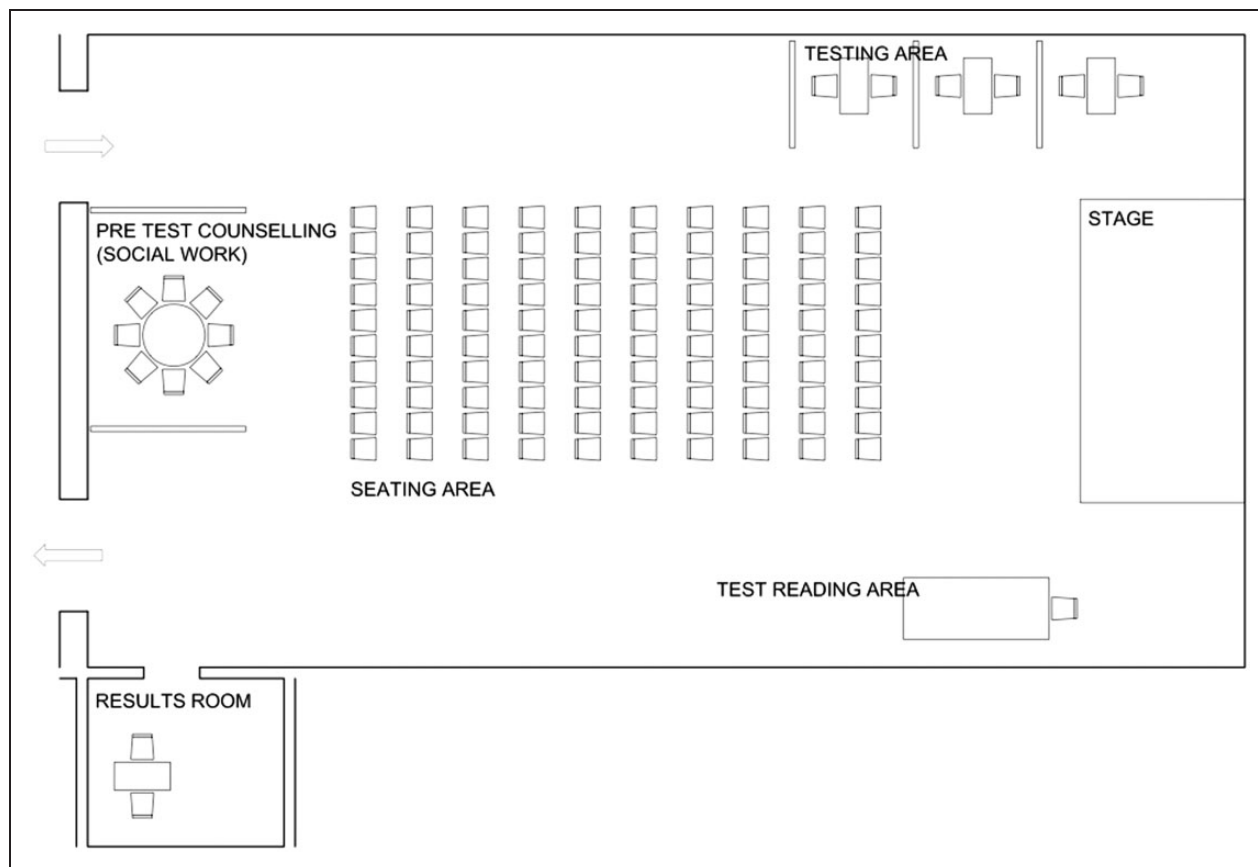


Figure 1. Figurative description of the one-way system for testing in Wheatfield prison.

Prisoners found the information campaigns ‘informative’, ‘well publicised’ and ‘definitely reduced stigma’. Prisoners said they were ‘more aware of HIV now’ that it was a ‘treatable’ condition and ‘not the end of the world’. Prisoners stated that HIV was ‘not really understood before this’ and was previously ‘a very taboo subject’. It was expressed that it was ‘better to know that you had it and not spread it’. Prisoners also stated they would ‘offer support to anyone who tested positive’. Prisoners now talk about HIV and openly request to be tested with the ethos that it is preferable to be aware of your status. During the campaign, HIV-positive inmates came forward and advocated testing uptake. All these measures served to reduce stigma regarding HIV.

In total, 741 inmates were tested in three major Irish prisons. One reactive HIV result was recorded. A total of 43 inmates had persistent invalid test results on point-of-care testing requiring formal laboratory serum testing for HIV. Four false-positive results were obtained on point-of-care tests. All four tested negative on formal laboratory testing.

In 2010, over four days of testing in Wheatfield prison, 248 inmates (50% of the prison population) were tested. A total of 35 inmates required laboratory HIV testing due to invalid results. No positive HIV results were recorded.

In 2012, 139 inmates attended for testing over two days in Cloverhill prison. Testing of 33 inmates resulted in invalid test results. All inmates were offered a further point-of-care test; however, two inmates refused further testing. Following the repeat point-of-care test nine inmates had an invalid second test. Eight inmates consented to formal laboratory serum HIV testing, while one inmate refused further testing. All eight inmates tested negative for HIV. One inmate had a reactive rapid test and subsequently tested positive for HIV. He was immediately linked with the in-reach HIV services.

In 2013, testing was carried out over two days in Mountjoy Prison. At the time, 536 prisoners were in custody. Forty-five were excluded due to a known HIV-positive status or a recent viral screen. A total of 354 (72%) of a target population of 491 were tested. Four inmates had a reactive result on the screening test, all of whom subsequently tested negative following formal laboratory serum testing. An overview of testing from all three prisons is outlined in Table 1.

Discussion

We demonstrated that an awareness campaign coupled with large-scale point-of-care testing for HIV in the Irish Prison Service is achievable. Work performed by the Irish Red Cross raising awareness of HIV and

Table 1. Summary overview of testing in the three prisons.

	Wheatfield	Cloverhill	Mountjoy
Year of testing	2010	2012	2013
Total prison population	495 ^a	433 ^a	536
Number tested	248	139	354
Invalid tests resulting in formal HIV testing	35	8	0
False positive results	0	0	4
Positive results	0	1	0

^aNumber obtained from the ‘average daily occupancy’ for the respective prisons in the respective testing year (Source: www.irishprisons.ie).

reducing stigma prior to testing at all three sites served to encourage the prison community to attend for rapid HIV testing and counseling.

A total of 741 prisoners were tested in total and one positive result was obtained. We were surprised that there were not more positive results. There are several limitations to our study.

As security is of paramount importance in the prison setting, we did go to individual cells for testing of some of the high-security prisoners. This had an impact on timing and efficacy of the testing process.

HIV testing was by antibody testing only, which may have missed prisoners who had recently acquired HIV infection and were in the window period. This is of particular importance in the remand setting where individuals frequently come from a period of chaotic drug use to the prison setting.

A number of prisoners declined HIV testing making it difficult to estimate the true prevalence of HIV infection in the prison setting, although this was not an objective of our study. Reasons for refusal were not recorded. No data were collected regarding previous testing for HIV, sexual practices or intravenous drug use.

A large number of invalid tests were recorded with the Hexagon HIV test, necessitating repeat point-of-care testing in a number of prisoners, resulting in delays in our testing schedules and requirement for formal laboratory serum testing of 43 prisoners in total. Three prisoners declined further testing following receipt of an invalid result. Reasons for the invalid tests are unclear, but were felt to be secondary to poor technique with the pipettes and difficulty obtaining the required blood volume for the test. As technique improved, the number of invalid tests declined.

Four false positive results were obtained with the OraQuick *ADVANCE*[®] Rapid HIV-1/2 Antibody test. The reasons for this are also unclear, but may have been due to inadvertent oral care product use or food and drink consumption within 30 min prior to testing.

Given the transient nature of the prison population, routine opt-out testing would be most appropriate for all prisoners; however, challenges remain in resources and linking patients to care both in prison and the community.¹⁴

Future studies should look to ensure continued and sustained surveillance for HIV in this high-risk population, coupled with surveillance for other BBVs, and rates of uptake of preventative measures such as hepatitis B vaccination. We have demonstrated the importance of peer education and collaborative work in achieving large-scale point-of-care testing in the prison setting.

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