Protective immunity in hepatitis C virus infection

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PROTECTIVE IMMUNITY IN HEPATITIS C VIRUS INFECTION

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Primary hepatitis C virus (HCV) HCV infection is associated with viraemia which induces cellular and humoral immune responses in the majority of individuals, regardless of subsequent outcome. HCV-specific immune responses have also been documented in subjects who appear to have never had infection, including in injecting drug users who are at high-risk of infection. These immune responses may be protective, either against incident infection, or against the development of chronic infection.

Minute amounts of HCV RNA at levels below detection by conventional assays have been detected in subjects who have cleared HCV infection. This 'occult virus' infection may also occur in high risk injecting drug users, and may therefore provide the antigenic stimulus for the observed immune responses.

This study examined the presence of HCV-specific cellular immune responses in a cohort of high-risk, seronegative, aviraemic prisoners using interferon-gamma enzyme linked immunospot assays. Detailed behavioural analyses to identify potential correlates of the presence of these immune responses, and with incident infection were undertaken. In addition, initial efforts to establish an ultra-sensitive, nested reverse-transcription polymerase chain reaction to detect both vegetative and replicative HCV RNA strands in high-risk apparently uninfected subjects in this cohort were undertaken.

A significant prevalence of HCV-specific cellular immune responses and a high rate of incident infection were found. There was a significant association of measured cellular immune responses with injecting crystal methamphetamine, and surprisingly a negative association between incident infection and tattooing. There was no reduction in the incident infection rate in those with HCV-specific immunity.

The studies in this thesis have demonstrated, HCV-specific cellular immune responses in a large cohort of high-risk seronegative subjects. The potential association with occult infection requires further assay development and investigation. The biological significance of the immune responses in protection requires further investigation.

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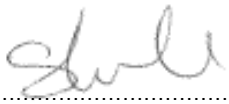

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ABSTRACT

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Minute amounts of HCV RNA at levels below detection by conventional assays have been detected in subjects who have cleared HCV infection. This 'occult virus' infection may also occur in high risk injecting drug users, and may therefore provide the antigenic stimulus for the observed immune responses.

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A significant prevalence of HCV-specific cellular immune responses and a high rate of incident infection were found. There was a significant association of measured cellular immune responses with injecting crystal methamphetamine, and surprisingly a negative association between incident infection and tattooing. There was no reduction in the incident infection rate in those with HCV-specific immunity.

The studies in this thesis have demonstrated, HCV-specific cellular immune responses in a large cohort of high-risk seronegative subjects. The potential association with occult infection requires further assay development and investigation. The biological significance of the immune responses in protection requires further investigation.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PRESENTATIONS & PUBLICATIONS DURING CANDIDATURE

Ratnarajah S, Teutsch S, Cameron B, Li H, Lloyd AR. Protective immunity against hepatitis C in high-risk IDU prison inmates. Oral presentation at Australian Centre for HIV & Hepatitis Virology Research Fourth Annual Workshop 2008

Post JP, **Ratnarajah S**, Lloyd AR. Immunological determinants of the outcomes from primary hepatitis C infection. *Cellular and Molecular Life Sciences* 2009 66(5):733-756

Sugden P, Pham T, **Ratnarajah S**, Cameron B, Bull R, White P, Michalak T, Lloyd A. Detection of low-level hepatitis C virus in leucocytes of individuals who are apparently uninfected. Abstract at HCV 2010 – 17th International Meeting on Hepatitis C Virus and Related Viruses. Japan

Sugden P, Pham T, **Ratnarajah S**, Cameron B, Bull R, White P, Michalak T, Lloyd A, HITS investigators. Two centre, masked replication study of occult hepatitis C virus in peripheral blood of high risk seronegative subjects who are apparently uninfected. Poster presentation at HCV 2011 – 18th International Symposium on Hepatitis C & Related Viruses, Seattle, USA.

Sugden P, Pham T, **Ratnarajah S**, Cameron B, Bull R, White P, Michalak T, Lloyd A, HITS investigators. Rare occurrence of occult hepatitis C virus in apparently uninfected injecting drug users. *Journal of Infectious Diseases* (in preparation).

Sugden P, Cameron B, Keoshkerian E, **Ratnarajah S,** Lloyd A. Hepatitis C-specific CD4 and CD8 T cell responses and protection against infection in high risk injecting drug users. *Journal of Hepatology* (in preparation).

1 INTRODUCTION

1.1. Hepatitis C virus

1.1.1. Introduction

Hepatitis C virus (HCV) infection affects 2%, or nearly 130 million, of the world's population [1, 2]. Acute infection is often asymptomatic but commonly progresses to chronic infection with ongoing hepatic inflammation in up to 80% of cases [3]. Over time the resultant fibrotic liver injury leads to the development of cirrhosis and an increased risk of hepatocellular carcinoma (HCC) [4, 5], even in the absence of cirrhosis [6]. These outcomes are associated with a substantive economic impact [7, 8], including in relation to liver transplantation for which HCV-associated complications remain the commonest indication [9, 10]. No effective vaccine exists, and although antiviral treatment is available, it is associated with considerable side-effects and incomplete curative success [11]. Pharmacological treatment in the acute setting reduces progression to chronicity [12]. However, this is complicated by the often asymptomatic nature of acute infection, and the ethical issues surrounding exposing patients to side-effects when clearance may have occurred without pharmacological intervention. The factors associated with spontaneous clearance after primary infection remain incompletely understood, although female gender, symptomatic disease, alcohol use, non-black ethnicity and the HLA DRB1*01 and HLA B857 have been positively associated, [13-18]. Coinfection with human

immunodeficiency virus (HIV) has been negatively associated [14, 15, 19]. One study has shown an association with Aboriginal ethnicity and spontaneous resolution of infection [19]. Over the last two years polymorphisms in the gene coding for interleukin-28B (IL-28B) have been shown to be associated with increased clearance after anti-viral treatment in subjects with chronic infection with genotype one infection [20-22]. These polymorphisms have also been shown to be associated with spontaneous clearance [23]. Improved understanding of these factors, especially those of the host immune response, will guide vaccine development and ultimately reduce the disease burden.

It should be noted that the experimental work upon which this thesis is based was undertaken between 2007 and 2008. However, the literature reviewed below covers studies published up to late 2011, and therefore includes a period of three-years after experimentation was completed.

1.1.2. Virology

HCV is the only member of the genus *Hepacivirus* in the family *Flaviviridae* [24]. It is an enveloped viral particle consisting of a single, positive strand of ribonucleic acid (RNA) which encodes a polyprotein of 3010 amino acids [25]. This is processed by host proteases into four structural proteins (Core, p7 and the Envelope glycoproteins E1 and E2) (Figure 1.1). In addition virally-encoded proteases (non-structural (NS)2-3 and NS3-4A) cleave the polyprotein into six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The latter are involved in virus assembly, release and replication [25, 26].

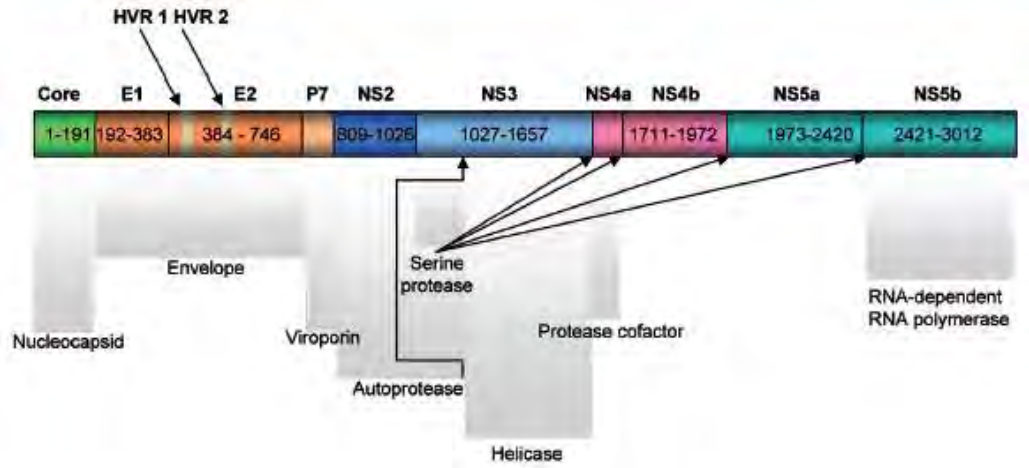

FIGURE 1.1. HCV polyprotein and protein function. From Post et. al [27]

1.1.3. Replication

HCV has no stable genomic intermediate and requires continual production of viral RNA and proteins in the cellular cytoplasm. The half-life of the HCV virion has been estimated at 3-5 hours, with a clearance and production rate of about 10^{12} particles per day. Based on the assumption that 10% of hepatocytes are infected, viral production occurs at a rate of 50 virions per hepatocyte per day [28, 29].

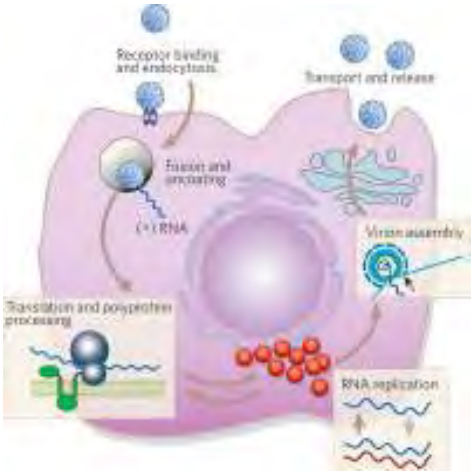

FIGURE 1.2. HCV replication cycle. From Lindenbach et al[30]

Replication occurs via NS5B, which is an RNA-dependent RNA polymerase. The activity of this enzyme is modulated by interaction with the helicase, NS3 as well as NS5A [25]. During transcription a negative strand of RNA is synthesised from the single positive strand genome (Figure 1.2). This serves as the template for synthesis of further positive strands. Detection of negative strand RNA in cells or tissues is therefore taken to indicate replicating virus (see further below – Occult Virus section 1.6). Lack of proof-reading ability in the RNA-polymerase causes poor fidelity and a high mutation rate, particularly in the hypervariable region 1 (HVR1) of the E2 gene [28, 29, 31, 32].

1.1.4. Genotypes

There are six, distinct genetic strains of HCV, or genotypes, which are further divided into over 50 closely related isolates termed subtypes (e.g. 1a, 1b) [33]. The high mutation rate produces a diverse range of more closely related sequences within these subtypes within an infected host, referred to as a quasispecies [26]. Further genotypes, 7-11, have been proposed, but it is thought that they would be more accurately classified as further subtypes of genotype 3 and 6 [34, 35].

1.1.4.1. Pharmacological treatments

Combination therapy with pegylated-interferon and ribavirin has been the standard of care since the early 2000s when pegylation of interferon was shown to increase SVR rates over standard interferon and ribavirin alone [36, 37]. Successful resolution of chronic infection after conventional pharmacological treatment with pegylated-interferon and ribavirin occurs in up 60 - 82% in those infected with genotypes 2 and 3, compared to just 27 – 52% in those infected with genotype 1 [38-40].

The treatment options for genotype 1 disease have increased markedly in the last two years with the licensing of boceprevir and telaprevir, which inhibit the NS3/4A protease [41], with more 'direct-acting agents' (DAAs) expected to become available in the next several years. Triple therapy regimens with boceprevir include a four-week 'lead-in' with conventional combination therapy followed by addition of boceprevir, whereas teleprevir-based regimens involve starting the three agents together and stopping teleprevir at week 12. Current recommendations for those who have not previously received antiviral therapy are triple therapy for up to 48 weeks with consideration of a shortened treatment duration in those without cirrhosis who have undetectable HCV RNA at weeks 8 and 24 for those treated with boceprevir, and weeks 4 and 12 for those treated with teleprevir [41].

1.1.5. Epidemiology

Prevalence estimates vary between geographic regions but are limited by the lack of systematic data collection in many countries (Figure 1.3). Egypt has the highest reported seroprevalence at 22% [42]. This has been attributed to a national schistosomiasis vaccination scheme conducted from the late 1950s to the early 1980s, which used inadequately sterilised glass syringes - thereby resulting in cross-infection [43]. Rates are also high for Africa and Asia, with lower prevalences in industrialised nations [44]. The UK and Scandinavia have the lowest rates (0.01 – 0.1%) with that for Australia reported as 1.1% [2, 45]. The asymptomatic nature of acute infection, inability of available assays to distinguish acute from chronic infection, and the lack of systematic data collection in most countries has made accurate estimations of incidence very difficult. In Australia, it has been estimated as 16,000 new cases per year, based on annual surveillance datasets at the end of 2000 [46].

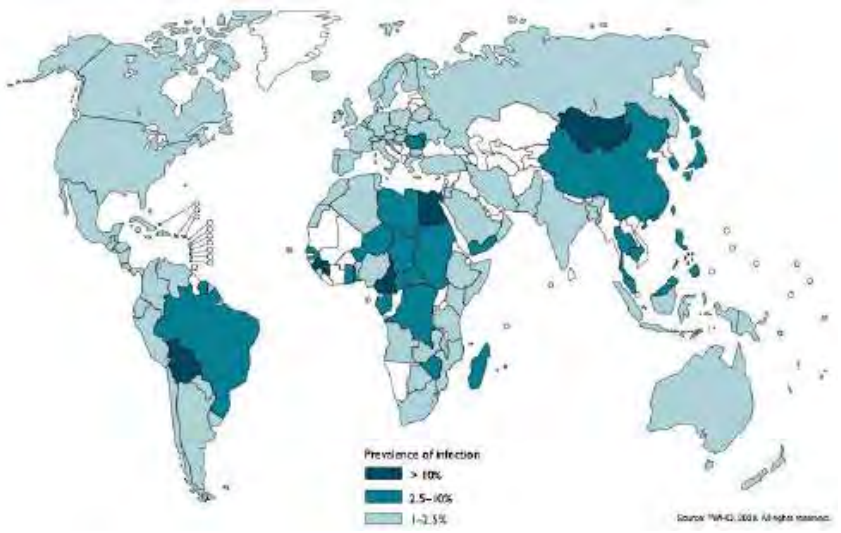

FIGURE 1.3. Worldwide prevalence of hepatitis C. From World Health Organisation 2008

The age-specific prevalence also varies, even between countries with similar overall prevalence. In the US, the prevalence is highest in those aged 30 – 49, with lower rates in those younger than 20 years and older than 50 years, suggesting two peaks of transmission, with a similar pattern observed in Australia [45, 47]. However in Turkey, Spain, Italy, Japan and China, the highest prevalence is in those aged over 50 years [2]. Chronic liver disease develops many years to decades after initial infection, and thus in countries such as the US and Australia, the burden of HCV-related morbidity and mortality has yet to peak [7, 44, 48].

1.1.6. Transmission

Humans are the only natural host, although chimpanzees are successfully infected for research purposes. Transmission is parenteral (*i.e.* blood to blood), with large volume or repeated exposure to infected blood increasing the risk of transmission, likely due to a higher 'infective dose' [2]. This is most commonly due to re-use and sharing of

contaminated equipment during injection drug use (IDU) in the Western world, and to sub-optimal healthcare in the developing world, including transfusion of unscreened blood products [2, 44]. The latter has become a very rare mode of transmission in developed nations since the introduction of antibody and nucleic acid screening [49].

Transmission can occur both to, and from, patients during surgery. The risk is thought to be low, even in areas of relatively high prevalence [50, 51]. However in one case, a surgeon diagnosed two years after operating on an infected patient, had transmitted HCV to 10 of 270 (3.7%) subsequent patients [52].

Perinatal transmission occurs due to mucosal and blood contact during delivery in 4 – 7% neonates born to mothers with HCV viraemia [53]. Co-infection with (HIV) has consistently been shown to increase risk, although this may be decreased in those mothers receiving antiretroviral therapy [54-57]. Most, but not all, studies demonstrate an increased risk with higher maternal viral loads, but reported associations with the method of delivery and prolonged rupture of membranes are not consistent [53, 55, 58, 59].

Other reported transmission routes include tattooing and fights involving blood-to-blood contact, ritual scarification, circumcision and even a human bite [60-63]. The risk of transmission is low, but possible, from sharing household objects such as toothbrushes and razors that may potentially be contaminated with blood [64].

Sexual transmission is generally thought not to occur through the heterosexual route [65]. One study of 967 monogamous, discordant couples with a 10-year follow-up found no evidence of intra-spousal transmission [66]. However sexual transmission, through the per-mucosal, rather than percutaneous, route in HIV-infected men who have sex with men is now recognised [67, 68]. The prevalence in this group is increasing, with rough sexual practices, increased number of sexual partners and IDU

being associated with increased risk of acquisition [67, 69]. More recently, a case-report identified one subject whose only risk-factor for acquisition of acute hepatitis C was vaginal intercourse, without clear disruption of mucosal integrity, with a HCV-positive former IDU partner [70]. Phylogenetic analysis demonstrated identical genotype 3a strains, suggesting that heterosexual transmission is possible, although likely to be very rare.

1.1.6.1. Transmission in IDU

HCV is the most common blood-borne viral infection affecting IDU [71]. Reported prevalence rates in this group vary dramatically both within, and between, geographic regions [72]. The prevalence in Australian IDU has been estimated between 50 – 88%, with IDU accounting for 80% of existing Australian cases, and 90% of new infections [46, 72-74]. Increased HCV prevalence has been associated with duration of injecting drug use, HBV infection and HIV infection [75]. Screening data from a prospective cohort study of IDU in three sites in New South Wales including south-western Sydney found that incarceration in the last year, duration of injecting, injecting in public and the presence of hepatitis B core antibody were positively associated with incident infection [74]. Recent initiates into IDU also had a higher incidence, which is supported by other studies, one of which showed a rate as high as 67% in this subgroup [76-79]. Other studies have documented that the incidence is highest in women, daily injectors and younger injectors [79-82]. A prospective study of IDU aged under 30 years found a positive association with pooling money with another IDU to buy drugs, and exchanging sex for money [83]. The few studies that have looked into the particular drug injected do not show concordant results. A positive association with use of heroin, over cocaine or amphetamines, was

demonstrated in a study of IDU recruited via outreach programmes in New Mexico [84]. By contrast, a study of IDU in Sydney found a positive association with cocaine, but not heroin, use [79].

IDU have previously often been excluded from antiviral therapy, mainly due to concerns regarding reinfection, compliance and concomitant alcohol use, all of which can adversely affect response rates. Information regarding treatment response is lacking as IDU are often excluded from trials. A review of treatment studies in this group found that none of the ten studies that compared IDU to non-IDU found a statistically significant difference between the rate of SVR between the two groups [85]. In addition, pre-treatment abstinence from IDU for six months or more did not have a significant impact on SVR attainment in two studies [86, 87]. Despite this, uptake of treatment remains low, with data from the annual Australian survey at needle and syringe programs reporting that between 2001 and 2007, only 0.9 – 2.4% of those who knew they were infected with HCV receiving treatment [72, 85].

Clearly interventions targeting high-risk behaviour in IDU are essential to limit ongoing transmission in this high-risk group. However, as reviewed above, there are some conflicting data, and no large-scale, detailed, behavioural analysis to guide potential measures has been carried out.

1.1.6.2. Transmission in prisoners

Imprisonment has been independently associated with HCV infection [88, 89]. This is primarily due to the close association between IDU and imprisonment for drug-related crimes, but associations between incident infection episodes with tattooing, fights involving blood-to-blood contact, anal sex and even reuse of barber's shears have been reported [90-93]. Almost half of all prison inmates in Australia report a

history of IDU and up to 90% of IDU have been imprisoned [94, 95]. Screening over one year in a prison in Paris detected no incident cases of HCV [96]. However a recent report from the Hepatitis C Incidence and Transmission Study (HITS) cohort (upon which the research for this thesis is based) which is a prospective study of 488 inmates with a history of IDU over 19 prisons in New South Wales, found an incidence of 31.6 per 100 person years, that is one in three IDU inmates became infected every year [95]. Positive associations were found with previous imprisonment, receiving methadone maintenance treatment (MMT) (potentially via being a surrogate marker for high risk behaviour), tattooing and daily injecting in the three months prior to incarceration. Previous imprisonment has been shown to be positively associated in other studies. In combination with the high incidence rate, this data highlights the importance of prisons as an ongoing reservoir for HCV infection and transmission [97].

1.2. Infection Outcomes

1.2.1. Introduction

HCV RNA can be detected by polymerase chain reaction (PCR) as early as two weeks after infection [98]. Serum alanine aminotransferase (ALT) levels may increase acutely, sometimes to more than ten times the upper limit of normal [99]. This is followed by 'seroconversion,' that is presence of antibody to HCV, detected by enzyme immunoassay, approximately six to seven weeks later [100]. Non-specific symptoms such as malaise and anorexia can occur, but acute symptomatic hepatitis occurs rarely [101, 102]. Chronic infection is defined as infection lasting more than six months, and occurs in 50 – 85% of cases [3]. If HCV infection resolves, HCV

RNA becomes undetectable, however the antibody remains positive for years to decades [103, 104].

Chronic HCV infection results in ongoing hepatocellular damage and inflammation, and accumulation of fibrosis - the rate of which varies widely between individuals. Estimates of progression to cirrhosis after 20 years ranged from 4 – 22% in a systematic review [105].

Some people develop viraemia without contemporaneous seroconversion [61, 106-110]. This has mainly been described in immunosuppressed subjects such as organ-transplant or haemodialysis patients, although it has also been described post-occupational exposure. In one study of 19 IDU undergoing seroconversion, HCV RNA was detectable in five subjects at low, intermittent levels for a mean of 40.8 months (range 13 to 94 months) before anti-HCV antibody became detectable [111]. In animal studies, one chimpanzee remained viraemic in the absence of an antibody response for 5 years post-inoculation [112].

Estimates of spontaneous clearance in acute infection vary, with the ability to perform accurate, prospective studies with large numbers hindered by the asymptomatic nature of primary infection. A systematic review of longitudinal studies involving almost 700 subjects estimated the rate of spontaneous clearance to be 26% [16]. Symptomatic infection is associated with a higher rate of clearance, with the symptoms likely to be due to more pronounced liver injury as a result of a more aggressive immune response [113]. The above mentioned review reported female gender, but not viral genotype, to be associated with clearance [16]. Alcohol consumption was associated with a lower rate of clearance in a study of 77 subjects [114]. Spontaneous clearance of chronic infection is reported, especially in those with

a low viral load, but becomes increasingly less likely with over 24 months post infection [115-118].

Clearance has traditionally been thought to reflect complete viral eradication, however some studies have shown low levels of HCV RNA in subjects thought to have cleared the virus, leading to the concept of 'occult' HCV infection (See further below - section 1.6) [119-121].

1.2.2. Reinfection

Unlike many other viral infections, clearance does not protect against reinfection, either with a homologous or heterogenous strain [122, 123]. However, data in both humans and chimpanzees suggests that those who have previously cleared the virus successfully are less likely to develop chronic infection than those infected de novo [123-127]. Most studies of reinfection in humans have been conducted in IDU. A prospective study of 262 IDU, 98 of whom had previously cleared infection, found 12% of the previously infected group became infected, compared with 21% of the previously uninfected group [124]. In addition, average HCV RNA levels in those who had previously cleared infection were 2 logs lower and the time to infection was significantly longer. A prospective study of 35 IDU who had achieved sustained viral clearance (SVR) after antiviral treatment, detected two cases of possible reinfection after a mean follow-up of two years [127]. If only the 16 who reported ongoing drug use were considered, this gave a reinfection rate of 5.3 per 100 person-years. Sequencing of the two isolates was not possible for one subject, therefore it is possible that the detected viraemia represented relapse rather than reinfection. However, the second subject was reinfected with a different genotype (1b) to that of their initial infection (3a) and subsequently cleared the second infection

spontaneously. A low rate of reinfection in those with ongoing exposure to HCV has been supported in another study, however clearance in this study was defined as one negative HCV RNA result as opposed to the conventional two results six months apart [128].

It is important to note that these studies predate the understanding of the association of IL28B polymorphisms with spontaneous clearance [23]. Therefore it is possible that there was an unwitting selection bias as the favourable polymorphism is likely to have been over-represented in the subjects who had previously undergone spontaneous clearance. In fact, in contrast to the above, the opposite finding was reported in a prospective study of IDU in metropolitan Melbourne [129]. Ten incident infections occurred in the 55 participants with no prior infection, translating to 15.5 cases per 100 person years, compared to 23 infections in the 50 who had successfully cleared previous HCV infection, corresponding to 46.8 cases per 100 person years. HCV Core region sequences were examined in those reinfected with the same genotype to exclude recrudescence as opposed to true reinfection. The time interval between HCV tests was shorter than in previous studies (median of 99 days compared with 15.6 months in [128]), and therefore episodes of reinfection followed by prompt clearance were less likely to be missed. Importantly however, high-risk behavioural factors were not analysed and may have been significantly different between the two groups. A similar finding was reported in a retrospective study in Sydney, with an incidence rate of HCV reinfection of 31 cases per 100 person years compared to 17 per 100 person years in naïve infection, however this was not statistically significant [130].

1.3. Seronegative-immune phenotype

The presence of HCV-specific cellular immune responses has been described in those with no evidence of seroconversion [131-142]. Many of these subjects are in high-risk groups, such as IDU, and hence one possible explanation is that these individuals become infected transiently but clear the virus rapidly and efficiently and hence do not remain viraemic for sufficient time to develop antibody seroconversion, or alternatively that prompt seroreversion occurs. Another alternative is that the observed HCV-specific cellular immune response is actually due to cross-reactive epitopes. For instance, a well characterised homologous epitope has been identified in the NS3 region of HCV and the Influenza virus neuraminidase [143].

A study of HCV-PCR and antibody negative subjects in a community in the Nile delta found those at high risk, defined as those with at least two household members who were anti-HCV antibody positive, were more likely to demonstrate cell-mediated immune responses to HCV peptides than those with no household members infected [134]. Thirteen of the 71 high risk individuals (18.3%) mounted an interferon-gamma (IFN-γ) Enzyme-Linked Immunospot (ELISpot) response to at least one of the seven peptide pools spanning 72% of the HCV polyprotein, - compared to just one of the 15 low risk subjects (2.9%). A further Egyptian study of aviraemic siblings of chronically infected children found IFN-γ ELISpot responses to at least one of the three peptide pools in 12 of the 45 (26.7%) tested [135]. In addition, the magnitude of the response was larger than that of the five known seropositive children tested. Similar cellular immune responses have been shown in spouses, household contacts, healthcare and laboratory workers, as well as uninfected children born to HCV-infected mothers [132, 136-139].

HCV-specific cellular immunity in the absence of apparent infection has also been reported specifically in prisoners and IDU [131, 133, 140, 141]. The most recent study looked at 43 IDU that had injected for less than ten years, making traditional seroreversion (which generally takes years) an unlikely explanation [133]. Inclusion criteria included injecting drug use at least once in the 30 days before enrolment. All were aviraemic, 26 were HCV-antibody negative and 17 were positive. Of the seronegative subjects, 12 (46%) had IFN-γ responses to at least one of the 21 peptide mixes spanning the entire genome. Six of these had intermediate to very strong responses to 10-20 of the pools. Ten of the 17 seropositive (but RNA-negative) subjects (59%) mounted a response with no significant difference in frequency or strength between the two groups.

This phenomenon may plausibly mirror the cellular immune response seen in sex workers in Nairobi who were heavily exposed to HIV, but who remained seronegative and aviraemic for HIV [144-147]. Interestingly, the duration of prostitution was associated with a decreased risk of seroconversion and an increased detection of HIV-1 specific CD8+ responses [144, 146]. Sadly, taking a break from prostitution actually increased the subsequent risk of incident HIV infection.

One possible mechanism for this phenomenon of seronegative immunity is that a low infective dose is sufficient to stimulate a rapid cellular immune response, followed by rapid viral clearance. This is supported by a study of 10 healthcare workers followed for up to 32 months after occupational exposure, thought to be associated with a low infective dose [142]. None became HCV RNA or antibody positive. CD4+ T-cell responses were detectable in two subjects at the time of injury and became detectable in a further three during follow-up, with transient CD8+ T-cell responses seen in two subjects. Additionally, work in chimpanzees has demonstrated cellular immune

responses in the absence of viraemia or antibody response in previously unexposed animals challenged with just one RNA virion [148]. An infective dose of ten virions resulted in a borderline, short-lived antibody response in both animals challenged, with 100 virion exposures causing a clear humoral response.

1.4. The immune response

1.4.1. Introduction

The definitive characteristics of a successful immune response against acute HCV infection remain unclear. Chronic infection can develop even in the presence of a seemingly strong immune response to acute infection. Those who clear incident infection are not protected against subsequent infection, but may experience an attenuated disease course and have a higher rate of clearance than those infected *de novo*. Possible immunological factors associated with clearance are reviewed below.

1.4.2. Innate immunity

Innate immune responses to viral infection generally include both cellular response components via natural killer (NK) cells and phagocytic cells (such as monocyte-macrophages) and cytokine production, particularly the type 1 interferons (IFN)-α and –β [149, 150]. The importance of the innate immune system in clearance of HCV has been illustrated in a study of four experimentally infected chimpanzees, two of which cleared infection, both without evidence of a HCV-specific T-cell response [151]. In addition, several components of the HCV polyprotein have been shown to alter innate immune system function, however this has largely been studied *in vitro* [152-154].

1.4.2.1. Interferon

The double-stranded RNA (dsRNA) produced during viral replication is recognised as a pathogen-associated molecular pattern (PAMP), initiating IFN synthesis [155]. Type-1 IFNs activate the adaptive immune system and also act via specific receptors to trigger the production of more than 300 IFN-stimulated genes (ISGs) through activation of Janus kinase-signal transducers and activators of transcription (Jak-STAT) [156]. These inhibit critical steps in viral replication [149].

Growing evidence for the importance of innate immunity in determining outcomes from HCV infection has been provided firstly by the recent demonstration of the association between clearance of primary infection and polymorphisms in the IL-28B (IFN-λ) gene [23, 157], and secondly with patterns of the interferon-stimulated gene (ISG) profile in the liver [158-160].

Recognition of extracellular dsRNA occurs through Toll-like receptor 3 (TLR3) and that of intracellular dsRNA by retinoic-acid inducible gene I (RIG-I) [161, 162]. RIG-I is a cytosolic protein which after binding with dsRNA undergoes a conformational change [155]. This allows association and activation of a mitochondrial antiviral signalling protein (MAVS), by 'caspase activation and recruitment domains' (CARDs) present in both proteins [155]. MAVS is also known as 'virus-induced signalling adaptor' (VISA), IFN-β-promoter stimulator 1 (IPS-1) and 'CARD adaptor inducing IFN-β [156]. Activation of MAVS causes phosphorylation and activation of IFN-regulatory factor (IRF) 3 by either TANK-binding kinase-1 (TBK-1) or IKKε, allowing IRF3 to translocate to the nucleus [155, 156]. IFN-β is induced by the cooperation of activated IRF3 with nuclear factor kappa B (NF-κB), another transcription factor activated downstream of MAVS.

Extracellular dsRNA from cell lysis binds to TLR3 which signals through Toll-IL-1 receptor adaptor protein (TRIF), again stimulating IRF3 and NF-κB, resulting in IFN-β synthesis. This process involves RIP1, TBK1 and TRAF6.

The importance of class III interferons in control of HCV infection is being increasingly recognised. Class III interferons, also known as interferon-lambda 1-3 (IFN-λ), display IFN-like activities but exert their action through a distinct receptor complex consisting of the IL-10 receptor 2 subunit and a unique subunit called the IFN-λ subunit [163-165], which is limited in expression to epithelial tissues [166]. The IFN-λ receptor gene is preferentially expressed on primary hepatocytes in normal liver. Although type I and III interferons act via distinct receptor systems, they activate the same signalling pathway and induce common ISGs [164, 167-169]. IFN-λ1 and IFN-α have been shown to induce equivalent levels of 2',5'-OAS and MxA gene expression in this cell type [170]. Interferon-λ has been shown to be induced by viruses including single-stranded RNA viruses [171].

Interferon-λ has been shown to inhibit replication of subgenomic and full-length genotype 1B HCV replicons in Huh-7 cells [169, 172]. This inhibition of replication has been shown to be both dose- and time-dependant in both genotype 1b replicons and genotype 2a cell-culture virus in Huh-7.5 cells [173]. The effect of IFN-α was more pronounced at the same concentration, and co-treatment with both IFN- α and IFN-λ in combination enhanced the antiviral activity. Cotreatment with IFN-α and IFN-λ has also been shown to have a synergistic effect on Jak-STAT activation [169]. STAT-1 phosphorylation in response to IFN-λ was unaffected by the presence of blocking antibodies to both the IFN-α and IFN-γ receptors, demonstrating that signalling through the type I and type II IFN receptors is not necessary for the IFN-λ-mediated STAT-1 phosphorylation [173]. Additionally, the patterns of STAT1 and

STAT2 phosphorylation, key mediators in the type I and II IFN signal transduction pathways, exhibited different characteristics in response to IFN-α and IFN-λ: phosphorylation of STAT1 and STAT2 after IFN-λ treatment occurred faster, peaked earlier and was eliminated more rapidly compared with the pattern seen after IFN-α simulation. In addition, mircoarray studies indicated that IFN-λ induced activation of most of the genes induced by IFN-α, and although the kinetics differed, after 12 hours, there were no genes induced by IFN-α that weren't also induced by IFN-λ [173].

HCV disrupts the interferon response at multiple levels and the relative contribution of each strategy is unclear. The NS3/4A protease has been shown to cleave MAVS [174-177]. Expression of NS3/4A in replicon cells demonstrated disruption of the molecular complex between the MAVS adaptor and IKKε, causing relocation from the mitochondria to the cytosol, thereby ablating ongoing signalling [176]. Further work in the replicon system has shown that in very high concentrations, pharmacological NS3/4A protease inhibitors can restore IRF-3 signalling in HCV-infected cells [178].

In addition, NS3/4A has been shown to cause proteolysis of TRIF in cell-free translation reactions, and accelerate degradation of TRIF in replicons, resulting in disruption of the TLR3 pathway [179]. This is thought to be due to a similar sequence to the HCV polyprotein NS4B/5A cleavage site.

NS3 has been shown to directly bind to TBK1 in the replicon system, leading to inhibition of the association of TBK1 with IRF3, and subsequent phosphorylation and activation of IRF-3 and IFN-β promoter activity [180].

Double-stranded-RNA-dependent protein kinase R (PKR) is an ISG which autophosphorylates in response to dsRNA, and subsequently phosphorylates its substrates, one of which is the α subunit of the eukaryotic translation factor 2 (eIF2α),

leading to a downstream general inhibition of viral protein synthesis [181, 182]. In addition, PKR activates IRF-1, resulting in transcription of ISGs [183]. Mutations within the discrete IFN- sensitivity determining region of NS5A have been shown to block PKR phosphorylation during RNA replication, disrupting induction of IRF-1 induced genes [152, 184].

The structural components of the HCV polyprotein have also been shown to interfere with the innate immune response. E2 has also been reported to bind to the kinase domain of PKR and inhibit IRF-1 activation [154]. The Core protein has been shown to induce suppressor of cytokine signalling (SOCS)-3 in hepG2 replicons, disturbing the Jak-STAT pathway by inhibiting activation and nuclear translocation of STAT1 [185]. IL-6 stimulation of the Core protein prevented phosphorylation of JAK1/2 and STAT3, and STAT3 mediated transcription, however the effect on phosphorylation of JAK1/2 and STAT1 and STAT1-mediated transcription was increased under IFN-γ stimulation [186]. The downstream effect is not clear, with earlier work showing that although expression of the Core protein did modulate components of the Jak/STAT signalling pathway, expression of the downstream effector genes IRF-1 and 561 remained unaffected [187]. Induction of SOCS-3 by the Core protein has been shown in further work in hepG2 replicons and Huh-7 cells [188, 189]. It was demonstrated in the replicons that expression of the full length HCV genome resulted in stronger STAT3 activation than the Core protein alone [188]. The Core protein was also shown to dose-dependently reduce expression of insulin receptor substrates (IRS) 1 and 2 in hepG2 replicons and Huh-7 cells, [189]. This downregulation of IRS1 and 2 by the Core protein was observed in the presence of primary mouse embryonic fibroblast cells from wild-type (SOCS3 +/+) mice, but not those derived from SOCS3 knockout mice [189].

Although these interactions have been shown *in vitro*, their significance *in vivo* needs to be evaluated in animal and human studies.

1.4.2.2. Natural killer cells

NK cells make up 5 – 20% of peripheral blood mononuclear cells but a larger proportion, 30 – 50%, of intrahepatic lymphocytes [190]. They can be divided into two phenotypic subsets, $CD56^{bright}$ and $CD56^{dim}$, depending on the level of expression of surface marker CD56 [191]. The latter make up over 90% of the circulating NK cell pool, express higher levels of killer cell immunoglobulin-like receptors (KIRs), Fc gamma receptor III (CD16) and perforin and have greater cytotoxic activity [191]. $CD56^{bright}$ cells express a high level of the inhibitory receptor CD94:NKG2A and generally do not express KIRs or CD16 [191]. Their primary function is cytokine production however they can be cytotoxic via expression of tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [192].

The antiviral functions of NK cells include direct cytotoxicity via degranulation of cytotoxic granules, surface expression of ligands such as Fas ligand and TRAIL that activate death receptors on target cells, and secretion of the T-helper 1 (Th1) cytokines IFN-γ and tumour necrosis factor (TNF)-α [193]. These cytokines prime the adaptive immune response and in addition, IFN-γ can have direct antiviral effects. NK cell effector functions are triggered when the signals from surface activating receptors exceed those from the inhibitory receptors [194].

Intrahepatic NK cells are often found in the Space of Disse adjacent to lobular sinusoids, and may be less responsive than NK cells elsewhere in the body [195, 196]. In a mouse model, liver NK cells displayed a dampened IFN-γ response to IL12/IL-18 stimulation compared to splenic NK cells [196]. In addition the liver contained a

significant population of functionally hyporesponsive NK cells that expressed high levels of the inhibitory receptor NKG2A. These cells were reduced in proportion by blockade of IL-10.

KIRs bind to their ligands, human leukocyte antigen (HLA) class I molecules, with varying affinity and specificity, leading to complex patterns of activating or inhibitory responses in NK cell sub-populations [197]. These interactions have been implicated in genetic association studies of acute HCV outcomes, however these data are complicated by the highly polymorphic nature of both the KIR genes and their ligands, leading to substantial diversity in this system [198]. Conclusions are therefore hindered by small sample sizes and different ethnic backgrounds in the reported studies. For instance, a weak inhibitory interaction between the KIR gene receptor 2DL3 and HLA-C1 has been shown to protect against chronic HCV infection in Caucasian subjects, with a more pronounced effect in those who reported IDU, rather than blood transfusion, as the mode of transmission [199]. This finding has been replicated [200]. Another study of 196 Caucasian Spanish subjects reported a higher prevalence of 2DL3 in those with persistence, compared to clearance (66.9% vs. 54.6%) [201].

An additional association was found between HCV clearance and the KIR receptor 2DL3 and the HLA class II alleles HLA-DRB1*1201 and –DQB*0501 [200]. A study in Puerto Rican IDU demonstrated a protective effect in HLA-C1 homozygotes of the inhibitory KIRs 2DL2 and 2DL3 in the presence of the activatory KIR2DS4 gene [202]. However another study of 146 subjects, 143 of whom had chronic infection, no significant association was found between inhibitory KIR genotypes and their HLA-C ligands and resolving infection [203].

1.4.2.3. Gamma delta T-cells

Gamma delta (γδ) T-cells are innate immune system cells with significant subset heterogeneity and functions that range from provision of help to conventional T-cells to antigen presentation [204]. In humans, these cells make up 1 – 10% of nucleated cells in peripheral blood, but are more abundant in epithelial-rich tissues such as skin, liver, intestines and reproductive tract where they can comprise up to 50% of T-cells [205, 206]. In the mouse thymus, strong signalling, generally considered a consequence of agonist-ligand binding, commits immature CD4-CD8- cells to the γδ lineage whereas weak signalling commits them to the αβ lineage [207, 208].

In humans, γδ cells are sub-divided into Vδ1 or Vδ2 depending on the variable regions of the T-cell receptor-δ [209], with the former found at mucosal surfaces and the latter in the peripheral blood [204]. A particular subset found only in humans and higher primates, Vγ9Vδ2+ cells, are unique in the ir ability to recognise low-molecular weight non-peptide phosphoantigens, enabling response to a diverse range of pathogens [210]. In addition, $T_{naïve}$, $T_{central\ memory}$ and $T_{effector\ memory}$ Vγ9Vδ2+ subsets have been identified, however it is unclear whether these are comparable to those subsets observed in conventional αβ T-cells [211].

In addition, despite being a component of the innate immune system, both Vδ1 and Vδ2 cells display remarkable functional plasticity, with the Vγ9Vδ2+ subset *in vitro* producing IFN-γ-secreting Th1-like cells in the presence of IL-12 and anti-IL-4 antibody, and generating Th2-like cells in the presence of IL-4 and anti-IL-12 antibody [212]. The same subset have been reported to express Foxp3 and display regulatory activity after activation with IL-15 and TFG-β [213]. Vδ1 cells frequently express CD8 and display a cytotoxic Th1-like phenotype with IFN- γ secretion [214], may express CD57 which correlates with high expression of perforin [215] and

upregulate expression of the natural cytotoxicity receptors NKp30, NKp44 and NKp46 upon activation in the presence of IL-2 or IL-15 [216, 217].

An in vitro study looked at the presence of γδ T-cells in 21 subjects with chronic HCV infection contained [218]. T-cell lines established from liver biopsy specimens contained significant numbers of T-cells expressing the γδ form of the TCR after cytokine exposure in nine of the 21 cases (43%), compared to none of the cell lines from the five controls of four subjects with non-viral hepatitis. A cell line that contained >15% TCRγδ+ T-cells was considered to be significant as the average percentage of these cell types in peripheral blood is about 5%. Interestingly the T-cell line from a subject co-infected with HCV and hepatitis B virus contained 85% γδ T-cells. In addition, T-cells lines from the liver tissue of eight chronically-infected subjects demonstrated high percentages of TCRγδ+ T-cells in three cases, compared to none of the cell lines derived from the PBMCs from the same eight subjects. γδ T-cell lines from HCV-infected individuals had high levels of non-MHC-restricted cytotoxic activity against different targets including primary hepatocytes, and produced IFN-γ, TNF- α and IL-8 following activation by anti-CD3 [218], in comparison to αβ T-cell lines which had little or no cytotoxic activity to any of the target cells tested. Interestingly, none of the γδ T-cell lines recognised any of the HCV proteins and had no cytotoxic activity against cells infected with recombinant vaccinia viruses expressing different HCV proteins, but cross-linking of CD81 which has been shown to bind HCV particles and E2, resulted in significant levels of IFN-γ and TNF-α production by liver γδ T-cells. These findings are in keeping with mouse models of other virus and parasitic infections, where γδ T-cell involvement was seen at the local site of infection and not in draining lymph nodes or spleen, emphasising the need to analyse tissue from the local site of infection [205].

1.4.3. Adaptive cellular immunity

Both CD4+ T-helper cells and CD8+ cytotoxic T cells have been shown to be important in successful clearance of HCV [27, 98, 219, 220]. The immunological features that result in clearance have not been fully elucidated, however studies suggest that clearance is more likely in those mounting a strong, broad and sustained response. Current data is limited by the relatively small number of subjects studied, and a bias towards subjects with symptomatic illness (which is associated with a higher likelihood of clearance, [113] as well as restriction of the analysis to peripheral blood responses only (that may not accurately reflect intrahepatic responses where the virus primarily resides) [221].

1.4.3.1. CD4+ T cell responses

CD4+ T-helper cells predominantly differentiate into either a Th1 or Th2 subtype. Although this dichotomy is most apparent in the mouse, in humans, Th1 cells generally secrete IL-2, IFN-γ and TNF-β which promotes effector responses of both CD8+ cells and the innate immune system, specifically NK cells and macrophages. By contrast Th2 cells promote humoral immune responses and secrete other cytokines (IL-4, IL-5, IL-6 and IL-10) [222, 223]. Effective antiviral cellular immune responses feature induction of the Th1 subtype [224]. Th1 cells directed against HCV are identified by (IFNγ ELISpot) assays using recombinant HCV proteins or longer synthetic peptides (15 – 20 amino acids in length) to preferentially stimulate CD4+ (as opposed to CD8+ responses), or with proliferation assays .

The breadth of the CD4+ response has consistently been shown to be associated with clearance [225-232]. In a prospective study of 34 subjects with acute infection, five of the eight (63%) subjects who subsequently cleared the virus had a CD4+ T-cell

response directed against at least three of the seven peptide pools tested by IFN-γ ELISpot, compared to only eight of the 22 who developed chronic infection [226]. However, one subject who went on to clear infection demonstrated no detectable response, implying that this may not be essential for clearance.

A cross-sectional study of 22 subjects with resolved infection, using 301 overlapping peptide (20-mers) spanning the entire HCV polyprotein found that 89 of the 301 peptides were targeted by at least one subject (range 3 – 28 peptides targeted) [230]. In comparison, only seven of the 23 subjects with chronic infection showed one or more positive responses with a mean of one peptide targeted (range 0 – 8). Another cross-sectional study examining proliferative responses to Core, NS3, NS4 and NS5 showed that the 14 subjects who had cleared HCV infection mounted a strong and multi-specific response to all of the proteins whereas the 14 subjects with chronic infection responded weakly or not at all [233]. These findings are supported by work in other smaller human studies, and also in chimpanzees [229, 234].

The magnitude of the CD4+ T-cell response has also been associated with clearance with a prospective study in 31 acutely infected subjects, using an IFN-γ ELISpot assay with 33 peptide pools, showing that those who displayed more that 390 spot-forming units per 2.5 x 105 CD4+ T-cells had an eight-fold increase in the likelihood of clearance than those who did not reach this threshold [232]. Another prospective study found that the number of CD4+ T-cells producing Th1-cytokines in response to NS3 and NS4 proteins was higher in the three subjects who cleared infection rapidly than in the seven who developed chronic infection or the six with transient clearance, only one of whom eventually cleared infection [235]. These findings have been supported by other studies in humans [225, 228, 231, 233], and chimpanzees [234]. However, it should be noted that one study of five acutely infected chimpanzees

showed no difference in the CD4+ T-cell proliferative responses in the peripheral blood regardless of outcome, whereas there was a correlation between the intra-hepatic CD4+ T-cell responses and clearance [221].

The non-structural proteins appear to be preferentially targeted by the CD4+ responses in those who clear infection [225, 229, 230, 232, 233, 235-237]. An early cross-sectional study of proliferative responses in 29 subjects with chronic infection and 15 with resolved infection showed that significant responses to NS4 and NS5 (and Core) antigens were more frequently detected in latter group, although this was not statistically significant [237]. No difference in response to the Envelope antigens was observed between the two groups. Another cross-sectional study of proliferative responses to an overlapping panel of 20-mer peptides spanning the entire HCV polyprotein showed that at least three of the six non-structural proteins were targeted by all 22 subjects who cleared infection, with less frequent responses against the Core protein and variable regions of the Envelope protein [230].

In all 22 cases, one or more epitopes on NS3 were targeted, which is a consistent finding, suggesting that this protein may be immunodominant [231, 235, 238]. A study examining IFN-γ ELISpot responses to three peptide pools spanning NS3 (15-mers overlapping by 11 amino acids), found that all ten subjects that had recovered from infection mounted a strong CD4+ reponse to all three pools [231]. Similarly another cross-sectional study using 750 overlapping peptides (15-mers) that covered the entire genome in 33 peptide pools found that the 25 subjects with resolved infection displayed on average twice as many CD4+ responses as the 25 subjects with chronic infection. Of these, NS3-specific responses comprised 31% of the cumulative magnitude of the overall response in those with resolved infection compared with

22% in chronic infection. After normalisation for the relative length of the HCV proteins, NS3 remained the most immunogenic [225].

The kinetics of the onset of the CD4+ immune response has also been studied, with a strong initial response suggested to be important [235]. A prospective study of 20 subjects with acute infection found that the number of Th1 cytokine-producing CD4+ cells was highest in the first 12 weeks in the subjects with rapid viral clearance compared to those with transient or no control of viral replication [235]. However, both human and chimpanzee studies have demonstrated a rebound in viraemia and progression to chronic infection even in some of those who initially mount a strong response [221, 239]. In a study of five healthcare workers with acute infection post-needlestick injury, four developed chronic infection despite a strong initial CD4+ response with concomitant control of viraemia [239]. In addition, the strongest CD4+ T-cell response has been shown to occur within 6 months of acute infection, regardless of outcome [228, 235, 236], suggesting that in addition to being early, strong, multi-specific and targeting the non-structural proteins, a successful HCV-specific CD4+ immune response needs to be sustained to achieve viral clearance.

1.4.3.2. CD8+ T-cell responses

CD8+ T-cells act by direct cytolysis of infected cells, and secretion of antiviral cytokines such as IFN-γ and TNF-α [240]. HCV-specific responses are generally measured via IFN-γ ELISpot assays using shorter peptides (8 – 11 amino acids) than those used to assay CD4+ responses [27]. This approach has largely superseded the traditional cytotoxic T-lymphocyte (CTL) assay which measures CD8+ T-cell killing of target cells expressing HCV proteins, but has a lower sensitivity [241]. As the precursor frequency of HCV-specific T-cells in the peripheral blood is low, some

studies have examined responses in cell lines after expansion *ex vivo* using HCV antigens or non-specific stimuli, however the *in vivo* relevance of this assay is uncertain. Enumeration of antigen-specific T-cells is also possible in flow cytometry by detection with class I tetramers [27], although this requires knowledge of the HLA genotype and putative epitope, and the precursor frequency of HCV-specific cells in very low.

Similar to CD4+ cell responses, the breadth and kinetics of the CD8+ T-cell response appears to be important in the control of acute HCV infection. Results regarding the frequency of CD8+ T-cells have been discordant. One cross-sectional study found no difference in the number of HCV-specific IFN-γ producing CD8+ cells between seven subjects with resolved infection and 14 with chronic infection as determined by ELISpot [242]. However other studies have shown that HCV-specific CD8+ cells are more common in those with chronic infection, raising the possibility that ongoing antigenic stimulation is necessary to maintain CD8+ T-cell responses [233, 243, 244]. Additionally, CD8+ cells may have a weaker proliferative capacity in subjects with chronic infection, as suggested by a cross-sectional study that detected HCV positive tetramer cells more frequently in the unstimulated PBMC of 20 subjects with chronic infection, but more frequently in stimulated cell cultures from the 12 with cleared infection [244].

A cross-sectional study of cytotoxic T cell responses using 13 peptides from Core, E2 and non-structural epitopes found that five of the seven (71%) subjects with resolved infection had responses directed against one or more peptides, compared to only four of the 14 (29%) with chronic infection [242]. In a longitudinal study of acutely infected IDU, the four who cleared infection had IFN-γ ELISpot responses to a median of five epitopes (range 4 – 8) assessed by overlapping peptides spanning the

entire HCV polyprotein, compared to a median of four (range 0 – 10) in the 15 who developed chronic infection [243]. One subject demonstrated responses to seven peptides at day 138 with transient control of viraemia, however the response narrowed down to four peptides and chronic infection ensued.

Other human studies have also documented that the strongest CD8+ responses occur early in infection with a subsequent decline in both magnitude and breadth [245, 246]. This is supported by a prospective study in five chimpanzees which showed that the animal that cleared infection mounted an early and multi-specific peripheral blood CD8+ T-cell response as determined by IFN-γ production in response to seven of the 68 peptides tested, with response seen in Core, E2 and non-structural proteins [221]. This response was sustained for at least 68 weeks. An animal that transiently cleared the virus also showed a persistent response to eight of the peptides, with much less vigorous responses detected in the other three animals, one of which was also aviraemic.

The kinetics of this arm of the cellular immune response also appear to be important. In a chimpanzee study, the animals that cleared infection had an earlier intra-hepatic IFN-γ response than those that developed chronic infection [247]. The delay in the immune response to HCV may reflect the preferential intra-hepatic location of the virus, with HCV antigens remaining unavailable to antigen-presenting cells until the infection is established, by which time rapid evolution of the viral quasispecies may outpace the cellular immune response. Longitudinal analysis of a known immunodominant epitope on NS3 in samples from a patient treated unsuccessfully during acute infection demonstrated evolution of the sequence, with none of the wild-type sequence detectable by week 60 [248]. Other studies support the role of viral escape mutants [249, 250]. For instance, a prospective study found no substitutions in

T-cell epitopes for a year after infection in the subject who cleared viraemia [250]. However, substitutions were observed in 69% of T-cell epitopes in the seven subjects with viral persistence and T-cell responses, with at least one substitution in every subject. In addition, substitutions occurred 13-fold more often within than outside T-cell epitopes. It has been hypothesised that rapid viral evolution produces sequences similar to the initial epitope, stimulating immune cells that recognise the old epitope further instead of priming new responses [243].

1.4.3.3. Interaction between CD4+ and CD8+ T-cells

In chronic viral infection, helper CD4+ T-cells help maintain the effector functions of cytotoxic CD8+ T-cells, via co-stimulatory pathways and the production of cytokines (IL-2 and IFN-γ) [251, 252]. The few studies of this aspect of the immune response in HCV infection lend support to its importance [232, 253]. For example, CD4+ depletion prior to re-infection of two immune chimpanzees resulted in persistent, low-level viraemia, despite functional intra-hepatic memory CD8+ T-cell responses [254]. Additionally, purified HCV-specific, central memory (CCR7+) CD8+ T-cells from subjects with acute infection showed poor effector function *ex vivo*, but proliferated efficiently and differentiated *in vitro* when supplemented with IL-2 [255]. This is supported by other work [232, 233].

1.4.3.4. Regulatory T-cells

CD4+CD25+ T-cells comprise 2.5% of CD4+ T-cells in the peripheral blood [256]. The regulatory T-cell (T-reg) subpopulation which make up a significant proportion of CD4+CD25+ cells have the capacity to suppress the proliferation of both CD4+ and CD8+ T-cells, and are further subdivided into two main categories – those that

occur naturally and those that are induced by infection or other immunological challenge [257, 258]. Treg may play a role in the outcome of HCV infection, with higher numbers detected in subjects with chronic infection than in those who have cleared previous infection [258, 259].

The functional effects of Treg on HCV-specific immune responses have been studied *in vitro*. The addition of autologous CD4+CD25+ Treg cells to CD4-depleted PBMC of chronically infected subjects impaired the expansion of HCV-specific CD8+ cells after seven days of peptide stimulation [258]. In the same study, tetramer staining of CD4-depleted PBMC after culture with decreasing numbers of CD4+CD25+ Treg cells resulted in a dose-dependent suppressive activity. Similarly, depletion of CD4+CD25+ cells increased the HCV-specific IFN-γ ELISpot activity in samples from subjects with chronic infection [259].

However, a study of 27 acutely infected subjects found no significant difference in the proportion of CD4+CD25+ T-cells in the peripheral blood at baseline between the 15 subjects who developed chronic infection and the 12 that did not [260]. Although definition of the Treg phenotype in this study did not include the markers forkhead transcription factor 3 (foxp3) or low level interleukin-7 receptor (CD127) expression, both of which are responsible for the suppressive function of Treg [261, 262], functional activity was comparable between the two groups in early infection. Similarly work in two HCV-naïve, eight recovered and six chronically infected chimpanzees detected CD4+CD25+foxp3+ Treg cells were present in both recovered and chronically infected animals [263]. Interestingly, HCV-specific IFN-γ ELISpot responses to overlapping peptides after CD25+ depletion was particularly increased in the animals that had recovered from multiple, sequential infections compared to those that had recovered from a single infection or remained persistently infected [263].

1.4.3.5. Programmed-death receptor-1 (PD-1)

The PD-1 receptor molecule and its ligands PD-L1 and PD-L2 function as a co-stimulatory pathway to inhibit T-cell activation [264]. Expression of PD-1 has been linked to CD8+ T-cell 'exhaustion' in lymphocytic choriomeningitis virus (LCMV)-infected mice, [265, 266]. Blockade of the pathway *in vitro* has been shown to improve T-cell responses in this model, and in HIV [266, 267]. Upregulation of PD-1 has been shown to be induced by HCV Core protein [268], and *in vitro* blockade of the PD1/PD-L1 pathway in subjects with chronic infection enhanced CD8+ T-cell proliferation and cytokine production, although to a varying degree between subjects [269, 270].

A cross-sectional study found that the HCV-specific CD8+ T cells in the peripheral blood of 31 subjects with chronic infection had markedly increased PD-1 expression compared to the 11 who had previously cleared infection [269]. Another cross-sectional study supported this finding for both CD4+ and CD8+ T-cells [264]. In addition, higher expression of PD-1 was found in the liver biopsy samples of the three chimpanzees that failed to clear HCV re-challenge after administration of an experimental vaccine, than in the one animal that cleared [271].

However, a prospective study of ten subjects with acute infection found a high level of PD-1 expression on HCV-specific CD8+ T-cells during the acute phase of infection, regardless of outcome [272]. Expression remained high in those who developed chronic infection, but decreased in those who cleared it.

1.4.4. Humoral Immunity

Antibodies defend the host against pathogens within the extracellular space, with effector mechanisms for viral clearance including neutralisation, complement

activation, opsonisation and antibody-dependent cell-mediated cytotoxicity [27]. The role of the humoral immune system in control of HCV infection remains unclear, partly due to the delay in development of suitable *in vitro* assays to measure neutralisation of infectivity.

Current assay systems include use of recombinant HCV-envelope glycoproteins, HCV-like particles, HCV-pseudotyped particles and cell-culture derived infectious HCV [273]. HCV-like particles are generated by the self-assembly of the HCV structural proteins in insect cells, and have been shown to bind and enter human hepatoma cells as well as primary hepatocytes and dendritic cells [274-276]. Retroviral HCV-pseudotyped particles consist of unmodified HCV envelope glycoprotein E1 and E2 assembled onto retroviral or lentiviral core particles [277]. Cells are transfected with expression vectors encoding the full length E1/E2 polyprotein, retroviral or lentiviral core proteins and a packaging-component retro- or lentiviral genome carrying a marker gene [273]. These have been shown to infect Huh-7 cells and human primary hepatocytes [277]. Vesicular stomatitis viruses/HCV (VSV/HCV) pseudotypes have also been developed and unlike those developed from retroviruses, can also infect a broad range of mammalian cell lines, in addition to human hepatoma cell lines [278].

More recently, an infectious HCV clone derived from a viral isolate of a Japanese patient with fulminant hepatitis C, termed JFH-1, has been developed [279-281]. In addition to Huh-7.5.1 cells (a cell-line derived from Huh-7.5 cells which is more permissive for HCV genotype 1 replicon replication), non-HCV-adapted Huh-7 cells were also successfully infected with this genotype 2a strain. [279-281]. These infected cells secreted infectious HCV particles which successfully infected other Huh-7 derived cell-lines [279, 281] but not Hep-G2, HeLa or HEK293[281]. In

addition, naïve chimpanzees inoculated with JFH-1 HCV particles developed HCV infection [282].

The available evidence predominantly suggests that anti-HCV antibody responses alone are insufficient to control acute infection, and that clearance is not dependent on antibody responses, reviewed in [220]. Nevertheless, it is likely they have a role in clearance. A potentially successful humoral response is likely to involve a rapid development of antibodies, an IgG2 subclass predominance and preferential targeting of the hypervariable regions of the HCV genome [234, 283-285].

In general, the antibody response generated in HCV infection is generally of low titre, IgG1 predominant, and with the exception of responses against the Core protein, is delayed [286, 287]. A study in chimpanzees showed that in the first eight weeks post-infection, there was a delay in the appearance of antibody responses with an average of 0.66 +/- 0.3 optical density units/ml (Ortho HCV 3.0 ELISA test system, Ortho diagnostic system, NJ) in the four animals with chronic infection compared to 4.059 +/- 7.138 units/ml in the two that recovered, however the difference in this semi-quantitative antibody level was not statistically significant [234]. The peak of the humoral response was delayed by up to 150 weeks (range 50 – 150 weeks) in the chronically-infected animals compared to 16 – 24 weeks in those that recovered. A higher titre of anti-HCV antibodies was observed at 50 – 150 weeks post-infection in the four with chronic infection.

Similarly, in 26 subjects with acute post-transfusion HCV infection, the prevalence of E2 antibodies in the first month was significantly higher in the eight who resolved infection than in those who did not [288]. The prevalence was similar between the two groups thereafter. The early generation of an anti-HVR1 antibody response was also associated with viral clearance in a small study of acute infection in five subjects

with chronic renal failure on haemodialysis [289]. In another study of 51 subjects infected by the same HCV-contaminated anti-D immunoglobulin, the early appearance of anti-HVR1 antibodies was associated with clearance [290]. Antibodies with this specificity were also produced by the subjects that developed chronic infection, but at a later timepoint.

The humoral response to HCV infection is often restricted to the IgG1 subclass without the switching to IgG3 or IgG4 subclasses that typically occurs with maturation of the response [286]. There may be an association between the development of IgG2 antibodies and clearance, possibly linked to a Th1 bias in CD4+ T-cell responses [291]. In a small study, IgG2 responses against Core and NS3 antigens were more frequent in subjects with clearance in the four subjects who cleared infection, compared to the 23 with persistent infection [283]. In addition, the ratio of IgG2 to IgG1 antibodies against Core and NS5 was greater than one in those who cleared infection, whereas it was less than one for all antigens in those who did not.

Neutralising antibodies are important both for the control of acute infection and protection against reinfection. Antibodies with likely neutralising capacity have been documented in humans and chimpanzees, however the absence of reactivity across the quasispecies and delayed production suggest that they are unlikely to efficacious in viral control *in vivo* [98, 292].

Antibody mediated neutralisation upon re-exposure has been demonstrated for homologous strains for chimpanzees *in vitro* [32]. Neutralising antibodies have been demonstrated to target the HVR1 region of the E2 protein. The importance of this aspect of the humoral response is suggested by the fact that this area undergoes a high rate of mutation in acute infection, and mutations in this regions have been associated

with the evolution of quasispecies leading to escape from neutralisation [284, 285, 293]. In addition, a reduced rate of mutation has been shown in hypogammaglobulinaemic individuals [294], and the peak of viraemia was inversely correlated with anti-E2 antibodies in a chimpanzee vaccination model [295]. However, it should be noted that some animals cleared viraemia in the absence of significant antibody titres, and the anti-E2 titre was lower in women who cleared HCV after infection via contaminated anti-D immunoglobulin than in those who did not [296]. However this was not assessed acutely and may have been a consequence of the lack of ongoing viral replication (rather than the cause).

The limited studies of neutralising antibodies in acute HCV infection using the HCV-pseudotyped particle methodology have demonstrated conflicting results [297-304], likely to reflect that autologous viral sequences were not utilised. Further longitudinal studies in subjects with acute infection and using autologous viral Envelopes are required to resolve the role of neutralising antibodies in viral control in acute HCV infection.

1.5. Diagnostic tests for HCV

1.5.1. Serologic assays

The diagnosis of HCV is dependent on detection of antibodies via serology or RNA via nucleic-acid based molecular assays [305]. Antibodies are detected via enzyme-linked immunoassays (EIAs) which involve the capture of HCV-specific antibodies in the serum by recombinant HCV proteins, which are then detected by secondary antibodies against IgG or IgM. Second generation EIAs detected antibodies against epitopes from the Core, NS3 and NS4 region, with the third generation assay

including an antigen from the NS5 region and a more highly immunogenic NS3 epitope [306]. The third generation assay allows detection of anti-HCV antibodies six weeks after infection with a sensitivity of 99% [307].

The specificity of the EIA is hard to define as there is no gold standard. False-positive results are more common in populations with a low HCV prevalence such as blood donors, and those positive for rheumatoid factor. False negative results can occur in immunosuppressed subjects, such as those with haematological malignancies and those on haemodialysis [306]. However latest generation tests perform better than older tests in these situations [308].

1.5.2. Nucleic-acid testing

Nucleic acid tests directly detect the presence of RNA using a combination of amplification and detection techniques [305]. They are classified into qualitative and quantitative tests, with the former traditionally having a higher sensitivity, however recent quantitative assays utilising real-time PCR are comparable [306, 309].

Standardisation of measurement units to International Units (IU) was mandated by the World Health Organisation. The IU represents the amount of HCV RNA in a sample rather than the actual number of vial particles [103]. A conversion table for some older assays which used viral genomic equivalents (or copies)/ml, to allow comparison between studies, is given (Table 1.1).

TABLE 1.1. Conversion factors from former non-standardised HCV RNA quantification units in older assays

Assay	Company	Technique	Equivalence of 1 IU/ml
Amplicor HCV monitor v2.0	Roche diagnostics	Manual competitive RT-PCR	0.9 copies/ml
Cobas amplicor monitor HCV v2.0	Roche diagnostics	Semi-automated competitive RT-PCR	2.7 copies/ml
Versant HCV v3.0	Siemens medical solutions	Semi-automated bDNA amplification	5.2 copies/ml
LCx HCV quantitative assay	Abbott Diagnostics	Semi-automated competitive RT-PCR	3.8 copies/ml
SuperQuant	National genetics institute	Semi-automated competitive RT-PCR	3.4 copies/ml

1.5.2.1. Qualitative assays

Qualitative assays use either reverse-transcription polymerase chain reaction (RT-PCR) or transcription-mediated amplification (TMA) (Table 1.2). In RT-PCR, HCV RNA is used as a template to synthesise single-stranded complementary DNA (cDNA) via reverse transcriptase. This is then amplified by a DNA polymerase to produce multiple double-stranded copies. The DNA polymerase used in the Amplicor HCV 2.0 assay (Roche diagnostics) is Thermus thermophilus, and provides both polymerase and reverse transcriptase activity, allowing a single-step, single-tube procedure to minimise contamination [310]. The assay has equal sensitivity for all genotypes.

TMA is a single-tube procedure, involving hybridisation of a T7-containing primer with HCV RNA, resulting in reverse transcriptase-mediated cDNA synthesis. The second primer then binds to the cDNA, synthesising a DNA/DNA double strand via the reverse transcriptase. The T7 promoter is then recognised by T7 polymerase, and produces 100 – 1000 RNA transcripts which are returned to the TMA cycle, creating exponential amplification of the target RNA. These amplicons are then detected using chemiluminescent probes [305, 306]. The Versant HCV RNA qualitative assay (Siemens Medical Solutions) provides a superior sensitivity to RT-PCR techniques with a lower detection limit of 5 – 10 IU/ml, a sensitivity of 96 – 100% and a specificity of 99.5%, across all genotypes [311-313].

1.5.2.2. Quantitative tests

Quantitative tests involve amplification of the target RNA via competitive or real-time PCR, or by amplification of the signal via branched-chain DNA (bDNA) assay [305, 306], (Table 1.3). The Cobas Amplicor HCV Monitor 2.0 (Roche diagnostics)

TABLE 1.2. Assays available for qualitative analysis of HCV RNA

Assay	Method	Lower detection limit (IU/ml)
Amplicor HCV v2.0 (Roche diagnostics)	Manual RT-PCR	50
Cobas amplicor HCV v2.0 (Roche diagnostics)	Semi-automated RT-PCR	50
Ampliscreen (Roche diagnostics)	Semi-automated RT-PCR	<50
Versant HCV RNA qualitative assay (Siemens Healthcare)	Semi-automated TMA	10

Adapted from Ghany et al 2009

involves amplification of two templates, the target, and an internal standard which is an internal control RNA with nearly the same sequence as the target RNA, with a clearly defined initial concentration [306]. The two templates are amplified by the same primers in a single tube. The initial amount of HCV RNA is calculated by comparison of the final amounts of both templates.

Branched DNA hybridisation only requires 50µl of serum, and involves reverse transcription with subsequent binding of the cDNA to immobilised capture oligonucleotides with a specific sequence from conserved regions of the HCV genome [314]. Multiple oligonucleotides bind to the free ends of the bound DNA strands and are then hybridised by multiple copies of an alkaline phosphatase (ALP)-labelled DNA probe. The ALP-bound complex is then detected by a chemiluminescent substrate.

Real time PCR allows simultaneous amplification and detection of the target RNA,

TABLE 1.3. Comparison of available assays for quantification of HCV RNA

Assay	Company	Technique	Lower detection limit (IU/ml)	Dynamic range (IU/ml)
Cobas amplicor monitor HCV v2.0	Roche diagnostics	Semi-automated competitive RT-PCR	500	500 - 5000,000
Versant HCV v3.0	Siemens medical solutions	Semi-automated bDNA amplification	615	615 - 800,000
Cobas Taqman HCV	Roche diagnostics	Real-time PCR	10	40 - 10,000,000
Real-time HCV test	Abbott diagnostics	Real-time PCR	12	12 - 10,000,000

which is achieved by the use of target sequence-specific oligonucleotides linked to a fluorescent reporter molecule and a quencher (of fluorescence) molecule at the opposite end of the probe [306]. These bind the target cDNA between the two PCR primers and are degraded by the DNA polymerase during DNA synthesis. This separates the reporter and quencher molecules, resulting in an increase in fluorescence which is intensified during each round of amplification and is proportional to the amount of RNA in the starting sample.

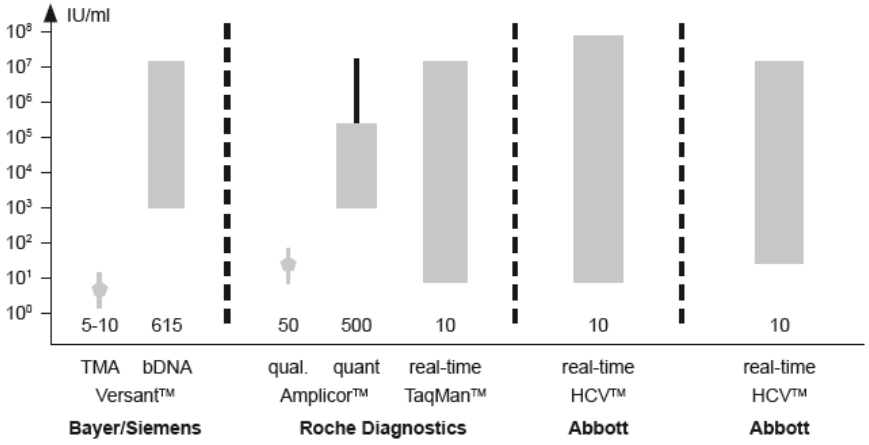

FIGURE 1.4. Detection limits and linear dynamic ranges of commercially available HCV RNA detection assays. From Mauss et al 2010

Both the Abbott diagnostics assay and the second version of the Roche diagnostics real-time assay are highly sensitive across all genotypes [315-319]. The detection limits of available quantitative assays are shown (Figure 1.4).

1.6. Occult HCV Infection

A consistently undetectable HCV RNA PCR in the diagnostic assays described above is generally accepted to represent successful resolution of HCV infection, either

spontaneously in primary infection or six months after antiviral therapy for chronic infection [320-322]. However over the last five years, evidence has emerged that residual HCV RNA at levels below that detectable by conventional assays may remain within the liver tissue of some subjects [119-121]. In addition, although HCV was initially thought to be purely hepatotropic, there is evidence of infection, and potentially also replication in cells of the immune system [323-328]. These extrahepatic sites may therefore also harbour residual, low levels of virus and thereby act as potential reservoirs for reactivation.

This phenomenon of 'occult HCV infection' was first described in subjects with persistently abnormal liver enzyme levels in whom all known causes of liver disease, including HCV, had been excluded [329]. Reverse-transcription polymerase chain reaction (RT-PCR) of 100 of these subjects with idiopathic hepatitis, detected intrahepatic HCV RNA in 57, with none detected in the 30 healthy control subjects. In situ hybridisation confirmed the presence of positive strand HCV RNA, and importantly negative strand RNA was also detected in 48 of the 57 (84.2%) implying active replication. The use of unrelated probes, omitting the probes, and predigestion of the slides with RNase abolished both positive and negative signals, lending weight to these representing valid results and not contamination. In addition, positive strand RNA was found by RT-PCR and confirmed by *in situ* hybridisation in the PBMCs of 40 of the 57 subjects with intrahepatic RNA, but not in the other subjects. Of these 40, thirty-five had also had negative strand RNA detected in liver tissue, however no correlation was found between intrahepatic presence of the replicative strand and infection of PBMCs. Sequencing of the amplicon to confirm HCV sequence and strand-specificity was not undertaken. Nevertheless, the potential clinical importance of this apparent ongoing infection was suggested by histological examination of the

liver biopsy specimens - higher necro-inflammatory activity and fibrosis scores were found in those with occult HCV infection compared to those without.

Further work using a strand-specific RT-PCR assay in 18 of the subjects with HCV RNA in their PBMCs found presence of negative strand HCV RNA in 11 (61%), implying that the PBMCs were not just a reservoir, but a site of active replication [330]. Sensitivity and specificity of the strand-specific RT-PCR assay were assessed using serial dilutions of synthetic HCV RNA positive and negative strands. Again the findings were confirmed with fluorescent in situ hybridisation (FISH) but not sequencing.

Lymphotropism of HCV is biologically plausible, given some of the extrahepatic associations of HCV such as non-Hodgkin's lymphoma and mixed cryoglobulinaemia, with resolution of the latter reported to accompany successful eradication of HCV [327, 331]. Some patients with cryoglobulinaemic vasculitis have no evidence of HCV infection, even when precautions are taken to minimise viral co-precipitation with cryoglobulins, and are classed as having true, essential cryoglobulinaemia [332]. Although anti-HCV treatment is not indicated in these patients, complete remission was obtained after interferon therapy in two HCV-negative patients, suggesting that they might have had underlying occult infection [333] (or an anti-vasculitic effect of IFN). Three other seemingly HCV RNA negative and anti-HCV antibody negative patients with cryoglobulinaemic vasculitis went on to demonstrate positive RNA results several months after the initial negative test, despite persistently negative anti-HCV antibody tests [334]. One patient had an acute infection with varicella zoster at the time of the first positive HCV RNA test, suggesting that an event such as brief immunosuppression may be sufficient for occult infection to become detectable by conventional assays.

Further support for lymphotropism comes from the detection of both positive and negative HCV RNA in B cells and T-cells, in addition to PBMCs, in patients with chronic infection [325, 335]. A recent study examined different immune cell subsets in seven subjects with chronic infection and seven with occult infection [336]. In both groups, positive and negative strand HCV RNA was found in CD4+ and CD8+ T-cells, B lymphocytes and monocytes, although different immune cells supported HCV to varying extents in different patients. In chronic infection, the highest quantities of viral nucleic acids were carried by monocytes, whereas in occult infection, this was found in B cells. Mitogen stimulation with phytohaemagglutinin (PHA) and interleukin (IL)-2 down-regulated both positive and negative strand RNA expression in those with chronic infection, and increased it in those with occult infection. In addition, when examined with confocal microscopy, the NS5A protein was detected in the different cell subsets in both patient groups.

Furthermore, bidirectional sequencing of the 5'-untranslated region (UTR) of clones from all investigated cell subtypes in two patients with chronic, and four with occult, infection has been reported. This region of the genome functions as the internal ribosomal entry site, is essential to viral RNA translation, and was chosen as it is highly conserved and thus would allow for reliable identification across genotypes and potential compartment-specific variants. Two of the single nucleotide polymorphisms detected in both occult and chronic infection had previously been described in the brain, dendritic cells and PBMCs from patients with chronic infection, lymphocytes in those with occult infection, and HCV passaged in lymphoblastoid cells and T-lymphocytes *in vitro* [121, 335, 337-340]. Even given the limitation of the small number of subjects evaluated, this finding lends support to the likelihood of the existence of extrahepatic HCV variants and their ability to propagate.

An immunologically important role for this putative reservoir, regardless of the presence of detectable HCV RNA in liver tissue and serum is supported by the demonstration of cellular immune responses against the occult virus. No HCV RNA was detectable in the PBMC of 11 of 20 subjects with cleared HCV infection, however it became readily detectable in these subjects, and was increased in the nine that had initially had detectable virus, after *in vitro* removal of CD8+ T-cells [341]. Removal of CD8+ T-cells also resulted in detection of negative strand RNA in three of the four subjects tested, all of whom had initially demonstrated presence of positive strand RNA. In addition, NS5 protein expression was inhibited in HCV replicon cells cultured with CD8+ T-cells. Finally, HCV replication was enhanced up to 30-fold in PBMCs from HCV-infected subjects that were treated with neutralising antibodies against IFN-γ, suggesting that endogenous antiviral cytokines may constrain replication of the occult virus.

Two groups have demonstrated evidence for occult virus in patients thought to have recovered from HCV infection [119, 121]. An in-house assay developed by one group with a sensitivity of ≤10 viral genomic equivalents/ml (IU not given), was used to examine PBMC, sera, and monocyte-derived dendritic cells in 16 subjects, including five subjects with spontaneous and 11 with treatment-induced resolution [119]. Briefly, this assay involved reverse-transcription of high quality, intact, total RNA from serum, plasma, PBMC or liver tissue, followed by a direct, and then a nested, round of amplification of the resulting cDNA by PCR using primers spanning different regions of the HCV genome, and then validation of the amplified products by hybridisation to a recombinant HCV DNA probe [328]. Positive strand RNA was found in either the sera, PBMC, or both, of all 16 individuals, and also in the dendritic cells in six of the seven tested. The negative (replicative) strand was detected in nine

out of 12 PBMC tested, including four subjects who attained a sustained virological response (SVR) after treatment five years previously.

Post-SVR liver biopsy specimens were available in 11 of the 17 subjects investigated by another group and HCV RNA was detected in three [121]. Importantly, the only two of the 11 biopsies that showed no histological improvement in fibrosis were both from subjects who were HCV RNA positive, again highlighting the potential clinical importance of occult virus. In addition, this was the first study to show that the genotype found in the liver was the same as that present in the serum prior to treatment. Real-time, strain-specific RT-PCR failed to detect negative strands in these liver specimens, though they may have been present at levels below the sensitivity of the assay which was able to detect 100 genomic molecules. Again HCV RNA was detectable in sera, lymphocytes and macrophages up to 9 years after SVR. In both studies, cells were stimulated with mitogens to increase RNA yield.

The virological relapse rate post SVR has been put as high as 8% in one study where subjects were followed for up to 8.8 years [322]. It should be noted however that sequencing to verify that the virus at relapse is related to that originally treated was not undertaken in this study – hence re-infection cannot be excluded and indeed is a more likely explanation.

Given the potential for the occult virus to cause an insidious chronic hepatitis, one group looked at the effects of antiviral treatment on subjects with idiopathic hepatitis and occult HCV [342]. Ten treatment-naïve subjects were treated with combination pegylated-IFN and ribavirin for 24 weeks. Three (30%) had an 'SVR' defined as those achieving both biochemical and virological complete response (i.e. normalisation of liver enzymes and undetectable HCV RNA in PBMC) at both the end of treatment and at follow-up 24 weeks later. One other subject who had

abnormal liver enzymes and detectable occult HCV RNA at the end of treatment achieved biochemical and virological response at the follow-up time-point. A further three had undetectable RNA in their PBMC despite persistently elevated enzymes.

Follow-up liver biopsies were performed in five subjects in this study - two complete responders, one virological responder (undetectable HCV RNA in PBMC at the end of treatment), one biochemical responder (ALT normalisation at the end of treatment), and one non-responder. Persistent evidence of occult HCV was demonstrated in all five cases. However, the mean intrahepatic viral load was significantly lower in the post-treatment biopsy than in the pre-treatment one and in three subjects, including the non-responder, necro-inflammatory activity and fibrosis score had improved, lending further support to the pathological nature of occult virus. All subjects in this study were infected with genotype 1, for which 48 weeks of treatment is usually given, hence it is possible that a full treatment schedule would have resulted in a higher rate of 'SVR' (i.e. clearance of occult virus). This has not yet been investigated, partly due to ongoing lack of information regarding the true consequences of occult virus infection.

Further data regarding the importance of occult virus comes from one group which has examined the presence of HCV-specific cellular immune responses in those with occult virus [343, 344]. HCV-specific CD4+ T-cell responses assayed by proliferation were detected in the PBMCs of 26 of 50 (52%) subjects with occult HCV compared to just 37 of 141 (26.2%) subjects with chronic infection [344]. The responses fluctuated between positive and negative in 17 of the 26 during the 6-24 month follow-up period, and were maintained as positive in nine. Proliferation was seen more commonly in response to the NS3 and NS4 proteins compared to the Core and NS5A/5B proteins. In the 20 of the 50 subjects that were HLA-A2 positive, HCV-

specific CD8+ T-cell responses were assessed by HLA class I multimer staining of PBMCs followed by flow cytometry. All 20 subjects had HCV-specific T-cells detected by one of the two tetramers investigated (both were NS3 epitopes) compared to only eight of the twenty (40%) subjects with chronic infection. Interestingly, the subjects with CD4+ T-cell responses had a significantly lower percentage of infected hepatocytes as assessed by positivity for the genome by *in situ* hybridisation, and significantly higher counts of HCV-specific CD8+ T-cells.

The same group showed similar results in those with a SVR lasting 12 months post treatment for typical chronic HCV infection [343]. Nine of the 15 (60%) with SVR had a positive CD4+ T-cell response in lymphoproliferation. These responses tended to decline over the follow-up period of 6 – 24 months. Consistent with earlier results from this group, the occult intrahepatic HCV RNA loads (measured by real-time PCR) were significantly lower in the subjects with positive responses than in those who never mounted a detectable response. Nine anti-HCV antibody positive subjects with negative HCV RNA results for over one year (and therefore presumably spontaneous viral eradication) were also enrolled. Six of these nine demonstrated T-cell responses, directed towards NS4 in five cases, in contrast to the SVR group, in whom NS3 was preferentially targeted in seven of the nine cases.

One other method used for the detection of HCV is measurement of the total Core antigen (HCV-cAg). This test is not routinely used in clinical practice, however its sensitivity in the pre-conversion 'window' phase of acute HCV infection is 100% compared with HCV RNA [345-347]. A retrospective study looked at the presence of HCV-Ag in subjects who had undergone treatment for chronic HCV infection, either successfully or unsuccessfully [348]. HCV-cAg was determined using a quantitative EIA Track-C ore antigen test (Ortho-Clinical Diagnostics, Raritan, NJ, USA) the

lower limit of sensitivity of which is 1.5pg/ml, equivalent to 12,000 IU/ml by PCR. This assay was positive in three of 71 (4.2%) of those who had had a successful response to treatment, at a mean concentration of 7.7 +/- 6.8 pg/ml, and 43 of 44 (98%) non-responders, at a mean concentration of 72.6 +/- 7.9 pg/ml. A linear correlation was found between serum HCV-cAg concentration and viral load, and the one non-responder with an undetectable HCV-cAg had a very low viral load. The chance of false positive results were reduced by analysing two further samples from each patient with a control, with the same results being achieved on these two further occasions. Interestingly, intrahepatic HCV RNA (measured by Amplicor v 2.0; Roche) was present in four of 104 (3.8%) of those with SVR, with HCV-cAg detectable in two of these subjects, albeit at a low concentration.

Despite these interesting observations, several groups have cast doubt on the existence of occult HCV. The first study involved analysis of PBMC using a modified, ultrasensitive assay (COBAS Taqman, Roche) with a detection limit of 15 IU/10^6 cells in 22 subjects with longstanding elevated liver enzymes of unknown origin, 21 with HCV-associated vasculitis (13 with a positive serum HCV RNA result), 27 with connective tissue disease (four positive for serum anti-HCV antibody and HCV RNA) and 20 positive controls with chronic HCV infection [349]. HCV RNA was only detected in the PBMCs of the four subjects with HCV RNA positivity and connective tissue disease and ten of the 13 with vasculitis who were HCV RNA positive. No HCV RNA was detected in the PBMC of any subject with a negative serum result. Liver tissue was not examined in this study so the absence of occult infection restricted to this tissue could not confidently be excluded. The sensitivity of the assay was validated by detection and dilution of quantified HCV-infected sera added to PBMC from patients without HCV infection. HCV genotype 1 serum quantified at

1,500 IU/ml was diluted by ten, and one ml of diluted serum was added to one million PBMC. This was quantified by the COBAS Taqman assay, giving a detection limit of 15 IU/million cells.

A further negative study used two different, sensitive assays, to look at PMBC of viraemic and aviraemic HCV-seropositive blood donors [350]. The transcription-mediated amplification (TMA) assay involved sample lysis with a detergent and denaturant buffer followed by the specific capture of HCV RNA with oligonucleotide-coated magnetic beads which were then rinsed and the HCV RNA amplified by an isothermal TMA. This assay has a 95% detection limit of 30 RNA copies/ml. It was adapted to detect viral RNA in PBMCs, and on dilutional testing had a sensitivity of 2-50 HCV RNA copies in 5 x 10^6 PBMC. The other assay used was a RT-nested PCR with a sensitivity of 15-150 HCV RNA molecules in 5 x 10^6 PBMC. No evidence for cell-associated HCV RNA was found in the 69 aviraemic seropositive subjects. In addition, the 13 of the 56 viraemic subjects with no detectable PBMC-associated HCV RNA all had very low viral loads.

The largest study of potential occult virus also questioned its validity [351]. PBMC samples were available for 156 of 344 subjects at a median of 3.0 years (range 0.5-18) post SVR after antiviral therapy for chronic HCV. The TMA assay used (VERSANT, Siemens Medical Solutions), had a sensitivity of <9.6 IU/ml (see table 1.1 above for sensitivity in vge/ml), and was not positive in a single subject. Paired pre- and post-treatment liver biopsies were examined in 126 subjects. HCV RNA of similar sequences (and identical genotypes) of the pre-treatment sample were found in the post-treatment biopsy of two subjects. The quantification varied markedly - 445 IU/mg of liver tissue and 10,945 IU/mg in the other. It was concluded that SVR is durable and should be considered to represent eradication of HCV infection. It was

accepted that the presence of HCV in the two post-treatment biopsies was due to residual infection rather than re-infection, which was dismissed as a rare occurrence.

Similar results were obtained by the most recent data which looked at 19 subjects who had spontaneously recovered and 98 who had recovered after treatment using the sensitive nested RT-PCR with a sensitivity of <40 copies/ml developed by one of the previous groups [119, 352]. In the spontaneously recovered group, no HCV RNA was detected in the plasma which was tested at a median of 20.8 years (interquartile range (IQR) 13.0–31.9) post clearance. Of the 14 who had PBMC samples, only one (7%) tested positive, at 11 weeks post-viral clearance. At this point, HCV-specific T-cell responses as assessed by IFN-γ ELISpot responses to 18 peptide pools spanning the entire genotype 1 polyprotein, were vigorous and multi-specific targeting both structural and non-structural proteins. A further PBMC sample from the same patient taken 28 weeks later was also weakly positive and there was a decrease in the intensity of the T-cell responses. HCV RNA was finally cleared from the PBMC compartment by 93 weeks at which point the T-cell responses decreased further.

In the group with SVR post treatment, 15 of the 98 (15%) tested positive for HCV RNA in the plasma and three of 76 (4%) tested positive in the PBMC compartment. The only one of the 15 with a viral load high enough to be quantified (at 40.5 million copies/ml), had a gap of over five years just preceding the follow-up sample that was positive, suggesting re-infection rather than reactivation, especially as no behavioural data was collected at follow-up. Sequencing of the amplified PCR products, showed that with the exception of two subjects, (not including the subject above) the post-treatment genotype matched the pre-treatment genotype.

Interestingly, the only factor that differed between subjects with, and without detectable HCV RNA was the length of time between cessation of treatment and

follow-up. The median follow-up for those with detectable HCV was 3.1 years (IQR 1.4-4.2) and in those without detectable HCV was 5.6 years (IQR 2.6-8.5). Fifteen of the 77 plasma samples within 8.5 years after cessation of treatment tested positive, whereas all 21 samples obtained from later time-points tested negative. When the first eight years after the end of treatment were split into two-year intervals, there was a decreasing trend in the prevalence of HCV RNA positive results.

This study also assessed humoral and cellular immune responses in those with occult HCV RNA. Neutralizing antibodies were assessed by testing the capacity of plasma samples from 11 subjects to block HCV infection in either JFH-1 virus cells with genotype 2a structural proteins or chimeric H-NS2/NS3-J virus cells with genotype 1a structural proteints. All samples blocked infection, with five neutralising HCV with either genotype indicating the presence of cross-neutralising antibodies. HCV RNA detected using RT-PCR with a sensitivity of <40 copies/ml, antibodies and T-cell responses assayed by IFN-γ ELISpot were analysed in subjects with samples available at multiple time-points. In some subjects, HCV RNA positive time-points were interspersed with HCV RNA negative time-points. Longitudinal samples for ELISpot were available in eight, and showed that T-cell responses were stronger at time-points when occult RNA were detected than at points without occult virus. However, antibody responses did not differ in strength or specificity at HCV RNA positive and negative time-points, and remained relatively constant in those who repeatedly tested negative for HCV RNA.

In addition, the dominant T-cell responses detected targeted non-structural HCV-sequences with this pattern being preserved over years, including when HCV RNA became detectable and T-cell responses increased. Non-structural proteins are only expressed in infected cells, therefore it is possible that the increased HCV-immune

response was in response to active translation of HCV proteins. However, the responses were sustained from during active infection and therefore this may simply represent memory and could be boosted by cross-reactive epitopes (e.g influenza).

The comprehensive, cross-sectional and prospective components of this study raise an interesting explanation for the variable, intermittent detection of occult HCV virus. After acute infection, there may in fact be three outcomes – spontaneous resolution, chronic infection characterised by persistently weak and ineffective immune responses, and an intermediate, slower form of resolution that can be expedited by antiviral therapy. In the latter, there may be a protracted, ongoing interplay between the immune system and the virus, with periods of viral suppression, until an outcome is reached, although it is possible that this may continue indefinitely. Thus, the detected occult HCV infection may reflect the ongoing natural history of resolving infection and not a chronic, persistent infection, providing a low-grade stimulus to the immune system.

In subjects who are continuously exposed to antigenic stimuli by virtue of their high-risk behaviour, the elicited immune response that resolves incident infection, may be in the 'waning' phase during subsequent re-infection. A reactivation of this effective immune response may occur more rapidly than in a subject who has not recently, or has never, been exposed to HCV. The role of lymphotropic HCV variants remain unclear, although again, detection of these variants may simply reflect part of the natural history of HCV, which is dependent on the individual host response.

Clearly, further studies are needed to further evaluate the existence, role, and importance of occult HCV infection. The majority of the data to date has been conducted in cross-sectional studies with relatively small subject numbers. The most

recent study is the largest to date and is comprehensive with both prospective and cross-sectional components, but again samples were only available from time-points after recovery from HCV infection [352]. No data exists regarding a role of occult virus in incident infection in seronegative subjects, and whether the presence of cellular immune responses targeting occult virus impact outcome.

The contribution of occult HCV replication to total viraemia is unclear as thus far, the detected quantities of HCV RNA have been very low. However, extrahepatic reservoirs may still have implications for transmission, disease progression, and treatment efficacy, as does the possibility that a SVR after antiviral treatment may not completely resolve the sequelae of chronic infection. Applying the sensitive assays that have been established to a further range of populations, especially those without chronic infection, evaluating the presence and nature of HCV-specific immune responses in these groups, and in much larger numbers, and if possible, assessment of the virological characteristics of the occult virus needs to be carried out.

A critical issue to be addressed is the establishment of a reliable, widely accepted, ultrasensitive assay where contamination can be confidently excluded, as to date, no 'gold standard' exists. Potential assays that do not require invasive acquisition of hepatic tissue have been developed, but need further validation before they are accepted [353-355]. More recently, it has been suggested that the low levels of HCV present in occult infection could be identified when clinical assays of enhanced sensitivity, such as real-time PCR are employed. The utility of quantitative HCV-cAg has not yet been adequately studied in relation to the occult virus phenomenon, however it may play a complementary role, especially when hepatic tissue is not available. Finally, reproducing the findings on samples taken from the same subjects, in more than one centre, and with sequencing to verify specificity would strongly

support the validity of the findings. However no multi-centre analyses of occult virus infection has yet been undertaken.

1.7. Hypothesis

Subjects that remain HCV seronegative and free of viraemia despite being at high risk demonstrate cellular immune responses to the HCV polyprotein. The antigenic stimulus for these immune responses may be due to the presence of low levels of occult HCV RNA, undetectable by conventional assays.

1.8. Aims

At the time of experimentation (2007 – 2008), no published studies had examined the seronegative immune phenotype in highly exposed, uninfected individuals, nor had an analysis of potential behavioural correlates of incident HCV infection been undertaken. The concept of occult virus was (and still is) a controversial phenomenon with the majority of work in support of its validity carried out by two groups. To date, there are no studies showing independent replication of results in separate centres, to support or refute, its importance in HCV infection.

Immunology

- To evaluate HCV-specific cellular immune responses in seronegative, uninfected high-risk subjects in comparison with low-risk subjects using HCV-specific enzyme-linked immunospot (ELISpot) assays
- To prospectively evaluate whether the presence of HCV-specific cellular immune responses protect against future HCV-infection and/or are correlated with subsequent clearance

Behavioural

- To evaluate behavioural characteristics associated with the presence of HCV-specific cellular immune responses in seronegative, uninfected high-risk subjects.

Virology

- To examine the presence of positive and negative strand HCV RNA in seronegative, uninfected high-risk subjects using reverse-transcriptase nested-polymerase-chain-reaction (RT-nPCR) and to correlate findings with cellular immune responses.

2 SUBJECTS AND METHODS

2.1. Introduction

These aims were carried out using data collected from a single cohort (Figure 2.1).

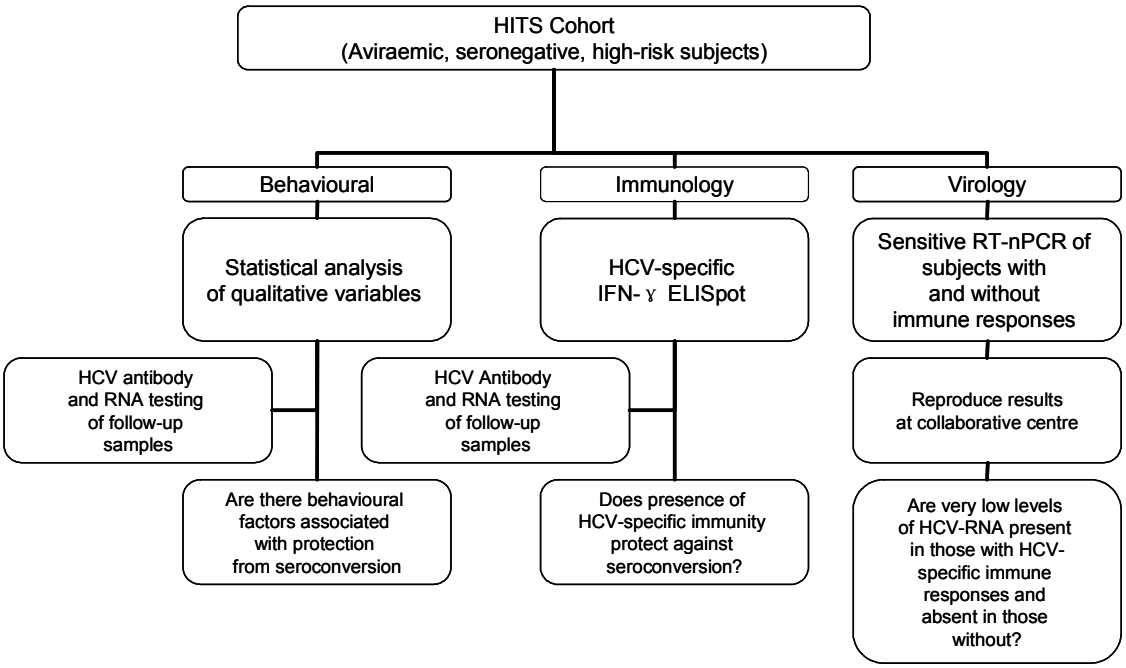

FIGURE 2.1. Schematic summary of subjects and research questions

2.2. Subjects

Samples from high-risk HCV-seronegative aviraemic subjects were selected from an ongoing prospective cohort of imprisoned IDU enrolled in the HITS cohort. This prospective cohort was established in 2005 to examine HCV incidence, and to identify behavioural, immunological and virological factors associated with incident HCV infection and the potential of immune protection against infection. Inmates across the 30 NSW prisons were eligible for enrolment in HITS if they: were aged at least 18 years (yrs), had a lifetime history of IDU, had been documented to be HCV-antibody negative within the last 12 months, knew adequate spoken English to be able

to participate in the interviews and gave provision of written, informed consent. Forensic inmates, pregnant women and those who were anti-HIV antibody positive were excluded.

A rolling cohort of 300 subjects has been recruited (with those released to freedom and those who became infected continuously replaced). Subjects provide detailed information regarding risk behaviour for blood-borne virus transmission, and blood samples for virological and immunological testing every six to 12 months.

Negative control blood samples for assay validation were acquired from low-risk seronegative subjects donating to the Australian Red Cross Blood Service (ARCBS). Samples and behavioural data from these subjects were analysed as outlined below.

2.3. Behavioural analysis

On enrolment into the HITS cohort, subjects were interviewed regarding risk factors for HCV infection, such as tattooing, body-piercing, blood transfusions and especially the nature of previous and current injecting drug-use - see Appendix A for interview schedule. This questionnaire was developed and validated for reliability by the study investigators [95]. It was administered to the subjects by one of two senior nurses trained to do so. This data was entered into a statistical database SPSS for Windows (v. 17.0, Chicago, IL). An analysis of the interview responses was undertaken in those subjects with, and without, HCV-specific cellular immune responses as measured by HCV-specific ELISpot to identify possible behavioural associations of HCV immunity, and with incident infection.

2.4. HCV serological and virological testing

HCV antibody testing was performed using the qualitative Abbott ARCHITECT

Anti-HCV chemiluminescent microparticle immunoassay (Abbott Diagnostics, Abbott Park, IL), and confirmed either using the INNOTEST HCV Ab IV ELISA assay (Innogenetics, Gent, Belgium) or Monolisa™ HCV Ag-Ab ULTRA assay (Bio-Rad Laboratories, Marnes-la-Coquette, France). Qualitative HCV RNA detection was performed either using the VERSANT HCV RNA Qualitative Transcription Mediated Amplification (TMA) assay (Bayer Diagnostics, Emeryville, CA) or COBAS AmpliPrep/ COBAS TaqMan HCV assay (Roche, Branchburg, NJ).

2.5. Separation and storage of peripheral blood mononuclear cells

Peripheral blood samples were collected from subjects by a research nurse using aseptic technique into acid-citrate dextran (ACD) anti-coagulant for plasma and PBMC, or without anti-coagulant for serum. Several anti-coagulated samples were pooled into 50 mL sterile centrifuge tubes (Greiner Bio-one, Germany) for density gradient centrifugation. Whole blood was utilised to record the total white blood cell, lymphocyte and monocyte counts using an automated haematology analyser (ActDiff, Beckman Coulter, USA).

The combined whole blood was spun at 1500 rpm for ten minutes with no brake. The separated plasma from this tube, and the serum from the clotted tube, were aliqoted into 1ml vials and stored at -80^{o}C. The blood remaining in the 50 mL tube was further divided into two 50 mL tubes, each diluted to 35 mL with Dulbecco's phosphate buffered saline (PBS) (Gibco Life Technologies, USA), before 15 mL of Ficoll-Hypaque (Amersham Pharmacia, Sweden) was gently underlayed. These tubes were spun at 1800 rpm for 25 minutes with no brake.

The mononuclear layer was harvested from these tubes into a separate 50 mL tube, and PBS added to make this solution up to 45 mL. This was spun at 1350 rpm for ten

minutes with the brake. The supernatant was discarded and the cell pellet gently resuspended. RPMI (RPMI 1640, 2mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin solution - all Gibco) was added, and the solution spun at 1350 rpm for ten minutes with the brake. The cell pellet was resuspended and 2 – 5 mL of autologous plasma added (depending on pellet size). The total and percentage counts of total white blood cells, lymphocytes, monocytes and neutrophils were again enumerated with the automated counter.

The cell count was adjusted to a concentration of 10 – 20 x 10^6 PBMC/mL and an identical volume of cold RPMI containing 20% dimethyl sulfoxide (DMSO) (Sigma, USA) was added dropwise for cryopreservation. 1ml aliquots of the cell suspension were transferred into cooled cryovials (Nunc, Denmark) and immediately placed in a cryofreezing isopropanol bath (Mr Frosty, Sigma) where they were stored overnight at -80^{o}C. The vials were then moved to vapour-phase nitrogen for long-term storage. The PBMC were thus stored in final concentrations of 10% DMSO and 50% autologous plasma. Subject and sample details were entered into an in-house, custom-built electronic specimen database, the 'Blood and Tissue Sample Inventory System' (BATSIS).

2.6. Thawing of frozen PBMC

After removing the PBMC vials from liquid nitrogen storage, the vials were immediately put in a waterbath at 38–40°C and monitored closely until only a small amount of frozen cell suspension was left. The contents were then very gently mixed with a transfer pipette and added to 7-10 mL of RPMI in a 15 mL polypropylene tube. The vial was rinsed with a small additional amount of media and recombined in the 15 mL tube to ensure a maximal yield of frozen cells.

The tube was spun for ten minutes at 1350 rpm and the supernatant decanted. The cell pellet was gently resuspended in 10mL of R+ (RPMI 1640, 2mM L-glutamine, 100 units/mL penicillin and 100 microg/mL streptomycin solution with 10% heat inactivated foetal calf serum (FCS)). The tube was spun again for ten minutes at 1250 rpm and the supernatant decanted. The cell pellet was gently resuspended and 1 mL of R+ added. A manual cell count was performed with Trypan blue to assess viability, recovery and yield.

2.7. HCV-specific gamma-interferon (IFN-γ) enzyme-linked immunoassay (ELISpot)

One hundred µL of anti-human IFN-γ monoclonal antibody (Mabtech, Sweden) at a concentration of 2.5 µg/mL in carbonate buffer was added to each well of a 96 well nitrocellulose-base plate (Millipore, USA). The plates were incubated overnight at 4^{o}C to allow coating with the cytokine capture antibody (Figure 2.2).

The next day the plates were washed six times with 100 µL PBS and blocked for at least one hour with R+(5% FCS) at 37 ^{o}C. After thawing, PBMCs were adjusted to a final concentration of $4x10^{6}$ PBMC/mL using R+. HCV peptides (see below) were used at a final concentration of 1 µg/mL. PHA at a final concentration of 2.5 µg/L was used as a positive control. Media containing 0.8% DMSO (final concentration in the well equivalent to the maximum concentration in the peptide pools) was used as a negative control. The blocking media was discarded and 50 µL of PBMC (2×10^{5}) was added to each well. 50 µL of media, or stimulant peptide pool solution were added to each well. Stimulations were performed in triplicate. PHA (Sigma-Aldrich, St Louis, MO) was added to a single well as a positive control stimulus. The plates

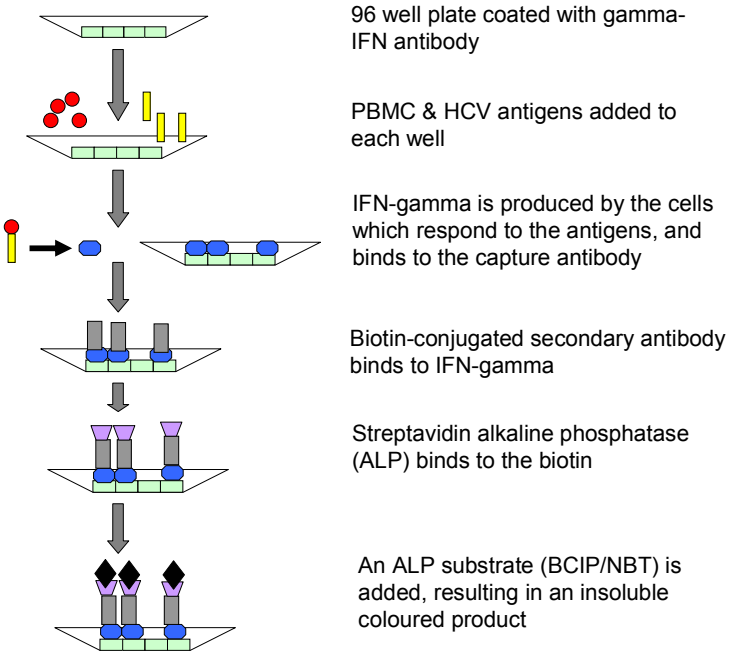

FIGURE 2.2. ELISpot assay

were wrapped individually in foil and incubated flat and without movement, at 37°C/5% CO2 for 18-24 hrs.

Following the overnight incubation, the PBMC and media were discarded. The plates were washed six times with PBS. The wells were incubated at room temperature for two hours with 100 µL of a second biotinylated anti-human IFN-γ monoclonal antibody (Mabtech) at a concentration of 1 µg/mL in PBS (Sigma). The plates were then washed six times with PBS. 100 µL of streptavidin-alkaline phosphatase (Sigma) was added to each well at a concentration of 1 µg/mL in PBS for one hour at room temperature. The plates were washed six times with PBS. Spots were then developed by adding 100 µL of BCIP/NBT (made up by dissolving one BCIP/NBT tablet (Sigma) per 10 mL of QH_2O in a light-proof container) for at least 20 min. The colour reaction was stopped by washing the plate in water. After air-drying for 24 hr, the

spots were counted using an AID ELISpot reader (Autoimmun Diagnostika, Germany).

2.7.1. HCV peptides

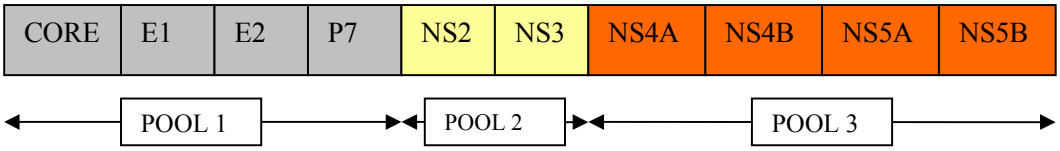

FIGURE 2.3. HCV peptide pools for ELISpot assay

Peptides spanning the entire HCV polyprotein (NIH AIDS Reagent Bank; 18-mers overlapping by 15 amino acids) were stored in 15 µL aliquots at a concentration of 100 µg/mL. Prior to use in the IFN-γ ELISpot assay, the peptides were diluted to 2 µg/mL using media (therefore yielding a final concentration of 1µg/mL in the ELISpot well - diluted 1:1 with cells). In assays run using ten peptide pools, each pool covered one HCV protein (*i.e.* Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). In assays run using three peptide pools, pool 1 covered Core, E1, E2 and p7, pool 2 covered NS2 and NS3, and pool 3 covered NS4A, NS4B, NS5A and NS5B (Figure 2.3).

2.7.2. Interpretation of IFN-γ ELISpot results

Assays were performed in triplicate. Outliers were excluded from the analysis using the method defined by Stone et al [356]. In brief, this outlier exclusion involved initially identifying the median of the triplicate values. A range was then calculated with the minimum acceptable value being 50% less than the median, and the maximum being 50% more than the median. Any values falling outside this range

were discarded and the remaining values were averaged. If two values fell outside the range, the entire triplicate was discarded.

Results were expressed as spot forming units (SFU)/10^6 PBMCs. A positive result was defined using a threshold for significant detection based on the assay results from ten low-risk seronegative ARCBS donors. A 'positive' result was designated as a test value greater than twice the numbers found in the media wells (*i.e.* background), and more than 35 spot forming units/10^6PMBC (mean + 3 standard deviations above the results of the ARCBS donors).

2.8. RNA extraction

RNA was extracted from sera and PBMC using the QIAmp Viral RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturers instructions. Five hundred and sixty µL of buffer AVL containing carrier RNA was added to a 1.5 mL microcentrifuge tube. 140 µL of serum or cell supernatant was added and mixed by pulse-vortex for 15 seconds. The sample was then incubated at room temperature for ten minutes to achieve viral particle lysis.

The tube was briefly centrifuged. 560 µL of 100% ethanol was added and mixed by pulse-vortex for 15 seconds. The tube was spun briefly. Six hundred and thirty µL of this sample was carefully applied to a QIAmp mini-column resting in a 2 mL collection tube which was then centrifuged at 8000 rpm for one minute. The filtrate was discarded. The QIAmp mini-column was placed inside a fresh 2 mL collection tube and centrifuged again at 8000 rpm for one minute.

500 µL of buffer AW1 was added and the mixture centrifuged at 8000 rpm for one minute. The filtrate was discarded and the mini-column placed inside a fresh collection tube. Five hundred µL of buffer AW2 was added and the mixture

centrifuged at 14,000 rpm for three minutes. The mini-column was placed in a new collection tube and centrifuged at 14,000 rpm for one minute to eliminate any chance of buffer AW2 carryover.

The filtrate was discarded and the mini-column placed in a 1.5 mL microcentrifuge tube. RNA was eluted by addition of 60 μL of buffer AVE equilibrated to room temperature. After incubation at room temperature for one minute, the Eppendorf tube was spun at 8000 rpm for one minute. The eluted RNA was stored at -80^{o}C.

2.9. Masked case-control samples

Reproducibility of detection or absence of occult HCV infection by ultrasensitive nested RT-PCR (see Section 3) was evaluated by carrying out the assay on the same samples in two different centres. Twenty subjects from the HITS cohort - 10 with, and 10 without, evidence of HCV-specific cellular immunity by IFN-γ ELISpot assay, with sufficient PBMC for the two centre study were identified from the stored sample set. Negative control samples were acquired from the PBMC of low-risk, seronegative subjects donating to ARCBS. In addition, ten HCV-antibody positive subjects from the HITS cohort, five who had previously cleared infection (*i.e.* HCV RNA negative), and five who had chronic infection (*i.e.* HCV RNA positive), were included.

PBMC and plasma from these 35 subjects were assayed by an in-house RT-PCR assay, and by the collaborative group in Canada. Both groups were masked regarding the anti-HCV antibody, RNA and immune status of each subject.

2.10. Statistical analysis

This was undertaken using two-tailed Fisher's exact tests and Yates' chi-square tests for categorical values, and t-tests for continuous variables. The threshold for significance was set at $p<0.05$, although it is recognised that multiple comparisons were being made in the behavioural analyses.

3 DEVELOPMENT OF A SENSITIVE NESTED RT-PC

3.1. Introduction

To detect both positive and negative (replicate-intermediate) HCV RNA strands, an ultrasensitive, strand-specific, nested RT-PCR (nPCR) was required. Due to the substantial number of amplification cycles in a nested approach, contamination is a critical issue, and has been particularly raised in querying the validity of the existence of occult HCV infection. To develop a suitable, specific, robust assay, the chosen starting point was an in-house nRT-PCR assay developed in the virology laboratory of A/Professor Peter White, which has previously been validated on subjects with chronic HCV infection [357]. This assay targets the highly conserved 5'UTR region and has been shown to detect genotypes 1a, 1b, 2a, 2b, 2c and 3a. It has a sensitivity of 28 copies/reaction (or 1,400 copies/mL). This assay was modified (see below), with reference to the negative strand assay pioneered by the leading group (Michalak and colleagues) who have published evidence supporting the validity of occult HCV. After optimisation in subjects known to have chronic HCV infection, the plan for this project was to assay aviraemic subjects to determine the presence or absence of occult HCV.

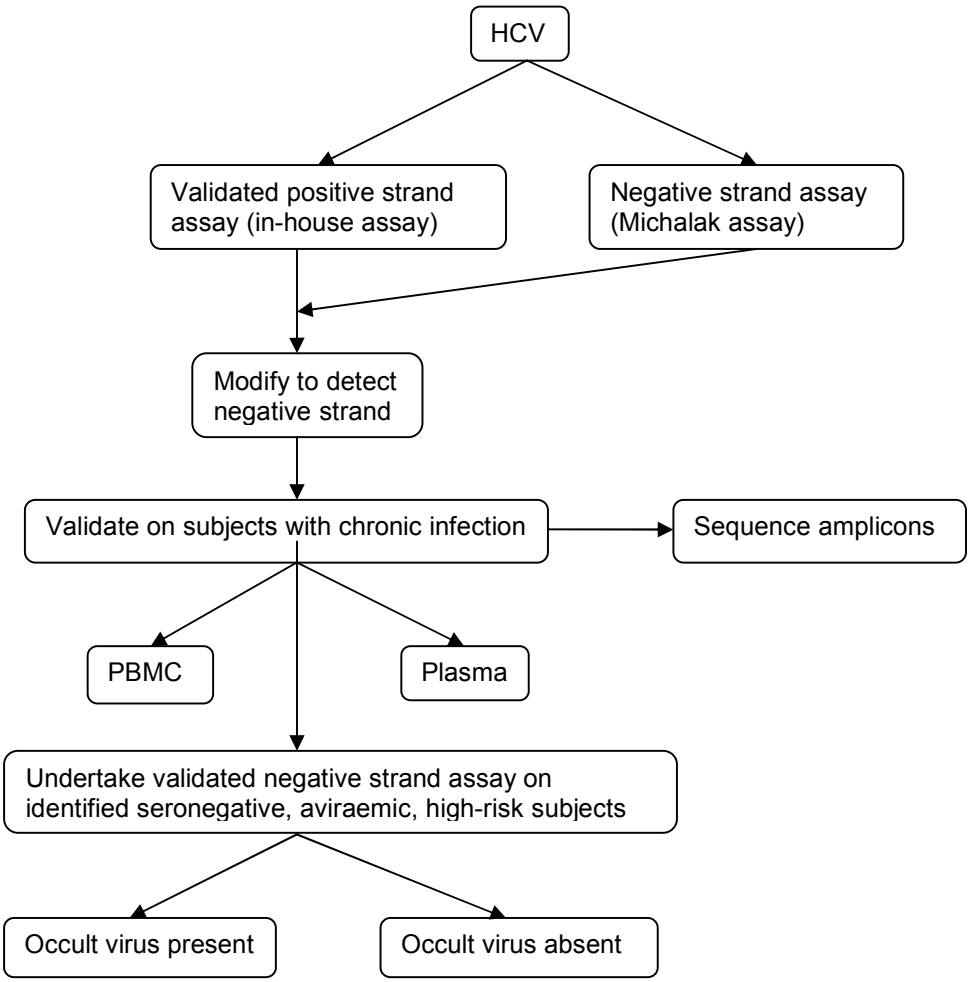

FIGURE 3.1. Schematic of nRT-PCR arm of study

3.2. In-house nRT-PCR

The assay was carried out as previously described [357]. Briefly, RT-PCR was performed with 5 µL of template RNA added to a 15 µL reaction mix containing 10 µL SYBR Green RT-PCR reaction mix, a 0.5 µM concentration of each primer (hep 14 and hep 15; Table 3.1), 4.4 µL of water, and 0.4 µL of 50x iScript RT enzyme (all reagents Bio Rad).

The MyiQ real-time detection system (Bio-Rad Laboratories, Berkeley, USA) was used at the following settings: reverse transcription at 50^{o}C for ten minutes, then

denaturation at 95°C for 5 minutes, followed by 15 PCR cycles, each cycle consisting of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by final extension at 72°C for seven minutes. This was held at 4°C.

The second round PCR (nPCR) was run with 2 μL of the first round product added to an 18 μL PCR reaction mix made up with 10 μL iQ SYBR Green Supermix, a 0.5 μM concentration of each primer (hep 21b and hep 22) and 7.8 μL water. After incubation at 95°C for 30 seconds, 40 cycles of PCR were performed, each cycle consisting of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for one minute. Melt curve analysis was then performed by 70 cycles at 55°C for ten seconds. The nPCR product was held at 4°C.

TABLE 3.1. In-house assay primers

	Primer sequences
Hep 14	5'GCA GAA AGC GTC TAG CCA TGG CGT 3'
Hep 15	(5'CTC GCA AGC ACC CTA TCA GGC AGT 3'
Hep 21b	5'-GAG TGT YGT RCA GCC TCC AGG-3'
Hep 22	5'- GCR ACC CAA CRC TAC TCG GCT-3'

3.2.1. Detection of PCR product

PCR products and the pGEM DNA marker (Promega) were separated on a 1.5% agarose gel (3 g agarose added to 200 mL 0.5% Tris Buffered Saline), stained with 10 μL of ethidium bromide, for 30 minutes at 110 Volts. The separated products were visualised under ultraviolet transillumination (GelDoc 2000, Bio-Rad).

3.3. nRT-PCR assay for detection of negative strand HCV RNA

This negative strand HCV assay was developed by the Michalak lab, who collaborated in this two-centre project [119], and involves nRT-PCR followed by Southern blot hybridisation of the amplified products to virus specific probes. This assay has been shown to detect HCV RNA in both plasma and PBMC, using both 5'UTR or E2 region-specific primers with a sensitivity of ≤10 vge/mL. This assay has previously been validated by sequencing of resultant amplicons [119, 358].

RNA template (10 μL) was denatured at 95^{o}C and held at 70^{o}C, before being added to 10 μL preheated (70^{o}C for two minutes) reaction mix (RT mix) containing 2 RT buffer, 2 mM $MnCl_2$, 1.6 mM deoxynucleoside triphosphates (dNTP), 15pM primer (UTR1), 5 U rT*th* DNA polymerase and 1.5 μL DEPC-treated water. This was cycled to 60^{o}C for 2minutes then 70^{o}C for 15 minutes.

Eighty microlitres of preheated PCR reaction mix (PCR mix) was added, containing 10 mM chelating buffer, 2.5mM $MgCl_2$, 15 pM primer (RTU1) and 62.5 μL DNase-free water. Three minutes of denaturation was followed by 45 cycles of 94^{o}C for one minute, 60^{o}C for two minutes and 72^{o}C for three minutes, then final extension at 72^{o}C for seven minutes. Ten to 20 μL of the direct PCR product was used as the second round template, under the same conditions, using primers UTR2 and RTU2.

TABLE 3.2. Pilot assay primers

	Primer sequences
UTR1	5'-CTG TGA GGA ACT ACT GTC TTC -3'
RTU1	5'-GCG GTT GGT GTT ACG TTT -3'
UTR2	5'-GCA GAA AGC GTC TAG CCA TGG CGT -3'
RTU2	5'-CTC GCA AGC ACC CTA TGA GGC AGT -3'

3.4. Assay optimisation

3.4.1. Introduction

The goal of this project was to examine evidence for positive and negative strand HCV RNA in individuals at high risk of HCV infection, including subjects with and without detectable viraemia in standard diagnostic assays, and with and without evidence of HCV-specific cellular immune responses. Optimisation of an ultra-sensitive nRT-PCR assay was undertaken in the local laboratory, with the described Michalak assay as the starting point. Further support to the current data regarding occult virus would be provided if multicentre reproducibility of nRT-PCR results, with sequencing, was shown.

3.4.2. Validation of primers

Two simultaneous in-house assays were run, one using in-house primers, and the other using primers from the Michalak group (termed here 'pilot primers'), on RNA extracted from six subjects with chronic HCV infection. Water was used as a negative control.

Appropriately-sized positive bands were detected on the agarose gel for all six subjects, except one sample run using pilot primer (subject 89) (Figure 3.2). Stronger bands were detected with the in-house primers, suggesting a higher efficiency of amplification and greater yield of PCR product. This result validated the in-house current assay, suggested that the pilot primers were suitable, and indicated that the RNA samples from these subjects, except subject 89, could be used as positive controls for further assay validation.

3.4.3. Pilot assay

In the initial experiment to establish the assay from the Michalak laboratory, the above protocol for negative strand detection was conducted on RNA extracted from

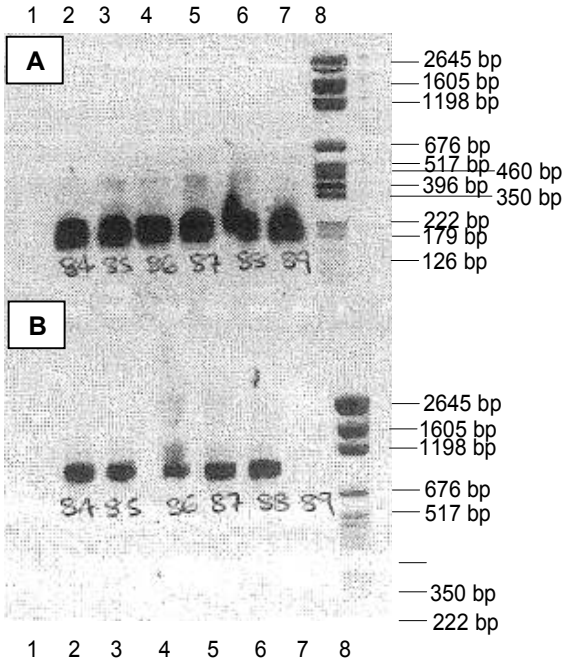

FIGURE 3.2. In-house nRT-PCR of six subjects (84 – 89) with chronic HCV infection. Agarose gel showing nRT-PCR amplification of 5'UTR region specific primers**.** A) In-house primers (Table 3.1); B) Pilot primers (Table 3.2). Lane 1, negative control; lane 2, subject 84; lane 3, subject 85; lane 4, subject 86; lane 5, subject 87; lane 6, subject 88; lane 7, subject 89; lane 8, pGEM marker (Promega).

two of the previously tested subjects with chronic infection, two subjects who had cleared infection ('clearers'), and water. No visible fragments were amplified, with identical results when the assay was re-run. Given this outcome, a troubleshooting process was undertaken.

3.4.4. Trouble-shooting the assay

Simultaneous in-house assays were run with both sets (in-house and pilot) of primers on the samples tested above. As expected PCR-products of the expected size were

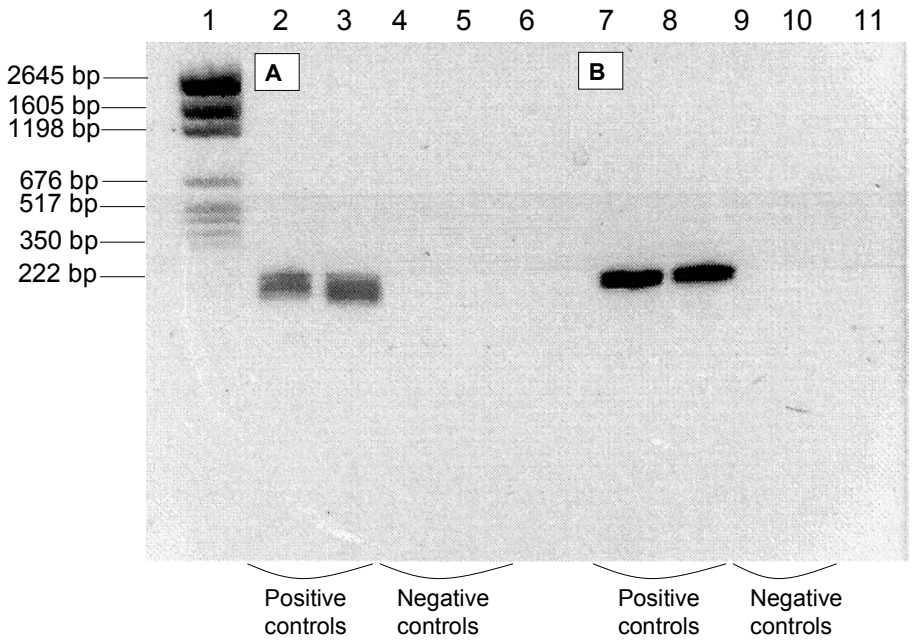

FIGURE 3.3. In-house nRT-PCR assay to establish positive and negative controls. Agarose gel showing nRT-PCR amplification of 5'UTR region specific primers**.** A) (lanes 2 – 6) In-house pimers (Table 3.1); B) (lanes 7 – 11) Pilot primers (Table 3.2). Lane 1, pGEM marker (Promega); lane 2, positive control one; lane 3, positive control two; lane 4, negative control one; lane 5, negative control two; lane 6, water; lane 7, positive control one; lane 8, positive control two; lane 9, negative control one; lane 10, negative control two; lane 11, water.

detected in the two subjects with chronic infection (*i.e* the positive controls), with both sets of primers, and no products were detected in the 'clearers,' or water (Figure 3.3). This indicated that samples from clearers would produce expected results in this assay and could be used as negative controls controls to exclude contamination. Subjects with chronic infection will be referred to as positive controls, and clearers as negative controls.

3.4.4.1. Sensitivity

Prior to optimising pilot assay sensitivity for detection of the negative strand, its ability to detect positive RNA, which is present in greater concentrations that negative strand RNA was attempted. The RT step was run using the RTU1 primer,

and the first and second round PCR steps carried out as normal in the same subjects (*i.e.* two positive controls, two negative controls and water). There was a failure to generate amplicons in any tested sample, including the two positive controls .

Initial modification were made sequentially to the pilot assay to sensitivity for positive strand RNA detection as outlined below:

- Both RT mix and PCR mix made up on ice to avoid possible degradation of primers due to 5'–3' exonuclease activity of Tth polymerase
- Primers and dNTP added to RNA *before* denaturing to improve specificity of primer binding to the template
- Addition of an RNase inhibitor (1 μL RNasin, Promega,) prior to denaturing
- Annealing temperature decreased to 55^{o}C
- Final volume of PCR mix decreased to 80 μL
- PCR-products separated on agarose gels of different concentrations to allow for smaller/larger fragments than are suited to a 1.5% gel
- Gel electrophoresis run for more than 30 minutes
- Different dilutions and volumes of primers used
- Amount of cDNA increased
- Number of PCR cycles increased
- Different combinations of in-house and pilot primers (for example, pilot RT and first round PCR primers with in-house second round PCR primers)

No PCR products were detectable with any of these experiments, even for the positive control

3.4.4.2. Reverse-transcription

After failure of the basic initial modifications described above, trouble-shooting focussed on the separate components of the assay *(i.e.* RT, PCR and nPCR), to see if the problem could be isolated to one stage. Superscript III (Invitrogen), a dedicated reverse transcriptase, was substituted for the Tth polymerase in the pilot assay RT step.

Eight microlitres of RNA template was added to a 2μL RT reaction mix containing 10mM dNTP and 2 μM outer reverse primer, incubated at 65^{o}C for five minutes then placed on ice for one minute. Ten microlitres of cDNA mix containing 10 x RT buffer, 4 μL 25mM mgCl2, 2 μL 0.1 DTT, 1 μL RNaseOUT and 1 μL superscript III reverse transcriptase was added and the mixture incubated at 55^{o}C for 25minutes, followed by five minutes at 85^{o}C (to terminate reverse transcription). RNA template was removed with RNase H (1 μL) and incubated for twenty minutes at 37^{o}C. The first and second round PCR steps were conducted as per the in-house assay.

Different primer combinations were trialled, all without successful detection of PCR-products. As the in-house PCR steps had been shown to work, trouble-shooting now focussed on the components within the RT step.

Degradation of RNA was excluded by running the original in-house assay with two positive controls, water, cDNA produced by the RT step of the previous experiment, and validated cDNA from a colleague (E cDNA). Bands of appropriate size for the desired PCR-products were detected for the two positive controls, and two samples of cDNA (Figure 3.4). This result was confirmed by reproducing the results in a separate experiment (data not shown).

A modified in-house assay was run using Superscript III for the RT step on two samples from one positive control. In one experiment, RT was conducted using the normal in-house RT primer (hep 14), to detect the positive strand, and in the second experiment RT was conducted using the primer hep 15 to detect the negative strand. The first and second round PCRs were run as normal. No PCR-products were detected for the lane run with water, and a band was detected for the assay to detect positive-strand RNA (Figure 3.5). Surprisingly, the assay to detect negative-strand RNA yielded a strongly positive band, suggesting that attempts to improve sensitivity had compromised specificity, resulting in inaccurate amplification of the positive strand in the negative-strand assay. This was clarified using primers upstream of the RT primers, with subsequent sequencing planned if this did not work (Figure 3.6).

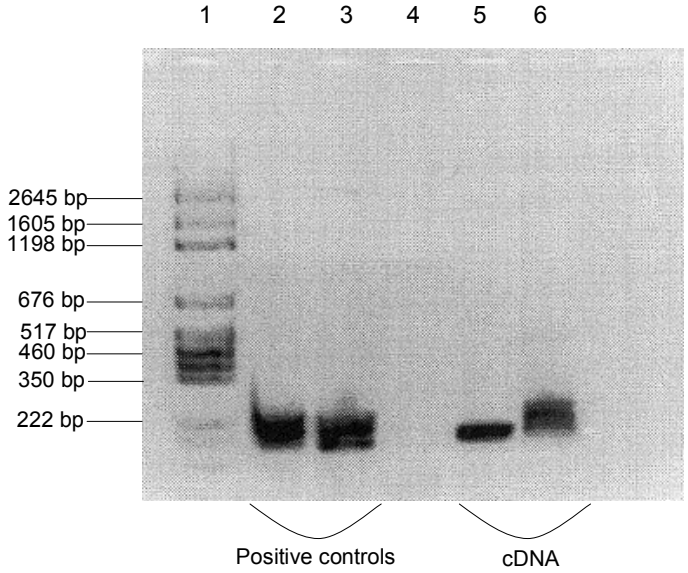

FIGURE 3.4. Agarose gel showing nRT-PCR products of modified in-house assay to exclude RNA degradation. (Superscript III RT; in-house assay PCR and nPCR); amplification of 5'UTR region specific primers (in-house primers table 3.1)**.** Lane 1, pGEM marker (Promega); lane 2, positive control one; lane 3, positive control two; lane 4, water; lane 5, PCR-product from previous cDNA; lane 6, PCR-product from E cDNA.

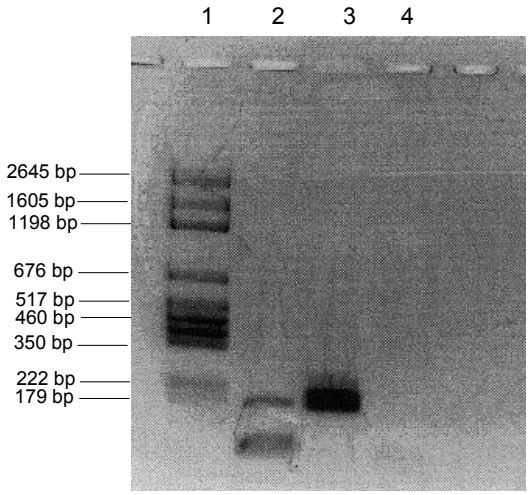

FIGURE 3.5. Agarose gel showing nRT-PCR-products of modified in-house assay to detect positive and negative strand HCV RNA. (Superscript III RT; in-house assay PCR and nPCR); amplification of 5'UTR region specific primers (in-house primers table 3.1)**.** Lane 1, pGEM marker (Promega); lane 2, nRT-PCR product of assay to detect positive strand RNA (hep 14 RT primer); lane 3, nRT-PCR product of assay to detect negative strand RNA (hep 15 RT primer); lane 4, water.

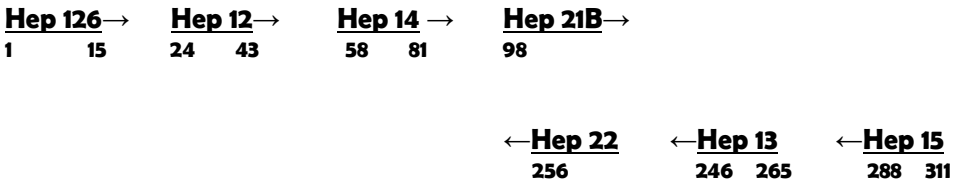

FIGURE 3.6. Schematic of upstream primers

After Superscript III cDNA synthesis using *hep15* for the positive strand reaction, and *hep 14* for the negative strand reaction, three sets of in-house assays were run using different first and second round PCR primer combinations (Table 3.3). To ensure that appropriate termination of RT occurred, each experiment included a lane run with the product of RT conducted without primers.

Bands were detected in the sample run with no RT primer, for each of the three sets of PCR primers, implying continuation of RT during PCR, or that the detected bands represented primer-dimers (Figure 3.7).

TABLE 3.3. Primer combinations and expected results

Set	PCR primers	nPCR primers	Strand amplified	Detectable amplicons expected?
1	UTR1 & Hep 15	Hep 14 & Hep 22	Positive	Yes
1	UTR1 & Hep 15	Hep 14 & Hep 22	Negative	No
2	Hep 12 & Hep 15	UTR1 & Hep 22	Positive	Yes
2	Hep 12 & Hep 15	UTR1 & Hep 22	Negative	No
3	Hep 14 & Hep 15	Hep 21b & Hep 22	Positive	Yes
3	Hep 14 & Hep 15	Hep 21b & Hep 22	Negative	Yes

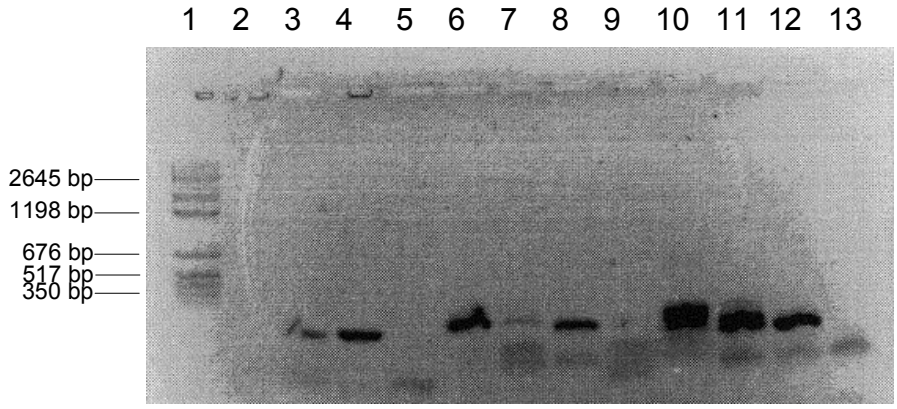

FIGURE 3.7. Agarose gel showing nRT-PCR-products (Superscript III RT, in-house assay nPCR), and different primer combinations. Lane 1, pGEM marker (Promega); lane 2, positive strand assay, primer set 1; lane 3, negative strand assay, primer set 1; lane 4, no RT-primer, primer set 1; lane 5, water, primer set 1; lane 6, positive strand assay, primer set 2; lane 7, negative strand assay, primer set 2; lane 8, no RT-primer, primer set 2; lane 9, water, primer set 2; lane 10, positive strand assay, primer set 3; lane 11, negative strand assay, primer set 3; lane 12, no RT-primer, primer set 3; lane 13, water, primer set 3.

Inactivation of Superscript at 90^{o}C for ten minutes was attempted, however bands for the assays run with no RT primers were not completely eradicated. The assay was re-run with freshly synthesised cDNA with similar results. Purification of cDNA was undertaken with Wizard® PCR clean-up system (Promega). Twenty microlitres of cDNA was incubated with 80 μL of resin for five minutes, and passed through

Wizard® minicolumns, followed by 250 µL of ethanol and allowed to dry. cDNA was eluted in 20 µL of nuclease-free water.

An assay using purified cDNA, with the same three primer combinations was run, with the following experiments for each primer combination:

- Positive strand RNA detection
- Negative strand RNA detection
- No primers in RT step
- RNA in the place of cDNA
- Water.

PCR-products were again detected for the samples run with no primers (Figure 3.8). Additionally, the presence of primer dimers were suggested by the detection of two

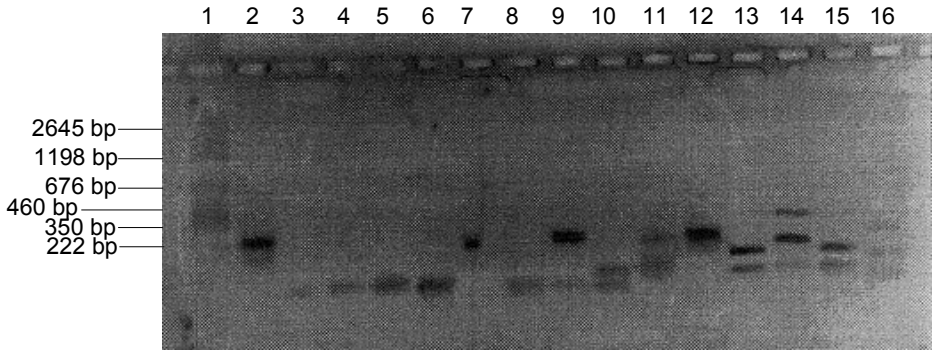

FIGURE 3.8 Agarose gel showing nRT-PCR-products (Superscript III RT, in-house assay nPCR with purified cDNA), and different primer combinations. Lane 1, pGEM marker (Promega); lane 2, positive strand assay, primer set 1; lane 3, negative strand assay, primer set 1; lane 4, no RT-primer, primer set 1; lane 5, PCR carried out with RNA instead of cDNA, primer set 1; lane 6, water, primer set 1; lane 7, positive strand assay, primer set 2; lane 8, negative strand assay, primer set 2; lane 9, no RT-primer, primer set 2; lane 10, PCR carried out with RNA instead of cDNA, primer set 2; lane 11, water, primer set 2; lane 12, positive strand assay, primer set 3; lane 13, negative strand assay, primer set 3; lane 14, no RT-primer, primer set 3; lane 15, PCR carried out with RNA instead of cDNA, primer set 3; lane 16, water, primer set 3.

bands in some experiments. Faint amplification products were seen in the experiments run with RNA, although this was less marked for the in-house primers,

again suggesting continued RT in the PCR step. Sequencing was attempted but was not possible due to two overlapping sequence reads, suggesting contamination.

3.5. Discussion

Attempts to recreate the sensitive Michalak RT-nPCR (pilot) assay to detect negative-strand HCV RNA in the local laboratory were unsuccessful with the described assays. There are several possible explanations for this unresolved outcome, and potential stages of continued assessment are discussed. It was not clear that the RT step had been terminated. As cDNA purification had been unsuccessful, adding chelation buffer on its own, following by the remaining PCR mix would have been the next step in the trouble-shooting process.

After termination of RT step had been successful, measures to increase the sensitivity of the pilot assay for negative-strand HCV RNA detection would be investigated. Subjects with chronic infection have detectable HCV RNA in serum and liver, and the viral load in these sites are highly correlated [357], suggesting that in these subject the liver is the primary, or perhaps only, site of replication. Negative-strand HCV RNA may potentially only be present in sites of active replication. The assay described here was carried out using RNA extracted from plasma, and hence negative-strand RNA may not, have actually been present. Liver tissue from subjects with chronic HCV would be expected to have the highest concentration of replicating virus, and would have been an excellent control, but was not available during this project. Once verified in liver, the pilot assay would have been performed using RNA extracted from PBMC in the same subjects.

Up-regulation of HCV RNA expression using mitogen stimulation with PHA and IL-2 has been reported [359]. However, as this only up-regulated expression in subjects

with occult virus, and actually down-regulated expression of both positive and negative strands in samples from chronic infection, it is unlikely to have been helpful in the experiments described here, until the assay was consistently shown to detect negative-strand RNA in those with chronic infection [358]. A final option to facilitate optimisation of the assay would be to generate synthetic HCV RNA (either negative or positive strand from cDNA templates using *in vitro* cDNA expression systems utilising SP6 and T7 polymerases followed by DNAse digestion of the cDNA template (e.g. MAXIscript, Ambion). Once synthesised, serial dilutions of synthetic positive or negative strand HCV RNA into negative control samples could be used to resolve the specificity and sensitivity of the assay.

Assays for detection of active replication of other RNA viruses are also similarly dependant on accurate amplification of a negative-RNA strand intermediate, raising the same concerns regarding sensitivity and specificity identified here. Detection of hepatitis E negative-strand RNA is particularly similarly problematic [360-363]. However assays for detection of the negative-RNA strand of Dengue virus have been consistently more successful. In addition, in one of the earlier assays, the final detection step was performed using Southern blotting [364] similar to that utilised by our collaborative group, however this was successfully removed in later assays [365, 366] as attempted by the HCV-pilot assay. However concerns regarding specificity have been raised due to self-priming of the reverse-transcriptase in the absence of exogenous primers at the RT step [366, 367]. Although none of the assays completely eliminate the potential for self-priming, two other steps utilised in other RNA-virus assays that may be appropriate for consideration in further development of the pilot assay include tagged PCR, with a unique tag sequence at the 5' end of the RT primer

to allow specific amplification at the PCR step, and use of a thermophilic RT approach [367].

Once the pilot assay had been validated on the positive and negative controls, it would have been conducted on both plasma and PBMC of the high-risk seronegative immune subjects that had been assessed by HCV-specific IFN-γ ELISpot.

4 RESULTS – HCV immunity and its correlates

4.1. Subjects and samples

Seronegative, aviraemic samples were identified from the HITS cohort. Samples from comparison subjects (*i.e.* those with chronic HCV infection and those who had cleared infection -'chronics' and 'clearers,') were also utilised. HCV-specific IFN-γ ELISpot assays, which predominantly detect CD8+ T-cell responses were conducted using PBMC from seronegative subjects in order to identify 10 subjects with and 10 without HCV-specific cellular immune responses who also had sufficient PBMC samples stored to run RT-nPCR assays.

4.2. HCV-specific IFN-γ ELISpot assay

4.2.1. Designation of positive value cut-offs

HCV-specific IFN-γ ELISpot assays were conducted on 10 low-risk ARCBS donors to determine background levels in a low-risk uninfected population. This was calculated as the mean plus 3 standard deviations of the 10 ELISpot assays (Table 4.1). A 'positive' result was thus designated as a value greater than 35 SFU/10^6 PBMC, and more than twice the value for the negative control (media alone). An assay was excluded from the analysis if the background result, *i.e.* the result for the negative control, was 50 SFU/10^6 PBMC or above. In addition, the mean result for each individual peptide or peptide pool was discarded if two of the triplicate values lay outside the range as previously described (see section 2).

TABLE 4.1. Determination of cut-offs for a positive result

	Pool 1	Pool 2	Pool 3
Mean (SFU/10^6 PBMC)	7.06	4.19	8.95
SD (SFU/10^6 PBMC)	7.39	5.01	8.49
Mean + 3SD (SFU/10^6 PBMC)	*29.23*	*19.22*	*34.42*

4.2.2. Validation of the 10-pool versus 3-pool assay

Samples from subjects were assayed by ELISpot using either 10 or three peptide pools, spanning the entire HCV genome, depending on the quantity of PBMC available for each subject. Prior to this, parallel experiments using both three and 10 peptide pools were run with samples from the same subjects to confirm that the results would be comparable. To increase the likelihood of a positive response, samples for this quality control assay were taken from subjects with chronic infection.

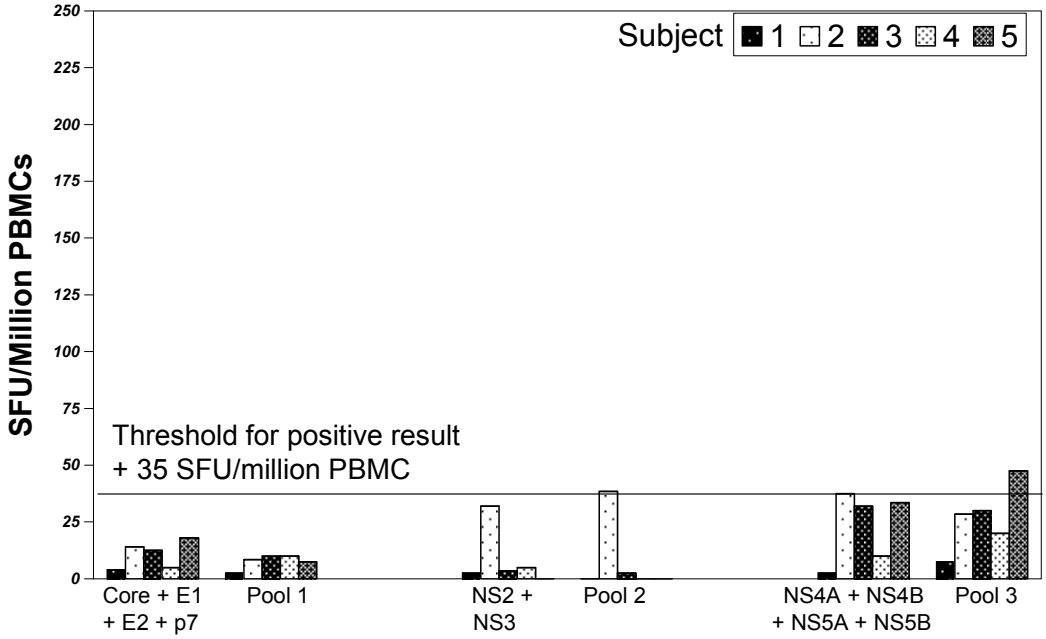

FIGURE 4.1. Comparison of HCV-specific ELISpot with 3 and 10 peptide pools

The results were mostly comparable. However, in two cases the results were positive for the 3-pool assay but not the sum of the relevant parts of the 10-pool assay. In one case this corresponded to a difference of only 8 SFU/million cells. This suggests that in the 3-pool assay, test results which are just above the positive threshold should be interpreted with caution. Further comparison between these assays have been conducted in the lab (data not shown) revealing closely correlated results.

4.3. HITS cohort

PBMC obtained from 84 subjects at their enrolment into the HITS cohort were assayed for the presence of HCV-specific cellular immune responses using the ELISpot assay. 17 assays were discarded due to high background values, some were re-assayed, giving a final total of 74 subjects with valid results.

Of the 74 subjects, 33 (45%) demonstrated HCV-specific cellular immune responses targeting HCV peptides in either the 10- or three-pool ELISpot assay (Figures 4.2 and 4.3).

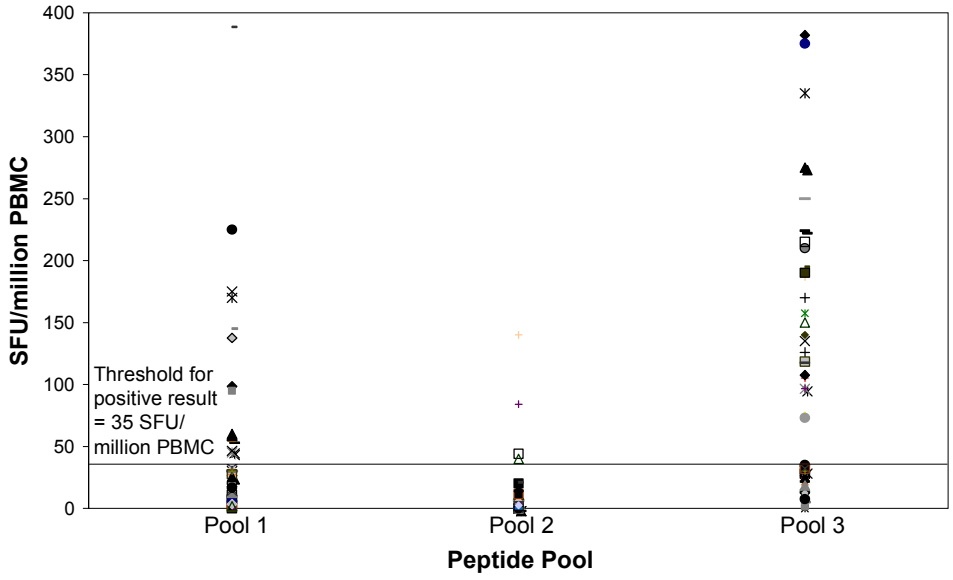

FIGURE 4.2. HCV-specific IFN-γ ELISpot assay with 3 peptide pools

N.B. Note that the Y-axis scale is 0 – 400 and not 0 – 250 as per other graphs

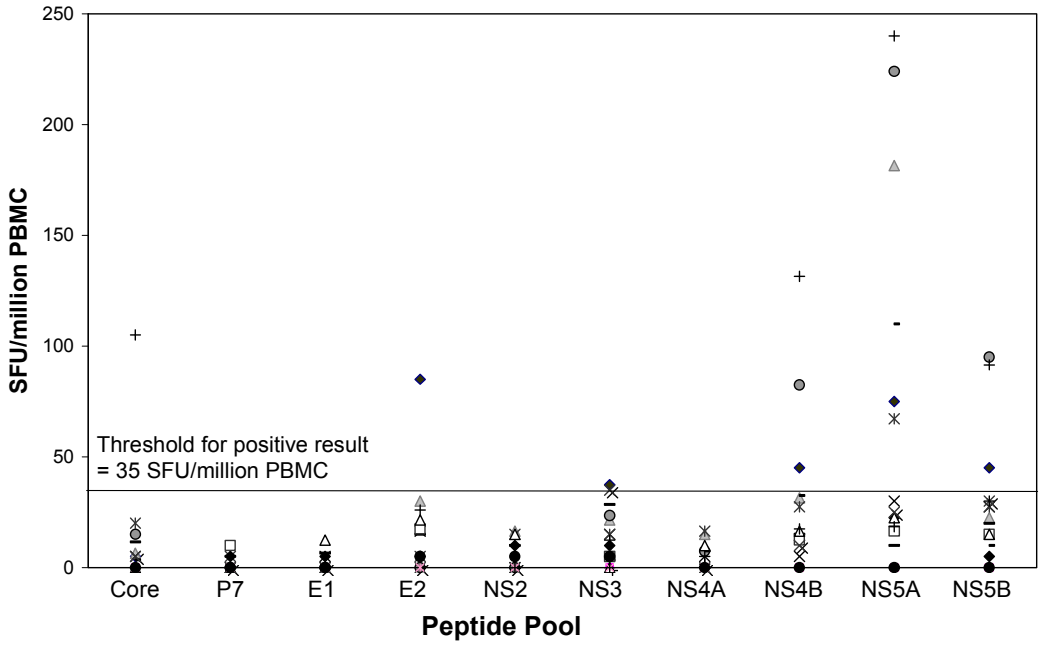

FIGURE 4.3. HCV-specific IFN-γ ELISpot assay with 10 peptide pools

4.4. Pattern of immune responses

4.4.1. Target specificity

The most-commonly targeted parts of the HCV genome in the ELISpot assay were NS4B, NS5A and NS5B. In the three-pool assay, 26 of the 44 (59%) positive immune responses targeted pool 3, corresponding to 49% (26 of 53) of all subjects with an acceptable assay in pool 3. Fourteen of the 44 (32%) positive responses were induced by pool 1.

In the 10-pool assay, six of the 15 positive (40%) immune responses targeted NS5A with NS4B and NS5B targeted by a further 20% each (3 of 15). No immune responses were induced by P7, E1, NS2 and NS4A proteins. Core, E2 and NS3 proteins were each targeted by one of the 16 subjects investigated with the 10-pool assay.

4.4.2. Breadth

Six of 16 subjects demonstrated HCV-specific immune responses in the 10-pool assay (Figure 4.4). At least three proteins were targeted by three of these six subjects, with a maximum of five proteins targeted by one subject.

Subject	Core	P7	E1	E2	NS2	NS3	NS4A	NS4B	NS5A	NS5B
1										
2										
3										
4										
5										
6										

FIGURE 4.4. HCV proteins targeted by positive immune responses in 10-pool assay

Of the 27 subjects with a positive result in the 3-pool assay, 14 targeted at least two of the three pools, with three subjects displaying positive responses to all three pools. Peptide pool 3 elicited positive responses in all of the 11 subjects with responses to two pools. In all but one case, the second targeted pool was pool 1 (Figures 4.5 and 4.6).

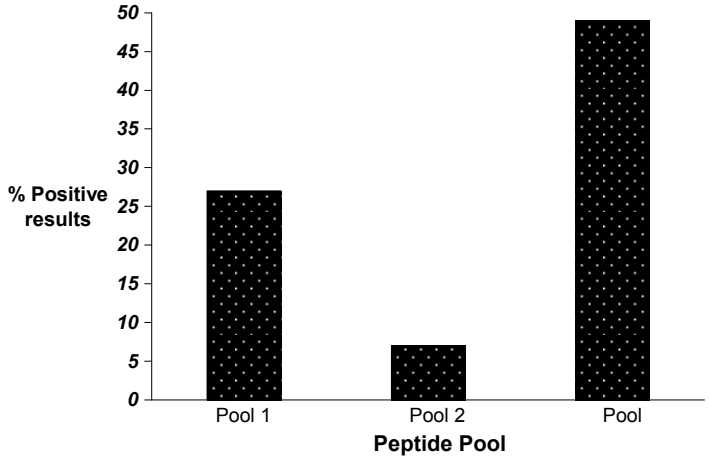

FIGURE 4.5. Bar chart of HCV proteins targeted by positive immune responses in 3-pool assay

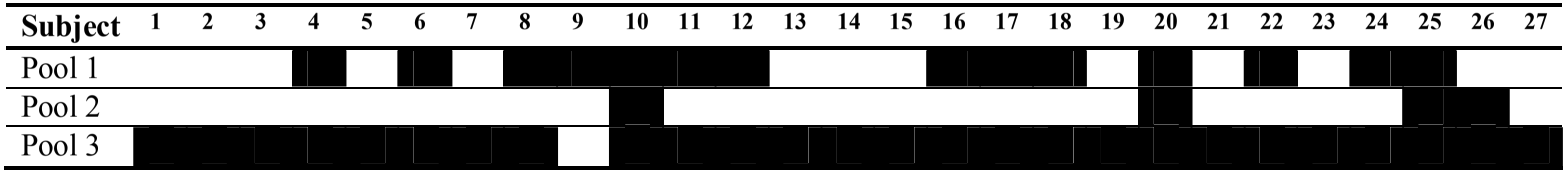

FIGURE 4.6. HCV proteins targeted by positive immune responses in 3-pool assay

4.4.3. Magnitude

Of all subjects with positive responses, the strongest responses were directed towards the non-structural proteins, however the number of individuals with positive responses in each of the 10 peptide pools was small (Tables 4.2 and 4.3).

TABLE 4.2. Magnitude of positive responses in the 3-pool ELISpot assays

	Pool 1 (n = 14)	Pool 2 (n = 4)	Pool 3 (n = 26)
Mean positive response	124	77	185
Range (positive responses only)	(37.5 – 388.5)	(40 – 140)	(75 – 381.85)

TABLE 4.3. Magnitude of positive responses in the 10-pool ELISpot assays

	Core (n = 1)	E2 (n = 1)	NS3 (n = 1)	NS4B (n = 3)	NS5A (n = 6)	NS5B (n = 3)
Mean positive response	105	85	38	86	150	77
Range (positive Responses only)				(45 - 131.5)	(75 – 240)	(45 – 95)

4.5. Clinical outcomes

All of the 74 subjects with acceptable assays had follow-up anti-HCV antibody and HCV RNA test results available to determine their subsequent infection status (Table 4.4). Of the group, 53 (72%) remained aviraemic and seronegative and 21 (28%) seroconverted and were deemed incident infection cases.

TABLE 4.4. Clinical outcome of subjects with and without HCV-specific immune responses

	HCV-specific immune response present	HCV-specific immune response absent	Total
Incident infection	6	15	21
Remained seronegative	27	26	53
Total	33	41	74

Of these 21 infected subjects, data at subsequent follow-up time-points were available for 14, to designate clearance or chronicity (Table 4.5). Seven resolved the acute infection, and seven developed chronic infection. Of the 21 subjects who developed

incident infection, 6 had HCV-specific immune responses as assayed by ELISpot and 15 did not (p=0.14).

TABLE 4.5. Clinical outcome of subjects with incident infection

	HCV-specific immune response present	HCV-specific immune response absent	Total
Cleared infection	1	6	7
Developed chronicity	1	6	7
Outcome awaited	4	3	7

Of those who cleared infection, one had a positive ELISpot test result and six did not. There was no statistically significant difference between outcomes in those with, and without, positive ELISpot results (p=1.0), although it should be noted that the numbers available for this analysis are very small.

The clinical outcome compared to the breadth of ELISpot response is shown in tables 4.6. and 4.7. In the ten-pool assay, none of the subjects with the responses targeting three or more pools developed incident infection, however again numbers were very small (n = 3). The association between a positive response in one or more pool and clinical outcome was not statistically significant (p=0.24).

In the three-pool assay, although none of those with a positive result in all three pools and the majority of those with responses in two pools, did not develop incident infection, a similar majority of those without ELISpot responses also did not develop infection. The association between a positive response in one or more pool and clinical outcome was again not statistically significant (p=0.37).

TABLE 4.6. Clinical outcome of subjects compared to breadth of response in 10-pool assay

Number of pools with a positive response	Number of subjects	Incident infection	No incident infection
0	10	6	4
1	3	1	2
2	0	0	0
3	1	0	1
4	1	0	1
5	1	0	1

TABLE 4.7. Clinical outcome of subjects compared to breadth of response in 3-pool assay

Number of pools with a positive response	Number of subjects	Incident infection	No incident infection
0	31	10	21
1	13	2	11
2	11	3	8
3	3	0	3

4.6. Behavioural analysis

4.6.1. Introduction

Of the 74 subjects with ELISpot data, 69 had completed baseline interviews. These were analysed for differences between those with, and without, positive ELISpot results; and in those who did, and did not, develop incident infection.

4.6.2. Behavioural correlates of positive ELISpot responses

4.6.2.1. Demographics

The majority of the 69 subjects with behavioural data and ELISpot results were male, all but four subjects had been born in Australia, and all but seven identified themselves as heterosexual (Table 4.8). A minority of subjects (24 - 32%) in each group had completed more than 10 years of schooling. One subject was hepatitis B surface antigen positive.

TABLE 4.8. Demographic data

	Positive ELISpot n = 31 (%)	Negative ELISpot n = 38 (%)	p-value
Mean age at enrolment (range)	29.1 (20 – 39)	26.7 (18 - 43)	0.17
Male gender	26 (84)	25 (66)	0.15
Aboriginal/Torres Strait Islander	7 (23)	10 (26)	0.92
Completed >10 years of schooling	6 (19)	9 (24)	0.89
Times incarcerated (range)	2.5 (1 - 8)	2.8 (1 - 15)	0.68
First time in prison	16 (52)	15 (39)	0.44
Using illegal drug at time of offence	25 (81)	32 (84)	1.00
Ever in juvenile detention facility	13 (42)	20 (53)	0.52
Ever received counselling for IDU	25 (83)	27 (71)	0.37

Values given as number and percentage of subjects except where specified, when given as number of subjects and range.

4.6.2.2. Risk factors for transmission of HCV

As per the inclusion criteria, all subjects had injected drugs. The two other most common risk factors for HCV transmission were having been tattooed and pierced (Table 4.9). The mean number of tattoos was similar in both groups (4.8 compared to 3.9) (p= 0.54). One subject in the group with positive responses had been tattooed over 25 times compared to a maximum number of 13 tattoos in the group with negative test results. Of the 48 subjects with tattoos, 14 (29%) had at least one performed whilst incarcerated. In-prison tattoos were not different in prevalence between the groups (p=0.89).

TABLE 4.9. Risk-factors for HCV transmission

	Positive ELISpot n = 31 (%)	Negative ELISpot n = 38 (%)	p-value
Tattoo	25 (81)	25 (66)	0.27
Piercing	22 (71)	28 (74)	1.00
Transfusion (prior to 1990)	1 (0.3)	1 (0.3)	1.00
Fight with blood contact	13 (42)	14 (37)	0.86
Stabbed	9 (29)	12 (32)	1.00
Haircut with breach of skin	12 (39)	7 (18)	0.11
Shared razor	7 (23)	6 (16)	0.68
Blood contact during sport	5 (16)	4 (11)	0.72
Accidental needlestick	5 (16)	5 (13)	0.74

The majority of subjects had had piercings performed (50/69 subjects; 72%) with a mean of 3.3 times (range 1 – 26). There was no statistical difference between the two

ELISpot response groups for the number of piercings (p=0.10), or whether any piercings had been performed inside a correctional facility (p=0.68).

4.6.2.3. IDU-specific behavioural factors

A significant minority of the subjects (29/69; 42%), had injected drugs whilst in prison or a juvenile detention centre (Table 4.10). Over half had previously shared a part or all of their injecting equipment, and 70% (48/69) reported (ever) having been injected by someone else. None of the frequencies of the IDU variables were statistically different between ELISpot positive and negative groups. All but seven subjects had taken a break from injecting in the last twelve months. Neither this, nor the length of the break were statistically different between groups.

TABLE 4.10. IDU-specific risk factors

	Positive ELISpot n = 31 (%)	Negative ELISpot n = 38 (%)	p-value
Mean age at first injection (range)	19.4 (12 – 33)	18.8 (13 - 41)	0.64
Ever injected whilst incarcerated	12 (39)	17 (45)	0.79
Ever injected by someone else	24 (77)	24 (63)	0.31
Ever shared injecting equipment	18 (58)	26 (68)	0.52

The majority of subjects reported a period of injecting at least daily. There was no statistical difference in the report of highest frequency - daily or more often was chosen for this analysis (p=0.76) (Table 4.11).

TABLE 4.11. Highest frequency of IDU

Frequency of injecting	Positive ELISpot result (n = 31)	Negative ELISpot result (n = 38)
More than once/day	22	17
Daily	3	9
Weekly or more often	2	7
Monthly or more often	1	2
Less than monthly	3	3

The most commonly injected drug in both groups was 'ice' (*i.e.* crystal methamphetamine) (Table 4.12), and was the only drug used that was statistically

TABLE 4.12. Main drug injected

	Positive ELISpot n = 31 (%)	Negative ELISpot n = 38 (%)	p-value
Heroin	19 (61)	27 (38)	0.55
Buprenorphine	10 (32)	10 (26)	0.78
Ice	30 (97)	28 (74)	**0.02**
GHB/GBH/Liquid E	1 (3)	2 (5)	1.00
Cocaine	11 (35)	13 (34)	0.89
Benz	3 (10)	2 (5)	0.65
Steroid	2 (6)	1 (3)	0.58
Opiates	4 (13)	6 (16)	1.00
Hallucinogens	3 (10)	3 (8)	1.00
Ecstacy	5 (16)	5 (13)	0.74
Ketamine	1 (3)	2 (5)	1.00

Ice – Crystal methamphetamine

GHB/GBH – gamma hydroxy butyrate

different in frequency between the two groups. A higher frequency of ice injecting was seen in those with a positive ELISpot response (p=0.02).

Of the 69 subjects, 21 (30%) reported injecting since being incarcerated on this occasion (Table 4.13). All but two of these 21 had had used equipment that they reported was not likely to be sterile, corresponding to 28% of the whole sample group. Twelve subjects, six in each group, had received help to inject from someone else (p=0.37).

TABLE 4.13. IDU during this period of imprisonment in those with and without positive ELISpot results

	Positive ELISpot n = 31 (%)	**Negative ELISpot n = 38 (%)**	**p-value**
Injected	8 (26)	13 (34)	0.62
Injected by someone else	5 (16)	7 (18)	0.89
Helped to inject by someone else	6 (19)	6 (16)	0.37
Used potentially unsterile equipment	8 (26)	11 (29)	1.00

4.6.3. Behavioural characteristics associated with incident infection

There was a trend towards association with younger age at enrolment and incident infection (p=0.07) (Table 4.14).

Table 4.14. Demographic data in those with and without incident infection

	Incident case n = 19 (%)	Remained seronegative n = 50 (%)	p-value
Mean age at enrolment (range)	25.3 (18 – 39)	28.7 (18 - 43)	**0.07**
Male gender	12 (63)	39 (78)	0.23
Aboriginal/Torres Strait Islander	7 (37)	10 (20)	0.21
>10 years schooling	3 (16)	12 (24)	0.53
Times incarcerated	3 (1 – 15)	2.56 (1 – 7)	0.5
First time in prison	5 (26)	26 (52)	1.00
Using illegal drug at time of offence	17 (89)	40 (80)	0.49
Ever in juvenile detention facility	12 (63)	21 (42)	0.19
Ever received counselling for IDU	13 (68)	39 (80)	0.35

There was a statistically significant association between ever having been tattooed and incident infection (p=0.01) (Table 4.15), although in the unexpected direction (*i.e.* more tattoos, less likely to become infected). No association was seen with the number of tattoos (p=0.79), and whether they had been performed whilst incarcerated (p=1.0).

There was no significant association seen between risk variables specific to IDU and incident infection within prison (Table 4.16).

TABLE 4.15. Risk factors for transmission in those with and without incident infection

	Incident Case n = 19 (%)	Remained seronegative n = 50 (%)	p-value
Tattoo	9 (47)	41 (82)	**0.01**
Piercing	14 (74)	36 (72)	0.86
Transfusion (prior to 1990)	0 (0)	2 (4)	1.00
Fight with blood contact	8 (42)	19 (38)	1.00
Stabbed	9 (47)	12 (24)	0.11
Haircut with breach of skin	4 (19)	15 (30)	0.66
Shared razor	4 (21)	9 (18)	1.00
Blood contact during sport	2 (11)	7 (14)	1.00
Accidental needlestick	3 (16)	7 (14)	1.00

TABLE 4.16. IDU-specific risk factors in those with and without incident infection

	Incident Case n = 19 (%)	Remained seronegative N = 50 (%)	p-value
Mean age at first injection (range)	18.5 (12 - 31)	19.3 (12 - 41)	0.59
Ever injected whilst incarcerated	9 (47)	20 (40)	0.78
Ever injected by someone else	12 (63)	36 (72)	0.67
Ever shared injecting equipment	13 (68)	31 (62)	0.82
Taken a break from IDU in last 12 months	16 (84)	46 (92)	0.38
Injected since this incarceration	6 (32)	15 (30)	0.86
Injected by someone else during this incarceration	3 (16)	9 (18)	1.00
Used non-sterile equipment during this incarceration	6 (32)	13 (26)	0.78

No association was found between becoming infected and the frequency of injecting Table 4.17).

TABLE 4.17. Highest frequency of injecting in those with and without incident infection

Frequency of injecting	Incident case (n = 19)	Remained seronegative (n = 50)
More than once/day	13	26
Daily	2	10
Weekly or more often	2	7
Monthly or more often	0	3
Less than monthly	2	4

There was no association with the drug injected, including 'ice' which reached significance with incident infection (Table 4.18).

TABLE 4.18. Main drug injected in those with and without incident infection

Variable	Incident Cases n = 19 (%)	Remained seronegative n = 50 (%)	p-value
Heroin	12 (71)	34 (68)	0.92
Buprenorphine	5 (26)	15 (30)	1.00
Ice	16 (84)	42 (84)	1.00
GHB/GBH/LIQUID E	0 (0)	3 (6)	0.56
Cocaine	7 (37)	17 (34)	1.00
Benz	2 (11)	3 (6)	0.61
Steroid	1 (5)	2 (4)	1.00
Opiates	2 (11)	8 (16)	0.72
Hallucinogens	3 (16)	3 (6)	0.34
Ecstacy	3 (16)	7 (14)	1.00
Ketamine	0 (0)	3 (6)	0.56

5 DISCUSSION

HCV is an important cause of significant morbidity and mortality worldwide. IDU is the most common mode of transmission in developed countries [2, 44, 71], and although both the prevalence and incidence is high in this group, no study has reported rates of 100% [46, 72-74, 368]. This may be due to "staying safe" behavioural factors [369], some of which may be amenable to public health intervention. Additionally, it is possible that regular, low-level antigenic exposure may prime the immune response sufficiently to clear incident infection before viraemia is detectable by conventional assays, accounting for the HCV-specific cellular immune responses demonstrated in high-risk aviraemic IDU [131, 133, 140, 141]. This concept of 'protective immunity' has been described in HIV, but has not been fully investigated in HCV [144, 146] [145].

The prospective, prison-based cohort studied here provided a unique opportunity to reliably identify incident infection, which usually passes undetected, and unique access to detailed behavioural data and longitudinally-collected samples in both those who did, and did not, remain uninfected. A significant rate of incident infection was found (28%; 21 of 74). This substantive incidence highlights the public health importance of prisons as ongoing reservoirs for HCV, and the need to allocate target preventative and therapeutic measures to this setting.

A significant prevalence of HCV-specific immune responses was found, with 45% (33 of 74) of assayed subjects demonstrating a response. This is similar to a rate of 46% found in one study of 26 IDU [133], but lower than other rates of 62 – 76% which have been reported [140, 141]. However, in the latter of these studies HCV-specific responses were assessed by T-cell proliferation as well as by ELISpot - if

IFN-γ production as assessed by ELISpot was taken alone, the prevalence dropped to only 23% (six of 26) [141]. The other study used lymphoblastoid cell lines infected with vaccinia virus constructs as antigen-presenting cells, with the HCV polyprotein encoded by only two constructs – therefore there was more antigenic stimulus per construct than in each of the peptide pools used in the assays in this study [140]. In addition, the sample size in these previous studies were markedly smaller (n = 4 – 29), than the dataset described here. Taken together, these data suggest that the prevalence of HCV-specific cellular immunity is high in IDU, and is likely to be higher than in other previously examined groups such as household contacts (although this remains to be confirmed in a direct comparison study).

In this cohort, the non-structural proteins NS4B, NS5A and NS5B were the most commonly targeted, and with a higher magnitude than other proteins, which is consistent with the literature in seronegative, aviraemic subjects [140], although one study in IDU found that the strongest and most frequent responses were observed towards peptides spanning the envelope glycoproteins 1 and 2 [133]. Preferential targeting of the non-structural proteins has also been consistently described in those with successful resolution of acute infection (see section 1.4.3).

Although the presence of immunity has previously been described in those who have apparently never been infected, this is the first dataset that has explored the potential consequences of this immune response (*i.e*. the concept of 'protective immunity' in HCV infection). A higher percentage of those without HCV-specific immune responses developed incident infection than those with HCV-specific immune responses (37% vs. 18%; table 4.4), however this difference was not statistically significant. This may reflect a true absence of 'protection,' but may also be due to the small sample size. There were equal numbers of those who cleared incident infection

and those who developed chronic infection within the group with ELISpot responses, and those that did not. This strongly argues against a role for these immune responses in increasing the likelihood of clearance, but it should be noted again that the numbers were small (n = 2 for the ELISpot positive group). Further analysis with additional follow-up data for more subjects may clarify the presence or absence of an association. However, it is clear that HCV-specific immune responses have been demonstrated in subjects who subsequently become infected and develop chronic infection. This suggests that the presence of HCV-specific ELISpot responses in not a simple correlate to protective immunity against HCV infection.

The immune response of the only subject with positive 10-pool assay results who developed infection, targeted NS5A only at a magnitude of 181.5 SFU/million cells. Although three of the other five subjects demonstrated a broader response, the remaining two also targeted NS5A alone, but more weakly. In the subjects with positive three pool assay results, none of the three subjects with responses to all three pools developed incident infection. However, as only two of the 13 with responses to one pool did become incident cases, no protective association with the breadth of the HCV-specific immune response, such as that generally observed in those who clear incident infection, can be concluded.

One potential alternative explanation is that the chosen assay, HCV-specific IFN-γ ELISpot,is an inadequate surrogate marker for protective immune responses. This context is very similar to the recent outcomes of a randomised controlled trial of a candidate T-cell based HIV vaccine which was terminated early due to higher infection rates in the treatment than the placebo arm, despite clear demonstration in ELISpot assays of high magnitude cellular immune responses against HIV-type 1 peptides in healthy adults [370]. ELISpot responses were also measured in a sample

of trial participants at various points of the vaccination schedule, with no difference found in frequency, magnitude or target specificity of responses between those who developed HIV infection and those who did not [371]. The absence of correlation between immune responses to the vaccine, and the acquisition of HIV infection questions the intrinsic assumption that ELISpot positivity is a simple correlate of protective immunity *in vivo*.

Finally, the ELISpot assay measures only one potential component of the 'protective immune' phenomenon, which is likely to include elements of the other arms of the immune system, which have been shown to be associated with control of HCV in primary infection, including HCV-specific CD4+ T-cell responses and neutralising antibodies.

The importance of the both CD4+ and CD8+ T-cells, and their interaction, has been mentioned above (section 1.4.3.), with spontaneous clearance most often associated with early, broad strong responses from both of these arms of the cellular immune response. Loss of HCV-specific CD4+ T-cells within the first months of infection has been associated with relapse of viraemia [236], and whereas protection from re-infection in chimpanzees was lost when memory CD8+ T-cells where depleted prior to rechallenge [372], depletion of CD4+ memory T-cells resulted in persistence of virus despite presence of CD8+ memory T-cells [254]. One study that delineated potential functional T-cell thresholds in subjects with acute infection found that if five or more of the 33 evaluated peptide pools (spanning approximately 15% of the HCV genome) were targeted by CD4+ T-cells early after infection, the chance of recovery was seven-fold higher than if that threshold was not reached [232]. This is in comparison to a threshold of at least two peptide pools targeted by CD8+ T-cells, however the likelihood of clearance was not increased to the same degree. It is clear

that both CD4+ and CD8+ T-cells are required for effective control of HCV infection, however other elements are required as infection can persist even when an early and robust cellular response is made.

A potential protective role for NK cells in high-risk subjects has recently gained interest. In a study of 25 IDU, *in vitro* expression of the natural cytotoxicity receptor (NCR) NKp30, which is involved in NK cell activation and cytolytic activity, was significantly upregulated in the 11 subjects that remained uninfected [373]. A correlation was seen between expression of this receptor and lymphokine-activated killing in the uninfected subjects. In addition, unstimulated NK cells expressing high levels of NKp30 from four normal subjects, prevented JFH-1 infection of Huh-7.5 replicons more effectively, demonstrated more efficient degranulation and expressed more perforin, than those with low or negative expression. Another high-risk group that may be protected from HCV infection are infants born to HCV-positive mothers, with a strikingly low rate of vertical transmission of up to 7% [53]. Another study examined placental and cord blood of 12 treatment-naïve pregnant women with chronic HCV using flow cytometry [374]. Placental NK T-cell proportions and cytotoxicity (assessed by CD107a expression) was increased in those with HCV infection. Additionally, another innate system population, gamma delta T-cells, were also increased in HCV-exposed placentas compared to controls. Further studies are required correlating development of incident infection in relation to the presence of integrated HCV-specific multi-cellular and humoral responses.

In the behavioural analysis, a statistically significant association was found between injecting methamphetamine and the presence of HCV-specific cellular immune responses. Methamphetamine has been identified as a risk factor for HIV infection, partly due to its effects on high-risk sexual behaviour [375-379], but also due to

modulation of the immune response to HIV [380, 381]. An *in vitro study* using HIV-infected macrophages cultured from monocytes isolated from healthy donors showed that treatment with methamphetamine resulted in a significant time- and dose-dependant increase in HIV reverse transcriptase (RT) activity [380]. Dopamine-1 receptors (D1R) were shown to be expressed on macrophages, and antagonists for this receptor completely blocked the effect of methamphetamine on HIV RT activity. Methamphetamine-induced upregulation of macrophage expression of the primary co-receptor for HIV entry, CCR5, was demonstrated by flow cytometry, however D1R antagonists did not block this effect. Methamphetamine treatment of macrophages also resulted in a significant decrease of endogenous IFN-α at both mRNA and protein levels, an effect that was again blocked by D1R antagonists. The potential downstream effect on IFN-signalling was demonstrated by the finding that macrophages treated with methamphetamine also expressed lower levels of STAT1 proteins than untreated macrophages.

The role of methamphetamine in HCV infection is less well known, with most of the available research focussing on its effects on the clinical sequelae of HCV infection [382, 383]. Whilst the above-mentioned effect of methamphetamine on CCR5 should not influence HCV transmission, its suppressive effect on IFN-α and STAT1, a crucial factor in mediating IFN-dependent responses, may play a role in failure of the host immune system to control acute HCV infection therefore increasing the likelihood of progression to chronic infection. Also, methamphetamine exposure has been shown to significantly inhibit Th1 cytokine production in splenocytes and significantly increase the expression if TNF-α and IL-6 [384]. Therefore those who inject methamphetamine may be less likely to demonstrate HCV-specific cellular immune responses, which was not the finding from the data presented here.

In addition, although sexual transmission is not a common route of HCV transmission, documented links between risky sexual behaviour and drug use practices make [385] an association plausible.

A study of 219 street-involved youth in Canada reported that the 62 IDU with HCV infection were more likely to use methamphetamine than the 157 who did not have infection (odds ratio 2.43; confidence interval 1.33 – 4.43) [386]. However, infection was determined by anti-HCV antibody positivity, and incident infection was not examined. Another study of 732 methamphetamine-dependent users who sought outpatient treatment between 1999 and 2005 reported a HCV prevalence rate of 15%, with 44% of those who injected the drug (as opposed to smoking and intranasal use) affected [387]. The study is limited by its retrospective design, determination of HCV infection status from medical examination records, failure to assess other important risk factors such as unsafe sexual practices, tattooing, body piercing and drug-sharing practices, and the fact that the population was drawn only from methamphetamine users who had sought treatment.

This is the first prospective data in seronegative IDU to identify a possible association with methamphetamine and HCV-specific immune responses. As mentioned above an immunomodulatory effect of methamphetamine has been shown *in vitro* [388], although it is generally regarded as immune-suppressing. Although methamphetamine had no cytotoxic effect on Huh7 cells, it was found to suppress IFN-α expression and significantly increase HCV RNA in cells infected with JFH-1 in a time-dependent manner, as well as decreasing the anti-HCV effect of recombinant IFN-α on both JFH-1-infected Huh7 cells (representing acute infection) and stable Huh7 cell chromosomally integrated with JFH-1 (representing chronic infection). A potential mechanism for this inhibitory effect on the innate immune

system was suggested by the same study by demonstration of lower levels of STAT-1 and IRF-5 in cells treated with methamphetamine.

Methamphetamine has also been shown to decrease IL-2 and IFN-γ secretion, decrease IgG level, decrease proliferation of mitogen-stimulated B and T-cells, suppress dendritic cell and macrophage function and increase TNF-α expression and NK cell activity in murine cells *in vitro* [384, 389, 390]. Additionally, the balance of thymic T-cells was altered, with an increase in CD4+ cells, and decrease in CD8+ and CD4+CD8+ cells [389].

An association with injecting of ice and incident infection was not found, but this possibility is particularly concerning as methamphetamine use is increasing, with the prevalence of its use amongst IDU suggested to be as high as 50% [391].

Tattooing has consistently been shown to be associated with HCV infection [91, 392-398], with some data specifically focussing on tattooing in prison [91, 393, 397, 398]. Interestingly, the behavioural analysis revealed a statistically significant *negative* association with tattooing and incident infection. Given the volume of long-standing data to the contrary, this is unlikely to represent a valid finding and may be due to the fact that only 19 of the subjects with behavioural and ELISpot data developed incident infection and thus the numbers available for this analysis were small (*i.e.* a Type I error). It is also important to note that this dataset was a pilot study, with multiple statistical comparisons made. Multivariate analysis performed on the entire HITS data-set (publication in progress) may well yield different and more robust associations.

It has also been suggested that a low infective dose, such as that incurred by accidentally-exposed healthcare workers, [142], may induce an immune response in the absence of viraemia. It is possible, especially with the increasing awareness of the

need for safe practices to avoid HCV transmission, that those infected by contaminated tattoo needles, received a 'dose' of HCV that induced transient viraemia and cellular immune responses, without seroconversion, which then decreased the likelihood of developing chronic infection if subsequent high-dose exposure occurred, and hence the negative association demonstrated by this study reflects an uncommon phenomenon.

In addition, tattooing of DNA has been shown to induce strong immune responses, due to the high prevalence of antigen-presenting cells in the form of Langerhans cells in the epidermis and dendritic cells in the dermis [399-403], an observation that has been utilised in the development of DNA vaccines [404]. Vaccination via tattooing was shown to be associated with stronger humoral and cellular immune responses to a codon modified gene encoding a human papilloma virus type 16 protein than via an intradermal needle in mice [403]. However work using an *ex vivo* human skin model demonstrated that DNA tattooing was an inefficient process, with only approximately 2% of epidermal cells transfected [405]. In addition, longitudinal measurement of expression of skin tattooed with firefly luciferase reporter plasmid showed that expression was restricted to areas of tattooing, with expression peaking at 2 – 18 hours and no longer detectable after 2 – 3 days [405].

Tattooing of RNA has not been studied, however electroporation, a technique involving brief electrical pulses that create transient 'pores' in the cell membrane has been used to facilitate entry of both DNA and RNA into the cell cytoplasm *in vitro* and in animal models [404, 406-409] and has been shown to enhance humoral and cellular immune responses in animals [410]. Additionally, delivery of a HCV DNA candidate vaccine has been trialled in humans with electroporation [411].

It is therefore possible that a tattoo applied with HCV-infected equipment could induce HCV immunity via abortive infection of dendritic cells with minimal resultant viraemia but successful antigen presentation to T-cells.

Development of an ultra-sensitive assay for 'occult' HCV detection is difficult, partly due to concerns regarding contamination, but also due to uncertainty regarding whether negative results are due to insufficient sensitivity of the assay, or due to true absence of RNA, especially when a 'gold standard' does not exist. Many problems were encountered during optimisation of the occult virus assay in this study, some of which were addressed in further work conducted after completion of this thesis, (publication in progress). Ultra-sensitive RT-nPCR assays, with sequencing of amplicons in both directions, were performed on RNA extracted from the PBMCs and plasma of the subjects described in this thesis. The difficulties regarding contamination were demonstrated by detection of identical sequences in a chronically infected subject and a negative control in the Michalak laboratory. Importantly however, one seronegative subject met the stringent criteria for a positive occult virus result, defined as demonstration of matching sequences obtained from both plasma and PBMCs from both centres, without identity to known laboratory sequences or other possible contaminants. The genotype 1 origin of this occult infection was also confirmed from the Core region sequence. This finding argues strongly for the validity of the existence of occult infection, albeit as a rare phenomenon.

One of the previously discussed occult virus studies, found that in the longitudinally collected samples of recovered subjects with occult virus detected, the timepoints where HCV was detectable corresponded with the intensity of HCV-specific T-cell responses, with both RNA and cellular immune responses becoming less likely over time [352]. This led to the novel suggestion that there might be an unrecognised

infection outcome, characterised by protracted, possibly indefinite, periods of viral suppression, interspersed with periods of viral escape with resultant stimulation of the immune system. The findings from completion of the planned occult virus study, discussed above, are consistent with this theory.

In this situation, serial detection of occult virus may ultimately contribute to clinical decisions regarding the need for treatment in apparently aviraemic subjects, with ongoing elevation of hepatic enzymes and histological evidence of fibrosis and/or necro-inflammatory activity.

In conclusion, a large body of work has demonstrated HCV-specific cellular immune responses in apparently uninfected subjects. However the role of this immune response regarding clinical outcomes remains unclear, and the whether minute amounts of HCV RNA are required to maintain this response has not been resolved.

6. APPENDIX A – Behavioural questionnaire

HITS-p Baseline Interview

2x2 Code (Surname first)						
Date of Birth	D	D	M	M	Y	Y
MIN						
Current Prison [Interviewer – see code on page 2]						
Interviewers Initials						

Serology: (ensure that both the Date and Positive/Negative/Unknown fields are completed)

HIV, HCV, HBV status	**Date**	**Positive**		**Negative**		**Unknown/ Not done**	
HIV Ab			1		2		9
HbcAb (core antibody)			1		2		9
HbsAb (surface antibody)			1		2		9
HbsAg (surface antigen)			1		2		9
HCV Ab			1		2		9
HCV PCR			1		2		9

Eligibility criteria (tick relevant boxes)	**Yes**		**No**		**Unknown**	
Age 18 years or greater		1		2		9
Ever injected drugs?		1		2		9
Been in prison less than 12 months		1		2		9

Enrolment Criteria (tick relevant boxes)	**Yes**		**No**		**Date**
Signed Consent form		1		2	
Subject recruited?		1		2	
Intake Interview completed?		1		2	
Follow up Contact Form completed?		1		2	
Blood taken?		1		2	

HITS test results: (Admin use only)

	Date	**Positive**		**Negative**		**Unknown**	
HCV Ab			1		2		9
HCV PCR			1		2		9

Current Prison (please tick relevant box)

Bathurst		1
Berrima		2
Bolwara Transitional Centre		3
Brewarrina		4
Broken Hill		5
Cessnock		6
Cooma		7
Dawn De Loas		8
Dilwynnia		9
Emu Plains		10
Glen Innes		11
Goulburn		12
Grafton		13
John Morony I		14
John Morony II		15
Junee		16
Ivanhoe		17

Kirkconnell		18
Lithgow		19
Long Bay Complex		20
Maitland		21
Mannus		22
Mid North Coast		23
MRRC		24
Mulawa		25
Norma Parker		26
Oberon		27
Parklea		28
Parramatta		29
Silverwater		30
St.Heliers		31
Tamworth		32
Wellington		33

I. THIS SECTION SHOULD BE COLLECTED FROM OIMS/MEDICAL RECORD

1.	On reception was the client assessed to be intoxicated?	Yes (Go to Q 2) No (Go to Q 3) Unknown	☐ 1 ☐ 2 ☐ 9
2.	What substance caused the intoxication?	_____ Unknown	☐ 99
3.	When was the inmate arrested this time?	____/____/____ Don't Know	☐ 9
4.	When did the inmate come into prison this time?	____/_____/____ Don't Know	☐ 9
5.	Has the inmate been sentenced?	Yes (go to 6) No (go to 7) Don't know (go to 7)	☐ 1 ☐ 2 ☐ 9
6.	What is the total length of this sentence? *[Interviewer: Record the maximum sentence length]*	_____MONTHS Don't know	☐ 999
7.	What is inmate's current security Classification?	_____	
8.	How many times has the inmate been in an adult prison including this episode?	_____ Don't Know	☐ 9

9. What is the most serious offence that the inmate is in prison for this time?
[Interviewer: Circle one of the following options]

1 = Robbery
2 = Armed robbery
3 = Drug offences
4 = Goods in custody
5 = Sexual assault
6 = Aggravated assault
7 = Grievous bodily harm
8 = Malicious wounding
9 = Rioting
10 = Fraud
11 = Shop lifting
12 = Driving/traffic offences
13 = Break and enter
14 = Larceny
15 = Enter with intent to steal
16 = Other assault
17 = Prohibited weapon
18 = Breach of AVO
19 = Breach of parole
20 = Murder
21 = Assault and GBH
22 = Assault actioning actual bodily harm
34 = Attempt murder
35 = Conspiracy to murder
36 = Manslaughter
37 = Contempt of court
38 = Accessory after the fact
39 = Escape lawful custody
40 = Resisting arrest
41 = Perjury, bribery
42 = Falsify documents
43 = Aggravated robbery
44 = Battery
45 = Harassment
46 = Stalking
47 = Not reporting serious offense
48 = Home invasion
49 = Other offences
99 = Unknown

Other _____

23 = Trespass
24 = Malicious damage
25 = Arson
26 = Bail offences
27 = Breach of suspended sentence
28 = Breach of bond
29 = Other steal
30 = Rape
31 = Incest/carnal knowledge
32 = Indecent assault
33 = Buggery/bestiality

Instructions for interviewer:

- **Complete all questions, unless instructed to go on to a specific question.**
- **Read the question exactly as it appears.**
- **For all questions, await a response from the subject, then code according to the responses listed.**
- **If the subject does not understand the question, repeat the question and list all of the responses before requesting a response from the subject.**
- **Most questions require you to tick the box next to the appropriate response number. Those with line require an answer to be written by you.**
- Comments in *italics* explain aspects of the question.
- Don't let the inmate know that by choosing option "NO" they can skip a section, since it may increase the chance of lying to finish off quickly.

II. WE WILL START WITH SOME BACKGROUND INFORMATION [INTERVIEW]

10.. What is your gender?

Male ☐
Female ☐
Transgender ☐

11. Are you an Aboriginal?

Yes ☐
No ☐
Don't Know ☐

12. Are you a Torres Strait Islander?

Yes ☐
No ☐
Don't Know ☐

13. What country were you born in? _____

14. a. What country was your father born in?
[Interviewer: If unknown code (9) in the provided space] _____
b. What country was your mother born in?
[Interviewer: If unknown code (9) in the provided space] _____

15. What is the main language you speak at home? English ☐
Other (if not English) _____

16. Have you ever been in a juvenile detention centre?

Yes ☐
No ☐

17.	Were you using, or withdrawing from, an illegal drug when you committed the offence for which you are in prison?	Yes No Don't Recall	
18.	Would you describe yourself as:	**Straight** Gay/Lesbian Bisexual	
19.	How many years of schooling have you completed? *[Interviewer: for answers like "intermediate school", or "junior high", ask the number of the years and tick the appropriate box.* Tertiary Education	No formal education 1-6 years at school 7-10 years at school 11-12 years at school TAFE	

III. THIS SECTION IS ABOUT RISK FACTORS FOR SPREAD OF HEP C
[*Interviewer: all unsure responses, code as "Don't recall"]*

20.	Have you ever had a tattoo?	Yes (Go to 21) No (Go to 23) Don't recall (Go to 23)	
21.	How many different times have you been tattooed? [*Interviewer: consider each session of tattooing as a separate tattoo*] *[Interviewer: Include tattoos that have been removed]*	_____	
22.	Were the tattoos done inside or outside of prison or a juvenile detention centre?	Inside Outside Both Inside and outside Don't know	
23.	Have you ever had any part of your body pierced? [*Interviewer: must mention that body piercing include ear piercing as well*]	Yes (go to 24) No (go to 26) Don't recall (go to 26)	
24.	How many times have you had your body pierced? [*Interviewer: include piercings that do not currently have a ring in them*] _____ [*Interviewer: if only ears are pierced, write the total number of the piercing in both ears, i.e. 1 piercing each ear = 2 in total]*		

25.	Were the piercings done inside or outside of a prison or a juvenile detention centre?	Inside Outside Both inside and outside Don't recall	
26.	Did you have a blood transfusion prior to 1990?	Yes No Don't recall	
27.	Have you ever been in a fight where blood from another person may have come in contact with your mouth, eyes or an open wound?	Yes No Don't recall	
28.	Have you ever been stabbed? [*Interviewer: do not include self-stabbing*]	Yes No Don't recall	
29.	Have you ever had a haircut where your skin or scalp was cut?	**Yes** No Unsure/Don't recall	
30.	Have you ever shared the same razor as someone else?	Yes No Don't recall	
31.	Have you ever had someone else's blood on you during sport? (e.g. during a football game)	Yes No Don't recall	
32.	Have you ever been accidentally pricked by a needle? (e.g. needle stick injury) [*Interviewer: do not include IDU*]	Yes No Don't recall	

IV. THIS IS A SECTION ABOUT DRUG U

[*Interviewer: must tell the inmates that the same questions about drug use are referring to different times their life*]

33.	Have you ever used illegal drugs?	**Yes** No Don't recall	
34.	Have you ever injected drugs?	Yes (go to Q35) No (go to Q 85) Don't recall (go to Q 85)	
35.	Which of the following drugs have you injected? *[interviewer: read the options and emphasise "injecting"]*	**Heroin** Buprenorphine (Subutex) **Ice (crystal meth/amphetamine)** GHB/GBH/ liquid e/fantasy Cocaine/ Coke Benzodiazepines/Benzos Anabolic/Steroids Other opiates/codeine/pethidine/opium/omnopon Hallucinogens/LSD/ Acid/Magic/Mushies/Daitura Ecstacy/ E/MDA/MDMA Ketamine/Special K Oxycodone (e.g. Oxycontin, Endone) Methadone Morphine (e.g. MS Contin) Speed/base (or other methamphetamine Other (please specify)	
36.	How old were you when you first injected drugs?	_____ Years Don't recall	
37.	Think about the period when you were injecting most frequently. Which one of the following best describes how frequent that was? [*Interview: "injecting" refers to IDU*] *[Interviewer: check that "daily" and " more than*	Less than monthly Monthly or more often Weekly or more often Daily More than once a day	

	once a day" are distinguished]	Don't recall	
38.	Have you ever injected drugs in prison or a juvenile detention centre?	***Yes***	
		No	
		Don't recall	
39.	Has someone else ever injected you with drugs? (Given you a hit?) [*Interviewer: do not include medical injections*]	Yes	
		No	
		Don't recall	
40.	Have you ever shared any part of the injecting equipment? That includes the needle, syringe, spoon, swabs, filters, mix or tourniquet. [*Interviewer : mention that this includes sharing with their partner, and even once is a "yes"*]	Yes	
		No	
		Don't recall	

V. THIS SECTION IS ABOUT TAKING BREAK FROM INJECTING

[*Interviewer: Ask if drugs have been injected in the last 12 months. If so, proceed with the next two questions "Break" is absolutely NO injecting*]

41.	In the last 12 months have you had a break from injecting?	Yes (Go to Q 42)	
		No (Go to Q 43)	
		Don't recall (Go to Q 43)	
42.	***How long was the longest break?***	Less than one month	
		1- 3 months	
		3- 6 months	
		6-9 months	
		9-12 months	
		More than 12 months	

VI. THE NEXT SECTION REFERS TO DRUG USE IN THE 3 MONTHS BEFORE COMING INTO PRISON

[*Interviewer: Check OIMS or by asking the inmate to clarify if this is the first imprisonment*]
[*Interviewer: if the inmate been outside the prison less than 3 months Q43-Q64 should refer just to that perio*

43.	How long were you out before coming in to	3 months or more	

prison this time? _____

[Interviewer: If less than 3 months, write the duration and adjust the questions accordingly]

First time in prison

44. In the 3 months *[or as appropriate]* before coming into prison did you inject drugs?

Yes (go to Q45)
No (go to Q65)
Don't recall (go to Q65)

45. In the 3 months *[or as appropriate]* before coming into prison how often did you inject drugs?

[Interviewer: check that "daily" and " more than once a day" are distinguished]

Less than monthly
Monthly or more often
Weekly or more often
Daily
More than once a day
Don't recall

46. In the 3 months *[or as appropriate]* before coming into prison, compared to the rest of your life, has your injecting pattern been…
[Interviewer: assess the lifetime pattern of injecting and code yes if frequency, sharing behaviours or drug of choice have changed]

Stable
Increasing
Decreasing
Don't recall

47. In the 3 months *[or as appropriate]* before coming to prison which drugs did you inject?
[Interviewer: read the options and emphasise "injecting"]

Heroin
Buprenorphine (Subutex)
Ice (crystal meth/amphetamine)
GHB/GBH/ liquid e/fantasy
Cocaine/ Coke
Benzodiazepines/Benzos
Anabolic/Steroids
Other opiates/codeine/pethidine/opium/omnopon
Hallucinogens/LSD/ Acid/Magic/Mushies/Daitura
Ecstacy/ E/MDA/MDMA
Ketamine/Special K
Oxycodone (e.g. Oxycontin, Endone)
Methadone
Morphine (e.g. MS Contin)
Speed/base (or other

[Interviewer: Exclude medications prescribed and administered by health workers, e.g. morphine in hospital]

		methamphetamine	
		Other (please specify)	
48.	In the 3 months *[or as appropriate]* before coming into prison has someone else injected you with drugs? (Given you a hit?)	Yes No Don't recall	
49.	In the 3 months *[or as appropriate]* before coming into prison have you shared any part of the injecting equipment? That includes the needle, syringe, spoon, swabs, filters, mix or tourniquet? [*Interviewer: mention that this includes sharing with their partner, and even once is a "yes"*]	Yes No Don't recall	
50.	In the 3 months *[or as appropriate]* before coming into prison did you at any time use injecting equipment that was not new and sterile? [*Interviewer: include the needle, syringe, spoon, swabs, filter, mix, and code only one response – even once is a "yes"*]	Yes No **Don't recall**	
51.	In the 3 months *[or as appropriate]* before coming into prison did you use injecting equipment after someone else had used it?	Yes (go to 52) No (go to 61) Don't recall (go to 61)	
52.	In the 3 months *[or as appropriate]* before coming into prison how often did you use equipment after someone else had used it? *[Interviewer: check that "daily" and " more than once a day" are distinguished]* Don't know but did use after someone else	Less than monthly Monthly or more often Weekly or more often Daily More than once a day **Don't recall**	

In the 3 months* [or as appropriate] *before coming into prison which equipment did you share?

		Yes		No		Don't kno
53.	**Needle and syringe**		1		2	
54.	Spoon		1		2	
55.	Mix		1		2	
56.	Filter		1		2	
57.	Swab		1		2	
58.	Tourniquet		1		2	
59.	Other		1		2	

60.	In the 3 months *[or as appropriate]* before coming into prison did you attempt to bleach the shared equipment in any way?	**Yes, always** Yes, sometimes Never Don't recall	
61.	In the 3 months *[or as appropriate]* before coming into prison did someone else help you inject? [*Interviewer: This may include finding vein, drawing up or preparing.* *Do not include medical injections*]	**Yes, always** Yes, sometimes Never Don't recall	
62.	In the 3 months *[or as appropriate]* before coming into prison did you help someone else to inject drugs?	Yes No Don't recall	
63.	In the 3 months *[or as appropriate]* before coming into prison how often did you use a needle and syringe exchange?	Never Some times Most times I needed Every time I needed	
64.	In the 3 months *[or as appropriate]* before coming into prison did you inject in the Medically Supervised Injecting Centre? [*Interviewer: MSIC is in Kings Cross, NOT shooting galleries]* *[Interviewer: check that "daily" and " more than once a day" are distinguished]*	Never Less than monthly Monthly or more often Weekly or more often Daily More than once a day	

Don't recall

VII. THIS SECTION IS ABOUT INJECTING DRUG USE SINCE ENTRY INTO PRISO THIS TIME

65. Since coming into prison have you injected drugs?
[Interviewer: this includes injecting either self or someone else]

Yes (go to Q66)
No (go to Q84)
Don't recall (go to Q84)

66. Since coming into prison how often have you injected?
[Interviewer: check that "daily" and " more than once a day" are distinguished]

Less than monthly
Monthly or more often
Weekly or more often
Daily
More than once a day
Don't recall

67. Since coming into prison this time compared to the rest of your life, has your injecting pattern been…
[Interviewer: assess the lifetime pattern of injecting and code yes if frequency, sharing behaviour or drug of choice have changed]

Stable
Increasing
Decreasing
Don't recall

68. Since coming to prison which drug/s have you injected? *[interviewer: read the options and emphasize on "injecting"]*

Heroin
Buprenorphine (Subutex)
Ice (crystal meth/amphetamine)
GHB/GBH/ liquid e/fantasy
Cocaine/ Coke
Benzodiazepines/Benzos
Anabolic/Steroids
Other opiates/codeine/pethidine/opium/omnopon
Hallucinogens/LSD/ Acid/Magic/Mushies/Daitura
Ecstacy/ E/MDA/MDMA
Ketamine/Special K

[interviewer: Exclude medications prescribed and administered by health workers, e.g. Morphine in hospital]

		Oxycodone (e.g. Oxycontin, Endone)
		Methadone
		Morphine (e.g. MS Contin)
		Speed/base (or other methamphetamine
		Other (please specify)
69.	Since coming into prison has someone else ever injected you the drug? (Given you a hit?)	Yes No Don't recall
70.	Since coming into prison have you ever shared any part of the injecting equipment - that includes the needle, syringe, spoon, swabs, filters, mix or tourniquet?	Yes No Don't recall
71.	Since coming into prison did you use injecting equipment that was not new and sterile?	Yes No Don't recall
72.	Since coming into prison did you ever use injecting equipment after someone else had used it?	Yes (go to 73) No (go to 82) Don't recall (go to 82)
73.	Since coming into prison how often did you use equipment after someone else had used it?	Never Some times Most times I injected Every time I injected

Since coming into prison which equipment did you share?

		Yes		No		Don't kno
74.	**Needle and syringe**		1		2	
75.	Spoon		1		2	
76.	Mix		1		2	
77.	Filter		1		2	
78.	Swab		1		2	
79.	Tourniquet		1		2	

80.	Other		1	2
81.	Since coming into prison did you attempt to bleach the shared equipment in any way since coming into prison?	**Yes, always** Yes, sometimes Never Don't recall		
82.	Since coming into prison did someone else help you inject?	**Yes, always** Yes, sometimes Never Don't recall		
83.	Since coming into prison did you help someone else to inject drugs?	Yes No Don't recall		

VIII. THIS SECTION IS ABOUT DRUG TREATMENT

84.	Have you ever seen a Drug and Alcohol counsellor or received counselling for a drug problem? *[Interviewer: at least one complete session of counselling]*	Yes No Don't recall	
85.	Are you currently on a methadone program?	Yes (go to 86) No (go to 89) Don't recall (go to 89)	
86.	What is your current dose of methadone (1ml = 5mg) [*Interviewer: 4ml= 20mg = less than a tea spoon. Make sure to clarify the dose, if not clear , check the medical record. A typical dose range is 20-100mg*].	_____ mg	
87.	In the last month has your methadone dose been:	Stable Increasing Decreasing Don't recall	
88.	How many days have you missed your	_____	

	methadone since being arrested this time?	Never Don't recall	☐ ☐
89.	Are you currently on a Buprenorphine program?	Yes (go to Qu.90) No (go to Qu.93) Don't recall	☐ ☐ ☐
90.	What is your current dose of Buprenorphine? [tablets (0.4mg,2mg,8mg)] [*Interviewer: typical dose range is 2-16mg*]	_____mg	
91.	In the last month has your Buprenorphine dose been:	Stable Increasing Decreasing Don't recall	☐ ☐ ☐ ☐
92.	How many days have you missed your Buprenorphine since being arrested this time?	_____ Never Don't recall	☐ ☐
93.	Have you had a Naltrexone implant within the last 6 months?	Yes No Don't recall	☐ ☐ ☐
94.	Are you currently taking Naltrexone (Revia) tablets?	Yes No Don't recall	☐ ☐ ☐

IX. THIS SECTION IS ABOUT INJECTING DRUG USE IN THE LAST MONTH

95.	Have you injected drugs in the last month?	Yes No (end of interview) Don't recall	☐ ☐ ☐

96. In the **last month**, what has been the **main** drug you have injected?

- Heroin
- Ice (crystal meth/amphetamine
- Speed, base (or other meth/ amphetamine)
- Cocaine
- Methadone
- Buprenorphine (Subutex)
- Morphine (e.g. MS Contin)
- Oxycodone (e.g. Oxycontin, Endone)
- Other opioids (e.g. codeine, Pethidine, opium)
- Benzodiazepines
- Steroids
- Other (please specify)

96a. How often on average did you inject this (**main)** drug in the **last month**?

- Once a week or less
- More than weekly (but not daily)
- Once a day
- 2 to 3 times a day
- 4 to 5 times a day
- More than six times a day

97. In the **last month**, how many times have **you** used the same needle and syringe after someone else used it (even if it was cleaned)?

- None
- 1 time
- 2 times

3 to 5 times	
6 to 10 times	
11 to 20 times	
More than 20 times	
Can't remember	

PLEASE FILL IN CONTACT DETAILS SHEET

You remember that the plan for this study is to follow up with you every 6 months for

three years. If you are released we will need to contact you outside. We will need your contac
details.

[*Interviewer proceed to complete the Follow Up Contact Form*]

Hepatitis C Incidence and Transmission Study (HITS)-p

Contact Details Date ☐☐/☐☐/☐☐

We need to get some contact information. We may need this information in order to contact you during the follow-up period each year. All the information that you give us will be treated confidentially and stored in a secure location.

First Name	
Middle Name	
Surname	
'Street' names/ nicknames	
Previous surname	

Height ☐ Weight ☐ Hair Colour ☐

Distinguishing physical features e.g. tattoos	

What was your last address before coming into prison?

Number/Street	
Suburb	
Post Code	

How long have you lived there? ☐ ***Years*** ☐ ***Months*** ☐ ***Weeks***

How many different places have you lived in within the last 12 months? (include prison, refuges etc) ☐

Phone numbers:

Home: ( )

Do you live alone? ☐ YES/NO

If no, specify with whom:

Mobile:

Is this your own mobile? ☐ YES/NO

If no, specify?

Work:

Do you expect to be living in the _____ area for the next 3 years?

☐ Yes ☐ No

If NO give details:

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