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Acute Hepatitis C Infection in HIV Co-infection – Epidemiology, Natural History and the Host-viral Responses

A thesis submitted for the degree of
Doctor of Medicine
University College London

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Abstract

**Aim:** To analyse the epidemiological, clinical and immunological aspects of a recent epidemic of acute HCV in HIV co-infected individuals.

**Methods:** The epidemiology was characterised using combined molecular and clinical studies. A phylogenetic analysis of the E1/E2 region of the HCV genome was performed to determine HCV transmission. Transmission risk factors were then explored in a multicentre case-control study. Data was collected on the early clinical course of HCV in HIV. Longitudinal cell-mediated responses to HCV and HCV evolution were studied with PBMCs and serum collected during the acute phase.

**Results:** The studies revealed significant differences in the epidemiology, natural history, cell-mediated responses and HCV evolution between acute HCV with and without HIV. The seven clusters demonstrated in the molecular study is strong evidence for common source transmission. The case-control study identified permucosal factors, including high-risk mucosally traumatic sexual and drug practices, were significantly associated with the recent transmission of HCV. HCV persistence was the outcome in the vast majority of HIV co-infected individuals and these rates are significantly higher than those for HCV mono-infection. The CD4 T-cell responses lacked the magnitude and breadth of response for control of HCV. The virological data supported this, revealing quasispecies evolution that appeared not to be driven by immune pressure.

**Conclusions:** The phylogenetic clusters cross both HCV genotype and subtype implying that the HCV transmission is not due to a specific viral change, but that patient and/or environmental factors are responsible for the recent infections. Permucosal rather than parenteral behavioural risk factors are associated with this transmission. The immuno-deficient state associated with HIV results in very low spontaneous clearance of HCV and the vast majority of these patients become persistently infected. In order to mitigate this important and ongoing epidemic, these factors need to be the focus of a concerted effort on the part of public health specialist, clinicians and HIV-positive individuals themselves.
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In Oxford I would like to thank Dr Paul Klenerman and Dr Nasser Semmo for their support with the immunological aspects of the work; and Dr Oliver Pybus who provided invaluable insights into and support for the phylogenetic analysis.

Without the clinical cohort this study would not have been possible. Therefore, I have to thank Dr Mark Nelson and Dr Martin Fisher, lead HIV clinicians at the Chelsea and Westminster Hospitals and Brighton and Sussex Hospital, respectively. I also have to acknowledge the patients who gave their time by consenting to be part of this study.

This thesis was generously supported by the Special Trustees of the RFUCMS, the Peter Samuel Fellowship and the Pathological Society of Great Britain.

Last but not least, Lisa and Max have been through tough times to see this project come to fruition.
Statement of Originality

The studies that form the content of this thesis were designed and performed by myself except as stated below.

The final phylogenetic analysis, which is presented in Chapter 2, was performed on the cluster of computers in the Department of Zoology, Oxford University by Dr Oliver Pybus.

The cell-mediated immune responses, presented in Chapter 4, were studied in the Nuffield Department of Medicine, Oxford University. Peripheral blood mononuclear cells were collected and prepared at the Royal Free campus. These were then transported to Oxford where the analysis was performed in the laboratory of Dr Paul Klenerman. This was a collaboration between myself and Dr Nasser Semmo. This work involved performing ELISpots and CFSE assays, of which I performed approximately half.
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List of Abbreviations

AA  Amino acid
AI  Anal intercourse
AIDS Acquired Immunodeficiency Syndrome
ALT Alanine aminotransferase
anti-HCV Hepatitis C IgG
AST Aspartate aminotransferase
AUDIT Alcohol Use Disorders Identification Test
BSGH Brighton and Sussex General Hospital
CFSE Carboxyfluorescein succinimidyl ester
CHIC Collaborative HIV Cohort
CTL Cytotoxic T-lymphocytes
CWH Chelsea and Westminster Hospitals
DMSO Dimethyl Sulphoxide
dN/dS Rate of non-synonymous to synonymous amino acid change
DNA Deoxyribonucleic Acid
E Coli Escherichia coli
E1 First envelope region of HCV
E2 Second envelope region of HCV
EVR Early virological response
FACS Fluorescence-activated Cell Sorter
GHB Gamma hydroxybutyrate
GUM Genitourinary medicine
HAART Highly active anti-retroviral treatment
HAV Hepatitis A
HBV Hepatitis B
HCC Hepatocellular carcinoma
HCV Hepatitis C
HIV Human Immunodeficiency virus
HKY Hasegawa Kishimo Yano model
HLA Human leucocyte antigen
HVR1 Hypervariable region 1
IFN Interferon
IVDU Intravenous drug use
LSD Lysergic diethylamide
ML Maximum likelihood
MSM Men who have sex with men
NANB Non-A non-B
NJ Neighbour-joining
NS Non-structural region of HCV
NSU Non-specific urethritis
OLT Orthotopic liver transplant
PBMC Peripheral blood mononuclear cell
PCR Polymerase chain reaction
PI Protease inhibitor
RFH Royal Free Hospital
RNA Ribonucleic Acid
SFC Spot forming cell
STI Sexually transmitted infection
SVR Sustained virological response
UAI Unprotected anal intercourse
ULN Upper limit of normal
UTR Untranslated region
WHO World Health Organisation
Chapter 1: General Introduction

Hepatitis C virus (HCV) and Human Immunodeficiency virus (HIV) are two of the most prevalent persistent viral infections worldwide. Given their shared routes of transmission it is not surprising that HCV/HIV co-infection is relatively common. While the impact of HCV on the natural history of HIV infection is still controversial, HIV increases the pathogenicity of HCV, and leads to an increased morbidity and mortality. The introduction of highly active antiretroviral treatment (HAART) for HIV infection has led to dramatic reductions in morbidity and mortality due to AIDS (Palella et al., 1998). However, hepatocellular failure and hepatocellular carcinoma (HCC), complicating HCV-related cirrhosis, have become increasingly significant causes of morbidity and mortality in HIV infected individuals when expressed as a percentage of all deaths. The increase in the HCV-related burden of illness in HIV is the result of the combination of relatively high prevalence rates of HCV/HIV co-infection and the accelerated HCV-related hepatic injury. It is not yet clear whether there has been an absolute or proportional increase in morbidity following the introduction of HAART. In the developed world, end-stage liver disease has been reported as the leading cause of deaths in HIV-infected patients (Puoti et al., 2000; Bica et al., 2001). While there has been an overall decline in HIV-related hospital admissions, HCV/HIV co-infected patients are at increased risk of hospitalisation compared to HIV mono-infected patients (Mocroft et al., 2004).

The epidemiology, natural history, pathogenesis and optimal management of HCV/HIV co-infection are not yet fully elucidated. Consequently, research is needed into these aspects of HIV/HCV co-infection. Hence studies of the acute phase of HCV in HIV would improve our understanding of the epidemiology of co-infection and viral interactions in the short-, medium- and long-term. However, very little is known about the early stage of co-infection.
1.1 The problem

Acute HCV is an unusual diagnosis because only 20% of HCV monoinfections are clinically apparent (Hoofnagle, 1997). However, since the early 2000s, there has been a marked rise in the diagnosis of acute HCV in the HIV-positive population of London. This was first described at the Chelsea and Westminster Hospitals in London (Browne et al., 2004). In a review of case notes between 1997 and 2002, 25 cases were identified. While there was no increase in the amount of anti-HCV testing over this period there was a significant rise in the proportion of positive tests rising from 0.6% in 1997 to 9.3% (p<0.001) in 2002. While in part this may represent more appropriate testing and case ascertainment, it probably also represents the tip of the iceberg of as yet undiagnosed patients. Interestingly, whereas the majority of these cases might have been expected to have a percutaneous transmission risk, unsafe and high-risk sexual practices were in fact the main risk factor in 21 of these cases. Indeed, supporting the hypothesis of sexual transmission of HCV, nine cases were also diagnosed with syphilis around the time of the HCV diagnosis. Since this initial report from the Chelsea and Westminster Hospitals, more than 200 cases of acute HCV have been identified over the last three years in the South-Eastern UK (personal communication from Royal Free Hospital, Chelsea and Westminster, University College Hospital and Brighton HIV units). This epidemic is not limited to the UK; acute HCV in HIV infected individuals has been described by other groups in Europe and USA (Gambotti et al., 2005; Gotz et al., 2005; Luetkemeyer et al., 2006). In France, a cohort of 29 patients has been described from three centres (Gambotti et al., 2005). In the Netherlands, a cluster of seven acute HCV cases were described in association with a surveillance study of rectal lymphogranuloma, a recognised sexually transmitted disease (Gotz et al., 2005). All these groups report sexual rather than parenteral risk factors.

“Epidemics are a function of the people who transmit infectious agents, the infectious agent itself and the environment in which the infectious agent is operating. And when an epidemic tips, when it is jolted out of
equilibrium, it tips because something has happened, some change has occurred in one (or two or three) of those areas.” (Gladwell, 2001). To understand why there has been a dramatic increase in the diagnosis of acute HCV in the HIV-positive population of the UK, the epidemiological signature of the virus, its potential modes of transmission within the population and the current environment of this epidemic needs to be examined in detail. Identifying the factors responsible for this new epidemic of acute HCV, would allow specific strategies to be developed to mitigate and control the spread of this infection in the HIV infected population.

1.2 HCV mono-infection
A background understanding of the virology, immunology, epidemiology, natural history and treatment of HCV mono-infection and HCV/HIV co-infection is essential.

1.2.1 Virology:
In 1989 Choo and colleagues discovered hepatitis C virus (HCV) via recombinant DNA cloning. HCV has subsequently been shown to cause the vast majority of non-A non-B (NANB) hepatitis (Choo et al., 1989). HCV is an enveloped single stranded positive sense RNA virus belonging to the Flaviviridae family with a genome of 9.4kb length. The genome has a single open reading frame encoding a 3011 amino acid polyprotein, which is cleaved into three structural proteins located towards the amino terminus and several non-structural proteins towards the carboxy terminus. The structural proteins are the nucleocapsid and two glycosylated envelope proteins E1 and E2. The non-structural proteins consist of four major units, NS2, NS3, NS4, and NS5. The function of NS2 is unclear but is thought to be a zinc-dependent metalloproteinase. The NS3 protein carries out a number of biochemical functions including serine protease, NTPase, and RNA helicase activities. The NS4 domain consists of two sub-units NS4A, a co-factor for NS3, and NS4B, the exact function remains to be elucidated. NS5 also consists of two sub-units NS5A and B. NS5A is a phosphorylated protein and although the exact
function of this protein is unknown it appears essential for activity of the RNA dependent RNA polymerase, NS5B. The viral genome is flanked by un-translated regions at both 5' and 3' ends which play an essential part in viral replication (Figure 1.1). HCV leads to persistent infection in the majority of cases (Lauer & Walker, 2001).

**Figure 1.1: HCV genome.**

HCV has significant molecular diversity which can be elucidated at three different levels; genotype, subtype, and quasispecies (Simmonds, 2004). The major variation is between HCV genotypes, which differ by more than 30% of the nucleotide sequence. Six different genotypes, classified as clades by analysis of the NS5B region, exist in specific geographic or risk group populations (Simmonds et al., 1993). Genotype 1 is distributed in the USA and northern Europe, especially in the IVDU population. Genotype 2 is predominantly Mediterranean and Asian. Genotype 3 is widely distributed in the intravenous drug users (IVDU) population of Europe. Genotype 4 is distributed in the Middle East and regions of Africa. In Egypt this genotype has been particularly associated with previous medical treatment and instrumentation for schistosomiasis; genotype 5 is more common in South Africa; and Genotype 6 is most prevalent in South East Asia. Each of these genotypes contains subtypes which vary by between 20-25% in nucleotide sequence. Subtypes are an epidemiological phenomenon associated with relatively recent spread of the virus. Finally, quasispecies represent the variation in sequence diversity within a replicating population, usually an individual, at a given time point. A simple model that initially described viral diversity of HIV can be applied to HCV (McMichael & Rowland-Jones, 2001; Stumpf & Pybus, 2002). This model describes quasispecies viral diversity as a function of the selective pressure of the immune system and the effective viral population. This model is supported by Farci et al. who studied HCV quasispecies diversity and clinical outcome (Farci et al., 2000).
Molecular phylogenetic analysis uses nucleotide sequences to reconstruct the evolutionary relationships between the sequences. The evolutionary relationship is then described using a computer generated tree topology. Molecular techniques can be used to facilitate epidemiological studies, including disease surveillance, outbreak investigations, the identification of transmission patterns and risk factors in populations, and to characterise the host-virus interaction providing a better understanding of disease pathogenesis at a molecular level (Foxman & Riley, 2001). These techniques are particularly relevant to the study of viruses, such as HCV.

Phylogenetic patterns are the result of natural selection of genetic mutations favouring the survival of the virus. Viruses have exceedingly high replication rates. However, RNA encoded viral polymerases including HCV, lack a proofreading activity, resulting in high mutation rates and genetic variability. In viral genomes the same sequence often codes for more than one protein or regulatory function. Consequently, a large proportion of the mutations are harmful to the virus and reduce its fitness to survive. Two external processes modulate this variability; the host's immune response to the virus and the bottleneck at transmission. Intra-host viral diversity (quasispecies) is driven by humoral or cell-mediated immune responses (discussed below). In contrast, inter-host variation occurs as a result of the arbitrariness by which viral particles find a receptive cell in the passage of infection from one host to another (the bottleneck). There is little evidence for different intrinsic transmissibility among strains of HCV. As a result, the inter-host phylogenetic structure is not determined by immune selection but instead reflects the demographic or spatial history of transmission (Grenfell et al., 2004). Therefore, the viral diversity and phylogenetic lineages depend on the prevailing epidemiological and immunological forces. Recently, population dynamic analysis has allowed the rate and magnitude of an evolutionary process through time to be inferred from current DNA or protein sequence in a representative population (Drummond et al., 2003). The most commonly used regions in the HCV
genome for epidemiological study have been the hypervariable region 1 (HVR1) and non-structural protein 5 (NS5B).

1.2.2 Immunology:
Study of the acute phase of HCV has been difficult because it is usually not recognised clinically. While there is no specific serological marker of acute hepatitis C, it is conventionally defined as HCV infection diagnosed in the first six months of infection. This is characterised by documented seroconversion to anti-HCV and detection of HCV RNA in blood. Chronic HCV is usually defined as the persistence of HCV RNA for longer than six months. Up to 85% of individuals exposed to HCV will develop chronic infection (NIH, 1997; Communicable Disease Network of Australia and New Zealand, 1999; Lauer & Walker, 2001). However, in contrast to the conventional clinical definition, virological studies have suggested the change from acute to persistent HCV infection may occur between 3-4 months of infection (Gordon, 2003). HCV RNA is detectable in the blood two days after infection but usually peaks after several weeks, when a steady state of viraemia is then reached of between $10^4$-$10^7$ IU/ml (Thimme et al., 2002). Three patterns of HCV infection have been recognised in the early phase of infection. First, HCV viraemia falls precipitously within the first six months and then remains undetectable. This is consistent with viral eradication. Second, the viraemia reaches a steady state and persists beyond six months, consistent with chronic infection. Third, there may be early virological control within the first six months, however, viraemia rebounds and infection persists (Gerlach et al., 1999). The chronic state of the virus is the result of a balance between an individual’s immune response and the level of viral replication in that individual.

A humoral response with antibodies (anti-HCV) develops a mean of 6 (range 4-9) weeks after infection (Netski et al., 2005). In chimpanzee studies, antibodies have been shown to neutralise HCV infectivity in vivo (Farci et al., 1994). However, while there is a humoral response with neutralising antibodies, these antibodies are not protective in humans (Lai et al., 1994). Studies of the HCV quasispecies evolution in the acute
phase have demonstrated that antibodies to the E1/E2 region of the genome select for viral mutations and maybe associated with clinical outcome (Farci et al., 2000). However, humoral responses have been difficult to study because functional ex vivo studies have only been possible since the development of pseudovirus models.

It is clear that a broad, strong and persistent cell-mediated immune response is essential for control and eradication of the HCV infection in the acute phase. There are a number of observations that suggest CD4 T-cells are important for control of HCV. Antigen-driven CD4 T-cell proliferation, particularly to the NS3 protein, was observed in all patients with acute HCV infection who resolved but only in some of those who developed persistent infection (Diepolder et al., 1995; Gerlach et al., 1999). Gerlach et al. showed that persistence of these responses was important for eradication of the infection, describing a group of patients who initially controlled the infection and then relapsed with loss of CD4 T-cell responses (Gerlach et al., 1999). HCV viral load has been associated with MHC class II alleles suggesting indirectly the importance of T-cell responses (Fanning et al., 2001). The appearance of CD8 T-cells is kinetically associated with elevated serum aminotransferases and control of viraemia (Cooper et al., 1999; Thimme et al., 2002). Depletion of CD4 T-cells by monoclonal antibodies in chimpanzees led to increased susceptibility to re-infection compared with controls (Grakoui et al., 2003). Persistent broad CD4 T-cell responses have been demonstrated up to two decades after infection in individuals who successfully spontaneously eradicated HCV infection (Takaki et al., 2000).

Persistent HCV infection is thought to be the result of a failure of cell-mediated control. The most consistent feature of a successful immunological response is that it is sustained; in chronic HCV infection T-cell responses are diminished (Shoukry et al., 2004). Multiple studies including functional lymphocyte assays, such as quantitative ELISpots, indicated that CD4 T-cell activity is of low frequency and, if present, proliferates against a limited number of epitopes in patients with persistent infection. (Gerlach et al., 1999; Takaki et al., 2000; Day et...
al., 2003). Failure of CD4 T-cells is pivotal to viral persistence, and the inability of CD8 T-cells to control infection is the result of antigen specific CD4 T-cell failure. The mechanisms responsible for this failure remain unclear. An absence of response may represent an early defect in priming or expansion of naïve CD4 T-cells, while a transient response may represent contraction of CD4 population before memory T-cells have developed (Shoukry et al., 2004).

1.2.3 Epidemiology:
HCV is a global disease, with an estimated worldwide prevalence of 2.2%; perhaps as many as 170 million people infected worldwide (WHO, 2004). There is wide geographic variation in the prevalence of HCV, which ranges from approximately 1% in the developed world to more than 10% in parts of the developing world (Purcell, 1994). The World Health Organisation (WHO) has recently attempted to address the issue of the global burden of HCV by estimating its prevalence and incidence (WHO, 2004). The WHO estimates the prevalence of HCV in Europe is 1.7%, while the prevalence in the USA is 1.8% (Alter et al., 1999; WHO, 2004). The WHO has suggested that 6.4 million new HCV infections occur worldwide each year, based on an estimate of a 10-fold increase in the HCV incidence since 1950 (WHO, 2004). This number is highest in the African and Eastern Mediterranean regions. While the introduction of viral sterilization of blood products in the mid-1980s reduced transfusion linked transmission, this did not significantly impact the incidence of transmission by IVDU. (Alter et al., 1990). However, there was a marked but unexplained reduction in the incidence of IVDU-related HCV from 1989 on, which led to an 80% reduction in the overall incidence, with the number of estimated cases in the USA falling from 180 000 in the mid-1980’s to 28 000 in 1995 (Alter et al., 1997). Currently, the WHO estimates of the morbidity and mortality are difficult to interpret as the common outcomes of cirrhosis and HCC are shared by other aetiologies such as hepatitis B and excessive alcohol. However, an estimated 8-10 000 people die each year in the USA related to complications of HCV.
Chronic HCV now is the major indication for liver transplantation in the West.

The UK epidemiology of HCV is similar to that of Western Europe and the USA. A large seroprevalence study (n=8297) analysing pooled stored serum samples from pathology laboratories in England and Wales found an overall prevalence of 0.46% (Balogun et al., 2002). The highest prevalence (1.07%) was found in the samples obtained before 1986. Males were twice as likely to be positive than females and London had a significantly higher prevalence than the rest of England or Wales. Of note, samples collected before 1991 excluded immunocompromised patients as well as those samples tested for HBV and HIV. The Trent HCV study group prospectively followed a cohort (n=1128) of anti-HCV positive patients (Mohsen et al., 2005). The prevalence in this study, estimated from the regional population, was 0.05%, of which 81% were HCV RNA positive, with a sex ratio of 2:1 of males to females. IVDU was the commonest risk factor (65%). Liver-related mortality accounted for 47% of deaths, significantly higher than the expected rates for this population.

1.2.4 Transmission of HCV:

The majority of HCV transmissions are a consequence of parenteral exposure. Initially termed Non-A Non-B (NANB) hepatitis, HCV transmission was first described after transfusion of blood products (Prince et al., 1974; Alter et al., 1978). Following the discovery of HCV in 1989, and the expression of recombinant HCV antigens, specific antibodies became available to diagnose exposure. In a seminal paper, Alter et al. used an anti-HCV antibody to a recently cloned viral protein (C100-3) to characterise both acute and chronic HCV in blood transfusion recipients previously diagnosed with NANB hepatitis (Alter et al., 1989a). In this cohort of 20 patients, NANB hepatitis had been diagnosed clinically and histologically. Serum from four patients transmitted NANB to chimpanzees. Of 15 patients diagnosed with chronic NANB, all seroconverted to anti-HCV while 3 of 5 acute cases seroconverted. The authors concluded that HCV was the major cause of NANB hepatitis.
Following this, results from a large case-control study of blood transfusion recipients and donors suggested that HCV accounted for 91% of all the NANB hepatitis seen in samples tested retrospectively between 1976-1979 (Aach et al., 1991). As a result of lifelong factor concentrate transfusion, most haemophiliacs treated before 1985 are infected with HCV (Watson et al., 1992). Analysis of Factor VIII, found that 83% of samples tested by PCR were positive for HCV RNA. (Makris et al., 1993).

Pereira et al. explored HCV transmission from solid organ transplantation (Pereira et al., 1992). They demonstrated that HCV RNA negative recipients of solid organ transplants from HCV RNA positive donors developed infection in 100% of cases. Transfusion as a risk factor for transmission has been confirmed in a number of large epidemiological studies (Alter et al., 1990; Osmond et al., 1993; Alter et al., 1997). With the introduction of screening of blood and tissue products, transfusion, which pre-1990 accounted for 10% of transmission, is now an extremely rare route for transmission (Alter, 2002). It has been estimated that with blood product screening the current risk of infection is 0.001%/unit transfused (Schreiber et al., 1996).

Determining the mode of transmission of disease from one person to another is essential to understanding an epidemic. The mode of transmission must be biologically plausible and temporally related to infection. Prospective cohort studies and retrospective case-control studies of acute disease are the most reliable method of determining risk factors for transmission (Gordis, 2004). Other methods include cross-sectional or prevalence studies which examine both potential exposure and disease outcome simultaneously. However, these studies often have problems as the temporal relationship between the risk and disease is unclear. Furthermore, these methods often will not detect low frequency associations unless the studies are large.

While HCV transmission has been described via both parenteral and permucosal routes, the efficiency of transmission varies significantly between these routes. The burden of infection due to a transmission factor is a function of the efficiency of transmission and the size of the
population exposed to that factor. A measure of the efficiency of HCV transmission by different routes can be made by comparison with HIV and HBV, which share similar epidemiological characteristics. HCV is approximately ten times more efficiently transmitted than HIV by percutaneous exposure. The estimated transmission rate for HCV after a needle stick exposure is 1.5-3%, compared with 0.3% for HIV (CDC, 2001b). This is supported by the higher prevalence of HCV than HIV in the IVDU population. In contrast, vertical and sexual transmission of HCV appear to be significantly less efficient than for HIV. In a cross-sectional study of the heterosexual transmission of HCV, HBV and HIV transmission in inner city women in Brooklyn (n=599), Feldman et al. reported prevalences of HCV, HBV and HIV of 1.6%, 2% and 18.8% respectively (Feldman et al., 2000). The estimated transmission risk of HIV infection through a single heterosexual or homosexual intercourse is 1-5%, in comparison with a risk of 0.1-0.3% for transmission of HCV (Verucchi et al., 2004). In seroprevalence studies of anti-HCV in highly sexually active groups (female sex workers, homosexual males, STI clinic attendees) prevalence rates were lower for HCV than for HBV and HIV (Terrault, 2002). The vertical transmission of HIV is estimated at between 20-30% in those infants whose mothers were not on antiviral therapy (Zanetti et al., 1995). In contrast, HCV was transmitted to 2-5% of children born to HCV RNA-positive mothers (Gibb et al., 2000). The different efficiency of transmission of HCV in part explains the epidemiology.

IVDU is currently the major route of HCV transmission. HCV infection occurs through the transfer of HCV by sharing syringes and needles either directly or through contamination of the injecting equipment (Heimer et al., 1996). In the USA, 68% of new HCV infections occur among IVDUs, 18% among people with only sexual exposure to an infected person or multiple partners, 4% among health care workers whereas nosocomial, iatrogenic and perinatal exposure account for only 1% of cases (Alter, 2002). In 9% of patients, no source could be identified. In large case-control studies, IVDU stands out as the major transmission factor (Alter et al., 1990; Osmond et al., 1993; Alter et al.,
The prevalence of HCV in IVDUs is between 30-98% (Roy et al., 2002). Transmission occurs early in injecting drug use and after 5 years 90% of individuals are infected (CDC, 1998). The high prevalence of HCV in the IVDU population contributes to this rate of transmission (CDC, 1998). A large case-control study of HCV-seropositive (n=758) blood donors and matched seronegative (n=1039) blood donors, found that IVDU was the strongest risk factor for HCV seropositivity (Murphy et al., 2000). It revealed a final multivariable model where IVDUs had an odds ratio of 50 for HCV infection compared with controls. Interestingly, while drug inhalation and high number of sexual partners were associated with HCV, this effect disappeared when adjusted for IVDU. This strong association has been confirmed in other studies (Alter et al., 1990; Osmond et al., 1993; Conry-Cantilena et al., 1996). Interestingly, a population dynamic analysis based on a phylogenetic study of the HCV subtypes 1a and 3a in IVDU patients in the UK demonstrated exponential growth in HCV transmission in the second half of the 20th century (Pybus et al., 2005). This mirrored the growth of the IVDU population, explaining the increased transmission. With such a strong association, IVDU may cause residual confounding if it is unmeasured when assessing other factors such as inhaled drug use and sexual transmission.

Drug inhalation has been described as a source of HCV transmission. A USA-based study among blood donors found an independent association between intranasal cocaine use and HCV infection (Conry-Cantilena et al., 1996). It is likely that this is through sharing of intranasal implements, such as straws. A significant proportion of the population of the USA (up to 14%) have used intranasal cocaine (CDC, 1998). However, intranasal drug use is identified only uncommonly as a risk factor in acute cases of HCV. It has been difficult to separate inhaled from parenteral drug use as the two routes are so strongly associated (Rall & Dienstag, 1995).

Renal dialysis is the most common nosocomial route of HCV transmission (Niu et al., 1993). The prevalence of HCV in the dialysis population has been reported to be between 10% and 60% (Tokars et al., 1994). A number of outbreaks of HCV have been described in the dialysis
population (CDC, 2001a). Transmission was associated with the use of contaminated equipment and unsafe nosocomial injection practices. The prevalence of HCV increases with length of dialysis (Hardy et al., 1992; Niu et al., 1993). The most common occupational exposure route is needle stick injury with an average incidence of anti-HCV seroconversion of 1.8% of those exposed (CDC, 2001b). It has also been documented to occur through conjunctival blood splashes (Ippolito et al., 1998). Other percutaneous routes such as body piercing, tattoos, commercial barbering have been described in case reports. However, case-control studies of acute disease have not found an association between HCV and recent medical or dental procedures, tattooing, acupuncture or piercing (Alter et al., 1982; Alter et al., 1989b; Alter, 2002). Transmission by these routes seems rare, with recent exposure by these routes reported by fewer than 1% of both cases and controls.

Mother-to-infant HCV transmission is defined on the basis of persistence of anti-HCV beyond at least 12 months of age or the detection of HCV RNA on at least one occasion in the infant. Transmission can occur either by intrauterine, intrapartum or postnatal routes. Yeung et al., in a review of all published studies between 1990 and 2000, calculated a rate of mother-to-infant transmission from anti-HCV positive women was 1.7% (Yeung et al., 2001). In the same review, the estimated rate among HCV RNA positive women was 4.3%. A higher HCV viral load (levels >10^5 IU/ml) appears to increase this transmission risk (Ohto et al., 1994; Thomas et al., 1998). Other factors associated with higher transmission rates include HIV co-infection and mode of delivery. The transmission rate among those co-infected with HIV is 19.4% compared to 3.5% for women infected only with HIV (Yeung et al., 2001). The transmission rate appears to be slightly higher among women having vaginal deliveries when compared with women having caesarean sections. While HCV RNA has been found in breast milk, no definitive case of transmission from breast-feeding has been described.

The importance of sexual transmission of HCV remains controversial. While there is convincing evidence that sexual transmission does occur,
the magnitude of risk has been difficult to quantify (Terrault, 2005). The first study to suggest sexual transmission of HCV was an interview-based case-control study of acute NANB hepatitis in two county hospitals in the USA (Alter et al., 1989b). This study did not use virological testing to confirm the diagnosis of HCV. Of 140 patients with acute NANB hepatitis enrolled in the study, 53% had no identifiable risk factor. Comparison of this subgroup with controls selected from the community revealed significantly higher rates of multiple sexual partners (>2) and a history of hepatitis in the household/sexual contact, although this could have represented confounding with other unmeasured factors. Certainly, sexual transmission is biologically plausible because HCV RNA has been isolated from saliva, semen and vaginal fluids (Numata et al., 1993; Young et al., 1993; Leruez-Ville et al., 2000). Of 21 HCV RNA positive patients, 38% were demonstrated to have HCV RNA in the semen (Leruez-Ville et al., 2000). The median concentration of HCV in semen (1.78 log IU/ml) was significantly lower than in serum (5.63 log IU/ml). Sexual transmission has been demonstrated in a number of case studies by confirming viral homology between sexual partners following the exclusion of other possible transmission factors (Healey et al., 1995b; Sanchez-Beiza et al., 1996; Capelli et al., 1997; Halfon et al., 2001; Quer et al., 2003). Interestingly, these cases all described transmission from men to women. Mucosal trauma was implicated in these cases, related to anal intercourse or after vaginal instrumentation. Higher rates of transmission from men to women have also been suggested by other studies (Thomas et al., 1995). In partners of attendees at an STD clinic (n=309), the prevalence of anti-HCV was higher in female but not male partners of anti-HCV positive individuals. Currently, 20% of acute HCV cases in the USA have sexual contact as the only identifiable risk factor (CDC, 1998). This was either sexual intercourse with >2 partners, or with an HCV positive person in the preceding six months. It is often complicated by the coexistence of high-risk sexual practices, such as lack of condom use, anal and mucosally traumatic sexual practice, and multiple partners with drug use (in particular IVDU). In a review of anti-HCV studies, prevalence rates in partners of individuals with high-risk behaviours such as IVDU and sexual promiscuity were between 11 and
27% compared with partners of low-risk individuals where the prevalence rates were between 0 and 7%, suggesting that partners of high risk individuals may also participate in these high-risk behaviours themselves (Rall & Dienstag, 1995).

The magnitude of the risk of sexual transmission is not known. A number of studies have tried to assess this and early studies suggested high rates of sexual transmission. A Japanese cross-sectional study (n=154) found an anti-HCV prevalence of 18% in the spouses of patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma (Akahane et al., 1994). This prevalence increased with the length of marriage, which was attributed to increased sexual exposure. However, on review some believe that this actually represented a cohort effect, with an increased prevalence in older individuals (Dienstag, 1997). In a review of early studies, Rall and Dienstag reported the prevalence of anti-HCV in partners of HCV patients to be between 0 and 27% (Rall & Dienstag, 1995). Unfortunately, these studies assessed risk without performing adequate assessment of viral homology to confirm viral transmission. This is important because simply testing anti-HCV may overestimate the rate of sexual transmission. Zylberberg et al. highlighted the importance of HCV sequencing in epidemiological studies (Zylberberg et al., 1999). They performed a detailed epidemiological and molecular analysis of 24 anti-HCV couples, in whom, concordant genotypes were found in 12. Seven of these couples had sequencing of the NS5B region of the HCV genome. Only three couples had identical isolates. However, all three couples had a parenteral risk factor which could also have explained the transmission. They concluded that the risk of sexual transmission had been overestimated. Large prospective cohort studies using virological analysis have now been performed and suggest that sexual transmission rates are very low in monogamous heterosexual couples. Vandelli and colleagues followed an Italian cohort of 895 monogamous heterosexual couples for over 8060 person-years (Vandelli et al., 2004). Over this period three spouses acquired HCV; however viral sequencing revealed all the isolates differed from their respective partners, ruling out transmission from these individuals. Tahan and colleagues found none of
the 216 HCV-negative partners became infected over a three-year follow-up (Tahan et al., 2005). In conclusion, sexual transmission in low-risk heterosexual relationships may occur but at an extremely low rate. Current clinical guidelines do not recommend barrier contraception to prevent HCV transmission in monogamous heterosexual couples (NIH, 2002; Terrault, 2005).

Certain individuals appear to be at increased risk for sexual transmission. These groups include: sex workers, individuals with multiple sexual partners, men who have sex with men (MSM), HIV-positive individuals and sexually transmitted infection (STI) clinic attendees. Osmond et al. found that a high number of lifetime sexual partners was associated with HCV infection (OR 2.1, 95% CI 0.9-4.8) (Osmond et al., 1993). Higher HCV prevalence has been found in studies of this group (Nakashima et al., 1992; Wu et al., 1993). In a study of Taiwanese prostitutes (n=622), 12% were anti-HCV positive. On multivariable analysis, a history of paid sex for more than six months and transfusion were associated with anti-HCV positivity (p<0.001) (Wu et al., 1993). HIV infected patients have been reported to have higher rates of anti-HCV positivity (Terrault, 2002). Other studies have reported higher rates of anti-HCV positivity in STI clinic attendees, individuals infected with STIs, MSM and individuals taking part in traumatic sexual practices (Thomas et al., 1995; Ndimbie et al., 1996; Hershow et al., 1998; Filippini et al., 2001). In comparison to the general population anti-HCV prevalence of 1.8%, Terrault estimated median prevalence rates of anti-HCV of 6% among female sex workers, 4% among MSM and 4% among those attending STI clinics and HIV surveillance studies (Terrault, 2002).

Although sexual transmission of HCV in MSM has been documented, recent evidence suggests that the risk of sexual transmission of HCV in MSM is low. Alary and colleagues prospectively followed a cohort of Canadian HIV-negative MSM (n=1085) to assess seroconversion rates (Alary et al., 2005). The entry prevalence of HCV was 2.9%; if IVDUs were excluded it was 0.3%, less than the Canadian population prevalence of 0.8%. Over 2653 person-years of follow-up there was only one
seroconversion and this was attributed to IVDU on interview. They calculated an HCV incidence rate of 0.038 per 100 person-years. However, some studies have reported higher rates of HCV in MSM (Melbye et al., 1990; Thomas et al., 1995). In a Danish study, an 8-year culmulative incidence of 4.1% was found. (Melbye et al., 1990). It has also been suggested that HIV co-infection increases the risk of sexual transmission of HCV (Tor et al., 1990; Eyster et al., 1991; Osmond et al., 1993). Filippini et al. performed a case-control study examining anti-HCV in hetrosexual and homosexual contacts of HIV-positive patients (n=318). The study demonstrated that subjects with only sexual exposure as a risk factor had a higher prevalence of HCV if co-infected with HIV (18.7% versus 1.6%, p=0.008) (Filippini et al., 2001). They suggested this was evidence for enhanced sexual transmission of HCV with HIV.

Case-control studies have identified non-sexual HCV transmission within families (Donahue et al., 1992; Napoli et al., 1993; Hou et al., 1995; Nakashima et al., 1995). The actual transmission route is difficult to identify, but is likely to be the result of inadvertent percutaneous or permucosal exposure to HCV infected blood or body fluids. In a review of these studies, Alter estimated the overall prevalence of anti-HCV in non-sexual contacts to be 4% (Alter, 1995). In a review of intrafamilial transmission, Akerman et al. calculated the prevalence of anti-HCV in non-sexual contacts to range from 1.14% in siblings and household contacts of children with paediatric liver disease to 10.96% in parents of the same population (Ackerman et al., 2000). Of note, the prevalence of all non-sexual contacts in Japanese studies was higher which was attributed to the endemicity of HCV in Japan and the increased frequency of different cultural practices, such as acupuncture. However, intrafamilial clustering in countries of high endemicity may represent another exposure and not intrafamilial transmission.
1.2.5 Natural history of HCV mono-infection:

Unfortunately, there are a number of serious difficulties encountered when defining the natural history of an illness such as hepatitis C. Seeff outlined five criteria that are critical to studies of natural history (Seeff, 1997). The onset of disease must be accurately established, which requires the identification of the acute illness. It is important to identify the full spectrum of acute illness to avoid bias in focusing on more serious illness, which may have a different natural history. Third, the illness must be tracked to its resolution or end-points. This includes the determination of illness outcomes without any therapeutic intervention. Finally, matched controls that could be followed with the same vigour would need to be available. There are many reasons why these studies are impossible in hepatitis C. The acute illness is symptomatic in only 25-35% of cases and estimates of the timing of the illness are often inaccurate (Hoofnagle, 1997). This makes the identification of controls difficult. HCV-related liver disease progresses very slowly and often asymptptomatically, requiring decades of follow-up. Furthermore, effective treatment is now available with combination interferon and ribavirin, making such studies unethical. Consequently, we have to interpret the natural history of the illness through the retrospective and cross-sectional data that we have available. It is also important to realise that much of the available data are based on liver clinic series or selected populations (eg. haemophiliacs), which may have inherent referral and selection biases. Freeman and colleagues compared hepatitis C data from different referral sources and found significant differences in the rates of cirrhosis development (Freeman et al., 2001). The estimated prevalence of cirrhosis in a liver clinic population after 20 years infection was 23%, 28% in post-transfusion cohorts, 4% in blood donor cohorts and 7% in community-based cohort studies.

It is estimated that 21% of community-acquired acute hepatitis is caused by hepatitis C (Alter, 1995). However, as stated, only 25% of patients become icteric. There have been reports of fulminant hepatic failure associated with hepatitis C, although this is extremely rare (Villamil et al., 1995). A German study has provided important data on the early
natural history of HCV (Gerlach et al., 2003). Patients with acute HCV (n=60) were prospectively enrolled over seven years, and were either offered immediate or delayed treatment. Six patients opted for immediate treatment while 54 delayed treatment. Of those delaying therapy, 37 initially cleared HCV but 13 relapsed. This resulted in an overall spontaneous clearance rate of 44%. Those who cleared HCV were more likely to have been symptomatic as none of the asymptomatic patients cleared the infection. This contrasts with the overall rate of 15-20% spontaneous clearance that has been suggested based on seroprevalence data (Lauer & Walker, 2001; Seeff, 2002).

Although there is a large degree of variability with respect to disease progression, the development of fibrosis and cirrhosis is the major underlying cause of morbidity and mortality. This can follow a relatively linear course from chronic infection to cirrhosis to hepatocellular carcinoma. On the other hand, the majority of people with hepatitis C will probably not progress to advanced liver disease and overall progression is highly variable. Most long-term follow-up data has been obtained from patients with post-transfusion hepatitis, because of the availability of accurate data on duration of infection. Seeff et al. found that, in a comparison of individuals with post-transfusion hepatitis and age-matched controls over a mean of 18 years, there was no difference in overall mortality and only a small, but statistically significant increase in liver-related mortality (Seeff et al., 1992). Other studies with longer follow-ups have found significant morbidity and mortality in a 10 to 30-year period. In these studies the incidence of cirrhosis has ranged from 8-32%, HCC was identified in 0.7-1.3% of subjects and overall liver-related mortality ranged from 1.6-6% (Di Bisceglie et al., 1991; Tremolada et al., 1992; Koretz et al., 1993; Mattsson et al., 1993). It has been estimated that serious liver disease develops in one third of patients within 20 years and does not progress in another third for 30 years or more (Lauer & Walker, 2001). In a Japanese cohort, of the approximately 75% of patients who developed chronic HCV infection, the estimated times for progression to chronic hepatitis was 10 years, to
cirrhosis was 21 years, and hepatocellular carcinoma was 29 years (Kiyosawa et al., 1990; Tong et al., 1995).

Hepatic fibrosis is the final response of the liver to an insult, and is usually diagnosed histologically by liver biopsy. Hepatic fibrosis represents an increase of collagen fibre deposition in the liver, which can impede intrahepatic blood flow, and subsequently cause persistent and progressive hepatic dysfunction. Fibrosis was previously thought to have been irreversible, although there is mounting evidence that some fibrogenesis may be reversible (Benyon & Iredale, 2000). Many studies have shown that disease progression mainly depends upon the severity of fibrotic or cirrhotic change (Poynard et al., 1997; Niederau et al., 1998). Fibrosis progression per year can be defined as the ratio between fibrosis stage, using the Metavir or Ishak histological scoring units, and the estimated duration of infection (Poynard et al., 1997; Wright et al., 2003). This yields a score in fibrosis units per year. The fibrosis rate is probably modulated by a number of potential cofactors, which can be classified into host factors, viral factors and external factors. Viral factors include viral dose, viral genotype and quasispecies. Host-related factors include age, ethnicity and gender. Extraneous factors include alcohol, environmental factors, and geographical location. Unfortunately, fibrotic liver disease often presents late as a result of complications related to cirrhosis, and most studies are limited by lack of routine biopsy data. It is therefore important to identify risk factors associated with causation and progression of fibrosis.

Important host factors such as age, sex and ethnicity also need to be considered. Age seems to be an important predictor of progression of hepatitis C. In a study of patients who underwent repeat liver biopsy, Kage et al. found that liver fibrosis increased dramatically after the age of fifty (Kage et al., 1997). In addition, there was an accelerated development of cirrhosis in those aged over fifty years. This has been supported by evidence older age of acquisition of HCV, is associated with more rapid progression of fibrosis (Verbaan et al., 1998; Bonis et al., 1999; Danta et al., 2002). There may also be slower progression in those
infected in childhood. The evidence that gender has a significant effect is less clear. Poynard, in a large French study, identified men as having a higher risk of more advanced fibrosis (Poynard et al., 1997). However, another Swedish study did not confirm this relationship (Verbaan et al., 1998). Geographically, there appear to be wide variations in the progression of HCV and its complications. A large proportion of patients with hepatocellular carcinoma in high prevalence countries are positive for HCV. In Japan, for example, relative risks of progression of up to 52 fold for hepatocellular carcinoma have been described compared with estimates of 10.5 fold in the USA (Communicable Disease Network of Australia and New Zealand, 1999). This may reflect racial differences in progression of the disease or other confounding factors. Verbaan did identify immigrant status in Sweden as a risk factor for cirrhosis but did not specify the ethnic differences. (Verbaan et al., 1998). This may have been confounded by alcohol intake. Studies have also suggested a difference in response to combination therapy between African-American and white Americans, with poorer responses in African-Americans (Reddy et al., 1999).

Viral factors are more important as predictors of treatment response. Early studies suggested an association between genotype 1b and more progressive disease (Hatzakis et al., 1996). This association was based on an increased prevalence of genotype 1b among people with cirrhosis and hepatocellular carcinoma. However, other studies found no association with genotype (Bonis et al., 1999). This probably reflected a cohort effect confounding genotype with duration of infection, as those with genotype 1b had been infected for longer. The size of the inoculum appears not to be significant, with little evidence of difference between the natural history of HCV in those infected from blood transfusion (a large inoculum) compared with IVDU (a small inoculum), when adjusted for other cofactors such as age at infection (Alter et al., 1992). As discussed, Farci has shown that quasispecies diversity is associated with clinical outcome representing the interaction of the immune system and virus (Farci et al., 2000).
Alcohol is an independent risk factor for liver disease. There is an association between HCV and alcohol, with a higher HCV prevalence found in populations with high alcohol intake. This is thought to be explained through a correlation between injecting drug use and high alcohol intake (McHutchison et al., 1992). There is strong evidence that alcohol causes progression of hepatic disease in HCV, even at relatively low intake levels. There are two forms of possible interaction. First, is the interaction with the virus, with the recognition that alcohol may increase the serum viral load. Pessione et al. showed a dose-response relationship between moderate alcohol intake and increased plasma HCV RNA levels (Pessione et al., 1998). The second interaction occurs directly, affecting histological progression of liver disease. An epidemiological review gave summary odds ratios, from a number of studies, of the increased risk with high alcohol intake for chronic hepatitis (OR 5.8, 95% CI 2.1-5.5), cirrhosis (OR 3.4, 95% CI 2.1-5.5), and hepatocellular carcinoma (OR 2.5, 95% CI 1.5-4.2) (Communicable Disease Network of Australia and New Zealand, 1999). Furthermore, abstinence from alcohol has led to improvements in liver histology (Yoshida et al., 1991). The mechanism of injury, which may relate to iron storage or immune modulation, still remains to be elucidated (Pessione et al., 1998; Wiley et al., 1998). Current recommendations based on our understanding of an interaction between alcohol and HCV are for reduced intake or abstinence, in particular for people who have significant liver fibrosis.

The relationship between progressive fibrosis and raised serum aminotransferases in HCV infection is unclear. Alanine aminotransferase (ALT) is a cytosolic enzyme while aspartate aminotransferase (AST) is a mitochondrial enzyme. Both rise with damage to hepatocytes. In addition, there is marked fluctuation of transaminase levels over the course of the illness (Alter et al., 1992). Healey et al. found significant histological abnormalities in patients with normal AST although the changes were more marked in those with a raised AST (Healey et al., 1995a). Patients with elevated AST had higher alcohol intakes. Furthermore, Luo et al. showed no significant correlation between ALT levels and scores for portal, periportal and total inflammation, although
ALT levels were generally higher in those with severe intralobular necroinflammatory activity (Luo et al., 1998). In a large French study of liver clinic patients the activity and fibrosis scores were significantly higher in the group with elevated ALT, even when corrected for viral load and alcohol intake (Mathurin et al., 1998). The calculated fibrosis progression rate for the normal ALT group was less than half that of the group with raised ALT, with an estimated progression time to cirrhosis of over 80 years. Severe fibrosis in the normal group was associated with heavy alcohol intake. It is clear from these and other studies that significant inflammation and fibrosis can occur in the absence of abnormal transaminases. Although not reflecting the severity of the histological abnormalities, there does appear to be a weak association between ALT and intralobular inflammation.

1.2.6 Treatment:
The primary aim of HCV treatment is the eradication of the infection. Successful eradication of HCV abolishes the inflammatory activity, preventing progression to fibrosis, cirrhosis and HCC. It has also been associated with regression of fibrosis (Shiratori et al., 2000). There may be a benefit to treatment even if HCV is not eradicated (Shiratori & Omata, 2000), although these data require confirmation. Early studies demonstrated that interferon was effective against HCV (Zeuzem et al., 1996; Hoofnagle & Di Bisceglie, 1997). Interferon inhibits the replication of HCV by unknown mechanisms. The pharmacokinetics and efficacy of interferon have been significantly improved by the combination of a polyethylene glycol molecule (pegylation) to the interferon molecule. Ribavirin, a guanosine analogue, is phosphorylated within cells and incorporated into the RNA of virions, thereby increasing the mutation frequency and the infectivity of new virions (Crotty et al., 2001). Ribavirin on its own is largely ineffective but has a synergistic effect when combined with interferon (McHutchison et al., 1998). Recent HCV dynamic modelling strongly suggests a mutagenic rather than an immunomodulatory action for ribavirin; supporting a synergistic action with interferon in the reduction of infected hepatocytes (Dixit et al., 2004). Studies of viral kinetics reveal at least two phases of viral decay
on treatment. Phase 1 clearance of HCV represents the direct anti-viral effect of interferon, while phase 2 relates to clearance of HCV infected cells. Viral response during treatment has been shown to predict outcomes. Sustained virological response (SVR) is defined as undetectable HCV viraemia 24 weeks after the cessation of treatment. This is assumed to equate to eradication of HCV. However, there is a low recrudescence rate of 1-2%. Early virological response (EVR) is defined as undetectable HCV RNA or 2 log drop in viraemia at week 12 of treatment. Failure of an EVR predicts failure of an SVR with a high specificity, which is useful as a measure to stop further treatment (Fried et al., 2002).

Treatment of acute HCV mono-infection is very effective (Alberti et al., 2002). In a large study, Jaeckel evaluated immediate interferon monotherapy for 20 weeks in a German cohort (n=45) of acute HCV patients (Jaeckel et al., 2001). Almost all patients (98%) experienced a SVR, with only one patient failing therapy. Follow-up confirmed that long-term virological, biochemical and clinical responses were maintained (Wiegand et al., 2004). Gerlach and colleagues studied another German cohort (n=60) who had treatment delayed by 3 to 6 months to allow for spontaneous HCV eradication (Gerlach et al., 2003). Approximately half of the patients spontaneously cleared HCV and in those that were then treated with interferon monotherapy, 81% had an SVR. These results are comparable to the Jaeckel study, suggesting that a short delay before therapy does not significantly impact SVRs and may reduce patients’ exposure to expensive and potentially toxic treatment. Hofer et al. has suggested by viral kinetic studies of acute hepatitis C that viral persistence at day 35 was likely to become chronic and treatment should be initiated at this stage (Hofer et al., 2003). Pegylated interferon has also been shown to be successful. An Italian group treated 16 patients with documented acute HCV treated for 6 months with pegylated interferon (Santantonio et al., 2005). The SVR was 94%, with one patient failing therapy. Combination pegylated interferon and ribavirin for acute HCV has not yet been widely studied.
Large multinational studies have now demonstrated the effectiveness of pegylated interferon-α and ribavirin in chronic HCV infection. Two seminal studies evaluated the two currently available forms of pegylated interferon. Manns et al. (n=1530) compared combination pegylated interferon α-2b 1.5 mcg/kg/week plus ribavirin 800mg/day to standard interferon plus ribavirin (Manns et al., 2001). The SVR was higher in the pegylated interferon arm, particularly in those infected with genotype 1 HCV. The overall SVR was 54%, while it was 42% for those with genotype 1 HCV and 82% for those with genotype 2/3. In post-hoc analysis, SVRs were higher in patients who received >10.5 mg/kg of ribavirin. Similar results were obtained by Fried et al. (n=1121) studying pegylated interferon α-2a plus ribavirin compared to standard interferon plus ribavirin (Fried et al., 2002). The SVR in patients who received pegylated interferon was 56% versus 44% in the standard interferon arm (p=0.01), with SVR for those with genotype 1 being 46% and those with genotype 2/3 being 76%. These treatments have been shown to be effective in bridging fibrosis and cirrhosis. Finally, economic analyses have demonstrated that the treatment is cost-effective (Salomon et al., 2003; Siebert et al., 2003). As a result, a combination pegylated interferon and ribavirin is now the standard of care for individuals with chronic HCV.

Virological factors are the most important predictors of treatment response. A number of pretreatment factors have been associated with higher SVRs. These include HCV genotype 2 and 3, lower HCV viral load (<800 000 IU/ml), minimal liver fibrosis, female sex, age less than 40 years, and ethnicity. Most studies to date do not demonstrate an improvement in virological response with more than 24 weeks of combination therapy for individuals with genotype 2/3 HCV (McHutchison et al., 1998). Poynard et al. using multivariate analysis found that genotype 2/3, viral load less than 2 x 10^6 copies/ml, age less than 40 years, female sex, and minimal fibrosis were associated with better SVRs (Poynard et al., 1998). Finally, McHutchison and colleagues demonstrated the importance of compliance with treatment (McHutchison et al., 2000).
SVRs were significantly better in patients who took more than 80% of their treatment 80% of the time compared to patients who were less compliant than this. The maintenance of the full dose of ribavirin also appears to be important (Shiffman et al., 2004).

1.3 HCV/HIV co-infection

1.3.1 Epidemiology of HCV/HIV co-infection:
Given the shared transmission routes for HIV and HCV, it is not surprising that co-infection is common. The WHO estimate that approximately 10 million people worldwide are HCV/HIV co-infected, while up to 48% of European HIV patients in the multinational CAESAR study were co-infected (Amin et al., 2004). In the CAESAR study the strongest predictor of HCV/HIV co-infection was HIV exposure category, with an odds ratio of 365 (CI 179-742) for IVDU compared to homosexuals. The prevalence of HCV/HIV was low (3.7%) among homosexual men without reported IVDU. The UK has lower co-infection rates compared with Europe because of lower IVDU; the prevalence has been estimated to be 9% of the London HIV population (Mohsen et al., 2005). In this London cohort, the prevalence of co-infection varied with transmission factor, from 82% among those with a history of IVDU, 32% in those who had received blood products, 3.5% and 1.8% among those with homosexually and heterosexually acquired HIV respectively. Both HIV infection and high-risk homosexual practices probably contribute to increased HCV sexual transmission (Thomas et al., 1995; Hershow et al., 1998). Sexual rather than parenteral risk factors appear to be associated with the recent rise in acute HCV in HIV-positive individuals in London (Browne et al., 2004; Danta et al., 2005a).

1.3.2 Influence of HIV on HCV:
HIV is associated with higher HCV viral loads and an accelerated rate of liver injury (Eyster et al., 1994). In contrast to HCV mono-infection, liver injury in HCV/HIV co-infection is relatively rapid, with a median time to cirrhosis of 10 years (Soto et al., 1997). In a study of the liver histology of 914 patients, HCV/HIV co-infected patients had more severe fibrosis
with Metavir stages: F0 (no fibrosis) in 10%, F1 (minimal fibrosis) in 33%, F2 (portal linking fibrosis) in 22%, F3 (bridging fibrosis) in 22% and F4 (cirrhosis) in 13% (Martin-Carbonero et al., 2004). Fibrosis progression rates (change in fibrosis stage units per year) were calculated for HCV/HIV co-infected and HCV mono-infected patients. The French group found that the rate was significantly faster in the co-infected cohort than in those infected with HCV alone (0.153 vs 0.106 units per year, p<0.001) (Bedossa & Poynard, 1996; Benhamou et al., 1999). In addition to previously recognised factors such as age at HCV infection and alcohol, the accelerated injury appears to be related to lower CD4 counts (Benhamou et al., 1999). Haemophilic patients with HCV-related cirrhosis were shown to be more likely to develop decompensated liver disease if HIV-positive than if HIV-negative patients with rates of 50% versus 13% at 2 years and 70% versus 40% at 5 years (p=0.005) (Martino et al., 2002). This translated into higher mortality rates among this group. In a cohort of HCV infected haemophiliacs in the UK, HCC occurred earlier and with increased frequency if the patients were co-infected with HIV (Darby et al., 1997). In a meta-analysis comparing HCV/HIV co-infected to HCV mono-infected patients the relative risk of cirrhosis with HIV co-infection was 2.07 (95% CI 1.40-3.07) and that of hepatic decompensation was 6.14 (95% CI 2.86-13.20) (Graham et al., 2001). It should be noted that many of these studies were carried out before the introduction of HAART.

1.3.3 Influence of HCV on HIV:

In contrast to the clear impact of HIV on HCV the influence of HCV on HIV remains controversial. A number of studies have examined this issue but are confounded by the introduction of HAART in 1996. The influence can be examined using either clinical (AIDS defining illnesses) or immunological (CD4 counts) parameters. In the post-HAART era two large studies have suggested a more rapid progression of HIV disease in those co-infected with HCV (Greub et al., 2000; De Luca et al., 2002). The Swiss Cohort study followed 3111 patients starting HAART, of which 1157 were HCV/HIV co-infected (Greub et al., 2000). The relative risk of an AIDS-defining illness was 1.7 (CI 1.26-2.3) in HCV-positive versus
HCV-negative individuals; this was independently associated with HCV seropositivity. The authors attributed this difference to a slower rise of CD4 count on starting HAART. This was supported by the Italian anti-retroviral-naïve cohort study, which found that progressing to an AIDS-defining illness was independently associated with HCV seropositivity (De Luca et al., 2002). Other studies have provided evidence that HCV infection induces a defect in immune reconstitution in response to HAART, as the rise appears to be blunted in HCV infected patients (Soriano et al., 2001; Macias et al., 2003). However, there are two large studies that have shown no difference in progression (Rancinan et al., 2002; Sulkowski et al., 2002). In the USA, Sulkowski et al. studied 1955 patients infected with HIV, 44.6% were also infected with HCV, and found no difference between HCV-positive and HCV-negative patients in their progression to AIDS (Sulkowski et al., 2002). While there was an initial difference between the groups, this disappeared when adjusted for exposure to HAART. In contrast to the Swiss Cohort study the follow-up was shorter and significantly less patients were on HAART. In the Sulkowski study, progression to AIDS was more rapid and an HCV effect may have been missed because of the latency of HCV-related morbidity. A French cohort found no increase in overall mortality among HCV/HIV co-infection (Rancinan et al., 2002). However, only 600 HCV positive individuals were enrolled. While it is difficult to compare these four studies, the CD4 recovery was different between the studies showing an interaction between HCV and HIV and the studies refuting the interaction; this may relate to HAART exposure. A number of mechanisms have been postulated to explain the possible detrimental effect of HCV on HIV (Soriano et al., 2004b). HIV replication may be enhanced by the non-specific immune activation with chronic HCV. Infection of lymphocytes by HCV could lead to CD4 depletion and also blunt the CD4 response to HAART, as suggested in the Swiss Cohort study. Finally, the increased hepatotoxicity could result in more frequent HAART discontinuation with its impact on HIV.
1.3.4 Highly active antiretroviral therapy (HAART) and HCV/HIV co-infection:

Antiretroviral therapy is important for the control of HIV, however, its effect on HCV is less clear. HIV treatment has been reported to improve the course of HCV in co-infected patients. Improved survival in a cohort of 285 German haemophilic co-infected patients suggested that HIV treatment reduced liver-related mortality (Qurishi et al., 2003). The liver-related mortality rates were: 0.45, 0.69, and 1.70 per 100 person years for the HAART, antiretroviral therapy (ART) and untreated groups respectively. However, the effect of HIV treatment reported in this study may have resulted from survivorship bias; those surviving longer would have been more likely to receive HAART (Sabin et al., 2004). There is evidence that protease inhibitors (PI) may slow the fibrosis progression rate. In a French cohort (n=182) the Metavir fibrosis rate in patients receiving a PI and those not receiving these drugs was 0.095 and 0.154 fibrosis units per year respectively (p=0.002) (Benhamou et al., 2001). The estimated difference in cirrhosis at 15 years in the PI group compared with the non-PI group was 13% (p<0.001) respectively. In contrast to this, Macias et al. reported increased fibrosis with the use of nevirapine in HCV co-infected patients (Macias et al., 2004). Patients with low CD4 counts commencing HAART are also at risk of immune reconstitution, which may increase fibrosis progression. Benhamou et al. found that a baseline CD4 count <200 cells/μl was independently associated with a threefold increase in the development of cirrhosis after initiation of ART (Benhamou et al., 2001).

Both HCV and HAART are associated with hepatic injury, mitochondrial toxicity and metabolic abnormalities. The presence of HCV increases the risk of hepatotoxicity related to antiretroviral therapy by 2-3 fold (Saves et al., 1999; Nelson et al., 2005). Sulkowski et al. demonstrated that severe hepatotoxicity (ALT >5.1 x upper limit of normal) occurred in 10.4% of patients taking HAART with either HBV or HCV and HIV co-infection (Sulkowski et al., 2000). Both full-dose ritonovir and nevirapine (during the first 12 weeks of treatment) have been associated with
increased hepatotoxicity (Sulkowski et al., 2000; Martinez et al., 2001). Inhibition of mitochondrial DNA synthesis can lead to lactic acidosis. Mitochondrial damage is also associated with hepatic steatosis. Hepatic steatosis is now recognised as a pathogenic mechanism causing hepatic inflammation and fibrosis. This is particularly associated with the nucleoside analogues didanosine (ddI), stavudine (d4T) and zidovudine (AZT) (Walker et al., 2004). Hepatic steatosis is also linked to metabolic changes that can occur with HCV and PIs. Theoretically, steatohepatitis could worsen the HCV injury, particularly in genotype 3 HCV, which is independently associated with steatosis. Steatosis may also affect response of HCV to interferon and ribavirin (Kaserer et al., 1998).

Recently, the British HIV Association (BHIVA) has published its guidelines for the management of HCV/HIV co-infection (Nelson et al., 2005). They advise that HCV should be treated early, if possible before the need for ART as this reduces the risk of toxicity associated with concurrent HIV therapy. Prior to treatment co-infected patients should have a CD4 count >200 cells/µl as lower counts are associated with poorer HCV responses (Garcia-Samaniego et al., 2002). Therefore, ART should be initiated to attain a CD4 count >200 cells/µl before HCV treatment is commenced. This contrasts with the USA and Canada where HIV treatment initiation is recommended at higher CD4 counts (Braitstein et al., 2004). The rationale is to reduce the risk of immune reconstitution injury and improve the response to HCV treatment. Antiretrovirals associated with specific toxicities such as didanosine, stavudine, nevirapine and full-dose ritonovir should be avoided. The association between nevirapine and increased fibrosis led BHIVA to recommend that it is only used where necessary in HCV/HIV co-infection (Macias et al., 2004). Finally, after starting ART HCV disease should be monitored closely for progression and complications, particularly in those patients with low CD4 cell counts.

1.3.5 Treatment of HCV in HIV Co-infection:

Theoretically, there may be problems assessing early virological responses in HCV/HIV co-infected patients. This is due to differences in
viral kinetics of HCV in HIV-positive patients. Both phase 1 and 2 HCV RNA decay curves are slower in the HIV-positive patient (Torriani et al., 2003; Talal et al., 2004). However, Torriani and colleagues found that failure of an EVR was 98% predictive of failure of an SVR in the largest clinical trial of co-infected patients (Torriani et al., 2004). These results are the same as in HCV mono-infected patients. Interestingly, the slower phase 2 HCV kinetic decline may impact on the length of HCV treatment and some have suggested this may lead to an increase rate of relapse of HCV after treatment (Soriano et al., 2004a). Important interactions have been identified between HAART and combination HCV treatment. Ribavirin increases the intracellular phosphorylation of didanosine (ddI). This increases the risk of mitochondrial toxicity, and has been associated with multiorgan dysfunction and lactic acidosis (Lafeuillade et al., 2001). Based on the use of the data from RIBAVIC study, there is now an FDA recommendation against the combination of ribavirin and ddI. BHIVA recommend avoiding the combination of zidovudine (AZT) or ddI with ribavirin therapy (Nelson et al., 2005).

**Acute HCV in HIV co-infection:**

Preliminary treatment results of interferon with or without to ribavirin for acute HCV in HIV co-infection has emerged from the recent UK epidemic. Treatment of a London cohort of 27 HIV-positive patients with acute HCV, defined as documented seroconversion of anti-HCV antibody and positive HCV viraemia, for 24 weeks with combination pegylated interferon α-2a plus ribavirin resulted in a SVR of 59% (n=16) (Gilleece et al., 2005). These results were supported by a smaller German case series (Vogel et al., 2005). A significant proportion of the UK cohort eradicated HCV prior to treatment, probably pointing to the necessity to delaying treatment for a short period (12 weeks) to allow spontaneous clearance. While the SVR is lower than among individuals with acute mono-infection treated with interferon monotherapy, these rates are significantly higher than those among chronically co-infected patients treated with combination pegylated interferon and ribavirin (Jaeckel et al., 2001; Fried et al., 2002; Torriani et al., 2004).
Chronic HCV/HIV co-infection:

Three large randomised controlled trials have examined the effect of combination pegylated interferon-α plus ribavirin for treatment of chronic HCV/HIV co-infection. In contrast to HCV mono-infection, high relapse rates have been reported after 24 weeks of treatment for HIV-positive patients infected with genotypes 2 or 3. Consequently, 48 week therapy was used in these studies. The APRICOT study (n=868) compared the efficacy of 48 weeks of pegylated interferon α-2a (180 mcg weekly) plus ribavirin (800mg daily), pegylated interferon α-2a (180 mcg weekly) plus placebo, or interferon α-2a (3 million IU three times a week) plus ribavirin (800 mg daily) (Torriani et al., 2004). Patients treated with pegylated interferon α-2a plus ribavirin had significantly higher overall SVR rates compared with pegylated interferon α-2a alone or standard interferon plus ribavirin (40% versus 20% and 12% respectively, p<0.001). In the pegylated interferon α-2a plus ribavirin arm the SVR for those infected with genotype 1 was 29% and for those infected with genotypes 2 and 3 was 62%. Failure of an early virological response was predictive of treatment failure. The side-effects were comparable between the different arms and there were no significant adverse effects of therapy on the HIV parameters.

The French RIBAVIC study (n=412) randomised co-infected patients to 48 weeks of Ribavirin 800 mg plus either pegylated interferon α-2b (1.5μg/kg weekly) or standard interferon α-2b (3 million IU three times a week) (Carrat et al., 2004). The overall SVR was better in the pegylated interferon α-2b arm (27% versus 20%, p=0.047). In the pegylated interferon arm the SVR for those infected with genotypes 1/4 was 17% and for those infected with genotypes 2/3 was 44%. In the Aids Clinical Trials Group (ACTG) study 133 (ACTG 133) patients were randomised to receive either pegylated interferon or standard interferon with dose escalating ribavirin (Chung et al., 2004). The overall SVR for the pegylated versus the standard interferon group was 27% versus 12%
In the pegylated interferon arm the SVR was 14% for those infected with genotype 1 and 71% for those infected with genotypes 2/3.

While the outcomes of treatment of dually infected patients are lower than in HCV mono-infected patients, these studies have demonstrated that pegylated interferon and ribavirin is an effective therapy for the co-infected patient (Fried et al., 2002). In summary, the studies have demonstrated overall SVRs ranging from 27-40%. The SVR for genotype 1 HCV ranges from 14-29% and for genotypes 2/3 from 44-71%. It is often difficult to compare studies and the reason for the variation between these studies is debatable. The SVRs in the RIBAVIC study were significantly lower than those in the APRICOT study. This may have been the result of the high frequency of discontinuation in the RIBAVIC study due to side-effects of the regime and the proportion of intravenous drug users enrolled. There was also a significant difference in the proportion of individuals with severe fibrosis at randomisation (bridging fibrosis or cirrhosis), which was more prevalent in the RIBAVIC than the APRICOT cohort (40% vs 15%). Advanced fibrosis is known to be a predictor of poor virologic response (Manns et al., 2001; Myers et al., 2003). A number of reasons have been suggested to explain the poorer treatment responses in co-infected patients (Brau, 2003). These include higher HCV viral loads, increased HCV quasispecies diversity and lower dosing of ribavirin due to intolerance. In those that do not have a SVR, HCV treatment was shown to improve histology in 34% of patients (Chung et al., 2004).
1.4 Objectives

The objective of this study was to analyse three linked aspects of the epidemic of HCV in the HIV population of South-East England.

The major focus of the thesis is the characterisation of the epidemiology of acute HCV in the HIV population through linked molecular and clinical studies. The association with high-risk sexual practices and sexually transmitted infections suggests that, in contrast to the normal parenteral route of HCV transmission, sexual factors may be important in the recent epidemic. A phylogenetic study of the E1/E2 region of the HCV genome from identified cases explores the thesis of recent HCV transmissions. Phylogenetic analysis of the HCV genome has previously been used to determine viral relatedness and provide evidence of HCV transmission within populations (Cochrane et al., 2002). A case-control study using a specifically designed questionnaire instrument was performed in parallel to elucidate relevant transmission factors. Matched controls were generated from the same HIV clinics as the patients. By demonstrating transmission, and identifying the responsible factors, public health interventions can be developed to focus on specific measures to prevent further infections.

By Seeff’s criteria, this cohort is ideally suited to describe the natural history of HCV/HIV co-infection (Seeff, 1997). The cohort has acute disease documented by seroconversion of anti-HCV and/or HCV RNA. Clinically the cohort covers the spectrum of acute disease from the asymptomatic presentations to icteric acute hepatitis. Since the majority of individuals were diagnosed as outpatients there would appear to be no bias towards severe disease. The clinical course of these patients has been recorded over this time. As part of the case-control study, HCV negative controls have been matched to these patients. This provides an accurate picture of the early clinical course of HCV in HIV-positive individuals. A proportion of the cohort has been treated and study of this group provides valuable data on the influence of standard HCV treatment in early co-infection.
Cell-mediated immunity is the most important immunological mechanism for control of HCV. HIV infection causes a cell-mediated immune defect. The immunological responses in HIV co-infection have not been well studied. Multiple longitudinal blood samples have been collected from patients in the acute phase of HCV infection to study these responses. Using qualitative lymphocyte assays a picture of cell-mediated immune responses will be developed. These are correlated with both HCV evolution and clinical outcome over the early phase of the infection.

This cohort provides a unique opportunity, through the characterisation of the epidemiological, clinical and immunological aspects to improve our understanding of both HCV mono- and co-infection.
Chapter 2: Molecular Epidemiology

2.1 Introduction

Evolution is founded on the concept that organisms share a common ancestor and diverge through time. Phylogenies are an attempt to reconstruct this evolutionary history; thus sensitive molecular PCR techniques have transformed phylogenetic studies in viral infections as viral evolution is particularly suited to this type of study. Phylogenetic studies of viruses use accumulated genetic divergence over time to understand the origin and spread of the virus. In contrast to the evolution of eukaryotic DNA, viral evolution is rapid, prone to mutation and can therefore be studied in a relatively short time frame. These studies exploit statistical dependencies among sequences sharing a common ancestor. Of particular relevance to this thesis, transmission patterns can be established and evolutionary histories of epidemics can be reconstructed. Dramatic advances in phylogenetic analysis have also resulted from increased computational speed, more realistic DNA modelling, and better computer programs.

2.1.1 Viral evolution:

Viral evolution depends upon genetic mutation as a result of change in the nucleotides (adenine (A), guanine (G), thymidine (T) and cytosine (C)) that make-up the DNA or RNA genome. Four processes can contribute to viral evolution. Adaptive (Darwinian) evolution describes the genetic change resulting from external selection pressure, which leads to phenotypic change. Mutations will predominate where there is a concomitant survival or fitness advantage. Different sequences of nucleotides can code for the same amino acid (AA); for example GCC and GCA code for alanine. A nucleotide change which results in a different AA (a phenotypic change), is termed a non-synonymous change. However, if the nucleotide substitution does not result in phenotypic change this is termed a silent or synonymous change. By looking at the ratio of the number of non-synonymous to synonymous changes (dN/dS), evidence of positive selection pressure (dN/dS >1) can be found. The HVR1 in the
E2 region of the HCV genome is an example of a region under strong positive selection pressure from the immune system in acute infection (Farci et al., 2000).

In contrast to adaptive Darwinian evolution, the neutral theory of evolution, proposed by Kimura, holds that the great majority of genetic diversity is caused by random fixation (the nucleotide substitution becomes a universal feature of the population), through sampling drift, of selectively neutral mutations under continued mutation pressure (Kimura, 1991). These mutations have little effect on the phenotype, biology or fitness of either the species or the individuals that make up the species, and become fixed by chance. Kimura’s view is based on nucleotide sequences coding AAs leading to synonymous/non-synonymous change. He proposed that the mutations are selectively neutral or slightly deleterious and are maintained by a balance between mutational input and random extinction. Spontaneous nucleotide substitution leads to a new allele, which is then subject to drift. These new alleles or mutations usually decline or disappear, but very occasionally they become ‘fixed’. In this way the neutral substitutions accumulate and contribute to the evolution of the genome. Neutral sequence change accounts for the vast majority of the genetic divergence between the geographically or epidemiologically separated HCV genotypes (Simmonds, 2004). While there is up to 35% variation in the nucleotide sequence of the HCV genome between genotypes, the replication cycle and infection in humans remains very similar. The rate at which these single-nucleotide differences occur, termed the ‘molecular clock’, is determined by the HCV RNA polymerase. HCV mutates at rates a million times faster than DNA organisms; the result of short generation times and lack of proof-reading activity in the RNA polymerase (Saitou & Nei, 1986). The molecular clock for HCV has been estimated by sequencing longitudinal samples from infected individuals. In a study of American and Japanese patients chronically infected with HCV, Tanaka et al. calculated the mean evolutionary rate of all codon substitutions within the combined nucleotide sequences was 0.67 (0.53-0.79) x 10^-3 per site per year (Tanaka et al., 2002). The rate for synonymous substitutions
was higher than non-synonymous, supporting the neutral theory of evolution.

Transmission events are another important source of viral heterogeneity. During the passage of virus from one host to another there is an extreme evolutionary bottleneck, as only a few randomly selected virions are transferred. This arbitrariness contributes to a high rate of evolution because only the transferred virions can replicate to produce a persistent progeny population with further transmission of HCV. At the time of infection the majority of patients have a few HCV particles. In a study of 12 individuals with primary infection, Herring and colleagues found that only 4 of the 12 had been infected with multiple infectious particles at the time of infection, based on evaluation of the HCV quasispecies and known mutation rate of HCV (Herring et al., 2005).

Finally, recombination can produce viral mutation. This occurs when genetic material is passed between different viral genomes, usually as a result of template-switching during RNA genomic replication. While recombination is common with HIV it is rare in HCV infection, and has only been described in Russia and Peru (Colina et al., 2004; Kalinina et al., 2004).

2.1.2 Phylogenetic studies:
Phylogenetic studies of genetic sequences have two components. One is a phylogenetic tree, which is a topographical description of the relationships between the sequences and has the form of branching lineages connected by nodes. The horizontal distance is a visual representation of genetic distance; internal nodes are the points where lineages branch or diverge. Terminal nodes are situated at the end of the branches and represent the individual sequences or taxa. A tree is rooted if it has a node designated as the ancestor of the sequences that make-up the tree, giving the tree a temporal polarity from past to present. A group of taxa (>2 sequences) are considered monophyletic if they all derive from the same ancestor; also termed a clade. Often this is determined by comparing the sequences of interest (the ‘ingroup’; in this
case the sequences of acute HCV in the HIV-positive individuals) to other similar but unrelated sequences (the 'outgroup'). The second component is the model used to describe how sequences evolve by nucleotide or amino acid change along the branches of the tree. Evolutionary models can be either empirical or parametric. Empirical models use properties calculated through comparison of large numbers of observed sequences. Parametric models use chemical or biochemical properties of DNA or AA to describe the evolution through time of the sequences. These models allow a genetic distance between two sequences to be estimated, measuring the expected number of nucleotide substitutions per site that have occurred on each lineage between them and their most recent common ancestor.

Nucleotide substitution parameters are fundamental to these evolutionary models. The rate of evolutionary change, the 'molecular clock', was initially proposed to be constant (Zuckerkandl & Pauling, 1965). However, it is now clear that the "molecular clock seems to tick at different rates" with a number of factors influencing the nucleotide substitution rate; these parameters can be defined in the evolutionary models (Lio & Goldman, 1998). First, base frequency can be defined, which describes the frequency of purines; adenine (A) and guanine (G), and pyrimidines; thymidine (T) and cytosine (C), over all sequenced sites and over the tree. Certain bases may be more likely to arise when substitutions occur. Second, base exchangeability parameters can be used to describe the relative rate of bases substituted for each other. This incorporates the different tendency for transitions and transversions. A transition is a change between a nucleotide of the same chemical type (purine to purine or pyrimidine to pyrimidine). A transversion is a change from one to the other (purine to pyrimidine or pyrimidine to purine). Transitions are more common than transversions (Brown & Simpson, 1982). Another parameter relates to the nucleotide mutation rate which varies across the HCV genome, termed rate heterogeneity. It is usually described by a gamma distribution for a non-symmetric distribution. In a review of variation among cloned HCV sequences, Smith et al. estimated sporadic nucleotide substitutions across the genome: 5' Untranslated
region (UTR) 0.08-0.72 \times 10^{-4}, \text{NS3} 0.71 \times 10^{-4}, \text{E2} 0.61-1.42 \times 10^{-4}, \text{NS5A} 0.43 \times 10^{-4}, \text{NS5B} 0.17-0.37 \times 10^{-4} \text{ per site per year (Smith et al., 1997). The high rate of change in the E2 region relates to the HVR1. Models have also been developed based on codons to describe the evolution of sequences in terms of DNA substitutions and the selective forces acting on proteins. The third codon site, where 70% of mutations are synonymous, evolves nine times faster than the second codon site, where all changes are non-synonymous (Stumpf & Pybus, 2002). Nucleotide replacement is a dependent random event, described as a Markov process, where future possibilities are determined by its most recent values.

Using these evolutionary models different methods can estimate the phylogeny. Typically, the methods either grow the tree progressively during analysis (distance-based) or perform an exhaustive search of all the possible tree topologies (character-based). It must be understood, however, that a derived tree is a hypothesis of a relationship between the sequences rather than the actual tree. Furthermore, estimates of evolution are based on representative sequences and often not the entire population. Distance-based methods infer the relatedness between sequences by calculating the pairwise distance between them and tabulating a distance matrix. The distance between them is a measure of their genetic difference and this makes up the matrix. The tree is calculated by progressive clustering from this distance matrix. This model describes how the nucleotide substitutions have occurred since they last shared a common ancestor. A tree is then drawn from the data with the more similar sequences clustering more closely than less similar sequences. Distance methods include the Unweighted Pair Group Method (UPGMA) and Neighbour-joining (NJ) approach.

Character-based methods search for the optimal tree among the total number of possible trees; and infer relationships more closely from observed sequence. These include maximum likelihood (ML), maximum parsimony (MP) and Bayesian methods. The ML estimation of phylogeny is a statistical method which gives the most probable tree for the
observed data using the specified model of evolution. For the ML method, the user can determine the evolutionary parameters. The strength of the ML method is that because it is statistically based, estimates are statistically consistent and comparisons between trees, parameters and models can be made. The problem with ML methods is they are computationally demanding due to the number of tree topologies generated; these methods are practical for a few hundred sequences at most. Maximum parsimony holds that the simplest hypothesis is most likely to be correct. To explain the data, this method uses the least number of evolutionary steps to create trees. This method can be problematic if there is large variability between the sequences. However, it is suited to sequenced-based analysis of large numbers of sequences. Bayesian methods employ probability theory and the Markov Chain Monte Carlo methods to analyse and search for the best set of trees. The method of analysis is dependent upon the number of trees. If there are fewer than 10 taxa then an exhaustive tree search can be performed. However, as the number of taxa increases, the number of possible trees increases exponentially; even with n=10 taxa, the number of possible trees exceeds $2 \times 10^6$ (Felsenstein, 1978). Consequently, with larger groups of sequences heuristic methods are used to simplify the computations. Heuristic methods use problem solving techniques in which the most appropriate of several trees found by alternate methods is selected at successive stages of a program for use in the next step of the program.

It is important to be able to test the generated trees, or chosen parameters and models. The introduction of likelihood frameworks has allowed statistical comparison of different models. The likelihood of a hypothesis is defined as the probability of the data given that hypothesis. The hypotheses in phylogenetic studies are the reconstructed trees and parameters of the evolutionary models. Different hypotheses have different likelihoods: the higher the likelihood, the more plausible the generated data. Statistical methods now exist to estimate these likelihoods, typically requiring computations that account for the unobserved sequences at the internal (ancestral) nodes of hypothesised
trees. These likelihoods can also be used to compare evolutionary models. Likelihood ratio tests (LRT) are a class of powerful statistical tests that compare ML values of competing hypotheses. Another analytic method is non-parametric bootstrapping, which is used as a test of the inferred evolutionary tree. This is a numerical re-sampling method in which a new sequence alignment is compiled from the original alignment by random sampling with replacement. A column of nucleotides is chosen randomly and placed in a new alignment and then this is repeated; up to 1000 times. The frequency with which a specific branch is found after analyses of these pseudoreplicates is used to assess the reliability of the relationship of the branches. A bootstrap score >70 represents a cluster with robust statistical support (McCormack & Clewley, 2002). Sequences derived from this branch are considered monophyletic.

Population dynamic studies analyse sequences to give estimates of temporal change in population size, population structure and substitution rates. Evolutionary trees spanning longer periods of time are inferred from DNA sequences. These analyses use node height or branch length of the tree. Models used for this sort of analysis include birth-death and coalescence models. Assumptions are made that at any point in time all the lineages in the trees are assumed to behave according to the same stochastic rules, although these rules can change through time. A relationship between node height and speciation (new lineages) and extinction (loss of lineages) exists. As the birth rate (speciation) increases, the nodes are more frequent closer to the present, while an increase in death rate (extinction) leads to the nodes being situated closer to the root. However, studying trees that represent individual taxa belonging to the same population, a coalescent model is more appropriate. Coalescence describes the relationship between the demographic history of a large population and the shared ancestry of individuals randomly sampled from it; represented in a tree. During analysis of the tree, a number of different lineages can be followed. This number decreases when there is a common ancestor (a coalescent event) or increases when there is a sampled individual (a sample event). Because the probability that a coalescent event occurs at a particular
time is inversely proportional to the population size at that time, the
correlation of observed coalescence and sampling events can be used to
estimate the demographic history of the population. A molecular clock is
fundamental to this analysis as it combines temporal information with the
genetic information of the sequences. Using the nucleotide substitution
rate the date of origin, also termed ‘the most recent common ancestor’
(TMRCA), of clusters can be calculated. This correlates with the entry of
the virus into the transmission cluster. HCV is suited to population
dynamic analysis as it has very short generation times, which generate
many mutations. Because the mutation rate is so high, shorter sequences
can be used in these analyses. The temporal information in sequences
from multiple time-points enables phylogenies to be calibrated in
calendar timescales from which, the timing of epidemiological events can
be inferred, and in conjunction with coalescent theory, enables past
epidemiological dynamics to be estimated.

A phylogenetic analysis therefore involves a number of steps. First the
relevant sequence of interest is identified. The region needs to have
enough variability that differences will exist to differentiate between the
individual viruses. The length necessary will be in part determined by the
assay and the viral dynamics. High mutation rates allow shorter
segments to be examined. Finally, there needs to be data available on
unrelated similar regions to produce the comparative ‘outgroup’. The
HVR1 and NS5B regions of the HCV genome have been used extensively
for this type of molecular epidemiological study. With the interest in the
immunological aspect of acute infection, the E1/E2 region of the genome,
which incorporates the hypervariable region (HVR1) was chosen. This
longer segment of sequence increases the robustness of the analysis.
Importantly, there are many E1/E2 sequences available publicly (on
Genbank/Los Alamos HCV sequence bank) for comparison. Finally, work
has already been done that has identified the rate of nucleotide
substitutions of the E1/E2 region. It has also been recommended that the
HVR1 is suited to analysis of more recent HCV transmission, while
analysis of the more conserved and longer NS5B sequence was the
choice for the examination of transmission in the more distant past.
(Smith & Simmonds, 1997). Interpretation of the final trees and population dynamics will provide detailed insights into this epidemic at a molecular level.

2.2 Aims

This phylogenetic study had two aims:

1. To determine if there is evidence at a molecular level for transmission of HCV in the HIV population. Through an analysis of E1/E2 homology and comparison with similar but unrelated sequences, a search for monophyletic transmission clusters (clades) was performed. If found these data would provide powerful evidence for transmission.

2. A population dynamic analysis was performed on the largest monophyletic cluster to understand the evolution and dynamics of the HCV transmission.
2.3 Methods

2.3.1 Study cohort:
The study cohort consisted of HIV-positive patients diagnosed with acute HCV infection. Acute HCV was defined as a documented positive anti-HCV seroconversion following a negative assay within the prior 6 months; or detection of HCV-RNA by PCR following a negative assay within the prior 6 months; or a positive HCV-RNA by PCR and a more than 10-times the upper limit of normal (ULN) rise in serum alanine aminotransferase (ALT) with documented normal ALT during the preceding year. All the cases were recruited from three large urban HIV clinics in South-East England: the Ian Charleston Day Centre at the Royal Free Hospital (RFH); the Koebler Centre at the Chelsea and Westminster Hospitals (CWH); and the Elton John Centre at Brighton and Sussex General Hospital (BSH). These centres treat substantial numbers of patients with HIV, providing both inpatient and outpatient services. The study began enrolling patients in late 2003 and patients have been enrolled both retrospectively and prospectively. Patients had been diagnosed with HCV between 1999-2005. Patients participating in the phylogenetic study, gave informed consent for blood samples and clinical information to be obtained and analysed. All these cases have been enrolled into the study under the approval of each unit’s local research ethics committee (LREC references: RFH 6148; CWH 3247; BSH B03/83) and the Central Office for Research Ethics Committees (COREC).

Specific clinical viral testing was performed using the following: anti-HCV was tested with Vitros ECI ELISA (Ortho-Clinical Diagnostics, USA). If this was positive but the HCV RNA negative then a Chiron HCV RIBA (Chiron, USA) was performed to confirm a true positive anti-HCV assay. Up until March 2004, HCV RNA was tested with Bayer branched DNA or Bayer TMA assay (Bayer, Germany) with a lower limit of detection of 615 IU/ml. Since March 2004 an automated Abbott system has been used, initially Abbott LCX HCV PCR (Abbott, USA) with a lower limit of detection of 50 IU/ml and then Abbott Real Time HCV PCR with a lower limit of detection
of 23 IU/ml. The genotype was determined using a Bayer AutoLiPA (Bayer, Germany) on samples that were HCV RNA positive.

2.3.2 Target genome:

HCV RNA isolation and preparation

The first HCV RNA positive serum sample that was available was used for the phylogenetic analysis. These samples were obtained from each centre's virology service and had been stored at between -4 to -20°C. Some had been stored for up to 4 years before analysis.

QIAamp Viral RNA Mini Kits (QIAGEN™) were used for extraction of the HCV RNA from serum according to manufacturer's instructions. The technique uses silica-gel based membrane to actively bind the RNA. Briefly, the samples were lysed under highly denaturing conditions to inactivate RNA-ases and ensure isolation of HCV RNA. Serum was used to limit the amount of cellular DNA as DNA is not separated with these kits. Aliquots of 140μl of serum were used for the extraction. The extracted RNA samples were eluted to 50μl in water and stored at -80°C.

Reverse-transcription polymerase chain reaction (RT-PCR):

The development of the polymerase chain reaction (PCR) technique in the 1980s has allowed rapid advances in the field of molecular biology. Thermostable DNA polymerase is utilised to extend sequences from synthesised oligonucleotide primer pairs, which are designed to flank an area of interest. The PCR mixture is heated to separate the double stranded DNA and then cooled to a specific temperature to allow the primers to anneal to a DNA strand. A further rise in temperature allows extension to take place from the 5' end of each primer. The reaction is then heated which separates the newly formed DNA from its template and the cycle is repeated. Each cycle, at optimum conditions, allows the number of copies of the region of interest to be doubled and this exponential increase in DNA continues until the DNA polymerase is exhausted, or the primer pairs become limiting.
The addition of a reverse transcriptase step before PCR is carried out, has allowed the amplification and quantitation of RNA as well as DNA by this method. Reverse transcriptase is an enzyme utilised by RNA viruses to convert their genomic material (i.e. RNA) into transcribable DNA within the host cell. If added to RNA isolated from cells or tissues of interest, along with suitable primers to initiate reverse transcription, it will produce cDNA that can then be used in a subsequent PCR. Primers can be either random (which will produce cDNA of all the mRNA in the sample) or sequence specific which produces cDNA only from mRNA containing the sequence of interest.

Reverse transcription was carried out using a Superscript™ First Strand Synthesis System (Invitrogen, UK). Briefly 50ng random hexamer primers (Amersham Bioscience, UK) were added to 10μl of extracted RNA and the mixture was heated to 65°C for 5 minutes. 9μl of the following mix was added; 1x First Strand Buffer, 500μM dNTP’s, 40units RNaseOUT ribonuclease inhibitor (Invitrogen, UK), 50units Superscript II M-MLV reverse transcriptase. The reaction was then incubated at 37°C for 60 minutes and terminated by heating to 70°C for 5 minutes. The cDNA was stored at -20°C until use.

**Nested E1/E2 PCR:**

Nested PCR was performed using primers to amplify the E1/E2 section of the HCV genome (Farci et al., 1992). The product was 575 basepairs (bp) in length. The following primers were used:

- **F1E2** (between nucleotides 1285-1304): 5’-caccgcatggcatgggatat
- **R1E2** (between nucleotides 1881-1862): 5’-ggggctgggagtgaagcaat
- **F2E2** (between nucleotides 1291-1310): 5’-atggcatgggatgatgat
- **R2E2** (between nucleotides 1865-1846): 5’-caagcaatataccggacaca

To prevent contamination during the PCR each step the recommendations of Kwok were followed (Kwok & Higuchi, 1989).
The first PCR reaction was carried out as follows; 1x Taq buffer, 1.5mM MgCl₂, 0.2mM dNTP, 50pmole F1E2 and R1E2, 1 unit Platinum Taq polymerase (InVitrogen, UK) and 5µl cDNA in a total volume of 50µl. The reaction was heated to 94°C for 15 minutes to activate the polymerase, 35 cycles of denaturing at 94°C for 30 seconds, annealing 54°C for 30 seconds and extension at 72°C for 1 minute. A terminal extension step of 72°C for 4 minutes was included to ensure complete synthesis of the double stranded amplicon.

The second PCR reaction was then carried out using the nested primers F2E2 and R2E2 using the same conditions and adding 2µl of first round product.

**Gel electrophoresis:**

Once the reaction had been completed the reaction was subjected to agarose gel electrophoresis. The expected size of the PCR product was 575 bp in length.

The gel electrophoresis was performed as follows:
A 1.5% w/v agarose gel was prepared in 1x TBE Buffer (0.89 M Tris, 0.89 M Boric Acid, 0.02 M EDTA) and melted in a microwave at full power. Once cooled to 50°C 1 µl of ethidium bromide solution (0.05 µg/ml) was added and the gel poured into a perspex mould with a gel comb in situ and left to set. The PCR reaction was then added to 8µl of 6x Loading Buffer (15% w/v Ficoll 400, 0.06% w/v Bromophenol Blue, 30mM EDTA-Tris pH 8.0). The samples were added to the wells and the gel subjected to electrophoresis at constant voltage of 80-100volts. Appropriate size markers were included in each run. After completion the gel was visualised by ultra-violet trans-illumination on a UVP EpiChemi II Darkroom (UVP Laboratory Products, USA) and a digital image saved. Following confirmation of a product of appropriate size, the bands were excised under ultraviolet visualisation and purified using a QIAquick PCR purification kit (QIAGEN™, UK) and manufacturer’s instructions. The purified DNA was eluted in 30-40µl of elution buffer (10mM Tris-HCl pH 7.6). Once purified the E1/E2 DNA was stored at -80°C until sequencing.
**Sequencing of the PCR product-E1/E2 region:**
The 575bp E1/E2 DNA amplicon was directly sequenced using forward and reverse PCR primers. Sequencing was performed by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. 20ng of purified DNA and 3.2pmoles of primer were included in each sequencing reaction. The primers’ sequences were not included in the final analysed section, leaving a 495 bp sequence.

**Cloning of E1/E2 region to assess consensus sequence:**
It is now acceptable to use the direct sequence of the purified amplicon which represents the consensus sequence (Odeberg et al., 1995). To determine that this was the case with our samples we compared the consensus sequence from direct sequencing to cloned individual sequences from a number of individuals.

The 575 bp E1/E2 amplicon was cloned into pCR 4.0 TOPO vector using a TOPO TA Cloning® kit (Invitrogen, UK), following the manufacturer's instructions throughout. This method is suitable for Taq polymerase generated amplicons as it has a non-template-dependent terminal transferase activity, which adds a single adenosine to the 3’ end of the PCR product. The vector provided has an overhanging thymidine residue, allowing efficient binding of the vector and PCR product. The kit utilises Vaccinia topoisomerase, which specifically cleaves the phosphodiesterase backbone after 5’-CCCTT in one strand and a covalent bond is then formed between the 3’ end of the PCR product and the topoisomerase. The plasmid construct is then transfected into *E.coli* (TOP10). The vector contains a lethal gene ccdB fused to the C-terminus of the LacZa fragment which is disrupted by the insertion of the PCR DNA; only *E.coli* with the recombinant will grow.

Briefly, 4μl of eluted E1/E2 cDNA was mixed with 1μl of salt solution and then 1μl of TOPO® vector was added. This was incubated for 5 minutes at room temperature (22°C) and then placed on ice. 2μl of the reaction
was then added to chemically competent TOP10 E.coli and incubated on ice for 20 minutes. The cells were heat-shocked for 30 seconds at 42°C and again transferred to ice. 250µl of SOC culture medium (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose) was added to each tube and then placed in a rotary incubator for 1 hour at 37°C. 10 and 50µl volumes of each reaction were then spread evenly on LB agar selection plates (1% Tryptone, 0.5% Yeast Extract, 1% NaCl, 15g/L Agar, 50µg/ml ampicillin pH 7.0) and incubated at 37°C overnight.

**Plasmid purification and sequencing:**
At least 10 colonies were picked from each plate and re-cultured in 1.5ml of LB medium (1% Tryptone, 0.5% Yeast Extract, 1% NaCl pH 7.0) containing 50µg/ml Ampicillin at 37°C. Plasmids were purified from the cultures using a QIAprep™ kit (QIAGEN, UK) according to the manufacturers instructions, and stored at -20°C. 300ng of the purified plasmid was sequenced at The Sequencing Service (School of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using M13 forward and reverse primers (sequence was 535bp in length).

### 2.3.3 Phylogenetic analysis:

#### Sequence alignment:
The amino acid sequences were aligned and then converted to nucleotide sequences. This gives an in-frame alignment. The beginning of the E1 region was identified as an alignment point because the divergence at this point is generally low. Nucleotide sequences were edited and compiled using the Lasergene software (DNASTAR Inc, USA). The patient sequences were then aligned along with a data set of the same HCV region (n=330) collected from GeneBank (www.ncbi.nlm.nih.gov) and Los Alamos HCV Sequence Database (www.hcv.lanl.gov).

#### Phylogenetic reconstruction:
The Neighbour-joining (NJ) method was used to reconstruct the phylogenetic relationship of the sequences. This method was developed by Saitou and Nei (Saitou & Nei, 1987). The NJ methods reconstructs the
phylogenetic trees based on the principle of minimum evolution or maximum parsimony which chooses the tree that requires the smallest amount of evolutionary change. Simply, the NJ method starts with a star shape of the sequences. A distance parameter is then defined between the sequences and this is entered into a distance matrix. Neighbours are defined as sequences connected through a single interior node in an unrooted bifurcating tree. The two sequences with the lowest distance between them are defined as the initial neighbours. Then the distance matrix is re-calculated to define the distance between the new node and the other sequences. Then a new neighbour is defined and this process continues until all the sequences lie in the tree. The NJ method provides not only the topology but also the branch lengths of the final tree.

Using a NJ method the genetic distances were calculated using maximum likelihood, under a Hasegawa Kishimo Yano (HKY) model with a gamma-distribution model of rate heterogeneity among sites (Hasegawa et al., 1985). The HKY model uses base frequency and transversion/transition parameters for nucleotide substitution models. The phylogenetic trees were mid-point rooted using the estimated genetic distances. Unrelated sequences of the same region were extracted from GenBank and the Los Alamos HCV Sequence Database. After identification of the largest clade, the tree was re-estimated using PAUP* software for the computational analysis (Swofford, 2003). PAUP* uses a maximum likelihood (ML) approach. The statistical robustness of the ML topologies was assessed by bootstrapping with 1000 replicates. The dynamics of the largest clade were explored following the application of a molecular clock using both coalescent theory and life-death plots. The date of origin of each HCV cluster, which approximates the year of entry of HCV into the cluster, was estimated using a molecular clock approach. First, an evolutionary rate for our specific region of E1/E2 was estimated using the method of Verbeeck et al. (Verbeeck et al., 2006). Dates of origin for each cluster were subsequently calculated using a Bayesian MCMC approach in Beast v1.3 (Drummond & Rambaut, 2003). The final trees were interpreted in the context of the available clinical, epidemiological and serological data.
2.4 Results

2.4.1 Patient cohort:
Serum was analysed from 111 HIV-positive patients who had been diagnosed with acute HCV. The patients were recruited from: the RFH (n=50), CWH (n=50) and BSGH (n=11). The clinical and demographic data is outlined in Table 2.1. All the cases were HIV-positive men with a median age of 35 years. Their HIV was well controlled with 57% on HAART and a median CD4 498 cells/µl. The majority (83%) had genotype 1 with high HCV viral loads (median HCV viral load 6.1 log IU/ml). The median peak ALT was 285 IU/ml.

Table 2.1: Phylogenetic study patient parameters.

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>RFH</th>
<th>CWH</th>
<th>BSGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>111</td>
<td>50</td>
<td>50</td>
<td>11</td>
</tr>
<tr>
<td>Median Age (years)</td>
<td>36 (24-58)</td>
<td>35.2</td>
<td>35</td>
<td>42</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Median peak ALT (IU/ml)</td>
<td>285 (22-5104)</td>
<td>394.5</td>
<td>256.5</td>
<td>310</td>
</tr>
<tr>
<td>HCV genotype 1 (%)</td>
<td>83%</td>
<td>84%</td>
<td>84%</td>
<td>73%</td>
</tr>
<tr>
<td>Median HCV viral load (log IU/ml)</td>
<td>6.1 (3.0-7.6)</td>
<td>6.0</td>
<td>6.4</td>
<td>6.1</td>
</tr>
<tr>
<td>Median HIV length of infection (years)</td>
<td>4.1 (0-19.1)</td>
<td>4.0</td>
<td>4.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Median CD4 (cells/µl)</td>
<td>498.5 (33-2715)</td>
<td>549</td>
<td>433.5</td>
<td>496</td>
</tr>
<tr>
<td>HAART (%)</td>
<td>57</td>
<td>61</td>
<td>54</td>
<td>53</td>
</tr>
</tbody>
</table>

2.4.2 Sequences:
Of the 111 patients’ sera, complete E1/E2 sequences were obtained from 93 patients. The sequences analysed were 495 base-pairs long, spanning the E1/E2 region, including the HVR1, of the HCV genome. There were a total of 87 genotype 1 sequences (genotype 1a n=80; genotype 1b n=7); and 6 genotype 3a sequences. Of the 18 samples that could not be sequenced 6 were genotype 1, 6 non-genotype 1, and 6 could not be genotyped.
Figure 2.1: Gel of E1/E2 product following nested PCR.

Figure represents an example of a gel with the E1/E2 product (575bp length) situated near 600 molecular weight band.

Comparison of the divergence between the direct sequences and the cloned sequences was made. Between 6-12 cloned sequences were compared to the direct sequence from the same time-point from 7 random patients from RFH. All were genotype 1 HCV (six 1a and one 1b). The data are presented as a phylogenetic tree with the direct and clonal consensus sequences analysed by the Clustal method (Figure 2.2). When the median percentage divergence of the clones was compared with the direct consensus sequence for 7 patients there was no significant difference ($p=0.25$) (Table 2.2). Therefore, given that there was no significant difference between the two methods, the use of direct sequencing was validated in this study.
Figure 2.2: Phylogenetic tree of direct and consensus of clones from 7 patients.

Figure represents the phylogenetic tree using the Clustal method of the direct sequences and consensus of the clones from the same time point in 7 patients. The percentage (%) nucleotide difference is the horizontal branch length.

Table 2.2: Percentage divergence between direct sequences and individual clone sequences.

Table outlines the percentage divergence between the direct and consensus of clones from the same time point using the Clustal method with weighted residues. Comparison of the median divergence was made by Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Divergence between direct and individual cloned sequences (range) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>0.0 (0-0.6)</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.0 (0-0.6)</td>
</tr>
<tr>
<td>Patient 3</td>
<td>0.4 (0.2-0.8)</td>
</tr>
<tr>
<td>Patient 4</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>Patient 5</td>
<td>0.0 (0-0.8)</td>
</tr>
<tr>
<td>Patient 6</td>
<td>0.4 (0.2-0.4)</td>
</tr>
<tr>
<td>Patient 7</td>
<td>0.0 (0-0.6)</td>
</tr>
</tbody>
</table>

\[ P = 0.25^* \]

*comparison of median divergence using Mann-Whitney U test.
2.4.3 Phylogenetic analysis:

A total of 93 sequences were aligned and analysed as the 'ingroup'. The sequences were analysed in their relevant subtypes and compared to unrelated sequences downloaded from GenBank and the Los Alamos HCV Sequence Database. A total of 330 E1/E2 unrelated sequences were downloaded and used for the 'outgroup'; 96 genotype 1a, 183 genotype 1b, and 51 genotype 3a sequences.

The genotype 1a maximum likelihood phylogenetic tree, using an HKY and gamma model with NJ bootstrap values (1000 replicates) included 80 patient sequences and 96 unrelated sequences downloaded (Figure 2.3). Within the tree there were 5 monophyletic clusters, which included 73 of the sequences. The largest cluster is composed of 43 patient sequences (46% of all the amplified study cohort sequences). The bootstrap value of the root branch was 83%, strongly supporting the relationship of these sequences in the cluster. The second cluster had 7 cohort sequences with a 100% bootstrap value at its root. The third cluster had 9 cohort sequences and a bootstrap value of 99%. The fourth cluster had 3 cohort sequences with a bootstrap value of 100%. The fifth cluster contained 11 cohort sequences and a bootstrap value of 76%. The bootstrap values of between 76-100% support the robustness of the relationships of these monophyletic groups. There were 7 sequences that did not form part of a cluster with any of the other cases, suggesting that they are unrelated.

The genotype 1b maximum likelihood phylogenetic tree, using an HKY and gamma model with NJ bootstrap values (1000 replicates) included 7 cohort sequences and 183 unrelated sequences (Figure 2.4). All the cohort sequences formed a monophyletic clade. This had a bootstrap value of 100%, which strongly suggests that this is a true relationship.

The genotype 3a maximum likelihood phylogenetic tree, using HKY and gamma model with NJ bootstrap values (1000 replicates) included 6 cohort sequences and 51 unrelated sequences (Figure 2.5). The 3a phylogenetic tree revealed one monophyletic clade consisting of four sequences. The bootstrap value was 74% suggesting this is a
monophyletic clade. There were two sequences lying separately, although one was situated close to the one patient cluster.
Figure 2.3: Genotype 1a maximum likelihood phylogenetic tree.
Maximum likelihood phylogeny of the subtype 1a sequences. Case sequences are in red and unrelated reference sequences in black. Each HCV cluster among HIV positive MSM is identified and a bootstrap value is given at the root of each cluster. Each sequence’s centre of origin is given: •=RFH, □=CWH, * =BSGH.
Figure 2.4: Genotype 1b maximum likelihood phylogenetic tree.
Maximum likelihood phylogeny of the subtype 1b sequences. Case sequences are in red and unrelated reference sequences in black. The HCV cluster among HIV-positive MSM is identified and a bootstrap value is given at it’s root. Each sequence’s centre of origin is given: ●=RFH, ■=CWH, * = BSGH.
Figure 2.5: Genotype 3a maximum likelihood phylogenetic tree.
Maximum likelihood phylogeny of the subtype 3a sequences. Case sequences are in red and unrelated reference sequences in black. The HCV cluster among HIV-positive MSM is identified and a bootstrap value is given at the root of the cluster. Each sequence’s centre of origin is given: ● = RFH, ■ = CWH, * = BSGH.
The origin of all the sequences are indicated in the phylogenetic trees and outlined in Table 2.3. Only the genotype 3a cluster is composed of sequences from a single geographic unit (RFH). All the other clusters contain sequences from at least two of the HIV units that participated in the study. This would suggest that there was significant mixing of patients transmitting HCV between these units.

**Table 2.3: Origin of sequences in each monophyletic cluster.**

Table outlines the origin of the sequences for each clade and each HIV unit participating in the study.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>RFH</th>
<th>CWH</th>
<th>BSGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a n=43</td>
<td>21</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>1a n=7</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1a n=9</td>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>1a n=3</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1a n=11</td>
<td>2</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>1b n=7</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3a n=4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ungrouped* n=9</td>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

*sequences not forming part of any cluster.

The multiple monophyletic clusters in the phylogenetic analysis provides strong evidence of common source transmission of related viruses in these groups.

**2.4.4 Population dynamics:**

To understand the dynamics of the HCV transmission, a molecular clock was applied to all the clusters (Figures 2.6-2.8). The median date of origin for each cluster was estimated based upon the E1/E2 nucleotide evolution rates calculated by Tanaka and colleagues (Tanaka et al., 2002). The median estimated date of origin for each cluster is given in
Table 2.4 Four of the seven clusters have origins in the mid-1990s, implying the onset of transmission of these lineages around this time. The majority (64%) of all lineage divergences have occurred since 1995, indicating increased transmission from this time. HCV entered cluster 2, the largest cluster, in the early 1960s and since this time there has been a constant rise in the number of new lineages with an increased frequency of nodes (divergence events) at more recent time-points. A large subclade has its ancestral node approximately 20 years ago, suggesting a change in the transmission. A log scale of the number of lineages over time suggests that the rate of new lineages is increasing, consistent with an increase in the transmission rate (Figure 2.9). There has been an approximately 1 log\(_{10}\) increase in lineages over the last 30 years.

Attempts were made to model the dynamics of the infection in the largest cluster using both coalescent theory and a life-death plot. Unfortunately, problems related to the proportion of the HIV-population with acute HCV that had been sampled led to implicit assumptions in both models not being met. As a result neither model generated interpretable results. These issues will be explored in detail in the discussion.
Figure 2.6: Genotype 1a clusters with molecular clock applied.
A molecular clock using nucleotide substitution rates based on the E1/E2 region of the HCV genome has been applied to all the genotype 1a clusters to examine the evolution through time of these sequences. Shaded area represents last 10 years with horizontal scale (blue) in years.
**Figure 2.7: Genotype 1b cluster with molecular clock applied.**
A molecular clock using nucleotide substitution rates based on the E1/E2 region of the HCV genome has been applied to the genotype 1b cluster to examine the evolution through time of these sequences. Horizontal scale (blue) in years.

**Figure 2.8: Genotype 3a cluster with molecular clock applied.**
A molecular clock using nucleotide substitution rates based on the E1/E2 region of the HCV genome has been applied to the genotype 3a cluster to examine the evolution through time of these sequences. Shaded area represents last 10 years with horizontal scale (blue) in years.
Table 2.4: Date of origin for each identified HCV cluster.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>HCV subtype</th>
<th>Number of sequences</th>
<th>Year of origin (CI)*</th>
<th>Number (%) of divergence events since 1995</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1a</td>
<td>7</td>
<td>1994 (1988-1998)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>2</td>
<td>1a</td>
<td>43</td>
<td>1962 (1946-1972)</td>
<td>21 (50)</td>
</tr>
<tr>
<td>4</td>
<td>1a</td>
<td>3</td>
<td>1995 (1990-1998)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>5</td>
<td>1a</td>
<td>11</td>
<td>1983 (1974-1989)</td>
<td>8 (80)</td>
</tr>
<tr>
<td>6</td>
<td>1b</td>
<td>7</td>
<td>1999 (1996-2001)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>7</td>
<td>3a</td>
<td>4</td>
<td>1982 (1971-1989)</td>
<td>2 (66)</td>
</tr>
</tbody>
</table>

*Estimated using a Bayesian MCMC approach in BEAST v1.3.
Figure 2.9: Plot of log number of lineages over time in cluster 2.

The number of lineages at 10 yearly intervals has been plotted on a log scale from the estimated entry of the virus into this population in 1962.
2.5 Discussion

Phylogenetic analysis is a powerful tool for the study of viral transmission and HCV is ideally suited to this type of analysis. The recent epidemic of acute HCV in HIV-positive men presented a unique opportunity to explore the molecular epidemiology of HCV infection in this HIV population. The reconstructed phylogenetic trees reveal multiple monophyletic clades, which strongly suggests common source transmission within these clusters. However, before discussing these results in detail, a number of limitations need to be explored.

2.5.1 Limitations:

Sequences were not obtained from all the serum samples derived from cases enrolled into the study. Of the 111 serum samples, 93 (84%) sequences were obtained. All the serum samples (collected between 1999-2005) had been reported to be HCV RNA positive by the respective virology laboratories. Failure to amplify E1/E2 sequences from these samples could relate to problems with either the stability of nucleic acid in the sample and/or the amplification reaction. It should be stated that the missing sequences were of various genotypes and from different HIV units. While strengthening some of the clusters, these sequences would not have strongly influenced the final epidemiological conclusions.

HCV may not have been isolated from the serum as a result of the collection and storage method of the blood from the patients. Storage of whole blood or serum at room temperature has been shown to reduce the yield of cDNA over time (Cuypers et al., 1992). Within two weeks of whole blood stored at room temperature there was a 3-4 log decrease in the yield of HCV cDNA. There was also a small drop in blood refrigerated at 4°C. The samples used in this study were clinical samples and the collection/storage history was not known. Some samples may have been left at room temperature for significant periods. There are two hypothetical reasons this may have reduced the yield of HCV. It is recognised that haem molecules, released following haemolysis, can inhibit the activity of Taq polymerase, which was used in all our PCR
reactions (Cuypers et al., 1992). Second, activation and lysis of granulocytes liberates proteases and nucleases, which digest viral particles, thereby potentially reducing the RNA within the sample. Interestingly, this effect is more pronounced at 4°C than at room temperature. To optimise yield, the recommendation is immediate separation of serum and freezing of the sample.

Problems related to the amplification reaction may have contributed to some of the samples not yielding E1/E2 sequence. The nested E1/E2 primers used were derived from genotype 1 HCV (Farci et al., 2000). Theoretically, this could have impacted the yield of other HCV genotypes. However, a number of genotype 3 sequences were amplified and conversely, a number of genotype 1 samples were not amplified. To avoid false negative amplification, a number of different primers designed specifically for genotype 3 HCV were used with no improvement in the amplification of these samples. In addition, the reaction was manipulated by changing the annealing temperatures and magnesium ion concentrations, again with no improvement in the yield of E1/E2 sequence.

Phylogenetic analysis is based on the genetic differences between DNA sequences. However, nucleotide substitution errors can occur as a result of errors introduced during the amplification reaction when using reverse transcriptase (RT) or Taq polymerase. High error rates have been reported and so these need to be considered. The resulting nucleotide substitutions may be misinterpreted as viral heterogeneity, particularly with cloned sequences. It has been estimated that the error rate is between 0.5-1% of the sequence (Koop et al., 1993). Specifically, the estimated error rate for RT is $10^{-4}$ and for Taq is $0.2-2 \times 10^{-4}$ errors/base pair (Lundberg et al., 1991; Barnes, 1992). These reaction-related error rates are compounded by repeated cycles of PCR; 35 cycles were used in each PCR step in this study. The reported variation between studies may also relate to the amount of template used in the RT or PCR reaction. A large amount of template would lead to saturation after only a few cycles. This reduces the effective number of cycles and the number of
errors would be less. Using consensus sequences analysis in part corrects for this, however, in this study it was not possible to differentiate spontaneous from experimental nucleotide substitutions, although the patterns of phylogeny argue against undue amplification errors.

Contamination is always a consideration when analysing PCR-based sequence data. As a measure of internal consistency, the sequence data was compared to the reported genotype for that clinical sample. Overall, the sequences were consistent with the genotyping. However, there were two samples which had been classified as a genotype 4, using Bayer autoLiPA, that were situated in the genotype 1a tree. Unfortunately, the result of the original genotyping could not be checked, but presumably the sequencing was the more accurate result. The NS5B region could have been used as a control for contamination by comparing the NS5B tree topology with the reconstructed E1/E2 trees. However, there was consistency between the direct and cloned sequences obtained through different PCR reactions performed at different times. This comparative data, showing no significant difference between the direct and clonal sequences, also validated the use of direct sequencing for this phylogenetic study.

2.5.2 Detailed Discussion:
Phylogenetic studies can provide powerful and unique evidence for a defined route of transmission through the analysis of viral homology. Monophyletic clades or clusters are sequences that share a common ancestral root, implying that they are more closely related to one another than sequences situated outside of this group. The likely explanation for sequence homology of a cluster is common source transmission of HCV. In this analysis, the three reconstructed phylogenetic trees revealed seven monophyletic clusters, which included 84 patient sequences. The largest cluster, cluster 2 (n=43), was composed of just under half the total number of amplified patient sequences. The robustness of the sequence relationships in these clusters is supported by high bootstrap values of the ancestral branch. These tree topologies suggest that there are a number of different HCV variants
co-circulating in the HIV-positive population of London and Brighton. Significantly, the clusters cross both genotype and subtype of HCV. This is strong molecular evidence for transmission within these groups.

Interestingly, every cluster except the genotype 3a clade contained sequences from at least two of the three participating HIV units, with no specific geographic clustering. This reveals similar lineages of HCV are co-circulating between patients seen in the different HIV units. This is evidence that transmission is occurring between individuals from these different units or from a source common to the three units. Brighton and London are geographically close cities, and there is significant movement between these urban centres that would contribute to the transmission of HCV. To explore the specific interactions, performing contact tracing and sexual networks studies would have provided a significantly more detailed insight into these interactions between the individuals from the different units, particularly those contributing to the transmission clusters. Unfortunately, this was beyond the scope of this study. A way of expanding the study would be to enrol HIV-positive individuals diagnosed with acute HCV from other centres, as cases have also been reported from other centres in London and Manchester. Similar cohorts have also been described in urban centres in Europe. Given the increased movement between the UK and Europe this could be an important opportunity for understanding the flux of HCV transmission. The evidence that there is transmission occurring between patients at geographically separate units has important implications for the development of any public health intervention, which would need to have a broad geographic focus encompassing related urban centres.

The dates of origin and proportion of sequence divergence of the transmission clusters provides strong evidence of increased HCV transmission since the mid to late 1990s. Four of the seven clusters have their date of origin in the mid-1990s. The majority (64%) of the sequence divergence occurred since 1995. Interestingly this coincides with the introduction of effective treatment for HIV (HAART), which was associated with significant behavioural changes in homosexual men. The
largest cluster, cluster 2, had the earliest entry of HCV in the early 1960s. Examining the number of divergent events over time in this cluster suggests that there has been a constant increase in transmission events over time, particularly since the 1970s. Since that time it appears the infection rate has increased by approximately 1 log over the last 30 years (log number of new lineages over time). Within this clade, a large subclade diverged approximately 30 years ago involving 37 of the 43 sequences. It could be hypothesised that this represents a change in transmission pattern, related to a patient and/or environmental factor. Interestingly, the 1970s was a period where many social changes afforded both HIV and HCV a number of amplification opportunities: multiple sexual partnering in gay men of North American and Europe increased dramatically; concomitantly STIs increased; numbers using illicit drugs soared in the industrialised world; and the global blood product industry expanded dramatically (Garrett, 1995). Unfortunately, the modelling of the population dynamics could not provide further insights.

The population dynamic studies were problematic and complex and required some intrinsic assumptions within the models. The fundamental problem was one of sampling. Both coalescent theory and birth-death plots make assumptions about the proportion of the study population that has been sampled for analysis. Coalescent theory assumes that the sample is a fraction of a percentage of the whole population. In contrast, birth-death modelling assumes a high proportion (100%) sampling. Birth (speciation) and death (extinction) rate of lineages are estimated from node height or frequency. As the death rate increases the nodes move distally to the tips, as the earlier sampled lineages are more likely to become extinct. However, as the birth rate increases the nodes also move closer to the tips, with the emergence of new lineages. Unfortunately, birth-death analysis could not mathematically differentiate between the two. However, clinical data would strongly suggest that there has been increased incidence of acute HCV, which equates to an increase in the birth rate as the reason for the increased frequency of distal nodes. The birth-death plots assume that the majority of
sequences have been sampled and modelling fails when only a proportion is sampled. In contrast to this, the coalescent model implicitly assumes that the genealogy represents a small random sample from a large population. The nodal frequencies of such phylogenies depend on the dynamics of population size through time. Our cohort probably had a significantly higher proportion of sampling than is assumed by coalescent theory. Consequently, provisional modeling suggested a recent downturn in rate of infection which is not consistent with the clinical epidemiology. It is not yet clear what the denominator is for the study population (what proportion of the cohort has been diagnosed), but given that HIV-positive individuals are regularly screened in the clinics it is likely that a significant proportion (>5% and <100%) has been diagnosed.

When exploring the reasons for an epidemic, three areas need to be considered which could contribute to the increased spread of an infection. These include the infectious agent, the patient population and the environment; any or all of these factors could be contributory. The HCV clusters identified cross both genotypes and subtypes of HCV, which suggests that the recent epidemic is not due to a specific viral change. This implies that the recent transmission of HCV is more likely associated with patient and/or environmental factors. While this phylogenetic analysis provides strong evidence for HCV transmission in the study population, it does not provide insight into the specific transmission mechanisms. These factors were explored in a questionnaire based case-control study, detailed in the next chapter (Chapter 3).
Chapter 3: Case-control Study

3.1 Introduction

There is strong evidence from the molecular epidemiological analysis that transmission of HCV is occurring in the HIV-positive population of South-East England. Common source transmission has been demonstrated through the phylogenetic analysis of the E1/E2 region of the HCV genome. Modelling of the dynamics of this transmission suggest that the rate of transmission is increasing, particularly since the mid-1990s. Furthermore, the molecular HCV clusters, which cross subtype and genotype, suggest this epidemic is not due to a hepatitis C viral change, but rather to patient and/or environmental factors. Given the clinical impact of HCV/HIV co-infection, it is important that the reasons for this increase in transmission are identified. While HCV transmission is usually parenteral, preliminary data have suggested that the recent increase in HCV transmission in the HIV population is not due to IVDU (Browne et al., 2004).

As discussed in the introduction (Chapter 1), permucosal transmission of HCV remains controversial. Sexual transmission accounts for a proportion of HCV transmission but it is unclear how large this proportion is in different populations. Some have suggested that the higher prevalence of anti-HCV in the HIV population reveals increased sexual transmission. However, both HIV and HCV share common transmission routes. A recent study from the Swiss cohort of HIV-positive men found increased anti-HCV rates in those with high-risk sexual exposure when IVDU was excluded as a risk factor (Rauch et al., 2005). In the UK general population there has been an increase in high-risk sexual behaviour demonstrated by the National Survey of Sexual and Lifestyles (Natsal) 2000 study which reported an increased prevalence of high-risk behaviour compared with data collected in 1990 (Johnson et al., 2001). This may explain the observed increase in acute sexually transmitted infections (STIs) over the same period reported in UK genitourinary medicine (GUM) clinics between 1990 and 1999; an overall rise of 20% in men and 56% in women (PHLS (England, 2000). High-risk sexual
behaviours have also increased in men who have sex with men (MSM). High-risk practices in MSM include unprotected anal intercourse (UAI), rimming (anal/oral sex play), fisting (insertion of the hand into the anus), use of sex toys and sadomasochistic practices. Contributing to high-risk sexual activities is 'sero-sorting', a form of 'negotiated safety', as a means of risk reduction, where MSM have sex with men of same HIV status. High-risk sexual behaviours also frequently co-exist with recreational drug use. However, the interaction between sexual behaviour and drug use is controversial and complex. This chapter will describe the clinical epidemiological analysis that was performed to elucidate the putative transmission factors in this cohort. Key to this is an assessment of sexual and drug behaviour.

Epidemiological studies aim to achieve precision in estimating sources of infection and modes of spread. A key challenge in identifying putative transmission factors, particularly related to sexual and drug behaviour, is in generating unbiased and precise measures. A questionnaire based case-control study is an effective method of delineating transmission factors, particularly in the setting of rare acute diseases such as HCV. Early case-control data suggested a link between high-risk sexual practices and HCV transmission (Alter et al., 1989b). Case-control studies compare the difference in previous exposure to factors in a group with a condition to a group without the condition; in this study HIV-positive individuals with acute HCV were compared to HIV-positive individuals without HCV. The difference between the proportions in each group exposed to a particular risk factor can then be calculated. Controls should have the same probability of exposure to the factor of interest, if there is no relationship between the factor of interest and outcome, and should therefore come from the same risk population. The most efficient case-control study has equal numbers of cases and controls. However, when the number of cases is small, statistical power can be gained by increasing the number of controls. The largest gain in power is obtained by adding an extra control per case (2:1 ratio). Matching is a method whereby the cases are individually matched by a confounding variable (for example; age or sex) to each control. By forcing the cases and
controls to become more homogenous for confounding factors, matching increases the power of the study to find a true relationship with other transmission factors. These are retrospective studies because the information about risk factor exposure is obtained from the past history of the patient. As with all studies there are inherent measurement biases that need to be understood.

There are a number of reasons why case-control studies of sexual and drug behaviour may be biased. Measurement error can occur as a result of: sampling procedures or respondent variables such as survey response, participation, reporting and recall bias, and questionnaire design. Sampling procedures can produce bias because the sampled population is not representative of the true population. For example, there is evidence that volunteers in studies have a different experience and attitude to sex than the general population (Strassberg & Lowe, 1995; Dunne et al., 1997). Whilst random sampling is the best method, this is not appropriate for all studies, particularly when studying specific questions in a high-risk group. By definition, case-control studies are not random.

A high response rate improves the representativeness of the study and reduces participation bias. As a result, the generalisability of the results improves. A significant proportion of people refuse to participate in studies of sensitive topics such as sexual and drug behaviours (Dunne et al., 1997). Reasons for non-participation vary but may include difficulties with contacting participants, refusal to take part, or refusal by 'proxy' if the participant is ill, or has literacy/language barriers. Many individuals refuse to participate before they are aware of the topic of the survey (Catania et al., 1992). Participation bias occurs when there are systematic differences between those who respond to the questionnaire and those who do not. Factors that have been associated with this bias include sex, age, social class, beliefs and behaviour. With respect to surveys of intimate information such as sexual or drug behaviour, some have suggested participation bias increases while others have not reported this (Biggar & Melbye, 1992). In addition, there maybe certain
questions which individuals systemically are more or less likely to respond to. Copas and colleagues found that older age, ethnicity and comprehension were associated with not answering more detailed questions in the British Natsal survey (Copas et al., 1997).

Recall bias is the result of systemic differences between the cases and controls response to particular questions. The reporting of sexual behaviour has been shown to vary with a number of factors including age, ethnicity, number of partners and the time frame of reporting (McFarlane & St Lawrence, 1999). Reporting frequency (e.g. number or frequency of events) may be less reliable than incidence (e.g. first time of event). There is more consistency reporting number of partners than number of acts (Jeannin et al., 1998). Furthermore, the length of time elapsed and whether the behaviour is common also affects the accuracy of reporting (Saltzman et al., 1987). Inaccuracy and underreporting increases when attempting to recall events further back in time (Kauth et al., 1991). Differences also exist in reporting between the sexes, as men tend to report a higher number of partners in surveys compared to women. This probably represents a social desirability bias. However, in case-control studies many of these factors are controlled and are not an issue with this study.

Obviously, the design, content and method of administration of the questionnaire instrument may contribute to measurement bias. These factors are affected by the literacy of the respondent, the complexity of the questions, and the definition of terms. The method of administration is important, and can either be self- or interviewer-administered. Overall response rates tend to be lower with self-administered questionnaires (Catania et al., 1990). This may relate to the motivation to complete the questionnaire. In contrast, interviewer based surveys generally have higher response rates and more flexibility in the interpretation of the questions. However, when dealing with sensitive behaviours, self-completion questionnaires may result in more valid reports than with interviewers (Catania et al., 1995). Computer assisted interviews have been used to improve responses. Comparative studies of computer
assisted versus self-completion surveys have demonstrated that computer-assisted interviews have the potential to improve the quality of the data and increase the respondents willingness to report sensitive behaviours (Turner et al., 1998).

Methods have been suggested to identify and reduce some of these biases. To determine representativeness the study cohort can be compared with the population from which it is derived. The overall response rate provides evidence of the likely size of the participation bias. Improving response rates will reduce participation bias; incentives have been used such as payment. Better interviewing techniques and the use of computer assistance have improved results. Validity describes the extent to which an instrument measures what it purports to measure. The external validity of a questionnaire can be assessed by comparison to existing data. For example, the rate of STIs reported by respondents in a study can be compared to rates in the UK HIV-population. Objective measures such as rates of STIs from the laboratory or drugs screened from urine can also be used. Internal consistency of the instrument can be assessed by crosschecking questions within the questionnaire, looking for logical agreement. Re-administration of the questionnaire has been used to assess the stability of responses. However, this is both time and resource intensive.

This chapter describes the case-control study that was designed to explore the transmission factors for HCV in this cohort. While assessing the standard risk factors for HCV, the study also focused on potential permucosal factors as transmission routes for HCV.

3.2 Aims

Using a case-control study with a specifically designed questionnaire instrument, the putative factors will be identified for the recent transmission of HCV in the HIV-positive individuals.
3.3 Methods

3.3.1 Study cohort:

There was a clear methodological process for performing the questionnaire-based case-control study. This involved identifying the study population and sample, determining the method of data collection, designing the questionnaire instrument, running a pilot study, performing the actual case-control study, and the statistical analysis.

All the identified cases were homosexual HIV-positive MSM, so the study population was defined as all HIV-positive MSM in South-East UK. The cases were derived from the cases identified in the phylogenetic study (n=111), outlined in section 2.4.1. The case definition was defined in section 2.3.1. HIV-positive controls were identified from each of the HIV clinic's databases. Due to the small number of cases, two controls were matched to each case to increase the power of the study. Controls were matched for: age ± 5 years, length of HIV infection ± 1 year, homosexuality, race, and HAART status (ever/never exposed). HCV negativity was based on previous testing; a specific test was not carried out as part of this study. Individuals matched on all these criteria from the HIV clinic database were recruited. If they refused to participate in the study then the next matched control was approached and so forth.

Demographic parameters of the study cohort were compared with the UK Collaborative HIV Cohort (CHIC) database. The CHIC study is a collaboration of six of the largest HIV clinics in the UK including the RFH, CWH and BSGH. This study was initiated in 2001 with the aim of establishing a large database of HIV infected individuals in order to provide detailed clinical data on large numbers of patients in the UK. The HCV study cohort was compared with the RFH, CWH and BSGH patients in the CHIC database. Permission was obtained from CHIC investigators at these clinics.
3.3.2 Questionnaire instrument:

Based on data suggesting that self-administered questionnaires improve responses when evaluating sensitive behaviours, this method was used for the data collection. A questionnaire was specifically developed using two validated questionnaires to inform its design. First, the National Survey of Sexual and Lifestyles (Natsal) instrument was used (Johnson et al., 1994). This study was a UK-wide self-administered questionnaire study of the sexual behaviours and attitudes of the general population. It was initially performed in 1990 and re-done in 1999/2000. The questionnaire was obtained from the lead investigator, Professor Anne Johnson, Head of the Department of Primary Care and Population Sciences, Royal Free and University College Medical School, London. The Urban Men’s Health survey (UMHS), an American instrument from San Francisco, was a telephone-administered questionnaire examining the lifestyles of urban gay men. It was obtained from one of the lead investigators, Dr Diane Binson, Assistant Adjunct Professor, Center for AIDS Prevention Studies, University of California, San Francisco.

The questionnaire designed for this study assessed six areas of over the 12 months preceding the diagnosis of HCV: place of meeting, sexual practices, drug practices, alcohol use, STIs and attitudes/understanding of HCV (Appendix A). A ‘date of interest’ was specified on the front of the questionnaire, which referred to the date when the case was diagnosed with HCV. Cases were specifically instructed to answer the questions referring to this date throughout the questionnaire. The matched control was given the same ‘date of interest’ as their case. This attempted to control for any temporal changes in behaviour as a confounding factor and also to reduce the possible impact of recall bias that may have arisen if controls were systematically describing their behaviour over a more recent time period than cases. A 12-month period of time from the ‘date of interest’ was chosen because the exact date of transmission was often unclear. It was also important to understand risk behaviour over a significant period before the diagnosis was made.
Participants were asked about their sexual behaviour and practices, including high-risk mucosally traumatic practices. This section included questions about where MSM met other men and the number of partners that they had sex with. Participants were then asked about the number of partners with whom they had participated in each particular practice. There is evidence that this is more accurately recorded than frequency of acts in questionnaires (Jeannin et al., 1998). Each of the practices was defined in the questionnaire using simple terminology. Participants were also asked about public and group sex practices, as these practices are common among the MSM community and are potential sources of high-risk behaviour. Finally, given the relationship between sexual practices and drug use, data was collected on participation in sexual activity under the influence of alcohol and drugs.

Alcohol use was explored using the Alcohol Use Disorders Identification Test (AUDIT) (Saunders et al., 1993). This was developed initially by the World Health Organisation (WHO) for use in primary care. The AUDIT instrument consists of 10 questions with scaled responses to specific questions regarding alcohol consumption. A score of $\geq 8$ is highly sensitive for hazardous drinking. It was designed to provide information on drinking behaviour over the preceding 12 months. It has previously been incorporated into validated questionnaires and is sensitive for the detection of hazardous drinking. Recreational drug use was explored by collecting data on individual drugs and their frequency of use. Data on the route, method of administration and shared routes of administration was also collected.

Standard HCV transmission risk factors were evaluated, including IVDU, blood product transfusions, tattoos and body piercings. Given the relationship between sexual risk behaviour and STIs, a lifetime history of STIs was obtained, including herpes, syphilis, gonorrhoea, non-specific urethritis (NSU), genital warts, hepatitis B (HBV) and hepatitis A (HAV) infection. Finally, general questions were asked about participants attitudes and understanding of HCV using a scaled response to specific statements about HCV.
Before the questionnaire was finalised it was reviewed by a number of colleagues, including clinicians, epidemiologists, psychologists, and nurses. A small pilot study was performed on six randomly selected subjects in the Ian Charleston Day Centre (RFH). The questionnaire took between 20-30 minutes to complete. The questionnaire was successfully completed and was not modified after the pilot analyses.

The questionnaire was administered in the participating HIV clinics. Identified cases and controls were approached at clinic visits where they were introduced to the questionnaire either by myself or a clinic nurse who had been instructed on how to introduce the study. Each case was given a packet containing the questionnaire, an introductory letter and information sheet. There was also a self-addressed envelope for returning the questionnaire. Each questionnaire was coded with a unique identifier for the participant’s anonymity. Following the introduction and the participant reading the patient information sheet, the patient was consented as per protocol. In addition, the 'date of interest' was explained to the patient in the information letter, in the instructions on the questionnaire and by the person introducing the study. The same process was used at each of the clinics. If a patient had consented to take part in the study but their questionnaire was not returned the case or control was approached again at their next clinic appointment. The questionnaire study packets from those individuals who refused were discarded. The study was run between August 2004 and August 2005. The entire study was approved by each of the local ethics committees and was overseen by Central Office for Research Ethics Committees (COREC).

3.3.3 Statistical analysis:
Comparison of variables was related to case/control status. Initially, univariable analysis was performed to compare proportions using Chi-squared or Fisher’s exact test as appropriate. Numerical values were compared using Mann-Whitney U test. Factors found to be associated with the case/control status in univariable analyses (p<0.05) were then
entered into a multivariable logistic regression model to examine independent associations, with adjusted odds ratios generated as the measure of association. However, before variables that were significant in the univariable analysis were entered into the multivariable analysis, they were assessed for correlation with other variables. This was an attempt to simplify the number of variables that were entered into the final model. Because of the high correlation of many of the factors considered, for example individual sexual practices or illicit drugs used, these were categorised by number of different sexual practices participated in and number of different drugs used in the preceding 12 months rather than specific practice. Factors were included in a forwards fashion based on the overall p-value for all categories of a factor. A Pearson chi squared goodness-of-fit test was used to assess the final model. Missing data was accounted for by including an unknown group for each category in the analysis. Values were considered statistically significant if the p<0.05 or if the 95% confidence intervals did not cross unity. Statistical analysis was performed using SAS (version 8).
3.4 Results

3.4.1 Study cohort:
Overall, 190 individuals completed the questionnaire: 60 cases and 130 controls. Of 111 patients enrolled into the phylogenetic study, 60 patients returned questionnaires; a participation rate of 54%. The participating cases came from: RFH n=45, CWH n=10, and BSGH n=5. Of 130 controls entered into the study, these were recruited from: RFH 122 and CWH 8. No controls were recruited from BSGH. Attempts to match two controls to one case were made. At least one control per case was matched successfully for 52 (87%) of the cases. At least two controls were matched per case successfully for 43 (72%) cases. The demographics of the cases and controls are outlined in Table 3.1. Table 3.2 outlines the comparison between the case-control, phylogenetic and CHIC populations from the clinics. There were no significant differences between the cases and controls with respect to the median ‘date of interest’, median age, median duration of infection, percent ever exposed to HAART, median CD4 count (cells/μl), and percentage with HIV RNA <50 copies/ml. With respect to the standard HCV transmission risk factors (Table 3.3), there was a trend towards a higher proportion of IVDU in the preceding 12 months in the cases versus controls (17.2% versus 6.6%, p=0.08). However, the majority of cases (82.8%) did not have this as a risk factor. A greater proportion of cases compared to controls had had a percutaneous piercing in their lifetime (70% versus 52%, p=0.03).
Table 3.1: Patient demographics of case-control study cohort.

<table>
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<th>p-value*</th>
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</tr>
<tr>
<td>(% of total)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Median (range) date of interest</strong></td>
<td>10/03 (01/01-08/05)</td>
<td>09/03 (10/01-8/05)</td>
<td>11/03 (01/01-8/05)</td>
<td></td>
</tr>
<tr>
<td><strong>Median (range) age (years)</strong></td>
<td>36 (22-58)</td>
<td>36 (22-58)</td>
<td>36 (24-58)</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>Median (range) time since first positive HIV test (years)</strong></td>
<td>4.5 (0-18.7)</td>
<td>4.5 (0-18.2)</td>
<td>3.7 (0-18.7)</td>
<td>0.44</td>
</tr>
<tr>
<td><strong>Median (range) time since first visit (years)</strong></td>
<td>0.1 (0-18.9)</td>
<td>0.1 (0-16.1)</td>
<td>0.0 (0-18.9)</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Number (% ) ever exposed to HAART</strong></td>
<td>131 (68.9)</td>
<td>91 (70)</td>
<td>40 (66.7)</td>
<td>0.77</td>
</tr>
<tr>
<td><strong>Median (range) CD4 count at time of interest (cells/mm³)</strong></td>
<td>517 (27-1854)</td>
<td>521 (27-1854)</td>
<td>514 (61-1382)</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>Number (% ) with HIV RNA &lt; 50 copies/ml at 'date of interest'</strong></td>
<td>88 (54)</td>
<td>59 (51.8)</td>
<td>29 (59.2)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

* P-values calculated using Chi-squared test or Mann-Whitney U test as appropriate.
Table 3.2: Comparison of case-control, phylogenetic and CHIC populations.

<table>
<thead>
<tr>
<th></th>
<th>Case-control cohort</th>
<th>Non-participant cases</th>
<th>CHIC cohort (MSM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>190</td>
<td>51</td>
<td>4479</td>
</tr>
<tr>
<td>Median age (years)</td>
<td>36 (22-52)</td>
<td>35 (24-55)</td>
<td>40 (18-77)</td>
</tr>
<tr>
<td>Median duration of HIV(years)</td>
<td>4.4 (0-18.7)</td>
<td>6.2 (0-19.2)</td>
<td>7.3 (0-22.0)</td>
</tr>
<tr>
<td>Number receiving HAART (%)</td>
<td>116 (61)</td>
<td>27 (52.9)</td>
<td>3401 (75.9)</td>
</tr>
<tr>
<td>Median CD4 count (cells/μl)</td>
<td>517 (27-1854)</td>
<td>462 (33-2715)</td>
<td>425 (0-2494)</td>
</tr>
<tr>
<td>Number HIV RNA &lt;50 copies/ml (%)</td>
<td>77 (52.7)</td>
<td>27 (52.9)</td>
<td>2627 (60.4)</td>
</tr>
</tbody>
</table>

Table 3.3: Standard HCV risk factors of cases and controls.

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=60)</th>
<th>Controls (n=130)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDU in prior 12 months (%)</td>
<td>10 (17.2)</td>
<td>6 (6.6)</td>
<td>0.08</td>
</tr>
<tr>
<td>Tattoo (%)</td>
<td>36 (60)</td>
<td>57 (43.9)</td>
<td>0.06</td>
</tr>
<tr>
<td>Transfusion (%)</td>
<td>10 (16.7)</td>
<td>10 (7.7)</td>
<td>0.11</td>
</tr>
<tr>
<td>Percutaneous piercing (%)</td>
<td>42 (70)</td>
<td>68 (52.3)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* P-values calculated using Chi-squared test.

3.4.2 Univariable analysis of questionnaire results:

Question 1 examined where cases and controls met other men. The univariable analysis revealed that cases met other men more frequently in three areas. The median number of times in the 12 months preceding the diagnosis of HCV where cases versus controls met other men more frequently were: private parties (4 versus 2.5 times, p=0.04), sex clubs/bathhouses/sauna (3.5 versus 1 times, p=0.01), and internet sites (50 versus 7 times, p=0.003) (Table 3.4).
Table 3.4: Place where men meet other men.
Represents the median number of visits by cases and controls to meet men in specific locations over the preceding 12 months for cases and controls.

<table>
<thead>
<tr>
<th>Location</th>
<th>All patients</th>
<th>Controls</th>
<th>Cases</th>
<th>p-value (ever visited)</th>
<th>p-value (no. times)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ever visited</td>
<td>Median (range) no. times</td>
<td>Ever visited</td>
<td>Median (range) no. times</td>
<td>Ever visited</td>
</tr>
<tr>
<td>a) Bar, nightclub, danceclub</td>
<td>171 (90.5)</td>
<td>20 (0-250)</td>
<td>115 (88.5)</td>
<td>20 (0-250)</td>
<td>56 (94.9)</td>
</tr>
<tr>
<td>b) Private parties</td>
<td>132 (71.0)</td>
<td>3 (0-100)</td>
<td>82 (65.1)</td>
<td>2.5 (0-50)</td>
<td>50 (83.3)</td>
</tr>
<tr>
<td>c) Sex club, bathhouse, sauna</td>
<td>114 (60.3)</td>
<td>2 (0-150)</td>
<td>69 (53.5)</td>
<td>1 (0-52)</td>
<td>45 (75.0)</td>
</tr>
<tr>
<td>d) Public cruising area</td>
<td>79 (41.8)</td>
<td>0 (0-80)</td>
<td>49 (38.0)</td>
<td>0 (0-80)</td>
<td>30 (50.0)</td>
</tr>
<tr>
<td>e) Internet sites</td>
<td>125 (66.1)</td>
<td>12 (0-1000)</td>
<td>76 (58.9)</td>
<td>7 (0-400)</td>
<td>49 (81.7)</td>
</tr>
</tbody>
</table>

* The proportions who have ever visited each location are compared using Chi-squared tests (or Fisher’s exact test if appropriate); the number of visits in the two groups is compared using Mann-Whitney U test.
The cases had significantly more partners (Table 3.5) than controls. The median number (range) of partners of the cases versus controls in the preceding 12 months was 30 (0-1000) versus 10 (1-800), p<0.001. The majority of these were "one-night stands" for both cases and controls. There was no difference in the proportion of men that did not have sex in the preceding 12 months between the cases and controls (4.2% vs 6.2%, p=0.08).

**Table 3.5: Median number of sexual partners.**

Gives the median and range of number of different sexual partners in the preceding 12 months. Of the total number of partners, the number of one-night stands is given.

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Controls</th>
<th>Cases</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>15 (0-1000)</td>
<td>10 (0-1000)</td>
<td>30 (1-800)</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>One-night stands</strong></td>
<td>10 (0-1000)</td>
<td>8 (0-1000)</td>
<td>20 (0-400)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

**Individual sexual practice:**

Question 4 examined individual sexual behaviour over the 12 months preceding the diagnosis of HCV. The univariable analysis revealed that significantly more cases participated in high-risk sexual behaviour and with more partners than the controls (Tables 3.6). While there was a trend for active and passive unprotected oral intercourse with ejaculation to be more common in cases, this difference was not significant (62.7% versus 47.1%, p=0.07; 50.9% versus 35.3%, p=0.07, respectively). There was no difference between cases and controls with respect to protected (safe) anal intercourse (AI). However, cases were more likely than controls to have had unprotected receptive and insertive AI (89.8% versus 50.4%, p<0.001; 83.1 versus 47.9%, p<0.001, respectively). Furthermore, cases participated in other mucosal traumatic sexual practices, including rimming, fisting, use of sex toys and sadomasochism, more frequently than controls. The most significant practices associated with case/control status were insertive and receptive fisting and use of
sex toys (74.6% versus 26.3%, p<0.001; 57.6% versus 12.6%, p<0.001; 78% versus 42.9%, p<0.001, respectively). The median number of partners for these three practices were also higher in the cases than the controls over the 12 months preceding the diagnosis (2 versus 0 men, p<0.001; 1.5 versus 0 men, p<0.001; 4 versus 0 men, p<0.001, respectively).
Table 3.6: Individual sexual practices.

Gives the number of cases and controls participating in each sexual practice and the median (range) number of different partners for each practice of those who participated in the preceding 12 months.

<table>
<thead>
<tr>
<th>Practice Description</th>
<th>All patients</th>
<th>Controls</th>
<th>Cases</th>
<th>p-value (ever)*</th>
<th>p-value (no. men)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ever Median (range) no. men</td>
<td>Ever Median (range) no. men</td>
<td>Ever Median (range) no. men</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Active oral sex, no ejaculation</td>
<td>169 (94.9)</td>
<td>12 (0-400)</td>
<td>112 (94.1)</td>
<td>10 (0-250)</td>
<td>57 (96.6)</td>
</tr>
<tr>
<td>b) Active oral sex, ejaculation</td>
<td>93 (52.3)</td>
<td>1 (0-300)</td>
<td>56 (47.1)</td>
<td>0 (0-100)</td>
<td>37 (62.7)</td>
</tr>
<tr>
<td>c) Active oral sex, condoms</td>
<td>17 (9.6)</td>
<td>0 (0-20)</td>
<td>12 (10.1)</td>
<td>0 (0-10)</td>
<td>5 (8.5)</td>
</tr>
<tr>
<td>d) Passive oral sex, no ejaculation</td>
<td>161 (91.0)</td>
<td>10 (0-200)</td>
<td>109 (91.6)</td>
<td>10 (0-200)</td>
<td>52 (89.7)</td>
</tr>
<tr>
<td>e) Passive oral sex, ejaculation</td>
<td>72 (40.5)</td>
<td>0 (0-50)</td>
<td>42 (35.3)</td>
<td>0 (0-30)</td>
<td>30 (50.9)</td>
</tr>
<tr>
<td>f) Passive oral sex, condoms</td>
<td>13 (7.3)</td>
<td>0 (0-20)</td>
<td>11 (9.2)</td>
<td>0 (0-10)</td>
<td>2 (3.4)</td>
</tr>
<tr>
<td>g) Receptive anal sex, no ejaculation</td>
<td>113 (63.5)</td>
<td>2 (0-300)</td>
<td>60 (50.4)</td>
<td>1 (0-80)</td>
<td>53 (89.8)</td>
</tr>
<tr>
<td>h) Receptive anal sex, ejaculation</td>
<td>88 (49.4)</td>
<td>0 (0-450)</td>
<td>42 (35.3)</td>
<td>0 (0-100)</td>
<td>46 (78.0)</td>
</tr>
<tr>
<td></td>
<td>All patients</td>
<td>Controls</td>
<td>Cases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------</td>
<td>----------</td>
<td>-------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ever Median (range) no. men</td>
<td>Ever Median (range) no. men</td>
<td>Ever Median (range) no. men</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i) Receptive anal sex, condoms</td>
<td>127 (71.4) 2 (0-150)</td>
<td>83 (69.8) 2 (0-150)</td>
<td>44 (74.6) 4 (0-50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>j) Insertive anal sex, no ejaculation</td>
<td>106 (59.6) 1.5 (0-130)</td>
<td>57 (47.9) 0 (0-50)</td>
<td>49 (83.1) 10 (0-130)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k) Insertive anal sex, ejaculation</td>
<td>73 (41.0) 0 (0-50)</td>
<td>39 (32.8) 0 (0-50)</td>
<td>34 (57.6) 1 (0-40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>l) Insertive anal sex, condoms</td>
<td>122 (68.9) 2 (0-200)</td>
<td>82 (68.9) 2 (0-200)</td>
<td>40 (69.0) 2.5 (0-70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m) Passive rimming</td>
<td>150 (84.3) 5 (0-160)</td>
<td>92 (77.3) 3 (0-160)</td>
<td>58 (98.3) 10 (0-150)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n) Anal rimming</td>
<td>146 (82.0) 3.5 (0-150)</td>
<td>92 (77.3) 2 (0-120)</td>
<td>54 (91.5) 7 (0-150)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>o) Insertive fisting - with gloves</td>
<td>75 (42.4) 0 (0-180)</td>
<td>31 (26.3) 2 (6.5)</td>
<td>44 (74.6) 3 (6.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p) Receptive fisting - with gloves</td>
<td>49 (27.5) 0 (0-180)</td>
<td>15 (12.6) 0 (-)</td>
<td>34 (57.6) 1.5 (0-180)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>q) Sex toys - sharing</td>
<td>97 (54.5) 1 (0-130)</td>
<td>51 (42.9) 0 (0-100)</td>
<td>46 (78.0) 4 (0-130)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r) S&amp;M - piercings</td>
<td>42 (23.7) 0 (0-50)</td>
<td>22 (18.6) 0 (0-50)</td>
<td>20 (33.9) 0 (0-30)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The proportions who have ever had each type of sex are compared using Chi-squared tests (or Fisher's exact test if appropriate); the number of men in the two groups is compared using Mann-Whitney U test.
While there was no significant difference between the participation of cases and controls having sex in public places, the cases were involved in more high-risk practices than controls in public places (Table 3.7).

**Table 3.7: Public sex practices.**

Gives the number of cases and controls participating in sex in public places in the preceding 12 months. Of those participating, the number (percentage) of those involved in the specified high-risk sexual activities is given.

<table>
<thead>
<tr>
<th>Sex in a public place</th>
<th>All patients</th>
<th>Controls</th>
<th>Cases</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Any sex</strong></td>
<td>113 (63.5)</td>
<td>70 (58.8)</td>
<td>43 (72.9)</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>Receptive anal intercourse, no condoms</strong></td>
<td>61 (54.0)</td>
<td>27 (38.6)</td>
<td>34 (79.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Insertive anal intercourse, no condoms</strong></td>
<td>61 (54.0)</td>
<td>29 (41.4)</td>
<td>32 (74.4)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Receptive fisting</strong></td>
<td>19 (16.8)</td>
<td>4 (5.7)</td>
<td>15 (34.9)</td>
<td>0.0002</td>
</tr>
<tr>
<td><strong>Insertive fisting</strong></td>
<td>21 (18.6)</td>
<td>7 (10.0)</td>
<td>14 (32.6)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

**Group sex practices:**

The cases were more likely to have participated in group sex than controls in the 12 months preceding the diagnosis of HCV (88.1% versus 52.5%, p<0.001). Cases participating in group sex were also more likely to be involved in unprotected receptive and insertive anal intercourse (94.2% versus 41.3%, p<0.001; 84.6% versus 47.6%, p<0.001, respectively) and receptive and insertive fisting (55.8% versus 14.3%, p<0.001; 67.3% versus 15.9%, p<0.001, respectively) when compared with controls (Table 3.8).
Table 3.8: Group sex practices.

Gives the number (percentage) of cases and controls participating in group sex in the preceding 12 months. Of those participating, the number (percentage) of those involved in the specified high-risk sexual activities is given.

<table>
<thead>
<tr>
<th>Sex in a group of &gt;2 people</th>
<th>All patients</th>
<th>Controls</th>
<th>Cases</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any sex</td>
<td>115 (64.3)</td>
<td>63 (52.5)</td>
<td>52 (88.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Receptive anal intercourse, no condoms</td>
<td>75 (65.2)</td>
<td>26 (41.3)</td>
<td>49 (94.2)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Insertive anal intercourse, no condoms</td>
<td>74 (64.4)</td>
<td>30 (47.6)</td>
<td>44 (84.6)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Receptive fisting</td>
<td>38 (33.0)</td>
<td>9 (14.3)</td>
<td>29 (55.8)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Insertive fisting</td>
<td>45 (39.1)</td>
<td>10 (15.9)</td>
<td>35 (67.3)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Drugs and alcohol use:

There was no difference between the cases and controls with respect to sex under the influence of alcohol. However, significantly more cases than controls engaged in sex under the influence of recreational drugs (91.7% versus 61.5%, p<0.001) (Table 3.9). The AUDIT survey did not reveal any significant differences in hazardous alcohol use between the cases and controls (median AUDIT score for cases 4 versus controls 6, p=0.12) (Figure 3.1). Overall recreational drug use was higher in the cases than controls in the preceding 12 months (96.7% versus 70.9%, p<0.001). The drugs more frequently used by cases compared with controls were: poppers, ecstasy, psychedelics, methamphetamines (crystal meth), ketamine and Gamma Hydroxy Butyrate (GHB) (Table 3.10). There was no difference in the use of heroin between the cases and controls. While there was no difference in the oral, nasal or intravenous route of taking drugs, there was more anal use of drugs in the cases (42.1% versus 20.5%, p=0.009) (Figure 3.2). Interestingly, cases were more likely to share the implements for the intranasal and
rectal routes of administration more than controls (79.3% versus 48.9%, \(p<0.001\); 21.4% versus 5.4%, \(p=0.007\), respectively) (Figure 3.3).

Previously, more cases had used intravenous drugs than controls (23.3% versus 7.7%, \(p=0.005\)). There was no difference between cases and controls in terms of sharing needles, concerns about recreational drugs use or receiving money for drugs or sex.

**Table 3.9: Sex under the influence of alcohol and drugs.**

Gives the number (percentage) of cases and controls participating in sex under the influence of either alcohol or illicit drugs in the preceding 12 months.

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Controls</th>
<th>Cases</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sexual activity with a man whilst feeling effects of alcohol</td>
<td>124 (65.3)</td>
<td>88 (67.7)</td>
<td>36 (60.0)</td>
<td>0.38</td>
</tr>
<tr>
<td>Sexual activity with man whilst feeling effects of drugs</td>
<td>135 (71.1)</td>
<td>80 (61.5)</td>
<td>55 (91.7)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Cases n=60
Controls n=130

Figure 3.1: Median AUDIT scores for cases and controls.
Represents the median AUDIT questionnaire score for hazardous drinking. A score of ≥8 has a high sensitivity for hazardous drinking.

Table 3.10: Recreational drug use.
Outlines the percentage of cases and controls using specific illicit drugs in the preceding 12 months.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cases %</th>
<th>Controls %</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any</td>
<td>96.7</td>
<td>70.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Methamphetamines</td>
<td>56.9</td>
<td>24.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ketamine</td>
<td>82.8</td>
<td>48.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GHB</td>
<td>51.7</td>
<td>25.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Poppers</td>
<td>89.7</td>
<td>67.4</td>
<td>0.003</td>
</tr>
<tr>
<td>LSD</td>
<td>34.5</td>
<td>13.7</td>
<td>0.005</td>
</tr>
<tr>
<td>Ecstasy</td>
<td>82.5</td>
<td>60</td>
<td>0.006</td>
</tr>
<tr>
<td>Cocaine</td>
<td>79.3</td>
<td>64.2</td>
<td>0.07</td>
</tr>
<tr>
<td>Cannabis</td>
<td>69</td>
<td>70.5</td>
<td>0.98</td>
</tr>
<tr>
<td>Heroin</td>
<td>6.9</td>
<td>8.4</td>
<td>0.98</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>10.3</td>
<td>10.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* P-values calculated using Chi-squared test.
Figure 3.2: Routes of recreational drug use in the preceding 12 months.
Bars represent the percentage of cases and controls using recreational drugs by each route in the preceding 12 months. Other includes intra-anal.

Figure 3.3: Routes of shared drug implements.
Bars represent the percentage of cases and controls sharing specific routes of drug administration in the preceding 12 months. Other routes includes intra-anal.
**Sexually transmitted infections:**
The cases had more lifetime STIs than the controls (92% versus 78%, \( p=0.005 \)). The infections that occurred in a larger proportion of cases than controls were syphilis (42% versus 19%, \( p=0.001 \)), gonorrhoea (73% versus 48%, \( p=0.002 \)), and non-specific urethritis (55% versus 33%, \( p=0.007 \)).

![Figure 3.4: Lifetime sexually transmitted infections (STIs).](image)

Bars represent the percentage of cases and controls ever having had STIs and the differences in specific infections between cases and controls.

**Attitudes and beliefs about HCV:**
Question 21 examined attitudes and beliefs about HCV. The most significant differences arose in a question about the availability of treatment and a vaccine for HCV (Table 3.11). The cases were more likely to know that treatment for HCV was available and that a vaccine was not available. Furthermore, cases were more likely to believe that: "Someone with HCV can feel fine" and that "alcohol should not be drunk if you have liver problems". Both groups believed that HCV could be
transmitted sexually. They also believed that there is a negative interaction between the viruses.

3.4.3 Multivariable analysis of questionnaire results:
The multivariable analysis was complex. To simplify the number of variables, new variables were created which indicated the number of different responses that each individual had given to each question (e.g. the number of different types of locations that they had gone to in the previous 12 months, the number of different types of oral sex that someone had experienced over the past 12 months, the number of different drugs experienced etc.). After generating these variables, the patients grouped into between 3-5 categories for each variable that included roughly equal numbers of patients (or that were biologically reasonable). The percentage of cases for each category for each variable was used to determine how well each of these new categorical variables discriminated between cases and controls. Where adjacent categories discriminated similarly, the groups were combined to reduce the number of categories. Table 3.12 summarises the final categorical variables that were generated.

Once these variables had been created, correlation was assessed. Groups of similar variables were included in multivariable logistic regression models to try to further reduce the number of variables. Initially, variables that related to drug use were considered (ANY11A, NASALGRP, ORALDRGRP, OTHERGRP) as well as the two existing variables that asked whether patients had ever shared drug implements for nasal or other use. Of these, only NASALGRP came out as significant. Sexual behaviours were then considered (ORALGRP, ANALGRP2, FISTGRP, TOTALSM, SEMENGRP, BLOODGRP), and of these, only ANALGRP2 and FISTGRP were significant. Finally, a model was fitted that included both Q3AGRPN and Q3BGRP – of these, Q3BGRP seemed to be the stronger predictor of case/control status.

Therefore, the variables NASALGRP, ANALGRP2, FISTGRP and Q3BGRP were included in the same model – at this stage, both NASALGRP and
Q3BGRP became non-significant, suggesting that exposure to drugs that are used nasally and the number of one-night stands were weaker risk factor than exposure to high-risk sexual behaviours. The remaining variables were added in turn to a model containing ANALGRP2 and FISTGRP. The vast majority of them did not add significantly to these two variables. However, when GROUP2 was added to the model, GROUP2 was highly significantly associated with case/control status, whereas ANALGRP2 and FISTGRP became non-significant. Despite these two variables being strong predictors of case/control status, having group sex seems to discriminate better. Therefore, the final model revealed that participation in group sex with more than 3 high-risk sexual behaviours (UAI and fisting) was strongly associated with acute HCV infection. The estimated odds ratio is 23.5 (95% confidence interval 9.47-58.33) (Table 3.13).
Table 3.11: Attitudes and beliefs about HCV.

Presents the mean and median scaled responses ('Strongly agree'=1 to 'Strongly disagree'=4 and 'Do not know'=5) to specific statements about HCV.

<table>
<thead>
<tr>
<th>Statement</th>
<th>All patients</th>
<th>Controls</th>
<th>Cases</th>
<th>p-value non-parametric</th>
<th>p-value parametric</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Hepatitis C is an important health problem.</td>
<td>1</td>
<td>1.14</td>
<td>1</td>
<td>1.17</td>
<td>1.08</td>
</tr>
<tr>
<td>b) Someone with HCV can feel fine.</td>
<td>2</td>
<td>1.81</td>
<td>2</td>
<td>1.89</td>
<td>1.66</td>
</tr>
<tr>
<td>c) HCV can be transmitted sexually.</td>
<td>1</td>
<td>1.37</td>
<td>1</td>
<td>1.38</td>
<td>1.33</td>
</tr>
<tr>
<td>d) HCV can make HIV worse.</td>
<td>1</td>
<td>1.40</td>
<td>1</td>
<td>1.31</td>
<td>1.59</td>
</tr>
<tr>
<td>e) HIV can make HCV worse.</td>
<td>1</td>
<td>1.46</td>
<td>1</td>
<td>1.42</td>
<td>1.54</td>
</tr>
<tr>
<td>f) There is treatment available for HCV.</td>
<td>2</td>
<td>1.85</td>
<td>2</td>
<td>1.99</td>
<td>1.60</td>
</tr>
<tr>
<td>g) There is a vaccine available for HCV.</td>
<td>3</td>
<td>3.24</td>
<td>3</td>
<td>3.03</td>
<td>3.60</td>
</tr>
<tr>
<td>h) Most patients with HCV have chronic (persistent) infection.</td>
<td>2</td>
<td>2.26</td>
<td>2</td>
<td>2.24</td>
<td>2.28</td>
</tr>
<tr>
<td>i) HCV can cause the liver to stop working.</td>
<td>2</td>
<td>1.61</td>
<td>2</td>
<td>1.62</td>
<td>1.58</td>
</tr>
<tr>
<td>j) Alcohol should not be drunk if you have liver problems.</td>
<td>1</td>
<td>1.46</td>
<td>1</td>
<td>1.40</td>
<td>1.61</td>
</tr>
</tbody>
</table>

* P-values calculated using Mann-Whitney U or unpaired t-test test as appropriate.
### Table 3.12: Creation of new variables for multivariable analysis.

<table>
<thead>
<tr>
<th>New variable name</th>
<th>New categories</th>
<th>New Variable name</th>
<th>Controls; n (%)</th>
<th>Cases; n (%)</th>
<th>Percentage of group who are a case</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of locations visited in previous 12 months</td>
<td>0-2</td>
<td>LOCATIONS</td>
<td>44 (33.9)</td>
<td>7 (11.7)</td>
<td>13.7</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>3-4</td>
<td></td>
<td>69 (53.1)</td>
<td>32 (53.3)</td>
<td>31.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td>17 (13.1)</td>
<td>21 (35.0)</td>
<td>55.3</td>
<td></td>
</tr>
<tr>
<td>Number of sexual partners in past 12 months</td>
<td>&lt;2</td>
<td>Q3AGRP</td>
<td>22 (18.3)</td>
<td>1 (1.7)</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-6</td>
<td></td>
<td>26 (21.7)</td>
<td>8 (13.6)</td>
<td>23.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7-14</td>
<td></td>
<td>21 (17.5)</td>
<td>9 (15.3)</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15-30</td>
<td></td>
<td>27 (22.5)</td>
<td>17 (28.8)</td>
<td>38.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥31</td>
<td></td>
<td>24 (20.0)</td>
<td>24 (40.7)</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td>Number of one-night stands in past 12 months</td>
<td>&lt;2</td>
<td>Q3BGRP</td>
<td>31 (26.1)</td>
<td>3 (5.2)</td>
<td>8.8</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>2-6</td>
<td></td>
<td>21 (17.7)</td>
<td>6 (10.3)</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7-14</td>
<td></td>
<td>27 (22.7)</td>
<td>13 (22.4)</td>
<td>32.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15-30</td>
<td></td>
<td>17 (14.3)</td>
<td>14 (24.1)</td>
<td>45.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥31</td>
<td></td>
<td>23 (19.3)</td>
<td>22 (37.9)</td>
<td>48.9</td>
<td></td>
</tr>
<tr>
<td>Number of different oral sexual risks</td>
<td>0-3</td>
<td>ORALGRP</td>
<td>36 (27.7)</td>
<td>8 (13.3)</td>
<td>18.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>33 (25.4)</td>
<td>12 (20.0)</td>
<td>26.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td>34 (26.2)</td>
<td>17 (33.3)</td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6-8</td>
<td></td>
<td>27 (20.8)</td>
<td>23 (38.3)</td>
<td>46.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Number of different anal sexual risks</td>
<td>0-2</td>
<td>ANALGRP2</td>
<td>68 (52.3)</td>
<td>7 (11.7)</td>
<td>9.3</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>3-6</td>
<td></td>
<td>62 (47.7)</td>
<td>53 (88.3)</td>
<td>46.1</td>
<td></td>
</tr>
<tr>
<td>Number of different fisting/sex toy risks</td>
<td>0</td>
<td>FISTGRP</td>
<td>61 (51.3)</td>
<td>8 (13.6)</td>
<td>11.6</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>31 (26.1)</td>
<td>8 (13.6)</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td></td>
<td>27 (22.7)</td>
<td>43 (72.9)</td>
<td>61.4</td>
<td></td>
</tr>
<tr>
<td>New variable name</td>
<td>New categories</td>
<td>New Variable name</td>
<td>Controls; n (%)</td>
<td>Cases; n (%)</td>
<td>Percentage of group who are a case</td>
<td>p-value</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------</td>
<td>-------------------</td>
<td>-----------------</td>
<td>--------------</td>
<td>-----------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Any S&amp;M</td>
<td>No</td>
<td>TOTALSM</td>
<td>96 (81.4)</td>
<td>39 (66.1)</td>
<td>28.9</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td></td>
<td>22 (18.6)</td>
<td>20 (33.9)</td>
<td>47.6</td>
<td></td>
</tr>
<tr>
<td>Number of different semen risks</td>
<td>0-5</td>
<td>SEMENGRP</td>
<td>44 (33.9)</td>
<td>4 (6.7)</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6-7</td>
<td></td>
<td>26 (20.0)</td>
<td>10 (16.7)</td>
<td>27.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8-9</td>
<td></td>
<td>25 (19.2)</td>
<td>13 (21.7)</td>
<td>34.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-11</td>
<td></td>
<td>24 (18.5)</td>
<td>18 (30.0)</td>
<td>42.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12-14</td>
<td></td>
<td>11 (8.5)</td>
<td>15 (25.0)</td>
<td>57.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Number of different blood risks</td>
<td>0</td>
<td>BLOODGRP</td>
<td>57 (47.9)</td>
<td>8 (13.6)</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td></td>
<td>45 (37.8)</td>
<td>20 (33.9)</td>
<td>30.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-4</td>
<td></td>
<td>17 (14.3)</td>
<td>31 (52.5)</td>
<td>64.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>Number of types of sex in a public place</td>
<td>None or 1 type only</td>
<td>PUBLIC2</td>
<td>107 (82.3)</td>
<td>27 (45.0)</td>
<td>20.2</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>2 types</td>
<td></td>
<td>17 (13.1)</td>
<td>19 (31.7)</td>
<td>52.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3/4 types</td>
<td></td>
<td>6 (4.6)</td>
<td>14 (23.3)</td>
<td>70.0</td>
<td></td>
</tr>
<tr>
<td>Number of types of sex in a group</td>
<td>None or 1 type only</td>
<td>GROUP2</td>
<td>94 (78.3)</td>
<td>11 (18.6)</td>
<td>10.5</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>2 types</td>
<td></td>
<td>14 (11.7)</td>
<td>15 (25.4)</td>
<td>51.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3/4 types</td>
<td></td>
<td>12 (10.0)</td>
<td>33 (55.9)</td>
<td>73.3</td>
<td></td>
</tr>
<tr>
<td>Any use of marijuana</td>
<td>No</td>
<td>ANY11A</td>
<td>28 (29.5)</td>
<td>18 (31.0)</td>
<td>39.1</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td></td>
<td>67 (70.5)</td>
<td>40 (69.0)</td>
<td>37.4</td>
<td>0.98</td>
</tr>
<tr>
<td>Number of different intranasal drugs*</td>
<td>0-1</td>
<td>NASALGRP</td>
<td>33 (34.7)</td>
<td>5 (8.6)</td>
<td>13.2</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td></td>
<td>37 (39.0)</td>
<td>16 (27.6)</td>
<td>30.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-6</td>
<td></td>
<td>25 (26.3)</td>
<td>37 (63.8)</td>
<td>59.7</td>
<td></td>
</tr>
<tr>
<td>Number of different oral drugs</td>
<td>0</td>
<td>ORALDRGRP</td>
<td>37 (39.0)</td>
<td>8 (13.8)</td>
<td>17.8</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td></td>
<td>58 (61.1)</td>
<td>50 (86.2)</td>
<td>46.3</td>
<td></td>
</tr>
<tr>
<td>Number of different 'other' drugs</td>
<td>0</td>
<td>OTHERGRP</td>
<td>65 (68.4)</td>
<td>29 (50.0)</td>
<td>30.9</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>1-3</td>
<td></td>
<td>30 (31.6)</td>
<td>29 (50.0)</td>
<td>49.2</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.13: Final multivariable model.

<table>
<thead>
<tr>
<th>Number of types of sex in a group</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>None or 1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>9.16</td>
<td>3.51-23.90</td>
<td>0.15</td>
</tr>
<tr>
<td>3/4</td>
<td>23.50</td>
<td>9.47-58.33</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
3.5 Discussion

Using a specifically designed questionnaire the case-control study identified a number of putative factors associated with the recent epidemic of acute HCV in HIV-positive individuals. In contrast to the usual parenteral routes of HCV transmission, permucosal factors are significantly associated with this epidemic. These factors will be discussed following an outline of the limitations of the study. The discussion will attempt to disentangle the complex interaction between these and other factors.

3.5.1 Limitations:

There are several limitations in the methodology, which need to be understood when examining these findings. Retrospective data collection is an obvious limitation, which could potentially lead to recall bias. In order to limit the potential for this bias, cases and controls were given a ‘date of interest’, which was the date of diagnosis of HCV for cases or the matched date for their control. By definition, there was no difference between the median ‘date of interest’ for cases and controls. There was also no difference in the recall period between the ‘date of interest’ and date that the questionnaire was completed for cases and controls (456 versus 500 days, p=ns). However, this was a long recall period, which in itself may have led to inaccuracies in the estimated numbers and events. It is also possible that there could have been systematic differences in the recall of events between the cases and controls. For example, the cases’ knowledge of the HCV diagnosis may have led to better recall of potential transmission events around the time of HCV acquisition. In contrast, the controls may have had more difficulty recalling specific events over the same period. If controls underestimated risk behaviour over this period, this would have exaggerated differences between the two groups. Given the incubation period of HCV and inaccuracy in the estimated infection date, a 12-month period from the date of diagnosis was used to study transmission factors. Twelve months is, however, a long period of time to estimate drug and sexual behaviours and may have contributed to inaccuracies in the recall of events. Finally,
significant drug use during this period may have compromised the accurate recall of events.

As with any case-control study, participation bias is important. The overall response rate was low at 54% (60/111 cases) and there are a number of reasons for this. The response rate was different between the three institutions; the majority of cases and controls were recruited from the RFH. At the RFH the response rate was 88% (44/50) for the cases. In contrast, the response rate at the BSGH was 54% (6/11) and 20% (10/50) at CWH. There were no controls recruited from BSGH and only 8 from CWH. The major reason for this was logistic difficulties with recruitment and follow-up of the cases at these centres. No specific individual was identified at these centres to complete questionnaires. It was not clear that there were any reasons that patients and controls from these centres were less likely to respond to the questionnaire. A comparison between the phylogenetic study and case-control populations did not reveal any significant differences. In addition to the logistic problems, reasons for the cases not returning questionnaires included refusal to participate, and lack of further clinic attendance or follow-up.

The study participants were recruited from large urban HIV units in London. Controls were appropriately recruited from the clinics databases, representing the same population from which the cases were derived. HIV-positive men not attending these hospital-based clinics would include men with early HIV managed by a local clinic or GP, disenfranchised groups such as IVDUs and immigrants, and undiagnosed HIV-positive men. This urban study population may not be representative of the wider HIV-positive MSM population. The comparison with the CHIC cohort of MSM suggests that the study cohort is younger and more recently infected with HIV (higher CD4 counts and less likely to have been on treatment. It is also not clear whether the study population is similar to other cohorts of acute HCV in HIV-positive men in large European urban centres such as Paris, Berlin or Amsterdam. However, all these groups described similar high-risk sexual transmission risk factors. Therefore, the generalisability of the results would be affected by the fact that the
majority of patients come from one centre and that they may not be representative of the wider HIV-positive community.

Finally, the sample size (n=60) was relatively small which limited the analysis. An attempt to control for confounding factors was made by matching two controls to each case. Unfortunately, while the majority (87%) of cases could be matched to at least one control, this was incomplete. However, there was no significant difference in any of these parameters between the cases and controls when the groups were compared. There were a large number of factors that were identified as significant on the univariable analysis. It is usually accepted that for each factor in a multivariable model at least 10 cases are required. In addition, many of the significant factors were highly correlated adding further complexity to the multivariable analysis. In some respects the studied behaviours are so inter-related that it is impossible to disentangle them. Therefore, rather than attempt to identify specific behaviours associated with acute HCV infection, the study revealed general patterns of behaviour associated with the transmission of HCV.

3.5.2 Detailed discussion:
It is noteworthy that all the cases identified have been HIV-positive MSM. This is despite the fact that all the cases came from HIV clinics that treat substantial numbers of heterosexual HIV-positive patients. This suggests that factors specific to MSM are important in the recent transmission of HCV.

A key finding in this study is that parenteral risk factors did not explain the vast majority of the HCV transmission; only 6.6% controls versus 17.2% cases (p=0.08) had IVDU as a risk in the preceding 12 months. Therefore, 82.8% of the cases did not have this as a risk factor, which is the reverse of the more usual current pattern of HCV transmission. In this cohort, permucosal factors were significantly more important. For permucosal transmission of HCV to occur there needs to be disruption of the mucosal barrier with exposure to infected body fluids. There are a number of possible mechanisms identified in this study that could disrupt
the mucosa. It could arise through mucosally traumatic sexual practices or instrumentation used when sharing drugs. In addition, STIs leading to mucosal/skin lesions could potentiate transmission. Second, these disrupted mucosa need to be exposed to infected body fluids, which can occur in a number of ways. Unprotected intercourse would lead to exposure to semen or blood. Recently, a group has demonstrated that HCV/HIV co-infection leads to detectable and higher concentrations of HCV in the semen compared with HIV negative men (Briat et al., 2005). Oral sexual practices would lead to exposure to saliva from which HCV has also been isolated. It is also plausible that blood or secretions related to shared drug implements and sex toys is another potential source.

It has been assumed until recently that individuals did not purposely seek to have unprotected sex. However, HIV seroconcordant sexual partnering has changed sexual behaviour. Unprotected intercourse between HIV seroconcordant men can be considered a 'harm reduction' strategy. In the MSM community it is now recognised that some individuals intentionally seek unprotected intercourse; termed 'barebacking'. However, unprotected sex puts these men at increased risk of STIs and HIV superinfection. Macdonald and colleagues at the Health Protection Agency, UK, found that the rates of HIV and other STIs, including syphilis, gonorrhoea, genital herpes and chlamydia, have increased substantially in the MSM community (Macdonald et al., 2004). The rates in London were twice those of other parts of the UK. Interestingly, there has been a disproportionate rise in the diagnosis of syphilis in MSM compared to heterosexual men in the UK, driven by large outbreaks in London and Manchester (Simms et al., 2005). HIV-positive men are a group prone to high-risk sexual activities. Repeated surveys of the homosexual community in London suggest that there is increasing risk behaviour in this group (Nardone et al., 1997; Dodds et al., 2000). High-risk sexual behaviours include: multiple partners, unprotected anal intercourse (UAI), and other mucosally traumatic sexual practices such as fisting, rimming, use of sex toys and sadomasochic practices. In a cross-sectional cohort study of gay men (n=8052) from venues in London between 1996 and 2000, Dodds and colleagues reported increasing levels
of UAI (Dodds et al., 2004). HIV-positivity is important and one study reporting sexual practice of HIV-positive MSM over the preceding 3 months found that if their partner was HIV-positive unprotected anal intercourse occurred in 91% of partners as opposed to 34% of HIV-negative or unknown status (McConell J, 2003). Halkitis and colleagues studied HIV-positive seroconcordant MSM in an attempt to determine factors predicting high-risk behaviours (Halkitis et al., 2005a). The three factors associated with high-risk behaviours were: lack of belief that STIs and HIV superinfection were a problem; less evaluation of sexual behaviours with more hedonistic expectations and higher level of sexual compulsivity; and recreational drug use. The same research group analysed the practice of intentional unprotected intercourse ('barebacking') and found that this was associated with higher levels of sexual compulsivity, lower responsibility for safer sex and increased drug use (injection and non-injection) (Halkitis et al., 2005b). Those identifying as 'barebackers' have more risk in terms of substance abuse, HIV transmission behaviours and combining drug use with sexual risk taking.

Sexual partners sought via the internet was an important factor in our study that was highly associated with the cases. This supports the increasing evidence that the internet is an emerging risk environment for STIs. McFarlane and colleagues found that individuals (n=135) seeking sexual partners on the internet were more likely to have had an STI, have a greater number of partners, be MSM and have HIV-positive sexual partners (McFarlane et al., 2000). They concluded that the internet has a role in the solicitation of high-risk sex and is a potential risk factor for STI/HIV. In a study of an outbreak of syphilis in the New York MSM community, the internet was determined on multivariable analysis to be an independent risk factor for transmission of syphilis (Wong et al., 2005). The internet provides a convenient and confidential way of meeting sexual partners. HIV-positive men may be able to disclose their status in a relatively safe environment thereby establishing seroconcordance more easily. While it is controversial if the internet leads to an overall increase in high-risk behaviour, there is good evidence that
in HIV-positive MSM it does increase risk behaviour (Bolding et al., 2005; Hospers et al., 2005). The cases in the study met men via the internet seven times more often than controls in the 12 months preceding the diagnosis of HCV. The strong correlation between being a case and meeting men on the internet supports the hypothesis that that the internet is an environment that foments high-risk sexual behaviours. Seroconcordance was not assessed in this study. It is likely that the high-risk behaviours were in HIV-positive seroconcordant couples. Seroconcordant coupling was controlled to some extent by using HIV-positive controls. Therefore, the internet probably represents an epiphenomenon, associated with HCV transmission by a third direct transmission factor, such as high-risk sex.

It is also apparent that the cases sought sexual partners offline. They met men significantly more often at sex clubs, bathhouses, saunas and private parties. This may be indicative of sexual compulsivity and is consistent with the higher number of partners. The questionnaire instrument was not designed to identify where specific sexual partners of the study cohort came from. Cases in our study had significantly more partners than the controls, the majority of partners in both groups however, were once only “one-night stand” partnerships.

Observational studies from other European groups have suggested that sexual factors are important in the recent increase in HCV transmission in HIV-positive men (Gambotti et al., 2005; Gotz et al., 2005). Our case-control study revealed distinct differences between the cases and controls with respect to their individual sexual practices. A larger proportion of cases participated in high-risk sexual practices and with more partners than controls. The unsafe sexual practices of unprotected anal intercourse (UAI), rimming, fisting and sharing of sex toys were all associated significantly with acute HCV infection. Theoretically all these practices could be associated with permucosal transmission of HCV as a result of mucosal trauma and exposure to infected secretions. The sexual factors identified in our study are highly inter-related and many would probably occur during the same session. The questionnaire did not
examine the pattern of sexual behaviour during these sexual encounters and there maybe a specific pattern to these behaviours. As a result the analysis has not been able to separate one from the other. To delineate this, specific qualitative research would be needed to evaluate in detail the behaviours of those involved in these practices. It should be stated that the controls also had a significant level of risk with more than one third participating in UAI. It would be informative to prospectively follow the controls who reported significant risk identified by the questionnaire for future incidence of HCV infection.

Group sex is a situation where these sexual factors could potentially amplify HCV transmission. Group sex practices were more common in the cases than controls. Of those participating in group sex, higher-risk behaviours were more common in the cases than controls with regard to UAI and fisting. Group sex is an environment that potentially leads to explosive transmission of STIs. Individuals are exposed to high-risk practices with multiple partners, increasing the risk of transmission. It is conceivable that multiple transmissions could occur in these situations. While the study only asked about high-risk practices of UAI and fisting, it is likely that the other practices were also involved. There is a paucity of data on group sex behaviour. Clatt and colleagues performed qualitative research among HIV-positive MSM participating in group sex parties in New York. Similar to our findings, they found individuals used parties as a means of serosorting with very high rates of unprotected intercourse and previous drug use (Clatts et al., 2005). There was little evidence of safe sex practice such as condom or glove use in our study. This practice is contributing to the increase in STIs and HCV transmission.

It is unsurprising that there were more STIs in the cases compared with the controls. There are two interpretations of this phenomenon. Most likely, this is a reflection of the high-risk sexual behaviour of the cases. As in our cohort, higher risk sexual behaviour is associated with the increased risk of STIs. Key to the transmission of STIs is the number of sexual partners. While both cases and controls had a high number of median sexual partners, the cases had significantly more (30 versus 10,
Analysis of the NATSAL study revealed that numbers and types of sexual partners were the dominant individual and population risk for the acquisition of STIs (Fenton et al., 2005). The study reported a disproportionate burden of STIs in highly sexually active individuals, those living in Greater London and those having homosexual partnerships, with almost 50% of the STIs occurring in the <10% of the population that reported >10 sexual partners. It is possible that STIs may also be contributing directly to HCV transmission through percutaneous/mucosal lesions and/or the presence of infected inflammatory cells. The relationship between STIs and HIV transmission has been recognised for some time (Stamm et al., 1988). Certainly, there were higher rates of STIs in the cases compared with controls. However, it should be noted that our questionnaire obtained details of lifetime STIs and not STIs in the period before the acquisition on HCV.

Interestingly, there was no difference between cases and controls in participation in sex under the influence of alcohol. However, cases were more likely to have participated in sex under the influence of drugs than controls. ‘Club drugs’ were more commonly used by the cases, including ketamine, metamphetamines, GHB, poppers, LSD and ecstasy. Many of these drugs are taken intranasally and this route of drug taking was shared significantly more often by the cases than controls. In addition, the cases used intra-anal drugs more frequently than controls. Unfortunately, it was beyond the scope of the study to explore the interaction of drugs and sex at the event-level. This would have required an in-depth qualitative analysis of the temporal relationship between drug and sexual activity of this group.

There are two possible mechanisms whereby drug use may increase HCV transmission. First, sharing of the instrument used to take the drugs could potentially lead to transmission. Interestingly, both intranasal and rectal drug use were more frequently used in the cases than controls. It is theoretically possible that infected implements led to transmission of the infection. However, intranasal transmission of HCV is rare (CDC, 1998). It may be that the rectal route of transmission occurs in the
context of mucosal trauma associated with sex. However, it is more likely that the effect of the drugs led to higher-risk behaviours due to disinhibition and sexual arousal. 'Club' drugs have been associated in other studies with high-risk sexual behaviour in the MSM community. Specifically, the increase in use of metamphetamines (crystal meth) has been reported to be contributing to a rise in the incidence of HIV in MSM (Boddiger, 2005). One study of crystal meth found that in HIV-positive MSM it increased sex through increased confidence, heightened sensation and loss of inhibitions (Semple et al., 2002). The dose of drugs and proportion of sexual interactions under the influence of the drug was not assessed by our questionnaire instrument.

Relating these findings to the phylogenetic trees there was no significant difference in risk factors identified in the questionnaires in the different clades. Among individuals lying separately in the phylogenetic trees (n=9), questionnaire data were available for 5. The numbers were too small for statistical analysis but descriptively these cases shared many of the same risk factors as those who formed part of the transmission clusters in the phylogenetic study. It is likely that some of these individual sequences are part of the same transmission route and that the other individuals forming the rest of the cluster were not identified for this study and remain either undiagnosed or under the care of a different HIV unit.

By definition, the matching in case-control studies controls for the matched confounders. As a result, the importance and relevance of HIV was not explored in this study. All these cases have been identified in the HIV-positive population and it is possible that HIV itself may favour the transmission of HCV. This is an important area of future study.

The interaction between sexual and drug behaviours is complex and controversial. Early studies suggested an association between alcohol and drug use and HIV seroconversion, UAI, and number of partners. Alcohol and drug use are strongly related to high-risk sexual behaviours such as inconsistent condom use and multiple partners (Siegel et al., 1989;
Ostrow et al., 1993). This is also the case with MSM (Stall et al., 1986; Woody et al., 1999). Studies have consistently shown that non-intravenous drug and alcohol use are common in the MSM population (Stall & Wiley, 1988; Stall & Purcell, 2000). Data from the Urban Men's Health Study in the USA, revealed increased levels of non-intravenous drug use in the homosexual community when compared with the heterosexual population (Stall et al., 2001). However, many of the studies had methodological problems because they used global measures of drug use and did not explore the direct temporal interactions between use and sexual behaviour. Our case-control study suffered similar problems. When this interaction is explored studies have both supported and dismissed a relationship. Overall, there is a lack of data at the event-level, describing drug and sexual practices. If there truly is a relationship, then it is unclear whether drugs are used to achieve unprotected sex, or whether unprotected sex is the result of clouded judgement secondary to drug use. Drug use and high-risk sex may also be linked through a third variable such as sensation-seeking, impulsivity or situational factors such as going to a bar/club/party where both occur. To unravel the complex interactions between drug use and sex, qualitative data is needed to examine the pattern and reasons for their use.

The exploration of HCV knowledge revealed very similar results between the two groups. The significant differences in median and mean results were that cases were more likely to believe that “you could feel fine with HCV”, “there is treatment available for HCV”, “there is no vaccine for HCV”, and “alcohol should not be drunk if you have liver problems”. These beliefs could all be the result of change in understanding following the diagnosis of HCV, a problem with retrospective data collection. Consequently, it is difficult to interpret this data. The most interesting finding, however, was that 62% of cases and 62% of controls “strongly agreed” that HCV could be transmitted sexually. This is important because it indicates that individuals are aware of this as a route of transmission. There would also appear to be a reasonable understanding that HCV is an important health problem, impacts HIV, develops persistence and causes liver disease. Unfortunately, because this was
evaluated retrospectively, it cannot be ascertained whether the cases had a poorer understanding of this before the acquisition of HCV, which may have contributed to transmission. It is of concern that cases may have had a good understanding of HCV but continued with risky activity. This level of insight will have an impact on how public health interventions should be focused.

This study has identified a number of potential factors associated with the recent transmission of HCV. These transmission factors form a complex interaction between sexual and drug risk behaviours. While significant in univariable analysis, many of these factors are highly correlated and have been difficult to disentangle in the multivariable analysis. The final multivariable model identified group sex as the only independent factor associated with acute HCV infection in the study cohort. While this is the strongest association, in itself it is not a specific mechanism for transmission of HCV. It is more likely to represent an epiphenomenon whereby a third factor within the milieu of sexual and drug factors is responsible for the HCV transmission. This study suggests that the mechanism may be either mucosally traumatic sexual practices or the sharing of drug implements, both of which biologically could transmit the infection. However, to define these specific factors a detailed qualitative study at the event level would be required. Figure 3.5 graphically represents the study cohort. A number of factors in the univariable analysis appear to be contributing to the recent transmission. First, mucosal traumatic sexual practices including UAI, rimming, fisting, use of sex toys and sadomasochistic practices could all theoretically transmit HCV. Internet use may contribute to this by facilitating the access of HIV-positive individuals to these high-risk practices. ‘Club drugs’ may increase the participation in high-risk sexual activities. The sharing of drug implements, particularly intranasally and intra-anally, may also lead directly to HCV infection. Finally, the role of STIs is unclear (represented by a two way arrow). They most likely represent the result of the high-risk sexual practices. However, STIs may also be potentiating HCV transmission through mucosal/skin lesions. Using the factors identified in this study, high-risk individuals such as those participating in group sex
could be identified. These factors need to be the focus of any public health intervention to prevent the spread of HCV in the HIV-positive population.

**Figure 3.5: Model of variable interaction and HCV transmission in HIV-positive MSM.**

Describes the complex contribution of factors to HCV transmission within the sexual and drug milieu.
Chapter 4: Natural History and Host-Virus Interaction in Acute HCV in HIV Co-infection.

4.1 Introduction

The natural history of HCV in HIV co-infected individuals can be better understood by studying the interaction between the virus(es) and immune response in co-infected individuals. Studies among individuals with chronic HCV/HIV co-infection have revealed significant influences of HCV on HIV infection and vice versa. As discussed in the introduction the liver injury from HCV is frequently accelerated in co-infection. However, the acute phase of HCV infection in those with HIV has not been studied because it has not often been recognised clinically. This cohort has provided the opportunity to study aspects of early phase HCV infection in HIV-positive individuals, which may lead to a better understanding of factors that are important in the long-term natural history of HCV/HIV co-infection.

The definition of acute HCV infection is based on the observation that most patients who clear HCV spontaneously will do so in the first six months of infection; usually within 35 days of the onset of symptoms (Gerlach et al., 2003; Hofer et al., 2003). In the largest reported series of acute HCV mono-infection, Gerlach and colleagues describe the course in 60 consecutive patients. There was a high spontaneous seroconversion rate of 44% in symptomatic patients within the first 3-4 months (Gerlach 2003). After this time there was no spontaneous eradication of HCV. Thus successful immune responses to HCV can be mounted in a proportion of individuals.

4.1.1 Cell-mediated immune responses in acute HCV mono-infection:

A successful response to HCV requires a strong, broad and sustained HCV-specific CD4 T-cell response (Diepolder et al., 1995; Gerlach et al., 1999). CD4 T-cells are vital for priming and sustaining CD8 cytotoxic T-cells, which leads to control of the infection. Gerlach and colleagues
demonstrated that patients who failed to clear HCV either did not mount CD4 responses or, after initial virological control, did not sustain these responses with a consequent relapse of HCV (Gerlach et al., 1999). This finding was reinforced by Thimme and colleagues who analysed CD4 T-cell responses in healthcare workers exposed to HCV (Thimme et al., 2001). Whilst two patients initially developed strong CD4 T-cell responses, following loss of these responses they developed persistent infection. These studies were performed on peripheral blood samples; intrahepatic CD4 T-cell responses in the acute phase in humans have not been studied because liver biopsy is not usually performed early in the course of HCV. However, in chimpanzee studies, intra-hepatic CD4 T-cell responses were identified only in those animals that controlled HCV (Thimme et al., 2002). Post-liver transplantation patients are a suitable and accessible study group because they undergo a number of routine liver biopsies as part of their routine management. In these patients, in whom HCV re-infection is almost universal, HCV specific CD4 T-cell responses have been reported in 62% of the studied patients (Schirren et al., 2001). These responses did not, however, correlate with prevention of recurrent infection. HCV specific T-cell responses have been identified against a wide range of epitopes, particularly the non-structural proteins; the most frequently recognized are HCV core (between nucleotides 21-40), NS3 1253-1272, NS4 1767-1786 and NS4 1909-1929 (Diepolder et al., 1997; Lamonaca et al., 1999). Significantly, these HCV-specific T-cell responses are persistent and have been detectable up to two decades following resolution of HCV in a group of women that had been infected from human Rhesus immunoglobulin (Takaki et al., 2000).

The appearance of CD8 T-cells is temporally associated with control of viraemia. Tetramer studies have shown that CD8 T-cells can take several weeks to expand after the onset of the infection (Shoukry et al., 2003). It has also been shown that these cells initially lack effector function, with limited production of interferon-γ (IFN-γ); this increased over the next few weeks (Lechner et al., 2000). Analysis of individuals following needlestick exposure to HCV found that CD8 T-cells at week seven were associated with significant elevations of serum ALT, but that they did not
produce IFN-\(\gamma\). Later, the CD8 T-cells recovered their ability to produce IFN-\(\gamma\), which was associated with a significant drop in viraemia and resolution of liver disease (Thimme et al., 2001). The reason for this is not clear. Interestingly, this delay was not apparent if memory T-cells responded to HCV (Shoukry et al., 2003). Chimpanzee studies have supported this finding, revealing that the expansion of CD8 T-cells is associated with an increase in serum aminotransferases and transient clearance of HCV from plasma (Cooper et al., 1999; Thimme et al., 2002). Those animals not developing responses developed persistent HCV infection. Similar to CD4 T-cell responses, CD8 T-cell epitopes have been identified to all HCV proteins. In those patients spontaneously clearing HCV these responses have also been shown to be vigorous and multi-specific (Gruener et al., 2000; Lechner et al., 2000). In a study of one individual’s response to HCV, Gruener and colleagues observed that contraction of the CD8 T-cell population responding to a single epitope was associated with loss of control of the infection (Gruener et al., 2001). Similar to the CD4 T-cell responses, studies have confirmed that CD8 T-cell responses are necessarily sustained for long-term clearance of HCV (Takaki et al., 2000; Thimme et al., 2002).

The importance of cell-mediated immunity is also demonstrated through its role in protective immunity. In a study of IVDUs, those who had evidence of previous exposure to HCV had a lower re-infection rate than those not previously exposed (12% versus 21%) (Mehta et al., 2002). There was also a reduced risk for the development of chronic infection. In chimpanzee studies, animals that had recovered from a previous exposure to HCV had a shorter course and milder disease when re-exposed to HCV which correlated with a strong memory T-cell response and intra-hepatic IFN-\(\gamma\) production (Bassett et al., 2001; Weiner et al., 2001; Major et al., 2002). Antibody depletion studies of CD4 and CD8 T-cells indicate that CD8 T-cells are the primary effector cell mediating protective immunity, while the CD4 T-cells are required to support this process (Grakoui et al., 2003; Shoukry et al., 2003).
4.1.2 Viral evolution in acute HCV:
It has been shown that HCV genetic changes can inhibit CD4/CD8 T-cell recognition (Chang et al., 1997). Interestingly, this occurs early in the infection and over time in chronic HCV mono-infection the virus did not diversify further, suggesting that viral escape mutations occur in the acute phase of infection. In a seminal study, Farci and colleagues described the role of escape mutation in early HCV infection (Farci et al., 2000). This study examined HCV diversity over time in four distinct clinical groups (n=12 patients); patients with resolved infection, fulminant hepatitis, chronic non-progressive HCV, and rapidly progressive chronic HCV infection. Patients were studied before and after seroconversion to anti-HCV. With antibody seroconversion, the resolving and fulminant hepatitis groups had decreased viral diversity, particularly in the HVR1, while progressive evolution of the HVR1 of E2 region was associated with chronicity. This suggested that the HVR1 region is under immune selection pressure and may play a role in viral escape. The highest rate of mutation was found at moderate levels of immune response and viral load.

4.1.3 Host-viral interaction:
The relationship between the immune response and HCV diversity can be described using a simple model that was initially applied to HIV (McMichael & Rowland-Jones, 2001) (Figure 4.1). This model contends that the level of viral diversity is determined by two factors: the size of the replicating viral population and the level of immune pressure. This then determines the level of selection pressure on the virus, with advantageous variants surviving. The Farci study elegantly confirmed that this holds true for HCV. This will be explored in this chapter.
Figure 4.1: Host-viral interaction.
Describes the relationship between the viral load (green) and cytotoxic T-lymphocyte activity (black) and escape mutations (red).

4.2 Aims
The aims of this section were:
1. To describe the early natural history of acute HCV in HIV.
2. To study the cell-mediated immune responses to HCV during the acute phase of infection in HIV-positive individuals.
3. To describe the viral evolution during the acute phase of HCV in HIV-positive individuals.
4.3 Methods

4.3.1 Clinical cohort:
Patients diagnosed at the Royal Free campus were enrolled into this study. The diagnosis of these patients has been previously outlined in Chapter 2. Clinical data was collected on the HCV, HIV and demographic parameters. Date of diagnosis was defined as the first documented positive HCV RNA, or anti-HCV seroconversion, or peak ALT followed by HCV viraemia. A period of 3-6 months before the date of diagnosis of HCV was defined as the pre-HCV infection period for the analysis of clinical parameters. Self-limited HCV was defined as the development of persistently negative HCV RNA by PCR and normalization of liver function tests without HCV treatment. Chronic HCV was defined conventionally as persistent HCV viraemia for more than six months. Clinical data were collected from clinical notes and also downloaded from the Royal Free Hospital database. A cohort of acute HCV mono-infected patients was used as a control group for the immunological study. This was an Italian cohort from Vincenza and the serum and PBMCs had been collected in Italy and sent to the Nuffield Department of Medicine, Oxford for analysis. Only basic clinical data were available on this cohort. Comparisons were performed using both SAS (version 8) and Prism (version 4) software.

4.3.2 Cell-mediated immunology:
Collection and Preparation of PBMCs:
Whole blood samples were collected on a 2-4 weekly basis over the acute phase of HCV infection. The PBMCs were prepared as follows:

- 50ml of fresh blood was collected at each time-point.
- Blood was layered over Lymphocyte H solution in a 1:2 ratio.
- Lymphocytes separated by centrifuging for 20 minutes at 800g at room temperature (22°C).
- Lymphocytes added to PBS.
- Centrifuged as above.
- Lymphocytes washed and re-suspended in PBS.
• Lymphocytes counted and re-suspended in 10% DMSO and 90% Fetal Calf serum (FCS).
• Samples were placed in cooling box (cooling by 1°C per minute) frozen in liquid nitrogen at -80°C

**ELISpot assay:**

ELISpot assays were performed using frozen PBMCs, which were analysed for interferon-γ (IFN-γ) production as per manufacturer's instructions (MABTECH, Sweden). IFN-γ ELISpot assays are a very sensitive ex-vivo measurement for low frequencies of cytokine-producing cells (potentially down to 1/300,000).

Briefly, multiscreen filtration plates (Millipore, UK) were coated with 50μl monoclonal antibody (mAb) to IFN-γ (Mabtech) diluted to 15μg/ml with sterile filtered phosphate buffer saline (PBS), and left overnight at 4°C. Plates were washed with 6x 200μl PBS before blocking with 200μl RPMI 1640 (Life Technologies) plus 10% heat-inactivated FCS (Sigma Aldrich) and incubated for 3 hours at 37°C. PBMCs were added to the plate at a concentration of 2 x 10⁵ cells/well. Protein or peptide was added to each well in duplicate. Peptides were added at a concentration of 10μg/ml and proteins at a concentration of 1μg/ml. Plates were incubated for 18 hours overnight at 37°C with 5% CO₂ and 100% humidity. Plates were then washed with 6x 200μl sterile filtered PBS before coating with 50μl biotinylated mAb (Mabtech) diluted 1 in 1000 in sterile filtered PBS and incubated for 2 hours at room temperature. Plates were washed with 6x 200μl sterile filtered PBS before coating with 50μl streptavidin-alkaline phophatase (Mabtech) diluted to 1:1000 in sterile filtered PBS and incubated for 1 hour at room temperature. Finally, plates were washed with 6x 200μl sterile filtered PBS, and 50μl substrate (BCIP/NBT Biorad) was added and incubated until dark spots appeared. Colour development was stopped by washing in tap water (3 x 200μl/well). The plates were left to dry and spots were counted on an ELISpot reader (ELISpot 3.1 SR program, AID Reader System, Germany). There is currently no consensus on the optimal way to identify and enumerate positive
responses in an ELISpot assay. In this study, a test was considered positive if the probability of a spot appearing in the test well was significantly different (p<0.05) from the probability of a spot appearing in the control well, assuming a Poisson distribution. Quantification was then determined by subtracting the mean background number of spots from the mean number of spots in the test well.

**Carboxyfluorescein Succinimidyl Ester (CFSE) assay:**
Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent cytoplasmic dye that can be used to measure cell proliferation using flow cytometry. For staining with CFSE (Molecular Probes, USA) PBMCs at a concentration of 1 x 10^7 cells/ml in PBS were incubated at 37°C for 7 min with 0.5μM CFSE. Staining was terminated by adding PBS containing 10% pooled human serum, cells were washed twice and re-suspended in culture medium containing 10% human serum at 2 x 10^6 cells/ml. Stained cells (1 x 10^6 cells/well, 1mL) were cultured in 48-well plates with medium alone, PHA as a positive control, core peptide pools 1-4 (10μg/ml final concentration) and non-structural proteins NS3-5 (1μg/ml final concentration). A recombinant yeast control protein (1μg/ml final concentration; Chiron, USA) and HIV p24 were also included as controls. After six days of culture, cells for each antigen were transferred into FACS tubes, washed in PBS and stained at 4°C with the following antibodies: anti-human CD4-APC, CD8-PE and Viaprobe (7-AAD) to exclude dead cells (BD Pharmingen, UK). The number of cells that had proliferated was determined by gating on the lineage-positive CFSE<sup>low</sup> subset. After normalisation for the cell input number the stimulation index (SI) was calculated using the following formula:

$$\text{Number of CD4}^+ \text{CFSE}^{\text{low}} \text{cells with antigen} \over \text{Number of CD4}^+ \text{CFSE}^{\text{low}} \text{cells without antigen}$$

A SI > 2 was considered to represent a positive proliferative response, as previously defined (Mannering *et al.*, 2003). The CD4 T-cell proliferative
frequency (%) was calculated only for those where the SI was positive, using the following formula:

\[
\frac{\text{Number of CD4}^+ \text{ CFSE}^{\text{low}} \text{ cells}}{\text{(Number of CD4}^+ \text{ CFSE}^{\text{low}} \text{ cells} + \text{number of CD4}^+ \text{ CFSE}^{\text{high}} \text{ cells})} \times 100
\]

The final proliferative frequency (%) was achieved by subtracting the proliferative frequency with antigen from the proliferative frequency without antigen.

4.3.3 Virology:

Analysis of the viral evolution in the acute phase of infection:

To explore the evolution of the virus in the early phase of infection the core and E1/E2 regions of the HCV genome were sequenced from amplicons at different longitudinal time points. The serum was collected at the same time as the PBMCs and stored. These were then extracted, amplified, cloned and sequenced using the methods described in section 2.3.2.

The average number of synonymous substitutions per synonymous site and the number of nonsynonymous substitutions per nonsynonymous site relative to the ancestral consensus sequence were calculated for each time point within each individual using SNAP (Synonymous/Non-synonymous Analysis Program), which calculates dN/dS rates based on a set of codon-aligned nucleotide sequences using the methods of Nei and Gojobori (Nei & Gojobori, 1986). The consensus sequence of the first time point was used as a reference.

HCV evolution was studied by characterizing the quasispecies’ diversity and complexity at each time-point. Genetic diversity, or the Hamming distance is defined as the number of amino acid differences between two sequences. The mean Hamming distance (that is, the average of the values taken for all sequence pairs derived from a single sample) was separately calculated using the formula \((1 - s) \times 100\), where \(s\) is the fraction of shared sites in two aligned nucleotide sequences (Ganeshan et
al., 1997). The mean Hamming distance was separately calculated for the entire E1/E2 sequence, the HVR1 and the sequence outside the HVR1. The complexity of the HCV quasispecies is defined as the number and importance of viral variants based on the nucleotide sequence of the clones from a single sample.

The core region was analysed using direct sequencing of the same longitudinal samples. The reactions were the same as for the E1/E2 region described in section 2.3.2. The primers used for the nested core PCR were:

Core primers (nucleotide position):

- HCVC1 (from nucleotides 62-42): cttgtggtactgcctgatagg
- HCVC2 (from nucleotides 1291-1269): cattgcagttcagggccgtgcta
- HCVC3 (from nucleotides 51-29): gcctgataggtgcttggtgtt
- HCVC4 (from nucleotides 1262-1243): tgccaactgccgttgggtt

4.3.4 Statistical analysis:
Clinical parameters within individuals were compared using paired t-tests. Differences between groups were determined using Student T-test, $X^2$ test or non-parametric test (Mann Whitney U) where appropriate. Statistics were performed using Prism V4 (Graphpad, USA).
4.4 Results

4.4.1 Natural history of clinical cohort:

The early natural history and host-viral interactions were studied using the RFH cohort (n=55) of co-infected patients. PBMCs were also available for comparison from an Italian cohort (n=8) of acute HCV mono-infection. The patient parameters are outlined in Table 4.1. The co-infection group used for the immunological and virological studies were relatively comparable to the entire co-infected cohort.

Following the diagnosis of HCV, co-infected patients were monitored for 12 weeks to allow for spontaneous seroconversion before initiation of HCV combination treatment. The median time between diagnosis and treatment of HCV was 14 weeks. Only 3 (5%) of the co-infected patients spontaneously cleared HCV, which is lower than rates (20-50%) reported for acute HCV mono-infection. The clinical parameters of the individuals that spontaneously cleared HCV and those that developed persistent infection are outlined in Table 4.1. Given the small number of spontaneous clearers, a formal statistical analysis was not feasible.

The vast majority (95%) of co-infected patients progressed to persistent infection. Interestingly, the three individuals who cleared HCV spontaneously were all jaundiced during their acute illness. These individuals appeared to have both higher peak ALT (2258 versus 366 IU/ml) and CD4 counts (847 versus 549 cells/µl). In contrast, the majority of patients who developed persistent HCV were asymptomatic with only 3 (6%) individuals becoming icteric. The majority of these patients were diagnosed as a result of the detection of abnormal liver function tests. The ALT measurements of the entire cohort are plotted in Figure 4.2. Comparison of the co-infected patients with historical controls revealed that the co-infected patients had higher viral loads (6.0 versus 4.5 log IU/ml) (Gerlach et al., 2003) (Figure 4.3). The vast majority (95%) of the co-infected patients seroconverted to anti-HCV positive during the course of their infection.
The interaction between HIV and HCV was explored by comparing the CD4, CD8 cell counts and HIV viral load before and after infection with HCV. These are outlined in Figures 4.4-4.6. There was no significant change in the median CD4 and CD8 cell counts in patients on HAART pre- and post-HCV (465 versus 568 cells/μl, p=0.91, and 1108 versus 1073 cells/μl, p=0.46, respectively). There was also no significant change in the median CD4 and CD8 counts in those not on HAART (518 versus 560 cells/μl, p=0.44, and 1195 versus 1272 cells/μl, p=0.76, respectively). The HIV viral load in patients not on HAART did not change before and after HCV infection (4.6 versus 4.9 log copies/ml, p=0.82). The number of HIV ‘blips’, defined as a detectable HIV viral load of between 50-400 copies/ml followed by a negative HIV viral load, were measured in those patients on HAART. In 18 individuals with available measurements there were 8 ‘blips’ found in seven patients between -103 days before HCV to +73 days post-HCV. Five (63%) of the blips occurred between 57-103 days before HCV infection. There were no reported HIV-related complications in the period following the diagnosis of HCV.
Table 4.1: Clinical parameters of entire RFH co-infected and Italian mono-infected cohorts analysed in natural history and immunological study.

<table>
<thead>
<tr>
<th>RFH cohort (RFH cohort) patients</th>
<th>Immunological study patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-infected</td>
<td>Co-infected (RFH)</td>
</tr>
<tr>
<td>Outcomes</td>
<td>Mono-infected (Italian)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Number (%)</td>
<td>55 (100)</td>
</tr>
<tr>
<td>Median age (years) range</td>
<td>35.6</td>
</tr>
<tr>
<td>Sex male (%)</td>
<td>55 (100)</td>
</tr>
<tr>
<td>Median peak ALT (range) (IU/ml)</td>
<td>389</td>
</tr>
<tr>
<td>Median HCV VL (range) (log IU/ml)</td>
<td>6.0</td>
</tr>
<tr>
<td>Anti-HCV seroconversion (%)</td>
<td>52 (95)</td>
</tr>
<tr>
<td>Genotype (non-1) (%)</td>
<td>11 (20)</td>
</tr>
<tr>
<td>Transmission Factor</td>
<td>Permucosal</td>
</tr>
<tr>
<td>Outcome – resolved (%)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Icteric (%)</td>
<td>6 (10)</td>
</tr>
<tr>
<td>Length of HIV (years)</td>
<td>4.6</td>
</tr>
<tr>
<td>Median CD4 count (range) (cells/µl)</td>
<td>554</td>
</tr>
<tr>
<td>HIV viral load (log copies/ml)</td>
<td>4.6</td>
</tr>
<tr>
<td>HAART (%)</td>
<td>35 (64)</td>
</tr>
</tbody>
</table>

- Not available
Figure 4.2: Lowess spline curve of all ALT measurements (red) in patients with acute HCV related to days from diagnosis in 55 co-infected patients. ALT ULN = 40 IU/ml.

Figure 4.3: Median HCV viral load (IU/ml) in co-infected patients (n=55) with mono-infected median viral load (broken blue line) from historical controls (n=54) (Gerlach et al., 2003).
**Figure 4.4:** Median CD4 and CD8 T-cell counts (cells/µl) pre-HCV (-194 to -54 days) and post-HCV (-30 to +26 days) in patients on HAART (n=24).

**Figure 4.5:** Median CD4 and CD8 T-cell counts (cells/µl) pre-HCV (-176 to -89 days) and post-HCV (-12 to +84 days) in patients not on HAART (n=13).
Figure 4.6: Median HIV viral load (log copies/ml) pre-HCV (-398 to -96 days) and post-HCV (-14 to +101 days) in patients not on HAART (n=11).
4.4.2 Cell-mediated responses:

Cell-mediated responses were assessed using PBMCs collected from the co-infected and mono-infected patients. A total of 14 co-infected and 8 mono-infected patients had PBMCs available for analysis. A further two patients (patients 15 and 16) had serum that was analysed for viral evolution. The clinical parameters of each group are outlined in Table 4.1 and individual patient characteristics in Table 4.2. The mono-infected (controls) and co-infected (cases) were distinct groups. They had different routes of HCV acquisition, with the cases acquiring HCV percutaneously, as described in chapter 3, compared to the Italian controls who were infected parenterally. The mono-infected controls were Italian hospitalised patients with apparently more liver inflammation, although this was not significant on comparison of the median ALT between controls and cases (1290 versus 427 IU/ml, p=0.79 by Mann-Whitney U test). The median HCV viral load was lower in the mono-infected controls (5.3 versus 6.0 log IU/ml). In addition, the controls included women (n=3). Overall, the spontaneous clearance rate of controls was significantly higher than the co-infected patients (37.5% versus 5%, p=0.004).

Cellular immune responses were detectable in some co-infected individuals. Comparative examples are given of a mono-infected patient and two co-infected patients relating longitudinal viral load, ALT and cell-mediated immune responses (ELISpots and CFSE assays) (Figure 4.7).
Table 4.2: Characteristics of individuals in the immunological and virological studies. The patient numbers are used consistently through the chapter.

Part A- Co-infected individuals.

<table>
<thead>
<tr>
<th>Co-infected Patient</th>
<th>Age</th>
<th>Symptomatic (Icteric)</th>
<th>HCV Genotype</th>
<th>HCV Viral Load (log IU/ml)</th>
<th>Peak ALT (IU/ml)</th>
<th>CD4 (cells/μl)</th>
<th>On HAART at diagnosis</th>
<th>Outcome (Clear)</th>
<th>Days between peak ALT and PBMC collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42.7</td>
<td>No</td>
<td>1</td>
<td>6.1</td>
<td>235</td>
<td>362</td>
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<td>No</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td>34.8</td>
<td>Yes</td>
<td>1</td>
<td>6.9</td>
<td>1504</td>
<td>821</td>
<td>Yes</td>
<td>No</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
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<td>1</td>
<td>4.6</td>
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<td>No</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>35.8</td>
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<td>3a</td>
<td>6.4</td>
<td>5104</td>
<td>847</td>
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<td>Yes</td>
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<td>5</td>
<td>30.6</td>
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<td>544</td>
<td>No</td>
<td>No</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
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<td>1</td>
<td>6.9</td>
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<td>1</td>
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<td>530</td>
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<td>No</td>
<td>15</td>
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<td>9</td>
<td>37.5</td>
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<td>5.2</td>
<td>498</td>
<td>266</td>
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<td>1</td>
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<td>10</td>
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<td>24.9</td>
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<td>13</td>
<td>39.5</td>
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<td>6.9</td>
<td>1415</td>
<td>337</td>
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<td>6.9</td>
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<td>1008</td>
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<td>NA</td>
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</table>
## Part B- Mono-infected individuals.

<table>
<thead>
<tr>
<th>Mono-infected Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Symptomatic (Icteric)</th>
<th>HCV Genotype</th>
<th>HCV viral load (log IU/ml)</th>
<th>Peak Al</th>
</tr>
</thead>
<tbody>
<tr>
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<td>42</td>
<td>M</td>
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<td>1a</td>
<td>5.1</td>
<td>1</td>
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<td>24</td>
<td>M</td>
<td>yes</td>
<td>1b</td>
<td>5.0</td>
<td>1</td>
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<td>32</td>
<td>M</td>
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<td>3a</td>
<td>6.6</td>
<td>1</td>
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<td>1b</td>
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<td>1b</td>
<td>?</td>
<td>1</td>
</tr>
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<td>8</td>
<td>25</td>
<td>F</td>
<td>no</td>
<td>1a</td>
<td>?</td>
<td>1</td>
</tr>
</tbody>
</table>
Fig 4.7: Examples of longitudinal HCV viral load, ALT and cell-mediated immune responses in acute HCV mono-infection and HCV/HIV co-infection.

Example 1 is a mono-infected patient who spontaneously cleared HCV. Example 2 is a co-infected patient who had early CD4 responses with relative virological control. Example 3 is a co-infected patient with no detectable cellular responses and uncontrolled viraemia. Both co-infected patients developed persistent HCV infection.
Comparison of IFN-γ ELISpots revealed significant differences between the cases and controls (Figure 4.8). Unfortunately due to the poor yield of PBMCs following freezing and storage, the PBMCs were incubated in most cases against pooled core peptides and pooled non-structural proteins 3-5. In the final analysis, 14 co-infected individuals were compared to 8 mono-infected individuals at the first available time-point. While more mono-infected PBMCs reacted to the core peptides compared with the co-infected individuals this was not significant (75% versus 36%, p=0.18). In contrast, significantly more mono-infected individuals had IFN-γ responses to the NS3-5 proteins than co-infected individuals (75% versus 14%, p=0.008). There was no significant difference in the median magnitude of mono-infected and co-infected individuals to pooled core peptides (65 versus 0 SFC/10⁶, p=0.13). However, there was a trend to stronger median responses to the pooled NS 3-5 proteins in the mono-infected patients compared to the co-infected patients (35 versus 0 SFC/10⁶, p=0.06). The individual ELISpot results are outlined in Table 4.3.
Table 4.3: IFN-γ ELISpot responses (SFC/10⁶) for individual patients.
Gives the SFC/10⁶ PBMCs for each mono-infected or co-infected individual for pooled core peptides and NS3-5 proteins.

<table>
<thead>
<tr>
<th>Co-infected Patients</th>
<th>Core (1-4)</th>
<th>NS 3-5</th>
<th>Mono-infected Patients</th>
<th>Core (1-4)</th>
<th>NS 3-5</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>110</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>300</td>
<td>2</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>60</td>
<td>20</td>
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<tr>
<td>4</td>
<td>0</td>
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<td>4</td>
<td>195</td>
<td>150</td>
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<tr>
<td>5</td>
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<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>25</td>
<td>170</td>
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<td>75</td>
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<td>0</td>
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<td>8</td>
<td>0</td>
<td>150</td>
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<tr>
<td>9</td>
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<td>0</td>
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<td></td>
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<tr>
<td>10</td>
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<td>0</td>
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<tr>
<td>11</td>
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<td>327</td>
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<td></td>
<td></td>
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<tr>
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<td></td>
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<td>65 (0-195)</td>
<td>35 (0-170)</td>
</tr>
<tr>
<td>(range)</td>
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<td></td>
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</tr>
</tbody>
</table>
Figure 4.8: Comparison of percentage IFN-γ ELISpot responses in acute HCV in mono-infected and co-infected individuals.

Comparison of percentage of mono-infected (n=8) and co-infected (n=14) individuals with IFN-γ responses to pooled core peptides by Fisher's exact test. The lower bar graph presents the IFN-γ responses to the pooled NS3-5 proteins in the same individuals. The mono-infected patients are in blue and co-infected patients are in red.
CFSE assays have previously been demonstrated to be sensitive for the detection of responses to HCV antigens. Figure 4.9 is an example of CFSE assays in a mono-infected and co-infected individual demonstrating responses. The proliferative assays using CFSE analysed 11 co-infected patients and 6 mono-infected patients. Similar to the ELISpot assays, the poor yield of PBMCs led to the core peptides and NS3-5 proteins being pooled for the incubation with antigens. There was no significant difference in CD4 T-cell proliferation to core between the co-infected and mono-infected individuals (14% versus 17%, p=0.94). There appeared to be a trend to more proliferation to NS3-5 in the mono-infected versus the co-infected individuals, although this was not significantly different (50% versus 9%, p=0.1). These results are outlined in Figure 4.10. There was no significant difference in the median percentage proliferation between co-infected and mono-infected individuals with respect to core peptides and non-structural proteins (0 versus 0, p=1.0; 0 versus 1.35, p=0.24, respectively). The individual CFSE percent responses are outlined in Table 4.4.
Table 4.4: CFSE responses (%) for individual patients.
Gives the percentage of CD4 proliferation of PBMCs for each mono-infected or co-infected individual for pooled core peptides and NS3-5 proteins.

<table>
<thead>
<tr>
<th>Co-infected Patient</th>
<th>Core (1-4)</th>
<th>NS3-5</th>
<th>Mono-infected Patient</th>
<th>Core (1-4)</th>
<th>NS3-5</th>
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</thead>
<tbody>
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</tr>
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<td>2</td>
<td>5.3</td>
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<tr>
<td>3</td>
<td>0</td>
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<td>4</td>
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<td>11</td>
<td>0</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>0 (0-5.3)</td>
<td>0 (0-67)</td>
<td>0 (0-5)</td>
<td>1.35 (0-31)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.9: CD4 T-cell proliferative responses detected in the CFSE assay.
This shows the proliferative responses for a co-infected and mono-infected individual, with a negative control.
Figure 4.10: Comparison of percentage CFSE proliferative responses in acute HCV in mono-infected and co-infected individuals.

Comparison of percentage of mono-infected (n=6) and co-infected (n=11) individuals with CFSE responses to pooled core peptides by Fisher's exact test. The lower bar graph presents the CFSE responses to the pooled NS3-5 proteins in the same individuals. The mono-infected in blue and co-infected in red.
4.4.3 Virology:

Viral evolution was studied in number of different ways. As the majority of detectable CD4 T-cell responses in the co-infected patients were to core peptides, this region was sequenced. A neighbour joining (NJ) phylogenetic tree of the direct sequences from the longitudinal samples was constructed (Figure 4.11). This revealed very little diversity, which would be expected as this is a relatively conserved region. The median time (range) in days between the longitudinal samples within each individual was 52 (17-144).

![Figure 4.11: Reconstructed phylogenetic tree of the core region from two or more time-points (TP) in co-infected individuals (n=9). Branch length is % difference in nucleotide sequence.](image)


Figure 4.12: Reconstructed phylogenetic tree of the consensus clone of E1/E2 region from two or more time-points (TP) in co-infected individuals (n=8).

The E1/E2 region of the HCV genome was evaluated in more detail using cloned sequences. Figure 4.12 is the reconstructed NJ tree of the consensus sequences from each individual's time-points. This reveals more genetic diversity between the samples than the core region. A total of 175 cloned sequences from 2-3 time-points from 8 individuals were analysed. The time-points studied ranged from 42 days before to 103 days after the peak ALT. The mean number of clones per time-point was 9.2. Each individual's time-points are outlined in Table 4.5.
Table 4.5: Longitudinal HCV evolution in co-infected patients in early phase of infection.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time-point</th>
<th>Peak ALT (IU/ml)</th>
<th>Days from peak ALT</th>
<th>Closest HCV viral load (log IU/ml)</th>
<th>Days from HCV viral load</th>
<th>Number of clones</th>
<th>Number of HCV variants</th>
<th>dN/dS E1/E2</th>
<th>dN/dS outside HVR1</th>
<th>dN/dS HVR1</th>
<th>Mean Hamming distance % (SD)</th>
<th>P-value**</th>
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*signifies same HCV viral load measurement for different time-points within the same patient; **signifies upper limit of calculation; **p-values for pairwise Hamming distance to previous time-point; * uses Wilcoxon Sign Rank test; both dN/dS and Hamming distances rounded to two decimal points.
The ratio of non-synonymous to synonymous nucleotide changes (dN/dS) was calculated for each time-point from each individual. A dN/dS ratio of greater than 1 is evidence for immune selection pressure resulting in viral mutation. The overall E1/E2 dN/dS ratios were all (100%) less than 1, which is strong evidence against significant immune selection pressure directed against this region leading to viral mutation. There was no significant difference between the mean dN/dS ratios inside or outside the HVR1 (211 versus 158, p=0.35). The dN/dS ratios are listed in Table 4.5. There was no correlation between the dN/dS ratio and HCV viral load, time from peak ALT, CD4 ELISpot responses or HIV parameters.

The diversity and complexity of HCV quasispecies were characterised for each individual’s time-points. Diversity was determined by calculating the Hamming (genetic) distance, which is the difference between two sequences. Complexity relates to the number and importance of the viral variants at each time-point. The mean Hamming distance was calculated by performing a pairwise comparison of every clone’s amino acid sequence for each individual time-point. The results are outlined in Table 4.5. The Hamming distances ranged from 0-18.63%. There was a significant increase in the median genetic distance from the 0.57% at the first time-point to 2.43% for the subsequent time-points, p=0.04. Seven patients had an initial genetic diversity of less than or equal to 1%. In five patients there was an increase in genetic diversity over time, while three patients had a decrease in viral diversity over time. The Hamming distances are graphically represented in Figure 4.13. These did not correlate with peak ALT, CD4 count or HCV viral load. The number of HCV variants at each time-point was determined by comparing the nucleotide sequence of clones from the same sample. These are outlined in Table 4.5 and Figure 4.14. There was a trend to an increase in the quasispecies’ complexity, from a median of 4.5 variants at the first time-point to a median of 8 variants at subsequent time-points, p=0.1. In combination with the low dN/dS ratios (<1) for the same region, the quasispecies data suggests that the viral evolution of this region is the result of random mutation associated with error-prone viral replication.
rather than specific immune selection pressure, which is consistent with the lack of cell-mediated responses.

Finally, the specific site of nucleotide and amino acid substitutions was determined. These are represented in Figures 4.15 and 4.16. Many of the areas with the most change (arbitrarily >2 non-synonymous changes per site) are in recognised HCV epitopes as summarised on Los Alamos HCV immunology database (Yusim et al., 2005). Cytotoxic T-lymphocytes epitope sites included in the substitutions are: AA336, 399-404, 408/412, 460 and 500/501. Changes in recognised antibody epitopes are: AA396-404, 408/412, 434-442 and 483, although antibody responses were not studied as part of this thesis. Sites that were not in recognised epitopes included: AA342, 344, 473, 475, 477, 481.
Fig 4.13: Longitudinal mean Hamming distance of entire E1/E2 sequence related to days from peak ALT for each patient. The patients with increasing Hamming distance over time are in red and those with reducing Hamming distance over time are in blue.
Fig 4.14: Number of HCV variants at each time-point in each individual (n=8).

Each colour represents a different variant within that individual at that time-point. The same colour does not represent the same variant between different time-points or individuals.
Figure 4.15: Synonymous and non-synonymous nucleotide changes by site in E1 and HVR1 regions of HCV.

E1 nucleotide changes (1313-1479)

HVR1 nucleotide changes (1486-1570)
Figure 4.16: Synonymous and non-synonymous nucleotide changes by site in E2 region of HCV.

E2 nucleotide changes (1571-1700)

E2 nucleotide changes (1701-1844)
4.5 Discussion

This cohort has presented a unique opportunity to study the early natural history of HCV in HIV-positive individuals. The data presented in this chapter indicate that the early natural history of acute HCV in HIV co-infection is different from acute HCV mono-infection. The major difference was the higher rate of persistence in the co-infected patients. This chapter set out to explore why HIV co-infected patients have higher rates of HCV persistence by studying the immunological and virological responses in acute disease. However, studies of clinical cohorts have a number of limitations that need to be discussed.

4.5.1 Limitations:

As with the study of other relatively rare conditions, the numbers were small. While the number of cases of HCV in HIV-positive individuals is increasing, acute HCV mono-infection is rarely recognised. Consequently, the identification of controls was difficult, particularly for clinical comparison. Fortunately, PBMC samples were available from an Italian cohort for the cellular assays. However, there were significant differences between the mono-infected and co-infected groups. The co-infected group was a predominantly outpatient UK cohort, with a broad clinical spectrum of HCV ranging from an acute icteric hepatitis to asymptomatic disease and a spontaneous clearance rate of 5%. In contrast, the mono-infected controls were hospitalised Italian patients; the majority were jaundiced, with a spontaneous eradication rate of 37.5%. In addition the transmission routes were different. Both cohorts were patients undergoing routine care and as a result, the collection of specimens and data in some cases was incomplete. Therefore, direct comparisons were difficult and the interpretation of the immune responses needs to be cautious. In neither group were the cellular immune responses correlated to outcome, but this probably relates to the small number of individuals studied. In an effort to standardise the analysis, immune responses were related to peak ALT. A larger number of co-infected individuals that spontaneously cleared HCV and mono-infected controls would have allowed analysis related to outcome to be performed.
The quality of the PBMCs was poor in many of the samples and as a result, the yield of lymphocytes was small. The assays were performed on frozen PBMCs that had been prepared and stored using standard cryopreservation techniques, which have been validated (Valeri & Pivacek, 1996). However, there is evidence that frozen samples may have different responses and cytokine secretion compared with fresh PBMCs. It has been demonstrated that cryopreservation of PBMCs can increase certain cytokine production, such as IFN-γ and IL-2, and T-cell viability may be reduced compared with fresh PBMCs (Venkataraman & Westerman, 1986; Venkataraman, 1995). This may then have had an effect on our lymphocyte assays. However, while collected at different centres, the method of collection and storage was the same for both groups. Finally, because the number of PBMCs and samples were limited and assays could not be repeated to check variability. Inter-assay variability could have been magnified, particularly in the co-infected cohort where the magnitude of PBMC responses was low. For this reason, the percentage with responses rather than the specific magnitude of responses was used for the statistical comparison of co-infected and mono-infected individuals.

An important concept is the compartmentalisation of the immune response. This study analysed peripheral PBMCs and there is probably discordance between the liver and peripheral blood responses due to the different tropism of lymphocytes. Minutello and colleagues described the differences in CD4 responses to HCV antigens between intra-hepatic and peripheral lymphocytes (Minutello et al., 1993). They found that a distinct subset of CD4 T-cells focused against NS4 antigen was found in the liver but not peripherally. Nuti et al. found that activated intrahepatic T-cells are biased towards a T-helper 1 response (Nuti et al., 1998). However, in the acute state the lack of access to liver tissue has meant that these studies have not been repeated. While peripheral responses are often studied these may not reflect the true responses in the liver.
Finally, issues related to the experiments could have influenced the results. Genotypic variations may alter the cellular responses to recombinant proteins. The cellular responses were tested using specific genotype 1 recombinant proteins and peptides for the ELISpot and CFSE assays. Unfortunately, specific non-genotype 1 recombinant proteins and peptides were not available. Of the co-infected cohort 29% versus 25% of the mono-infected cohort were non-genotype 1. This may have been a factor explaining some of the poor responses seen, specifically patient 4 who cleared genotype 3 HCV but did not develop any detectable CD4 T-cell responses. However, both groups had a similar distribution of non-genotype 1 HCV. Due to the small number of samples, it was not possible to assess if there was a statistical difference in the responses of genotypes 1 and non-1.

4.5.2 Detailed discussion:
Persistence is the most common outcome of HCV infection. The rate of persistence in HIV co-infection (95%) was significantly higher than the Italian mono-infected controls (62%) and reported historical controls (60-80%). This is important as it suggests that concurrent HIV infection favours HCV persistence. Our persistence rate of 95% contrasts with a rate of 76% in a similar cohort (n=50) of HIV-positive MSM with acute HCV from another London teaching hospital (CWH). Gilleece and colleagues reported a 24% spontaneous clearance rate, associate with higher CD4 counts and lower initial HCV viral loads (Gilleece et al., 2005). They reported HCV RNA results over a median of 14 months follow-up, the shortest period was one month. It is possible that a proportion with shorter follow-up may actually have had undetectable but persistent viraemia, especially with the less sensitive older assays (lower limit of detection= 615 IU/ml). In the largest study of the natural history of acute HCV mono-infection, Gerlach and colleagues reported that males and patients who were not symptomatic were significantly less likely to spontaneously clear HCV (Gerlach et al., 2003). All the patients in our co-infected cohort were male and the vast majority (90%) were asymptomatic, which would favour persistence. Those who cleared HCV were all jaundiced and had higher median CD4 counts. The Italian cohort
would appear to be much more typical of previously described mono-infected groups. The better rates of clearance in the mono-infected patients may represent a selection bias, as symptomatic individuals are probably more likely to clear HCV and present to medical care. However, it is also likely that specific immunological issues related to the host-viral interaction in the co-infected individuals contribute to increase rates of persistence.

Given the small numbers in the control group, detailed comparison of specific clinical parameters was difficult. However, it appeared that the median HCV viral load was higher by approximately 1 log IU/ml and median ALT was lower than both the Italian and historical mono-infected controls. This would imply poorer control of HCV in the HIV infected individual with higher viral replication. The hepatic lesion in HCV is usually immune-mediated and the apparent paradox of accelerated liver injury in an immunodeficient state has not been explained. However, it is likely that the higher HCV viral loads contribute to this.

With respect to the influence of HCV on HIV parameters some observations were made. There were no adverse clinical events related to HIV reported during the study period. There appeared to be no short-term impact of HCV on the CD4/CD8 T-cell counts, with no change before or after HCV infection. However, there is a suggestion that the HIV viral 'blips' may occur around the time of infection in those on HAART. There were 8 'blips' in seven (39%) patients over a relatively short period of time (260 days). One study detected 27.2% of patients had an HIV 'blip' over a median of 485 days follow-up (Sklar et al., 2002). The implication of HIV viral 'blips' is controversial, however it appears that they probably do not lead to increased HIV resistance (Nettles et al., 2005). It may be that the more frequent 'blips' in our study represents an early viral interaction between HIV and HCV, however, this would require a detailed study of the viral kinetics and evolution of both viruses that was not performed as part of this thesis. While long-term the influence of HCV on HIV remains controversial, it is difficult to relate findings in the acute phase to the long-term picture.
Cell-mediated immune responses to HCV are detectable in the early phase of infection in co-infected individuals. However, these responses lacked the breadth and magnitude that is probably necessary for control of acute HCV, which may explain the higher rate of viral persistence in this cohort. The responses of the co-infected individuals were focused against core but not the NS3-5 proteins. It has been shown that responses to the non-structural proteins are particularly important for the clearance of HCV (Diepolder et al., 1995). Our data reveal that the defect in cell-mediated immunity occurs early in the course of HCV infection. Failure to develop a vigorous, multispecific and persistent cell mediated response is postulated to occur as a result of primary T-cell failure/exhaustion, T-cell dysfunction, and viral escape mutation (Neumann-Haefelin et al., 2005). While any of these mechanisms is possible in HIV co-infection, it was beyond the scope of this study to explore the specific reasons for this. However, evidence in HCV mono-infection suggests that both primary T-cell failure and T-cell exhaustion play a role in failure to control the infection. Studies have shown that patients developing chronic infection have weak responses of the CD4 and CD8 T-cells in the acute phase. Importantly, if these responses were not maintained then persistence developed, which would support the hypothesis of CD4 T-cell exhaustion (Gerlach et al., 1999). Furthermore, Thimme and colleagues found that in health care workers exposed to HCV, patients with no significant cell-mediated responses to HCV developed persistent infection, suggesting primary T-cell failure (Thimme et al., 2001). The underlying mechanisms for this are unclear but it has been hypothesised that HCV may interfere with antigen presenting cells (APC) resulting in a failure to prime the T-cells (Sarobe et al., 2002). It has also been suggested that high HCV viral loads may contribute to deletion of virus-specific T cells, potentially an important reason in co-infection where the HCV viral loads are generally higher (Neumann-Haefelin et al., 2005).

CD8 T-cell responses were not studied as part of this thesis, however, CD8 T-cell dysfunction has also been shown to be important in persistent
HCV (Lechner et al., 2000; Wedemeyer et al., 2002). The defect can be in their proliferative, cytotoxicity or cytokine secretion capacity. Early recovery of responses led to control of viraemia, while lack of responses resulted in persistent disease (Thimme et al., 2001). High viral loads may lead to T-cell unresponsiveness. This has been shown in murine models where CD8 T-cell fail to produce IFN-γ after antigenic stimulation (Zajac et al., 1998). T-cell responses have been shown to increase on combination interferon and ribavirin treatment (Barnes et al., 2002). It has been suggested that impaired T-cell maturation may be important. Study of CD8 T-cell surface markers has revealed less differentiated phenotypes (CD28+ and/or CD27+) (Appay et al., 2002). Functionally, the production of IFN-γ by CD8 T-cells is initially impaired. Viral factors, such as HCV core antigen, may also interrupt cytokine pathways and impair T-cell activation, proliferation and IFN-γ production. Finally, it has been suggested that regulatory (CD4+/CD25+) T-cells suppress IFN-γ production in CD8 T-cells in chronically infected patients (Accapezzato et al., 2004). A combination of these mechanisms may contribute to T-cell dysfunction contributing to persistent infection. This is an area that should be a focus for future immunological work.

The replication of HCV is rapid and error prone and has been shown to play an important role in the evasion of host immune responses. However, the HCV quasispecies’ complexity (the number of viral variants and their importance) and diversity (genetic distance) in HIV has only been studied in the chronic state and the data are conflicting. In a study of the effect of early HIV on chronic HCV, 10 patients with chronic HCV and newly acquired HIV had analysis of HCV diversity determined before and after HIV acquisition (Mao et al., 2001). While the results were not statistically significant there was a tendency to lower dN/dS ratios supporting reduced immune pressure; this was more pronounced in HIV rapid progressors. Sherman et al. found the diversity of the HVR1 of E2 had increased diversity in HIV co-infected (n=10) patients than HCV mono-infected (n=7) patients (Sherman et al., 1996). If CD4 counts were <50 cells/µl the HCV complexity did not vary between mono-
infected and co-infected patients (Toyoda et al., 1997). In a Spanish study, with two samples analysed over a three year follow-up period, HCV quasispecies complexity in the E2 region was increased in co-infected patients (Roque-Afonso et al., 2002). A study of 52 patients with co-infection to assess whether HCV diversity impacted treatment outcome, found no difference between co-infected and mono-infected HCV viral diversity (Neau et al., 2003).

Early genetic diversity of HCV arises either from inoculation with multiple HCV variants, PCR reaction (taq polymerase) errors and/or post-infectious viral mutation. Herring and colleagues proposed that a genetic diversity of less than 3% supports an oligoclonal inoculum of HCV, assuming that taq polymerase errors may produce a 1% change and the nucleotide substitution rate of the E1/E2 region may produce a 0.47% change over 20 days (Herring et al., 2005). In our study of eight individuals, seven (88%) had a genetic diversity of ≤ 1% at their first time-point, suggesting that these patients were infected with a small number of HCV virions.

The longitudinal quasispecies analysis revealed that there was a significant increase in HCV diversity and a trend to increased complexity over time. However, for the same region the dN/dS ratios remained low (<1). This implies that while HCV evolution occurred over time it was not driven by immune selection pressure but more likely was the result of error prone replication. This is entirely consistent with the lack of immune response as demonstrated by the poor cell-mediated immune responses, which probably results in the inadequate control of HCV. The CD4 T-cell responses elicited in the co-infected patients were primarily against the core peptides. Despite this, there appeared to be no significant intra-individual change in the core region over time. It would be important for future work to explore the humoral responses in co-infected individuals.

The data presented reveal that HIV co-infection has a significant impact on the clinical, immunological and virological outcome of early HCV. Co-infected patients have significantly higher HCV persistence rates following
acute HCV. While there was no influence of HCV on objective HIV parameters such as CD4/CD8 cell counts and HIV viral load, the median HCV viral load was higher in the co-infected cohort. The detectable cell-mediated responses in the co-infected individuals lacked the breadth and magnitude that is probably required to control HCV. While the reason has not been elucidated, this defect was demonstrated early in co-infection. This also emphasises the importance of CD4 T-cells in the control of HCV. Finally, analysis of viral evolution reveals that there is little evidence for mutation as a result of immune selection pressure, which is consistent with the poor cell-mediated responses described. Future work should focus on specific mechanisms of this early defect, further detailing the host-virus interactions. HIV/HCV co-infection represents a major clinical issue and one where simple immunologic data in well-defined cohorts could provide important future insights.
Chapter 5: Conclusions, Recommendations and Future Work

5.1 Discussion

Since the identification of the hepatitis C virus in 1989, HCV infection and the associated liver-related morbidity and mortality, has been recognised as a worldwide pandemic. The Health Protection Agency recently addressed the issue of HCV in its report “Hepatitis C in England” (Health Protection Agency, 2005a). The focus of this report was on HCV mono-infection, particularly in IVDUs. However, an important subgroup are those individuals co-infected with HIV. The WHO estimates that 10 million people are co-infected worldwide. In the UK approximately 10% of HIV patients also have HCV. This is important not only because of the burden of infection but because of the accelerated course of HCV in HIV co-infection. Since the introduction of effective HIV treatments, there has been an unmasking of HCV-related morbidity and mortality and HCV is now a leading cause of death in HIV infected individuals in the developed world.

It is difficult to recognise acute hepatitis C, as the acute infection is often asymptomatic. While acute HCV can rarely be recognised clinically it has been increasingly diagnosed via surveillance and seroconversion amongst HIV-positive individuals in the UK, particularly in London and Brighton. Based on the most recent numbers of cases of acute HCV from each of the clinics participating in this study (RFH n=55, CWH n=85, and BSGH n=22), more than 1% of these clinics’ patient populations have become infected with HCV over the study period. This thesis explored two distinct aspects of the epidemic. First, the epidemiology was characterised using combined molecular (phylogenetic) and clinical (case-control) studies. Second, the natural history, cell-mediated responses and viral evolution of acute HCV in HIV were described. These studies reveal that acute HCV in HIV-positive individuals differs significantly from acute HCV mono-infection in its epidemiology, natural history, immunology and virology.
The key conclusions, resulting recommendations and future areas of research are discussed.

**5.2 Epidemiological aspects**

**Key conclusions:**

The epidemiological analyses confirmed that HCV transmission is occurring within the HIV-positive MSM population as a result of permucosal risk factors. The multiple clusters identified by the phylogenetic study of the HCV genome provide strong evidence for common source transmission. Each monophyletic cluster, except the genotype 3a cluster, was composed of sequences from at least two of the participating centres, supporting transmission between patients from geographically distinct HIV units. This implies that any intervention needs to include an appropriately broad scope to encompass the at-risk population. Finally, the application of a molecular clock to the individual clusters suggests that the majority of the sequence divergence, equating to transmission events, had occurred after the mid-1990s, which coincides with the introduction of effective HIV treatments. The introduction of HAART was associated with an increase in high-risk sexual behaviours in MSM. The fact that these molecular clusters crossed both HCV genotype and subtype, implies that the HCV transmission is not due to a specific viral change, such as the development of a more virulent strain, but that patient and/or environmental factors are contributing to the recent infections. The case-control study demonstrated distinct differences in risk behaviours between cases and controls, with permucosal rather than parenteral factors associated with the recent transmission of HCV in this cohort of patients. These factors include high-risk mucosally traumatic sexual and drug practices. It should be reiterated that the control group also had significant risk behaviours. This is strikingly different from the usual epidemiology of acute HCV, where the majority of HCV is transmitted parenterally via IVDU.
Recommendations:
The epidemiologic studies have important implications for interventions to mitigate and control the spread of HCV within the HIV-positive population. Given the evidence for transmission of HCV, early detection is vital. First, there needs to be enhanced surveillance of HCV within this population. The three centres involved in the study treat between 30-40% of the estimated 22 000 HIV patients in this region. These centres introduced annual screening for anti-HCV in 2002, following the recognition of increased HCV diagnoses. However, this process should be expanded to other HIV units. At present the collection of epidemiological data has been piecemeal, relying on individual units. It is clear that other units in the UK that were not part of this study are also seeing a similar phenomenon (personal communication with physicians at University College Hospital and St Mary’s Hospital). The data collection should therefore be centralised under an authority such as the HPA to develop a clearer picture of the epidemic.

All the cases identified were HIV-positive men suggesting factors specific to this particular group are important in the HCV transmission. The case-control study has identified a number of factors associated with case-control status, which could be used to identify high-risk individuals. Broadly, these include: high numbers of sexual partners, participation in group sex, specific mucosally traumatic sexual practices, use of illicit drugs (specifically ‘club drugs’), the sharing of drug taking implements, and use of the internet to meet other men. These factors could be used to identify individuals that are at higher risk for the potential acquisition of HCV.

Individuals need to be made aware of the transmissibility of HCV through the identified specific mucosally traumatic sexual and drug practices. Interestingly however, the assessment of attitudes in the questionnaire revealed that both cases and controls were aware of the potential for the sexual transmission of HCV. However, it was less clear whether they understood the implications of chronic HCV/HIV co-infection. Unfortunately, these data were collected retrospectively. As permucosal
transmission of HCV involves disruption of mucosal integrity with exposure to infected body fluids, interventions must aim at minimising this. Therefore, strategies should focus on reducing exposure to traumatic practices, promoting safe (protected) sexual practice through the use of condoms and gloves, minimising the use of 'club drugs', and education about the risk of sharing drug implements, particularly intranasally and intrarectally. While the specifics of the how and why these behaviours occur were not delineated, our data suggest that many of these behaviours are occurring in the milieu of group sex and this may be an important identifiable marker of risk around which interventions could be focused.

Education and health promotion campaigns need to be aimed at both the health professionals and at-risk individuals. There is growing awareness amongst clinicians of the risk of HCV transmission in the HIV-population, as a result of peer-reviewed articles, conference presentations and internet reports. However, specific programs for the clinicians involved in the care of HIV-positive patients are essential, particularly in the urban areas. Health promotion campaigns for patients would further increase awareness of the recent epidemic and its associated behavioural risks with the aim of reduced parenteral and permucosal risk exposure.

Specific strategies that have been used to prevent HIV/STI transmission would obviously be important. Attempting to fragment sexual networks has been effective (Wohlfeiler & Potterat, 2005). Sexual networks describe the sexual connection between individuals and the structure of these networks is important in the transmission of STIs. Through the provision of information and education, individuals can become better informed in the hope that they make decisions that reduce their risk of exposure. This aims to discourage lower-risk individuals interacting with high-risk individuals. Often because sexual networks are difficult to identify and because they overlap with social networks it may be more straightforward to intervene at the social level. This could, for example, involve venues such as sex clubs and the internet, both of which were factors that were associated with case/control status in our study.
Social interactions via the internet presents a new and significant public health challenge. The case-control analysis identified it as the most common location where cases met other men. In relation to STIs, a number of internet-based strategies have been used in an attempt to reduce individuals risk exposure for individuals. These include online partner notification of STIs, access to STI testing, banner advertising of health information, chat-room health outreach, internet epidemiology studies, behavioural interventions, and member profiles with associated computer algorithms (McFarlane et al., 2005). The outcomes of these various interventions have not yet been assessed.

Unfortunately, at present there is evidence that both unsafe sexual and drug practices are on the rise amongst MSM. The HPA outlined in a report “Mapping the issues” that the rate of sexually transmitted infections, including HIV, is increasing particularly among MSM (Health Protection Agency, 2005b). The data presented in this thesis supports this. Furthermore, there is increasing literature on the problem of drug use in this population, some suggesting a link between increasing use of crystal meth and new HIV diagnoses (Boddiger, 2005). This thesis provides further impetus for interventions to mitigate the transmission of not only HCV but also HIV and STIs.

Future work:
A collaboration between the identified cohorts in London, the Netherlands, France and Germany to extend the molecular phylogenetic study has been agreed. The work will be based at the Royal Free and University College Medical School. In association with clinical and epidemiological data this should provide fascinating insights into the interactions of the cohorts and the dynamics of the European epidemic. Cases will be enrolled both prospectively and retrospectively. By expanding the project, prospective data would be available for better stratification of risk. Finally, detailed studies at the event level are required to tease out the complex interactions between the sexual and drug practices that are contributing to the transmission of HCV. This
would require in-depth interviews with individuals with an exploration of these behaviours.

5.3 Natural history, virological and immunological aspects

**Key conclusions:**

HCV persistence was the usual outcome in the vast majority of co-infected individuals; these rates are significantly higher than those for HCV mono-infection. While the specific mechanisms were not elucidated, cell-mediated responses are important. The CD4 T-cells lacked the magnitude and breadth of response for control of HCV. The virological data supported this, revealing quasispecies evolution that was not driven by immune pressure. Therefore, HIV appears to impact the immune system’s ability to recognise and eradicate HCV.

**Recommendations:**

Treatment algorithms need to be drafted to ensure that infected individuals are treated in a timely fashion with appropriate antiviral regimens, as these evolve, to maximise the chance of viral clearance during the acute phase. Based on the high rates of persistence, early intervention with combination treatment should be recommended. While in HCV mono-infection, deferring treatment for a period of up to 16 weeks may be appropriate to allow for spontaneous eradication, this event is rare in HIV co-infection (Gerlach et al., 2003). Provisional treatment data reported by our group has shown that co-infected individuals with acute HCV treated with a combination regime of pegylated interferon and ribavirin have higher rates of sustained virological response than chronic HCV/HIV co-infection, but lower rates than acute HCV mono-infection (Danta et al., 2005b). Interestingly, ribavirin seems to be important for these outcomes when compared to pegylated interferon monotherapy. Therefore, early treatment is appropriate and should be recommended. Those individuals that cleared HCV spontaneously were icteric and had higher CD4 counts. It could be postulated that increasing the CD4 T-cell count with HAART during this period may favour eradication of the HCV. However, we did not have
sufficient data to test this hypothesis. Finally, there did not appear to be any significant interaction between HCV and HIV in the acute phase and so no conclusions could be made with regards to the long-term natural history.

**Future work:**
The long-term clinical follow-up of this cohort will provide a definitive insight into the natural history of HCV in HIV co-infection, in a similar way to the German and Irish HCV mono-infected anti-D cohorts. The strength of this analysis will be significantly increased by combining the European cohorts.

These findings emphasize the potential importance of HCV-specific CD4 T-cell responses in the control of HCV. Further analysis in particular should focus on the influence of HAART on HCV-specific CD4 T-cell responses, how this differs from responses to other antigens, and how this relates to any changes in HCV viral load observed. Finally, the sensitivity of HCV-specific CD4 T-cells in HIV may relate to features of their site, phenotype, activation status or turnover, which should also be studied. This would specifically involve tetramer studies and CD8 T-cell responses. In particular, important epitopes should be better defined, such as the non-structural regions. Humoral immune responses were not studied as part of this thesis. With the development of pseudo-virus models relevant antibody responses could be assessed. If the number of patients who spontaneously eradicate the infection increases (as more cases are identified) then specific immune features of these patients could also be studied. Combining the immunological studies with analysis of the evolution of both HCV and HIV in the acute phase would provide important insights into the reasons for persistent infection.

**5.4 Conclusion**
The current epidemic of HCV in HIV-positive individuals is attributable to permucosal transmission as a result of high-risk sexual and drug practices. The immunodeficient state associated with HIV results in very
low spontaneous clearance of HCV and the vast majority of these patients become persistently infected with HCV. This epidemic has come about as a result of significant change in patient behavioural risk factors. In order to mitigate this important and ongoing epidemic, these factors need to be the focus of a concerted effort on the part of public health specialist, clinicians and HIV-positive individuals themselves.
References


Appendix A
Case-control study questionnaire instrument

CONFIDENTIAL

HCV/HIV Co-infection Risk Questionnaire 2004

Confidentiality
The questions in this booklet are mostly very personal. Your answers will be treated in strict confidence. When you have finished the questionnaire, put it into the envelope and seal it. Your name will not be on the booklet or envelope.

How to answer
Just put a tick in the box opposite the appropriate answer like this: ✓
OR write a number in the box provided, like this: 8 6

Not all the instructions apply to you; follow arrows and instructions.

Please ask for help or explanations if you are not sure.

Date of Interest is the date of your diagnosis with hepatitis C or an allocated date.

Importance
It is very important to the study that you answer these questions as honestly and as accurately as you can.

Some things may be hard to remember, so please take your time.

Date of Interest

Questionnaire Number
QUESTION 1

We would like to ask you questions about your attendance at places where men go to meet or socialize with other men. In the 12 months prior to the date of interest how often have you gone to:

a) A bar, night club, or dance club? □ OR

b) Private parties? □ OR

c) A sex club or bathhouse/sauna? □ OR

d) A public cruising area, such as park, beach or cottage? □ OR

e) Internet sites such as gaydar, gay.com? □ OR

f) Other? Please specify

QUESTION 2

In the 12 months prior to the date of interest did you have sex with:

Only men □

Both men and women □

Did not have sex in the last 12 months □

GO TO QUESTION 9 (PAGE 6)

QUESTION 3

a) In the 12 months prior to the date of interest how many men have you had sex with?

write in number

b) In the 12 months prior to the date of interest how many of the men you had sex with were one night stands or someone you had sex with only once?

write in number
QUESTION 4
The following questions ask about different sexual activities you have engaged in with other men. In the 12 months prior to the date of interest, did you perform any of the following sexual activities and, if so, with how many different men?

a) Had active oral sex (had his penis in your mouth) without a condom, with withdrawal before ejaculation?  
   Yes □  -  write in number  
   No □

b) Had active oral sex where he ejaculated in your mouth, without a condom?  
   Yes □  -  write in number  
   No □

c) Had active oral sex where he wore a condom?  
   Yes □  -  write in number  
   No □

d) Received oral sex (had your penis in his mouth) without a condom, but with withdrawal before ejaculation?  
   Yes □  -  write in number  
   No □

e) Received oral sex where you ejaculated in his mouth, without a condom?  
   Yes □  -  write in number  
   No □

f) Received oral sex where you wore a condom?  
   Yes □  -  write in number  
   No □

g) Had receptive anal intercourse without a condom, without ejaculation inside you?  
   Yes □  -  write in number  
   No □

h) Had receptive anal intercourse without a condom, with ejaculation inside you?  
   Yes □  -  write in number  
   No □

i) Had receptive anal intercourse where he used a condom?  
   Yes □  -  write in number  
   No □

j) Performed insertive anal intercourse without a condom, with withdrawal before you ejaculated?  
   Yes □  -  write in number  
   No □

k) Performed insertive anal intercourse without a condom, with ejaculation inside him?  
   Yes □  -  write in number  
   No □

l) Performed insertive anal intercourse using a condom?  
   Yes □  -  write in number  
   No □
QUESTION 4 (Continued)

The following questions ask about different sexual activities you have engaged in with other men. In the 12 months prior to the date of interest, did you perform any of the following sexual activities and, if so, with how many different men?

m) Been rimmed by someone else?
   Yes □  -  write in number
   No □

n) Performed rimming on another man?
   Yes □  -  write in number
   No □

o) Actively fisted (had your fist in his anus) another man?
   Yes □  -  write in number
   No □

p) Been fisted (had his fist in your anus) by another man?
   Yes □  -  write in number
   No □

q) Used sex toys, such as dildoes or vibrators?
   Yes □  -  write in number
   No □

r) Engaged in bondage and discipline, or S&M practices?
   Yes □  -  write in number
   No □

QUESTION 5

a) In the 12 months prior to the date of interest, did you have sex in a public place, like a park, club, or bath house/sauna?
   Yes □  
   No □  -  GO TO QUESTION 6 (page 5)

b) If you had sex in a public place with a man, did you ever engage in:

   Receptive anal intercourse without condoms? Yes □  
       No □

   Insertive anal intercourse without condoms? Yes □  
       No □

   Insertive fisting (had your fist in his anus)? Yes □  
       No □

   Receptive fisting (had his fist in your anus)? Yes □  
       No □
QUESTION 6

a) In the 12 months prior to the date of interest did you have sex in a group with two or more other people?
   
   Yes □
   No □

b) If you had sex in a group, did you ever engage in:
   
   Receptive anal intercourse without condoms? Yes □
   No □

   Insertive anal intercourse without condoms? Yes □
   No □

   Insertive fisting (had your fist in his anus)? Yes □
   No □

   Receptive fisting (had his fist in your anus)? Yes □
   No □

GO TO QUESTION 7 (below)

QUESTION 7

Are you circumcised?

Yes □
No □
QUESTION 8

a) In the 12 months prior to the date of interest, have you engaged in any sexual activity with a man while feeling the effects of alcohol?

Yes □
No □

b) In the 12 months prior to the date of interest, have you engaged in any sexual activity with a man while feeling the effects of drugs?

Yes □  specify drugs →
No □

QUESTION 9

a) The next questions are about your use of alcohol and drugs. In the 12 months prior to the date of interest, on average, how often did you drink any kind of alcoholic drink?

Never □
Monthly or less □
2-4 times a month □
2-3 times a week □
4 or more times a week □

GO TO QUESTION 10 (page 8)

b) How many drinks containing alcohol do you have on a typical day when you are drinking? A "drink" is equivalent to: half pint (285ml) of ordinary strength beer or lager; 1 small glass (125ml) of wine; 1 glass (50ml) of fortified wine, eg. Sherry; 1 single measure (25ml) of spirits.

1-2 □
3-4 □
5-6 □
7-8 □
10 or more □

c) How often do you have six or more drinks on one occasion?

Never □
Less than monthly □
Monthly □
Weekly □
Daily or almost daily □
QUESTION 9 (Continued)

d) How often during the last year have you found that you were not able to stop drinking once you had started?

Never □  
Less than monthly □  
Monthly □  
Weekly □  
Daily or almost daily □  

e) How often during the last year have you failed to do what was normally expected from you because of drinking?

Never □  
Less than monthly □  
Monthly □  
Weekly □  
Daily or almost daily □  

f) How often in the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session?

Never □  
Less than monthly □  
Monthly □  
Weekly □  
Daily or almost daily □  

g) How often during the last year have you had a feeling of guilt or remorse after drinking?

Never □  
Less than monthly □  
Monthly □  
Weekly □  
Daily or almost daily □  

h) How often during the last year have you been unable to remember what happened the night before because you had been drinking?

Never □  
Less than monthly □  
Monthly □  
Weekly □  
Daily or almost daily □  


QUESTION 9 (Continued)
i) Have you or someone else been injured as a result of your drinking?

No □
Yes, but not in the last year □
Yes, during the last year □

j) Has a relative or friend or a doctor or other health worker been concerned about your drinking or suggested you cut down?

No □
Yes, but not in the last year □
Yes, during the last year □

QUESTION 10
In the 12 months prior to the date of interest have you used any drugs for recreational purposes?

Yes □
No □ [GO TO QUESTION 16 (page 11)]

8
**QUESTION 11**

In the 12 months prior to the date of interest have you used:

<table>
<thead>
<tr>
<th></th>
<th>Yes □</th>
<th>number of times</th>
<th>No □</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Marijuana or hashish?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) Poppers or inhalants like amyl, butyl or isopropyl nitrite?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>c) Crack cocaine?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>d) Cocaine, not including crack cocaine?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>e) Methamphetamines, also known as speed, crystal or crank?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>f) Other amphetamines or &quot;uppers&quot;?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>g) Ecstasy, also known as MDMA, or other forms of MDA (eg High Fives)?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>h) Psychedelics/ Hallucinogens, such as LSD?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>i) Downers including barbituates, sleeping tablets such as valium and rohypnol?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>j) Heroin, methadone, other opiates, or painkillers such as codeine or tramadol?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>k) Ketamine or K?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
<tr>
<td>l) Gamma Hydroxybutyrate or GBH?</td>
<td>Yes □</td>
<td></td>
<td>No □</td>
</tr>
</tbody>
</table>
**QUESTION 12**

In the 12 months prior to the date of interest have you taken recreational drugs:

<table>
<thead>
<tr>
<th>Method</th>
<th>Yes</th>
<th>No</th>
<th>Number of times</th>
<th>Which drugs?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orally</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Nasally</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By injection</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By other means</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**QUESTION 13**

In the 12 months prior to the date of interest have you shared recreational drug implements:

<table>
<thead>
<tr>
<th>Method</th>
<th>Yes</th>
<th>No</th>
<th>Number of times</th>
<th>Which drugs?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orally</td>
<td></td>
<td></td>
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<tr>
<td>Nasally (eg bullets)</td>
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<tr>
<td>By injection (eg needles)</td>
<td></td>
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<td></td>
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<tr>
<td>By other means</td>
<td></td>
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</tbody>
</table>

How was it taken?

With what implement?
QUESTION 14

a) Have you ever injected any recreational drugs?

Yes □ □ Date of last injection □

No □ □ □ GO TO QUESTION 16

b) Have you ever shared a needle with another person when you shot up drugs?

Yes □ □ Date of last shared needle □

No □ □ □ GO TO QUESTION 16

QUESTION 15

Have you ever been concerned about your use of recreational drugs?

Yes □

No □

QUESTION 16

During the 12 months prior to the date of interest, has anyone given you money or drugs in exchange for sex?

Yes □ □ How many different people?

No □ □ □ □
QUESTION 17

a) Do you have any tattoos?

Yes □  Year of first tattoo □  Number of tattoos □
No □

b) Have you ever had a transfusion of blood or blood products?

Yes □  Year of first transfusion □  Number of transfusions □
No □

c) Have you had or have body piercings?

Yes □  Year of first piercing □  Number of piercings □
No □

QUESTION 18

In the past year, other than an HIV test, have you been checked for a sexually-transmitted disease?

Yes □
No □

QUESTION 19

There are many diseases you can get from having sex. Some common sexually transmitted diseases, besides the AIDS virus, are syphilis, gonorrhea, anal or genital warts, crabs, genital herpes, non-specific urethritis (NSU) and others. Has a doctor, nurse or other health care worker ever told you that you have a sexually-transmitted disease?

Yes □
No □  GO TO QUESTION 21 (page 14)
QUESTION 20
Which sexually transmitted diseases have you previously had?

<table>
<thead>
<tr>
<th>Disease</th>
<th>Yes</th>
<th>No</th>
<th>Year of first diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genital Herpes</td>
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<td></td>
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<tr>
<td>Syphilis</td>
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<td></td>
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<tr>
<td>Gonorrhea</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Non-specific urethritis (NSU)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Genital Warts</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hepatitis B</td>
<td></td>
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<td></td>
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<tr>
<td>Hepatitis A</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
QUESTION 21

The following statements relate to your knowledge of hepatitis C. Please tick one box next to each statement to indicate how strongly you agree or disagree with the statement.

a) Hepatitis C is an important health problem.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know

b) Someone with hepatitis C can feel fine.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know

c) Hepatitis C can be transmitted sexually.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know

d) Hepatitis C can make HIV worse.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know

e) HIV can make Hepatitis C worse.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know

f) There is treatment available for hepatitis C.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know

g) There is a vaccine available for hepatitis C.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know

h) Most patients with hepatitis C have chronic (persistent) infection.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know

i) Hepatitis C can cause the liver to stop working.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know

j) Alcohol should not be drunk if you have liver problems.
   - Strongly Agree
   - Agree
   - Disagree
   - Strongly Disagree
   - Don't Know
Thank you for participating and answering these questions.
If there is anything that you would like to add, or comments that you would like to make, please write them below.

Now put the questionnaire in the envelope provided, and hand it to the interviewer.

Thank you
Publications and Presentations

Publications:


Presentations:
