THE SEVERITY AND ACTIVITY OF LIVER DISEASE IN CHRONIC HEPATITIS C INFECTION.

by

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A thesis submitted for the degree of Doctor of Medicine (M.D.)
At the University of Edinburgh
This work is dedicated to my parents for the extraordinary educational opportunities they have given me throughout my life.
“This is not the end. It is not even the beginning of the end. But it is perhaps, the end of the beginning.”

Winston Churchill (following the Battle of El Alamein), 1942.
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DECLARATION

I hereby declare that this thesis is based on the results of my own experiments and that this thesis is exclusively of my own composition. The data presented in this thesis have not been submitted previously for a higher degree.

Geoffrey H Haydon
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Figure 8.1 Aetiology of Hepatocellular carcinoma 1985 - 1994 (184).

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Infection with hepatitis C virus (HCV) is the cause of 90% of cases of non-A, non-B hepatitis. Chronic HCV hepatitis is at least initially an asymptomatic illness, but a proportion of patients will develop symptomatic, complicated disease. The most common complications are hepatic cirrhosis and hepatocellular carcinoma (HCC); presently it is unclear whether all infected patients will develop these complications.

Aims of the Thesis

There were two main aims. Firstly, to assess the clinical significance of staging investigations; in particular the significance of molecular virological investigations in terms of disease diagnosis and prognosis. The role of non-invasive investigations in staging the disease process was also considered. Secondly, to assess the impact of chronic hepatitis C infection was assessed in two populations; patients diagnosed as having hepatocellular carcinoma and those immunocompromised by chronic HIV infection.

Materials and Methods

A well characterised Scottish population of over 200 HCV infected patients was examined in detail to define the clinical significance and interpretation of serum
and intrahepatic hepatitis C virus levels, particularly in the context of biochemical, epidemiological, virological and histological parameters. The role of two non-invasive investigations in predicting the presence of hepatic cirrhosis was also assessed in these patients; firstly, a serum marker of perisinusoidal fibrosis, hyaluronic acid, and secondly, artificial neural network analysis of host and virus parameters.

Further, the impact of chronic HCV infection on two independent populations of patients was considered. Firstly, a population of 202 patients concurrently infected with the human immunodeficiency virus was examined in terms of clinical and immunological progression of disease. Secondly, the impact and association of chronic hepatitis C infection on the development of hepatocellular carcinoma in patients in Lothian (an area of low risk for the disease) over 10 years was investigated and compared with HCC associated with chronic HBV infection.

Results

In the Scottish population studied, both serum and intrahepatic virus levels were not determined by host factors (age of patient, mode or duration of infection) or by virus factors (HCV genotype). Likewise, there was no correlation between serum and liver HCV RNA levels demonstrated; however, these data did demonstrate that repetitive negative RT-PCR for HCV RNA in serum did not indicate absence of HCV from the liver. Pilot studies of the two non-invasive investigations, serum hyaluronic acid and ANN in this population showed both to be reliable in predicting the presence of hepatic cirrhosis.
Amongst the intravenous drug abusers with chronic HIV infection, HCV did not influence either the clinical progression of HIV disease to AIDS and it was not associated with a more rapid immunological decline. Chronic HCV infection was identified as a major risk factor for the development of hepatocellular carcinoma.

Conclusions

Molecular virological staging investigations should be interpreted with caution in chronic HCV infection; their most significant role is likely to be the initiation and monitoring of therapy rather than the inference of disease prognosis. Non-invasive investigations of hepatic cirrhosis are likely to be useful tests to monitor disease progression especially when a liver biopsy is contraindicated, although they should be first validated in larger, well described populations.
This thesis examines the severity and activity of liver disease in patients chronically infected with the hepatitis C virus. Its aims are twofold. Firstly, to assess the clinical significance of staging investigations; in particular the significance of molecular virological investigations in terms of disease diagnosis and prognosis. The role of non-invasive investigations in staging the disease process will also be considered. Secondly, to assess the impact of chronic hepatitis C infection will be assessed in two populations; patients diagnosed as having hepatocellular carcinoma and those immunocompromised by chronic HIV infection.

In the first part of the thesis (Chapter 1: Introduction), an overview of chronic HCV infection will be provided, including its history, virology, epidemiology, sequelae of infection and present treatment regimens. This background information will highlight the problems associated with interpretation of present methods of staging the disease process.

The second part of the thesis (experimental work) examines several questions regarding the clinical significance of staging investigations of chronic hepatitis C infection and the impact of the disease on specific populations. This section is divided into 5 chapters (Chapters 2 to 7).

Chapter 2 deals with sample availability, collection and molecular virology methodology used; it also compares the strengths and weaknesses of the techniques with rival methodology.

Each of the following chapters is divided into background and aim, materials and methods, results and conclusion. Chapters 3 and 4 examine in detail the clinical significance and interpretation of serum and intrahepatic hepatitis C virus levels,
particularly in the context of biochemical, epidemiological, virological and histological parameters. In Chapters 5 and 6, the role of two non-invasive investigations in predicting the presence of hepatic cirrhosis is assessed; firstly, a serum marker of perisinusoidal fibrosis, hyaluronic acid, and secondly, artificial neural network analysis of host and virus parameters. The potential for these investigations to replace liver biopsy, a procedure associated with significant morbidity and mortality is also considered.

In Chapter 7, the role of a proposed co-factor to HCV infection, chronic HIV infection, in the development of severe liver disease is considered. The reciprocal relationship of the effect of HCV on the clinical and immunological sequelae of HIV infection is also discussed in detail. In the final chapter (Chapter 8) in the experimental work section, the impact and association of chronic hepatitis C infection on the development of hepatocellular carcinoma (HCC) in patients in Lothian (an area of low risk for the disease) over 10 years is investigated and compared with HCC associated with chronic HBV infection.

The discussion section is in Chapter 9, and includes an overall critical discussion of the results, the new insights introduced by this thesis regarding the staging and severity of chronic hepatitis C infection in Scotland, as well as future studies emerging from this work.

References comprise Chapter 10.
1.1 Historical Aspects.

Following the development of commercial serological assays to diagnose hepatitis B and hepatitis A infections, in 1966 and 1973 respectively, it was evident that the 60-90% of patients with transfusion acquired hepatitis were both HBsAg and anti-HAV negative (Alter et al. 1975; Feinstone et al. 1975; Knodell et al. 1975). Consequently, the syndrome non-A, non-B (NANB) hepatitis was described, and for two decades the responsible infectious agent(s) remained elusive using conventional, immunological methods.

In 1989, antibody from a patient with posttransfusion hepatitis identified clone 5-1-1 in a random primed cDNA ygt11 expression library ($10^6$ recombinant phage) derived from the serum of a chimpanzee with NANB hepatitis (Choo et al. 1989). This clone did not originate from the host genome because the cDNA did not hybridise with either human or control DNA. Three further overlapping clones were subsequently isolated using 5-1-1 as a hybridisation probe to screen the original library and a 1089 nucleotide continuous open reading frame was reconstructed. The antigen (C100-3) for the first generation ELISA was prepared by expressing this ORF as a fusion polypeptide with human superoxide dismutase in yeast (Kuo et al. 1989). Most cases of NANB hepatitis (80% with posttransfusional hepatitis, 58% with
sporadic disease) were found to be associated with anti-C100-3 antibody and this response was used to define infection with a new virus, hepatitis C (Van der Poel et al. 1989; Esteban et al. 1989; Kuhnl et al. 1989; Roggendorn et al. 1989).

The ensuing seven years have brought characterisation of the complete virus, knowledge of its genetic variability, three generations of diagnostic antibody tests, identification of the virus by electron microscopy and increasing experience of viral nucleic acid detection, epidemiology, and antiviral treatment.

1.2 Genetic Heterogeneity of HCV: Quasispecies and Genotypes.

Hepatitis C virus (HCV) is classified as a prototype of a third genus of the Flaviviridae family of viruses; it has positive polarity and exists as a single stranded RNA genome. The genome consists of a single, large open reading frame of 9379-9481 nucleotides, flanked by 5' and 3' non-coding regions (NCR) (Choo et al. 1994; Kato et al. 1990; Takamizawa et al. 1991; Han et al. 1991). The 5'NCR is highly conserved and has a 92% homology among different HCV types; its probable function is translation of the viral genome and its highly conserved character renders it suitable for diagnostic detection of viral nucleic acid with polymerase chain amplification of HCV cDNA. The open reading frame encodes for 3 structural proteins at an aminoterminal end (the putative core (p22) and two envelope glycoproteins: E1 and E2) with 6 non-structural proteins at a carboxyl terminal end (Hijikata et al. 1991; Grakoui et al. 1993). The non-structural regions have a role in virus replication and encode for two serine proteases (NS2, NS3) (Tomei et al. 1993; Hijikata et al. 1993), a helicase (NS3) (Choo et al. 1994) and an RNA-dependent polymerase (NS5).
(Houghton et al. 1991). Finally, the 3' terminal regions show considerable variations, both in length and in sequence; in some isolates there is a poly-(rU) tail and in others a poly-(rA) tail. Several HCV isolates have been cloned, revealing wide sequence variation unevenly distributed over the genome (Figure 1.1). Apart from the highly conserved 5'NCR, the putative core and NS3 regions are relatively well conserved and antigens from these regions are used in anti-HCV assays. Hypervariable domains have been described at the N-terminal part of the E2 envelope region (Okamoto et al. 1992). Sequential mutations in this region probably have a role in genetic escape from the host immune response (Weiner et al. 1992). Indeed, a high rate of mutations during replication is common, since the RNA-dependent RNA polymerase is “error prone”, and there is a lack of an associated repair mechanism.

The hepatitis C virus demonstrates two forms of genetic heterogeneity, which are of clinical relevance. The first is the genetic heterogeneity amongst different HCV isolates (“Genotypes”) (Ogata et al. 1991), which can be classified as genetically distinct groups as a result of mutations accumulated during the evolution of these viruses. Secondly, the genetic heterogeneity of the HCV population within an infected individual (“Quasispecies”) (Martell et al. 1992); this is the means by which the virus can escape immune surveillance.

1.2.i Quasispecies Nature of Hepatitis C Virus.

HCV quasispecies result from mutations occurring during viral replication; the rate of mutations has been estimated at $1.92 \times 10^{-3}$ to $1.44 \times 10^{-3}$ base substitutions /
Figure 1.1: Organisation of HCV genome and functions of nonstructural viral proteins.

C=core E=envelope NS=nonstructural
HVR=hypervariable region
NTPase
RNA helicase
functions of nonstructural viral proteins

C

E1

E2/NS1

NS2

NS3

NS4A

NS4B

NS5A

NS5B

5ʹ

3ʹ

RNA-dependent RNA polymerase

cofactor for serine protease

Metalloprotease

function unknown

serine protease

Metalloprotease

{}
genome site/year (Martell et al. 1992; Okamoto et al. 1992). The structural regions, E1 and E2 have the highest rate of mutation.

The biological consequences of HCV quasispecies are that firstly, chronic HCV infection is established as a result of selection of mutants that escape neutralising antibodies or cytotoxic T lymphocytes and secondly, attempts at host vaccination have failed. Studies have assessed the clinical relevance of HCV quasispecies, and have hypothesised that the wider the species diversification, the more severe and progressive the resultant liver disease (Honda et al. 1994).

1.2.ii Genotypic Nature of HCV.

Phylogenetic analysis of NS5 and E1 nucleotide sequences from samples obtained world-wide indicate that there are at least six distinct HCV genotypes (these differ in nucleotide sequence by >30% over the complete virus genome), and at least 30 subtypes (differing by more than 20% in sequence) (Simmonds et al. 1993a; Simmonds et al. 1993b). This classification system was stipulated in 1994 and was based on 5'NCR and NS5 sequence analysis; it utilises Arabic numerals followed by lower case letters (Simmonds et al. 1994). Other researchers have described nine major genotypes with up to 30 subgroups, based on E1 sequence analysis (Tokita et al. 1994). Recent data have indicated that genotypes 7, 8, and 9 should be reclassified as genotype 6 subtypes and that type 6 appears to be diversified (Mizokami et al. 1996).

The geographical distribution of HCV genotypes has been documented from blood donor studies in different countries (McOmish et al. 1994; Davidson et al. 1996).
Type 1 is widely distributed throughout the world; 1a and 1b are predominant in North and South America and Europe. 1b is also common in Asia, whilst 1c is responsible for 20% of all HCV infection in Indonesia. Genotypes 2 and 3 are also common throughout the world, although, 3b is found only in Japan, Nepal, Thailand and Indonesia, whilst 3c, 3d, 3e and 3f are only present in the Nepalese population (Tokita et al. 1994). On the African continent, types 1, 2 and 3 are rare; type 4 is predominant in North and Central Africa and type 5 in South Africa; types 4 and 5 are only sporadically distributed in other countries. Genotype 6 has been described in Hong Kong and Vietnam; whilst 7, 8 and 9 (or the corresponding subtypes of 6) are endemic in Vietnam (Tokita et al. 1994). The presence of numerous subtypes of one of the major genotypes (e.g. genotype 3 in Nepal or genotype 6 in Thailand) is indicative of a long period of endemic infection.

1.2.iii Biological and Clinical Importance of HCV Genotypes.

Much of the interest in distinguishing between HCV genotypes has come from the possibility that they may have different clinical manifestations, either determining relative pathogenicity or response to interferon therapy.

Many authors have suggested that genotype 1b, cirrhosis and hepatocellular carcinoma are significantly associated. The largest European study examined 220 Italian and French patients and demonstrated the presence of genotype 1b in 75% of patients with cirrhosis and 84% of those with hepatocellular carcinoma, but in only 54% of those with less severe biopsy changes of chronic hepatitis (Nousbam et al. 1995). Genotype 1b was also associated with a longer disease duration and an age
greater than 40 years. Thus, 36 of 57 (63%) of patients with type 1b had had disease for 10 years or longer, whereas only 19 of 50 (38%) patients with non type 1b had had disease activity for more than 10 years. Therefore the increased severity of disease in patients with type 1b may reflect the cumulative effects of longer disease duration; this issue remains unresolved.

End-stage cirrhosis secondary to chronic HCV infection is currently an indication for orthotopic liver transplantation (OLT). The recognition that there is a high (perhaps universal) frequency of recurrence of HCV infection after OLT presumably following reactivation of extrahepatic sites of HCV replication and reinfection of the allograft, has provided a model to examine the relative pathogenicity of HCV genotypes (Konig et al. 1992; Feray et al. 1992). There is agreement that HCV genotype 1b is associated with an accelerated and more aggressive graft hepatitis, but the significance of this in terms of overall prognosis is unclear (Feray et al. 1995). Prospective studies examining these patients may provide evidence for a causal association between genotype 1b and hepatic cirrhosis.

In the example of interferon therapy, however, strong evidence exists that infection with genotype 1 (or in some studies, specifically 1b) predicts a lower rate of response to interferon therapy compared with infection with types 2a, 2b or 3a (Yoshioka et al. 1992; Mita et al. 1994). Multivariate analysis shows that these associations are independent of such other confounding variables as patient age and the duration or mode of acquisition of infection, which also differ between genotypes (Kasahara et al. 1995). Interferon response correlates with the level of circulating virus for persons infected with genotype 1b, but virus concentration does not explain
differences in interferon response between virus genotypes. Studies using branched chain DNA assays have shown that virus RNA levels are similar in individuals infected with different genotypes once variation in detection efficiency is accounted for (Smith et al. 1996). Recently, it has also been demonstrated that type 4 infections are similar to type 1 in response to interferon therapy (El-Zayadi et al. 1996).
1.3 Diagnostic Assays.

Patients with hepatitis C virus infection may be identified in a variety of clinical settings. These include (i) the discovery of abnormal liver function tests during the investigation of an illness; (ii) the discovery of abnormal LFT’s during a health screen; (iii) the discovery of HCV antibody in a healthy blood donor and (iv) the screening of high risk individuals (e.g. intravenous drug abusers) for evidence of previous exposure to the virus. Most newly diagnosed cases of HCV infection are asymptomatic and serological investigations, the reverse transcription polymerase chain reaction and liver biopsy, are required to make the diagnosis and stage the disease. The former two investigations are now considered.

1.3.i Antibody Screening Tests.

By generation of overlapping clones, the first HCV clone isolated (5-1-1) was extended to produce a recombinant antigen (c100-3) for first generation anti-HCV antibody testing with an enzyme-linked immunosorbent assay (ELISA) (Kuo et al. 1989). However, this initial mode of serological testing was associated with a high percentage of false positive results, amongst certain patient populations, particularly those with hyperglobulinaemia (MacFarlane et al. 1990). Subsequently, other HCV recombinant and synthetic peptide antigens have been expressed to produce respectively, second and third generation anti-HCV assays. Second generation ELISAs included recombinant antigens from the NS3 (c22-3 and c33c) and NS4 (c100-3) regions. Third generation anti-HCV ELISAs include antigens from the putative core, and from the NS3, NS4 and NS5 regions. ELISA-3 tests are widely
used in blood donor screening (they are 99.7% effective in preventing the transmission of HCV to recipients) and are more sensitive and specific than earlier generation tests (Courouce et al. 1994; Bousch et al. 1994). (Figure 1.2).

There are two situations in which antibody tests may not detect HCV infection. Firstly, it may take as long as 6 months after primary infection for an anti-HCV response to develop, the mean period between infection and detection being 12 weeks. Secondly, immunosuppressed patients may have infection without detectable antibodies; in such cases reverse transcription polymerase chain reaction is required to detect infection (Van der Poel 1994).

1.3iii Confirmatory Testing.

For confirmation of positive anti-HCV test results, recombinant immunoblot assays (RIBA, Chiron) have been developed. In these, the patient's sera is incubated with a nitrocellulose strip coated with HCV antigens. In the third generation RIBA (RIBA-3) system widely used in Europe, there are a total of five synthetic peptides from the core and NS4 regions and recombinant antigens from the NS3 and NS5 regions. RIBA-3 results are interpreted as "positive" when two or more of five bands are positive and "indeterminate" when one of five bands is positive.

A high proportion (75-80%) of RIBA-3 positive patients have viraemia as detected by RT-PCR for HCV RNA. RIBA-3 positive but RT-PCR negative patients may have cleared the virus from the circulation after a previous infection, may be viraemic below the RT-PCR detection level, or may represent false-positive anti-HCV reactivity. Previous studies have indicated that RT-PCR negative / anti-HCV RIBA
<table>
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<tr>
<th>Viral Antigens</th>
<th>Host Antibodies</th>
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<tbody>
<tr>
<td>NS5B</td>
<td>C200</td>
</tr>
<tr>
<td>NS5A</td>
<td>C100</td>
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<td>NS4B</td>
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Figure 1.2. Origin of cloned antigens used in anti-HCV diagnostic tests.
positive patients correlate with absence of inflammation on liver biopsy specimens, and this observation probably indicates clearance of the virus (Alberti et al. 1992). Likewise, most RIBA-3 positive, RT-PCR negative blood donors have a risk factor for parenteral exposure to HCV, so positivity of the RIBA-3 in these individuals probably represents true HCV infection rather than non-specific reactivity (Zaaijer et al. 1994).

During the initial acute antibody response (or even during chronic infection), there is occasionally indeterminate RIBA-3 reactivity. These indeterminate RIBA reactions should be reconfirmed using RT-PCR.

1.3.iv Qualitative Reverse Transcription Polymerase Chain Reaction (RT-PCR).

RT-PCR for HCV RNA has the advantage of establishing HCV viraemia. This test has evolved from a research tool into a routine diagnostic investigation; however, it remains limited by its expensive and lack of standardisation between laboratories (a European proficiency panel revealed that only 16% of laboratories yielded reliable RT-PCR results) (Zaaijer et al. 1993). Its methodology (described in detail in chapter 2) relies on reverse transcription of low titre HCV RNA into HCV cDNA, followed by amplification via the polymerase chain reaction. The sensitivity of the amplification stage depends on the primers used.

RT-PCR is invaluable as a diagnostic tool in four areas of HCV infection; (i) early diagnosis in acute infection; (ii) confirmation of infection in patients who do not produce an antibody response to the virus; (iii) follow up of drug therapy; (iv) monitoring perinatal transmission. RT-PCR can also be used to detect HCV RNA in
liver biopsy material, and negative RT-PCR in serum is thought to correlate with negative RT-PCR in liver tissue (Alberti et al. 1992).

1.3v Serum Aminotransferase Levels.

Clinical assessment of hepatitis C infection includes measurement of serum aminotransferases (ALT). Chronic hepatitis C infection is characterised by raised or fluctuating ALT or long-term marginal increases in ALT. 60% of HCV infected patients have normal ALT values in cross-sectional studies, and most (80%) asymptomatic individuals with HCV viraemia but normal ALT values have chronic hepatitis or even cirrhosis (Stanley et al. 1996). A normal ALT level in the context of hepatitis C infection is therefore of limited value.

1.4 Epidemiology and Transmission of HCV Infection.

1.4.i Geographical Prevalence of Infection.

HCV is encountered world-wide, with marked variation in seropositivity rates, not only between continents, but between towns and provinces, and even between ethnic groups. The highest prevalence of infection occurs in Japan, the southern states of the USA, the Mediterranean countries of Europe, Africa and the Middle East (Saudi Arabia has the highest overall prevalence at 3 to 4%, probably because of the very high prevalence of Egyptian residents in Saudi Arabia), where 0.5-2.0% of the blood donors are anti-HCV positive (by second generation enzyme immunoassay). In Northern Europe, the northern states of the USA, and Canada, prevalence amongst
blood donors is 0.01-0.05% (UK: 0.02% (Trent Regional Hepatitis C Virus Study Group 1994)).

There are few studies on the prevalence of HCV infection in representative samples of the population. A serological survey of a population based sample of 10,132 persons in the USA (between 1989 and 1992) found a prevalence of anti-HCV of 1.4%, which corresponds to a reservoir of anti-HCV of approximately 3.5 million anti-HCV positive persons nation-wide (Alter 1995). There are no such data available for the world’s population.

1.4.ii Incidence of HCV Infection.

HCV is a bloodborne virus, and the main route of transmission is parenteral; percutaneous spread of the virus is not well defined.

Reanalyses of prospective studies of transfusion recipients, who contracted NANB hepatitis have shown that 60-90% of these patients seroconverted to HCV (Esteban et al. 1990; Aach et al. 1991; Koretz et al. 1993; Tremolada et al. 1991). Prior to 1986, incidence rates of posttransfusion hepatitis C ranged from 5-13% (Aach et al. 1991); from 1986-1990, these rates declined to between 1.5 and 9% (Esteban et al. 1990) and following anti-HCV screening of donors in 1990, posttransfusion hepatitis C rates have been reported to be less than 1% (Alter et al. 1990).

However, most HCV infections occur outside the transfusion setting. The US Sentinel Counties Chronic NANB Hepatitis study team reported that 40% of their study patients had a history of intravenous drug abuse (IVDA); 5% were
posttransfusion patients; 15% household contacts and 40% had sporadic infection (no obvious risk factors for infection) (Alter et al. 1990; Alter et al. 1992).

Incidence data on community acquired hepatitis C infection are available from the US: the estimated incidence of acute hepatitis C remained relatively stable through much of the 1980s with an average of 15/100,000, but declined by more than 50% between 1989 and 1991. This decline has coincided with a decrease in cases associated with IVDA, possibly related to safer needle using practices (Alter 1993).

Acute hepatitis C infection may occur in all age groups, but most cases occur among young adults, presumably related to patterns of exposure (IVDA). There are no consistent sex predilections for HCV other than a higher frequency related to IVDA among men. In the US, the highest proportion of cases is among whites, but the incidence of disease is highest in non-white racial/ethnic groups, particularly Hispanics.

1.4.iii Prevalence of HCV Infection among Populations and Modes of Transmission.

Direct percutaneous exposures, such as transfusion of blood or blood products, or transplantation of organs or tissues from infectious donors and sharing of contaminated needles among injection drug abusers are associated with the most efficient transmission of HCV. Haemophilia patients who have received multiple factor VIII transfusions and IVDA have prevalence rates of anti-HCV exceeding 80% (Makris et al. 1990). The role of permucosal spread and covert percutaneous exposure in HCV infection are not well defined. Seroprevalence studies have reported
anti-HCV rates of 1% among hospital based care workers in the West; in one study, assessing risk factors for infection, a history of accidental needlesticks was independently associated with anti-HCV positivity (Polish et al. 1993). Likewise, higher seroprevalence rates have been reported among dentists (2%) and dental surgeons (9%) in the US than among blood donors (Klein et al. 1991). Nosocomial transmission of HCV is possible if disinfection procedures are inadequate. Studies of haemodialysis associated outbreaks of hepatitis have demonstrated an association between anti-HCV positivity and increasing years of dialysis, that was independent of blood transfusion (Niu et al. 1993; Jadoul et al. 1993).

Sexual transmission of HCV is again highly controversial. The strongest evidence in support of the transmission of HCV from sexual contacts is derived from epidemiological studies of NANB hepatitis. In a case control study comparing 52 NANB hepatitis patients with 104 uninfected control patients (Alter et al. 1989), the prevalence of NANB was significantly higher in patients either with a history of hepatitis in a household or sexual contact or with a history of 2 or more sexual partners in the previous six months. However, this and other studies (Steven et al. 1991; Osmond et al. 1993) have not found an association between homosexual activity and NANB (HCV) hepatitis; seroprevalence for anti-HCV is 4 to 8% in male homosexuals, compared with rates of 60 to 80% for HbsAg or anti-HIV positivity.

Other high risk sexual practices such as female prostitution are associated with a slightly higher anti-HCV seroprevalence rate, ranging from 4 to 8% (Lissen et al. 1993). Four recent European studies examining HCV seroprevalence among exposed heterosexual partners of individuals with HCV infection, all demonstrated a
seroprevalence for anti-HCV < 4% (Evehart et al. 1990; Brackmann et al. 1993; Bresters et al. 1993).

Rates of perinatal HCV transmission of HCV range from 0-5% (Reinus et al. 1992; Roudot-Thorval et al. 1993; Wejstal et al. 1992; Lam et al. 1993); this mode of infection is more likely to occur when the maternal virus level is high (Lin et al. 1994; Ohto et al. 1994). There are presently no WHO recommendations for prevention of perinatal infection by pregnant women.

It is unlikely whether any or even a combination of these modes of transmission accounts for the high incidence of sporadic HCV transmission. Instead, under reporting of drug abuse or extensive covert parenteral transmission of HCV by unsterilised needles (as has been reported in Japan) is a more likely cause (Kiyosawa et al. 1994).

1.5 Sequelae of HCV Infection.

1.5i Acute HCV Infection.

Acute hepatitis C infection is generally a benign event; following posttransfusion transmission of HCV, 70 to 80% of cases are anicteric and asymptomatic. In the NIH series of 86 consecutive posttransfusion cases, only 30% had a bilirubin greater than 2.5mg/dL and the mean peak ALT was 708u/l (Di Bisceglie et al. 1991). Fulminant hepatitis C is extremely rare; although, RT-PCR has established the acute appearance of HCV RNA coincident with the onset of fulminant disease (Fagan 1994). Quantitation of HCV RNA using the branched chain DNA assay demonstrated high levels of viraemia in this small series of patients with
fulminant hepatitis, although the significance of these levels and the pathogenesis of hepatocyte necrosis in fulminant hepatitis is unclear.

1.5ii Viral Persistence and Chronic Liver Disease.

Since fulminant disease is so rare, and acute HCV infection is generally benign, the paradox of HCV infection resides in its ability to become persistent and induce chronic liver disease. However, even here, the significance of chronic HCV infection has been a controversial issue. Initial retrospective analyses of NANB hepatitis patients using serum transaminase levels, indicated that resolution of infection occurred in 30-50% of patients, but that the acute infection evolved into chronic liver disease in about 50% of infected individuals (Di Bisceglie et al. 1991; Matsson et al. 1988; Realdi et al. 1982). Among those patients biopsied, the vast majority had chronic hepatitis or cirrhosis. Later an association with hepatocellular carcinoma became evident and the potential severity of NANB hepatitis was increasingly accepted. In 1992, a prospective study was published that had enrolled and followed transfusion associated hepatitis cases and controls from five major prospective studies that defined posttransfusion NANB hepatitis in the 1970s (Seef et al. 1992). After a mean follow up of 18 years, there was no increase in mortality comparing transfusion related NANB/C (n=568) with control patients (group I: n=526; group II: n=458) who were similarly transfused, but had not developed hepatitis. The authors of this study concluded that there was very little evidence that the clinical entity of NANB hepatitis resulted in severe life threatening disease; the study group mortality was 51% compared with 52% and 50% for groups I and II

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respectively. However, by 1994 (Seef et al. 1994), a significant excess of liver related mortality was emerging in the posttransfusion population (3.3% compared with 1.1% and 2.0% respectively), and of equal importance, an excess of morbidity; among NANB/C cases, 35% had biochemical evidence of chronic hepatitis as compared with 1% of controls and of 40 patients biopsied, two thirds had severe histological lesions including chronic hepatitis, cirrhosis and HCC. These two reports were the first evidence that the natural history of chronic liver disease secondary to HCV infection is a measure of decades rather than years. The lack of mortality in the NANB population at 18 years may be population dependent: most patients were elderly heart surgery patients whose life expectancy was less than 18 years.

There have been a series of other excellent follow up studies published, predominantly from the US. The first, which examined 80 patients who developed posttransfusion hepatitis, concluded that the probability of developing clinical evidence of cirrhosis (defined as decompensated disease) in patients with chronic hepatitis C was 20% after a mean interval of 16 years (Koretz et al. 1993). The second, from Spain, following patients with both transfusion associated and sporadic hepatitis, found that only 6% to 10% had apparent remission, whereas 30% to 39% developed cirrhosis, 13% to 15% experienced hepatic decompensation and 2% to 7% developed HCC (Sanchez-Tapias et al. 1990). The overall liver related mortality during the course of the study was 4% to 9%; there was no significant difference in disease outcome between transfusion acquired and sporadic hepatitis. In the NIH transfusion study (Alter 1995), 92 consecutive cases of hepatitis C have been enrolled since 1970; of these 33 (36%) have had liver biopsies. On the initial or subsequent
biopsy the majority had mild to moderate chronic hepatitis and 8 of 33 (24%) had cirrhosis. During long-term follow up, 3 of 8 with cirrhosis died of end stage liver disease and three had very severe liver disease when they died of intercurrent causes. The overall liver related mortality was 3%, but could have been as high as 6%. In the Centres for Disease Control (CDC) sentinel counties study of community acquired hepatitis (sporadic transmission), chronic hepatitis developed in 60 of 97 (62%) HCV infected patients followed for 9 to 48 months (Alter et al. 1992). This was the first large scale study to demonstrate that although biochemical evidence of hepatitis was noted in only 62%, HCV RNA was detected in serum by RT-PCR in 15 of 15 patients with normal serum transaminase levels. The authors concluded that in sporadic hepatitis, the frequency of chronic hepatitis exceeds 60%, but the frequency of persistent infection may exceed 90%.

There are other means of assessing the relationship of HCV infection and chronic liver disease. The first is to determine the proportion of cases that evolve into end stage liver disease requiring transplantation. In the US, HCV related end stage liver disease is now the primary indication for transplantation; however, it is difficult to derive these numbers in a prospective fashion, and it should be noted that there many factors which favour selection for transplantation (Alter 1995). Secondly, examination of multicentre interferon trials for the treatment of HCV infection attests to the large number of patients with severe liver disease; although, this method also involves a selection bias. In the US multicentre trial (Davis et al. 1989), 45% of patients had chronic hepatitis and 55% cirrhosis. The final method is to examine asymptomatic populations for severity of liver disease. In the most remarkable of
these studies, the entire population of two towns in Northern Italy were asked to enrol for a study to determine the prevalence of chronic liver disease (Bellantani et al. 1994). 69% of citizens enrolled, of these, 17.5% had persistent evidence of chronic liver disease including 1.1% with cirrhosis and 0.07% with HCC. The prevalence of anti-HCV in the population was 3.2% and HCV was the second leading cause of liver disease after alcohol. The combination of HCV infection and alcohol resulted in a ten fold increase in cirrhosis and a six fold increase in HCC compared with alcoholics who were anti-HCV negative. Among 78 patients with biopsy proven cirrhosis, 28% were related to HCV; 26% to alcohol and 8% to alcohol in combination with HCV.

Asymptomatic anti-HCV positive blood donor populations have also been sampled. In a study of one such population in Barcelona, 8% had minimal changes at liver biopsy, 83% chronic hepatitis and 9% active cirrhosis (Estaban et al. 1991).

These studies suggest that a significant number of patients will develop cirrhosis after several decades of infection. However, they do not explain why some patients, even those with cirrhosis, have a relatively good prognosis, while others progress rapidly to a fatal outcome in less than 5 years. There have been many suggestions made: the more severe cases may be infected by a more virulent strain of HCV; they may have higher virus levels in their liver; they may have a different immune response to the virus or they may have cofactors which enhance the severity of HCV infection. The distinction between host and virus factors in the evolution and severity of HCV infection is examined in detail in the studies which follow.(Table 1.1)
Table 1.1: This table lists the patient, virus and co-factors affecting the severity and progression of hepatitis C infection.

<table>
<thead>
<tr>
<th>Patient Factors</th>
<th>Virus Factors</th>
<th>Co-Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increasing age</td>
<td>Increased virus level</td>
<td>HBV infection</td>
</tr>
<tr>
<td>Mode of transmission</td>
<td>HCV genotype 1b</td>
<td>HIV infection</td>
</tr>
<tr>
<td>(blood transfusion)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of infection</td>
<td>Diverse quasispecies</td>
<td>Alcohol abuse</td>
</tr>
<tr>
<td>Host HLA status</td>
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</table>

The final line of evidence comes from the studies described in the previous session. In these post-transfusion patients there appear to be a stepwise progression from infection, to chronic hepatitis, to cirrhosis, and finally to HCC (Kiyosawa et al. 1990). However, the true causes of this progression are not well understood and these appear to be no reliable predictors of prognosis in infected patients.
1.5iii Hepatitis C Infection and Hepatocellular Carcinoma.

There are several lines of evidence supporting a link between chronic HCV infection and the development of hepatocellular carcinoma. The first is the observation that a significant number of patients with HCC have positive serology for anti-HCV. The prevalence of positive anti-HCV serology varies from 0% (Alaska) to 75% in Spain (Bruix et al. 1989). Although many of these data were collated using first generation EIA for anti-HCV, they have subsequently been confirmed using RIBA testing (Mangia et al. 1994).

The second line of evidence is the demonstration of HCV RNA in the serum of HCC patients by RT-PCR (Ruiz et al. 1992). More recently, HCV RNA has also been demonstrated by RT-PCR in both the surrounding liver tissue and the tumour tissue itself (Gerber et al. 1992; Takeda et al. 1992). Interestingly, the nucleotide sequence of the viral genome from these two sites has been identical where studied (Chou et al. 1991), indicating that the appearance of malignant hepatocytes is not dependent on replication of a different form of HCV than is found in non-malignant liver. Other studies have found positive and negative strands of HCV RNA in malignant tissue and have concluded that HCV must be replicating within tumour tissue (Horiiike et al. 1993). However, these studies do not prove a causal association.

The final line of evidence comes from the studies examined in the previous section. In these posttransfusion cohorts there appears to be a stepwise progression form infection, to chronic hepatitis, to cirrhosis and finally to HCC (Kiyosawa et al. 1990). However, the time course of this progression is measured in decades and there appear to be no reliable predictors of prognosis in affected patients.
The mechanism by which cirrhosis develops in these patients is unclear. One possibility is that the development of HCC is simply related to the presence of cirrhosis; malignant hepatocytes occurring through the mechanism of chronic liver injury, regeneration and repair (Craig et al. 1991). One recent exciting study has described small numbers of patients with chronic HCV infection who have developed HCC on a background of normal liver tissue, indicating that HCV may be oncogenic per se (Demitri et al. 1995). There has been much interest in the effect of virus factors on the development of HCC; at least one report has suggested that the level of HCV RNA in serum is directly related to the severity of liver disease (Kato et al. 1993). However, these initial findings have not been confirmed (Lau et al. 1993). Likewise infection with genotype 1b appears to be associated with more severe liver disease, although this may simply be a “cohort effect”, a hypothesis that is discussed in detail later on (Nousbam et al. 1993; De Mitri et al. 1995).

Sufficient evidence has accumulated that chronic infection with HCV substantially increases the risk of HCC, particularly in the presence of cirrhosis. Indeed, HCV appears to be the major underlying cause of HCC in southern Europe and Japan, regions where HCC incidence is intermediate, whereas its impact is smaller in sub-Saharan Africa and China, where HBV related HCC still predominates.

1.6 Histopathology of HCV Infection.

1.6i Acute HCV Infection.

The hallmark of classical acute viral hepatitis is a combination of diffuse hepatocellular injury, regeneration and the associated inflammatory response. Lobular
disarray is a term used to describe the parenchymal alterations due to the loss of the uniformity of the liver cell plates.

Two forms of liver cell injury are seen in acute viral hepatitis: ballooning and acidophilic degeneration. These degenerative changes reflect different pathogenic mechanisms of hepatocyte damage, rather than a specific aetiology, and are seen in varying proportions in all cases. Ballooning degeneration is characterised by swollen hepatocytes with indistinct cell membranes and pale granular cytoplasm. Ballooned liver cells may lyse and disappear from the liver cell plates, and when this process involves a group of cells, disruption and condensation of the reticulin network occurs.

Acidophilic degeneration is the histological manifestation of apoptosis and is detected morphologically by progressive eosinophilia and shrinkage of the involved hepatocyte. The acidohilic hepatocyte once removed from the hepatic cord, can be seen as a round often refractile body that may or may not contain nuclear material. Free floating acidophil bodies have been referred to as hyaline bodies or Councilman bodies.

Focal necrosis or spotty necrosis are terms used to describe the small intralobular foci of necrotic and apoptotic hepatocytes associated with a poorly circumscribed inflammatory focus of lymphocytes and macrophages. This necroinflammatory lesion is considered a hallmark of acute viral hepatitis.

Other histological characteristics of acute viral hepatitis in the active or fully developed phase include regenerative changes seen in surviving hepatocytes, lobular inflammation (mild diffuse mixed chronic inflammatory infiltrate of lymphocytes with occasional eosinophils and plasma cells), the sinusoidal reaction, hypertrophy of
Kupffer cells, portal tract changes and endothelial or subendothelial inflammation of the hepatic venules and portal veins. Macrovesicular steatosis may or may not be present.

1.6ii Chronic HCV Infection.

By nature of the presentation of the disease, the histopathology of chronic hepatitis C is better described than its acute counterpart; the histological descriptions of hepatitis C made in the 1990s largely supporting the earlier descriptions of non-A, non-B hepatitis (Lefkowitz and Apfelbaum 1989).

Within portal areas, lymphoid infiltrates are nearly omnipresent with lymphoid aggregates or follicles being present in about 50% of cases (Bach et al. 1992). Bile duct damage is generally lymphocytic and non destructive (Poulson and Christofferson 1969). The frequency with which these lesions are seen is about 30% (Lefkowitz and Apfelbaum 1989). While some degree of bile duct loss has been noted in up to 91% of cases (Lefkowitz and Apfelbaum 1989), this remains controversial and clinically significant ductopaenia is not a feature of HCV infection. Lymphocytic piecemeal necrosis (now referred to as “interface hepatitis”) may be present and, along with the presence of lobular inflammation and necrosis, defines disease activity or grade.

Characteristic albeit non-specific lobular alterations are also seen in chronic hepatitis C. Some degree of lobular disarray, inflammation and necrosis of single hepatocytes is common. Multinucleated hepatocytes are seen in 10-30% cases (Bach et al. 1992). Steatosis, usually macrovesicular is present in approximately 70% cases.
An increase in sinusoidal lymphocytes and macrophages, sometimes resulting in a “string of beads” appearance, is present in approximately 80% of cases (Lefkowitch and Apfelbaum 1989).

1.6iii Fibrosis and Cirrhosis.

Deposition of fibrous tissue is an invariable part of chronic hepatitis C infection. It is the progressive and irreversible component of the disease, since it is the fibrous scarring which leads to architectural distortion and cirrhosis.

There are two pathways that may lead to the fibrosis of chronic hepatitis. The first involves collagen deposition and capillarisation of the sinusoids that accompanies the progressive interface hepatitis. As the disease progresses, portal to portal fibrous bridges are formed, between adjacent acini. The second mechanism involves the formation of central to portal and sometimes central to central fibrous bridges.

Contraction of fibrous septa synchronous with nodular regeneration of surviving parenchyma produces architectural distortion, and when the nodules have formed surrounded by fibrous septa, the result is cirrhosis. Before the architecture is completely obliterated and areas maintain acinar structure, the disease state is regarded as “incomplete cirrhosis”.

1.6iv Neoplastic Transformation.

Hepatocellular carcinoma may be the terminal event in patients with chronic HCV infection. There are two principal lesions which are regarded as precursors of most cases of HCC; these are liver cell dysplasia and adenomatous hyperplasia.
Liver cell dysplasia refers to the hepatocyte nuclear changes that may be demonstrated in cirrhotic or precirrhotic liver disease, although whether these are true precursors of HCC remains controversial. Dysplastic nuclei are large and hypochromatic, often with an irregular shape and one or more nucleoli, similar to the nuclei of malignant hepatocytes. They have been described in association with hepatitis B infection, and also in NANB hepatitis (Lefkowitch and Apfelbaum 1987).

Adenomatous hyperplasia is more widely regarded as a premalignant lesion (Nakanuma et al. 1993). The term is often used interchangeably with macroregenerative nodule in referring to a cirrhotic nodule that is considerably larger than other nodules. Thus by definition this lesion occurs only in the setting on established cirrhosis. In a small series of patients undergoing liver transplantation in the US 23% to 30% of explanted livers with end stage liver disease had one or more of these nodules suggesting that this a frequent occurrence in the advanced stage of chronic HCV infection (Thiese et al. 1992; Ferrel et al. 1992).

1.6v Demonstration and Localisation of the Virus.

A variety of techniques have been used to demonstrate and localise the virus in tissue sections; however none have proven reliable enough to use routinely in diagnostic work.

Electron microscopy was used throughout the 1980s to attempt to identify the NANB virus; many virus particles were demonstrated but in retrospect, none were HCV (DeVos et al. 1983). It was not until 1996 that electron microscopy successfully
identified HCV, but clearly this remains a research tool at present (Shimizu et al. 1996).

Immunohistochemistry has been used to identify the viral antigen in the cytoplasm of infected cells. Frozen cryostat sections using immunofluorescence or immunoperoxidase stains, usually with one or more monoclonal antibodies, have had the greatest success; however, even with optimal technique, no more than 75% of patients with HCV have demonstrable antigen in a liver biopsy, whereas all have viral RNA in serum by RT-PCR (Krawcynski et al. 1992; Sansanno et al. 1993; Gonzalez-Peralta et al. 1994).

In situ hybridisation has been successful in demonstrating HCV RNA; frozen sections are required, and the results are comparable with immunohistochemistry (Tanaka et al. 1993). In situ RT-PCR has been used to improve the detection sensitivity of in situ hybridisation. This will undoubtedly become the standard by which other techniques are assessed in the future (Lau et al. 1996).

1.6vi Histological Activity Index (HAI).

The HAI or “Knodell score” is a semiquantitative method proposed to standardise interpretation of the biopsy, allowing comparison between subsequent biopsies in the same patient and between different patients in large studies (Knodell et al. 1981).

Numerical scores are assigned for each grade of the salient histological features and then totalled to yield the total HAI. The observations are still subjective,
involving grading of various histological features as mild, moderate or severe, but the method is fairly simple and the numerical result may be used in statistical calculations.

Other grading methods have been proposed to modify the HAI, but this remains the international standard scoring system (Desmet et al. 1994).

1.6vii An Overview of the Mechanisms of Liver Injury in Hepatitis C Infection.

In the absence of a high titre in vitro tissue culture system, the ability of HCV to cause cytopathic damage independently of any host immune response cannot be excluded. There is some evidence that HCV can, in certain circumstances, induce tissue damage directly. For example, a syndrome of rapidly progressive liver destruction has been reported in HCV-infected liver allograft recipients. Histologically, there is a relative paucity of lymphocytes, and these individuals have markedly depressed cell-mediate immunity, thus suggesting that it is the presence of HCV itself which causes liver damage.

Conversely, several lines of evidence suggest that it is the immune response that leads to tissue injury. First, cytopathic viruses rarely lead to persistent viral infections. Second, the typical histological picture is notable for lymphoid infiltration of the liver, not cytopathic changes in the infected cells. Third, there are many individuals with hepatitis C viraemia who have persistently normal transaminases and no apparent liver cell destruction. Fourth, studies have now reported transgenic mice which express the structural proteins of HCV, but none of these describe any evidence of liver cell damage in the animals (Kawamura et al. 1997; Pasquinelli et al. 1997). It may be possible that high level expression of some HCV gene products may even
enhance cellular susceptibility to cytokines or other mediators of liver cell destruction. In hepatitis B virus transgenic mice, high level expression of a retained form of HBs enhances cytotoxicity of these cells to interferon gamma (Chisari et al. 1995). Similarly, in vitro it appears that high level expression of HCV core leads to an enhanced sensitivity to the cytotoxic effects of TNF alpha (Ruggieri et al. 1997). Thus, high titres of HCV, such as those seen after liver transplantation, may sensitise cells to the lytic effects of cytokines. In general, therefore, it is most likely that the host immune response to the virus is responsible for the liver cell damage.

1.7 Current and Evolving Therapies for Chronic Hepatitis C Infection.

The rationale for therapy in chronic HCV infection is to arrest activity, improve symptoms and prevent progression to complicated liver disease. Throughout the past decade, the mainstay of therapy for chronic HCV infection has been interferon-alpha (IFN-α). The IFN-α family consists of greater than 20 subspecies of proteins and glycoproteins produced by many cell types, which have diverse antiviral, antiproliferative and immunomodulatory effects (Johnson et al. 1994). The precise mechanism of action of IFN-α in chronic HCV infection is unclear; however, it is likely to have one of, or a combination of immunoregulatory, anti-inflammatory or antiviral actions. World-wide, four forms are available for treatment of chronic HCV infection: IFN α-2b; IFN α-2a; IFN α-n1 and consensus interferon (CIFN). IFN α-2b is produced by recombinant DNA techniques using a strain of E. coli bearing a genetically engineered plasmid containing an IFN α-2b genome from human leucocytes. It is currently licensed for a dose of 3MU, three times weekly for 6 or 12
months therapy; the production and licensing of IFN α-2a is similar. IFN α-n1 is a mixture of 9 IFN subtypes produced from a human B lymphoblastoid line; its license is for 5Mu, three times weekly for 48 weeks. Finally, consensus interferon has been developed by scanning subtypes of α-IFN and assigning the most frequently observed amino acid at each position to form a consensus molecule; the DNA coding sequence was then synthesised and cloned in a recombinant system (Dhib-Jalbut et al. 1996). Its activity is as for IFN α-n1.

Assessment of the efficacy of interferon therapy may be undertaken by primary and secondary end points. Early trials examined the normalisation of ALT in treated cohorts both at the end of six months therapy and at least six months after the completion of treatment (biochemical response) (Davis et al. 1990); whilst later studies marked the success or failure of therapy by the presence of detectable HCV RNA in serum by the most sensitive assay available, usually the reverse transcription polymerase chain reaction (sensitivity > 10² virus copies /ml: virological response). Subsequently, a sustained response has been defined as a normal transaminase or no detectable HCV RNA at the end of therapy and throughout the observation period on at least two separate occasions. The interrelation, between biochemical and virological responses in terms of prognosis has been poorly defined. Secondary end points of therapy may also be assessed by undertaking liver biopsies before and after interferon. Meta-analyses of the efficacy of interferon therapy from the many multi centre trials already carried out suggest that after 3mu of interferon for 6 months, the biochemical end of treatment response ranges from 35 to 50%, whilst the sustained response ranges from 8 to 21% (Carithers and Emerson 1997; Lee 1997; Farrell...
Likewise, the virological end of treatment response ranges from 27 to 35%, and the sustained response from 8 to 12% (Carithers and Emerson 1997; Lee 1997; Farrell 1997). In studies in which a 12 month regimen was used, biochemical end of treatment response rates were not appreciably different from those with 6 month courses, but the likelihood of relapse was diminished; sustained response rates at 6 months, 19% to 42% were nearly twice those reported with 6 month courses of therapy (Carithers and Emerson 1997; Lee 1997; Farrell 1997). Conversely, the effect of varying the dose of IFN is unclear since the optimal dose needs to be standardised per product because of the different biological potency of agents.

Since the biochemical and virological response to IFN monotherapy has been almost universally less than 50%, it is likely that physicians choosing to pursue this treatment will use pre-selection criteria and selection criteria during therapy itself. Multivariate analyses assessing response to interferon have already indicated that the viral genotype (particularly genotype 1) or the presence of cirrhosis or fibrosis are independent factors influencing the response to six months of therapy (Lindsay et al. 1996; Martinot-Peignoux et al. 1995). Likewise, a low baseline HCV RNA level is associated with a sustained response but not necessarily a response after 12 weeks of therapy (Lindsay et al. 1996; Martinot-Peignoux et al. 1995). Conversely, the serum ALT level has been demonstrated by multivariate analysis to have little importance in predicting the outcome of therapy; indeed, there are now several studies suggesting that patients with a normal ALT almost universally have abnormal liver histology, and that the rate of sustained virological response compared with patients with an abnormal ALT is unchanged (Stanley et al. 1996). Finally, during the first decade of
therapy, many authors suggested that a mild hepatitis on liver biopsy is synonymous with a benign natural history; the evidence for this is sparse, and in the absence of large long-term follow up studies, patients presenting with any degree of hepatitis at the time of liver biopsy, must be presumed to be at risk of developing significant and progressive liver disease in the future. Paradoxically, nonfibrotic liver disease is also associated with a higher likelihood of a week 12 response to interferon than fibrotic liver disease, thus, treatment of patients with mild liver disease may improve interferon therapy results, prolong survival, and ultimately be more cost effective in the management of chronic hepatitis C infection.

Following contemporary successes using combination therapy to treat chronic hepatitis B and human immunodeficiency virus infections, similar modes of combination therapy have been suggested for HCV infection in the next decade. Current experience of combination therapy has been obtained through the use of IFN α-2a and ribavirin. Ribavirin is a guanosine analogue with broad spectrum activity against DNA and RNA viruses including flaviviridae; however, again the mechanism of action is poorly understood. There are two possibilities: the first is that ribavirin depletes intracellular triphosphate pools through inhibition of the 5 cap structure of viral messenger RNAs and inhibition of viral-dependent RNA polymerases. The second is that the drug acts as an immune modulator, altering the balance between Th1-like (pro-inflammatory) and Th-2 like (anti-inflammatory) cytokines. Prior to the initiation of combination therapy, primary, randomised, double-blind placebo-controlled trials examined the efficacy of ribavirin alone in the treatment of hepatitis C infection (Di Bisceglie et al. 1995). End of treatment biochemical responses ranged
from 21% to 43% compared with a placebo; however, no patients in any study achieved a virological cure. In addition, there was a histological improvement in some patients treated for a year; comparison of biopsies before and after therapy, suggested a reduction in necro-inflammatory activity in patients in whom the ALT improved. These early data also noted the not inconsiderable side effects of ribavirin: haemolysis, fatigue, depression, anorexia, nausea and pruritus.

Following the failure of ribavirin monotherapy to achieve virological cure, drug trials have assessed the combination of ribavirin and interferon in the treatment of chronic HCV infection. Pilot studies have concluded that up to an 80% sustained virological and biochemical response can be achieved in patients who had previously relapsed after an end of treatment response to a previous course of interferon (Brillanti et al. 1994). Conversely, a range of 0-25% of non-responders to a primary course of interferon had a sustained virological and biochemical response to combination therapy. A recent meta-analysis from Europe using individual patient results, concluded that patients with a traditional low response to interferon monotherapy (those infected with HCV genotype 1b or with cirrhosis on their liver biopsy) had a two to three times increased rate of sustained response compared with interferon alone (Schalm et al. 1997). Finally, there has been one published, randomised, double-blind, placebo controlled study of combination therapy in “naïve” patients (Reichard et al. 1996). Fifty patients were randomised to receive interferon and placebo, whilst fifty were randomised to receive interferon and ribavirin. The end of treatment virological and biochemical response rates were the same; however, the virological sustained response was significantly higher in the combination therapy
group of patients (47%) compared with the placebo group (23%). In summary, ribavirin alone is of limited efficacy in the treatment of chronic HCV infection; however, ribavirin-interferon combination therapy should be the treatment of choice for individuals who have relapsed after taking primary therapy. Currently, it is impossible to recommend combination therapy as a first line treatment in HCV infection; however, several multinational, multicentre, randomised controlled trials are under way comparing the value of different lengths of treatment (24, 48 and 57 weeks), the response of different HCV genotypes, virus levels and histology.

Pilot studies using additional novel antiviral drugs in the treatment of chronic HCV infection are also emerging (Table 1.2). Amantidine, a drug with partial efficacy in influenza, has a surprising effect in HCV infection at a much lower dose than is required to inactivate other enveloped viruses, once again suggesting a mechanism of action distinct from the expected antiviral one, and raising the possibility of its inclusion in combination drug therapies in the future (Smith et al. 1996). Thymosin is a short peptide of 28 amino acids derived from the thymus with immunomodulatory properties. In the largest randomised, double-blind trial, 110 subjects with HCV infection were randomised to receive interferon, interferon and thymosin or placebo; the end of treatment virological and histological response rates were 17%, 42% and 3% respectively (Sherman et al. 1996). The histological improvement was greatest in the combination therapy group; however, after several courses of treatment, the sustained virological response was 14.7% in the combination group and 7.9% in the interferon group. Other combination therapies incorporating thymosin have not yet been assessed.
<table>
<thead>
<tr>
<th>Host</th>
<th>Virus</th>
<th>Notes</th>
</tr>
</thead>
</table>
| Alpha-interferon | -↑number of Ab producing cells  
-↑number/activity of T-lymphocytes  
-↑upregulation of cytokine receptor expression | Effect on viral replication minimal in chronic HCV infection  
Exact mechanism of action unknown in HCV infection |
| Ribavirin     | Inhibits transcription of viral RNA                                    | Exact mechanism of action unknown in HCV infection                  |
| Ursodeoxycholic acid |                                                                  | Mechanism unclear; may prevent damage to hepatocytes by endogenous hydrophilic bile acids or may have direct cytoprotective effects on the hepatocyte |
| Protease inhibitors | -Inhibition of HCV serine protease and metaloprotease required for polyprotein processing |                                                                  |
| Thymosins     | -Regulation of T-lymphocyte maturation  
-Regulation of lymphocyte production  
-Stimulation of IL-2 expression |                                                                  |

Table 1.2: Summary of the modes of action of drugs currently under development for use in chronic hepatitis C infection.
In conclusion, in the next decade, combination therapy of interferon and ribavirin will become standard treatment for patients with recurring disease after interferon monotherapy and perhaps naïve patients who after staging, are likely to have a low response rate to interferon monotherapy (those infected with HCV genotype 1b or with cirrhosis on their liver biopsy). Secondly, emerging combination therapies, guilded by parallel successes in the treatment of chronic HBV and HIV infection are likely to further improve sustained responses; future triple therapy will perhaps consist of interferon, ribavirin and an HCV protease or RNA polymerase inhibitor.

1.8 Clinical Staging and Management of Chronic HCV Infection.

This section is an overview of a rational approach to the clinical staging and management of chronic HCV infection using widely available investigations and licensed therapy (Dusheiko et al. 1996).

1.8i Clinical Assessment of Chronic HCV Infection.

Most patients with chronic HCV infection are asymptomatic or at the most mildly symptomatic; the most common symptom described is fatigue. Usually, patients have no past medical history of acute hepatitis or jaundice and commonly there are no abnormal physical findings on examination. With more severe disease, signs of chronic liver disease may be elicited: spider naevi, palmer erythema, hepatomegaly or systemic manifestations of chronic HCV infection, e.g. cryoglobulinaemia. Serum transaminase
level may fluctuate over time, remain elevated or stay at a normal level. With the development of cirrhosis, weakness, wasting, oedema, ascites and variceal haemorrhage may become progressive problems. Older patients often present for the first time with complications of cirrhosis or even hepatocellular carcinoma.

1.8ii Routine Investigations Required to Stage Chronic Hepatitis C Infection.

Following confirmation of a positive anti-HCV serology result, the serum ALT is measured to define those patients who have a normal level, and the presence of HCV RNA determined by RT-PCR as a baseline to monitor future antiviral therapy and virological response. Liver biopsy should then be considered in all patients with abnormal transaminases and also in those who have a positive HCV RNA result (with or without a normal ALT level). Liver biopsy will ascertain the “grade” (severity and extent of hepatic inflammation) and the “stage” (degree of fibrosis or presence of asymptomatic cirrhosis) of the disease process. Patients repeatedly negative for HCV RNA by RT-PCR are not routinely biopsied and at present there is no consensus as to whether they require long-term follow up or not.

As discussed in the previous section, long-term response rates to interferon therapy have been disappointing, and one approach to improving response rates might be to categorise patients as likely “good or poor” responders at the time of staging the disease process. Thus, if HCV genotyping and quantification are routinely available, patients infected by genotypes 1a, 1b or 4, and / or a virus level > 10⁷ copies/ml might be selected as likely non-responders and either offered a longer duration of therapy or perhaps a trial of combination therapy (e.g. Interferon and Ribaviron). On the
otherhand, patients infected by other genotypes or with a virus level $< 10^6$ would receive a standard regimen of interferon therapy as described in the previous section.

1.8iii Assessment of a Successful Response to Therapy.

It is reasonable to infer that patients with a normal serum ALT, negative for HCV RNA and with histologically improved disease activity a year after stopping interferon, have had a meaningful response to therapy. Indeed, sustained clearance of HCV RNA is the most likely outcome to have tangible benefit in patients who have not developed cirrhosis. In practice, facilities are simply not available to repeatedly biopsy treated patients, and serum ALT and HCV levels are the usual parameters employed to assess response (Table 1.3).

In many patients there is a disparity between the biochemical, virological and histological response. Patients RT-PCR positive at the end of therapy (but with a normal ALT) usually relapse. Conversely, undetectable HCV RNA at the end of treatment does not preclude a relapse in serum ALT activity in patients after stopping interferon (presumably because replication of hepatic HCV has continued despite therapy), but it lessens the probability (Lau et al. 1993; Pawlotsky et al. 1994). Indeed, a comprehensive virological cure has been defined as the absence of HCV RNA from liver tissue, serum and PBMC (Berenguer et al. 1995); however, assessment of the presence of HCV RNA at all these sites by RT-PCR is again simply not practical in all patients a year after the completion of treatment.
<table>
<thead>
<tr>
<th></th>
<th>Non-response</th>
<th>Partial response</th>
<th>Sustained response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALT</strong></td>
<td>Remains abnormal</td>
<td>Transient decrease or normalisation</td>
<td>Normalisation</td>
</tr>
<tr>
<td><strong>HCV RNA in serum by RT-PCR</strong></td>
<td>Remains positive</td>
<td>Initially negative, then reverting to positive</td>
<td>Negative for six months after therapy</td>
</tr>
<tr>
<td><strong>HCV level by quantitative PCR</strong></td>
<td>Unchanged</td>
<td>Initial decrease, then reverting to pre-treatment levels</td>
<td>Undetectable</td>
</tr>
</tbody>
</table>

Table 1.3: Definitions of clinical response to alpha-interferon therapy
1.9 Summary.

(1) Hepatitis C virus is a prototype of the Flaviviridae family of viruses; it consists of a single stranded RNA genome.

(2) Hepatitis C demonstrates two forms of genetic heterogeneity. The first is genetic heterogeneity of the HCV population within an infected individual (“Quasispecies”). The second is genetic heterogeneity amongst different HCV isolates (“genotypes”).

(3) There are many data suggesting that HCV genotypes may have different clinical manifestations, either determining relative pathogenicity or response to interferon therapy.

(4) Individuals with hepatitis C infection may present through the discovery of abnormal liver function tests; the discovery of abnormal LFTs during a health screen; the discovery of HCV antibody in a healthy blood donor or the screening of high risk individuals.

(5) Third generation anti-HCV ELISAs include antigens from the putative core, and from the NS3, NS4 and NS5 regions. ELISA-3 tests are 99.7% effective at presenting the transmission of HCV to recipients. All positive ELISA tests are confirmed by RIBA testing.
(6) RT-PCR for HCV RNA confirms HCV viraemia and is invaluable in early diagnosis of acute infection; confirmation of infection in patients who cannot mount an antibody response; follow up of drug therapy and monitoring perinatal transmission.

(7) 60% of HCV patients have normal ALT values, and most (80%) asymptomatic individuals with HCV viraemia have chronic hepatitis or cirrhosis.

(8) The highest geographical prevalence of HCV infection occurs in Japan, the southern states of the USA, the Mediterranean countries, Africa and the Middle East. Seroprevalence of anti-HCV antibodies has been suggested to be 0.02% in the UK.

(9) Amongst populations, the highest prevalence rates are demonstrated in cohorts of posttransfusion patients; intravenous drug abusers and haemophiliacs. The role of permucosal spread and covert parenteral exposure in HCV infection are not well defined.

(10) Acute hepatitis C infection is usually a benign event; following posttransfusion transmission of HCV, 70 to 80% of cases are anicteric and asymptomatic.

(11) The natural history of chronic liver disease secondary to HCV infection is a measure of decades rather than years. Studies have suggested that the probability of developing cirrhosis was 20% after a mean interval of 16 years.
Evidence for an association between HCV infection and hepatocellular carcinoma is provided by data demonstrating a significant number of patients with HCC have positive serology for anti-HCV; by the demonstration of HCV RNA in the serum and tissue of patients with HCC; and the stepwise progression of disease in posttransfusion cohorts.

In general, histological findings, in acute and chronic HCV infection are not specific for the virus. Most biopsies will demonstrate portal inflammation; bile duct lesions; "interface hepatitis" and intra-acinar necroinflammatory changes.

The HAI ("Knodell Score") is a semiquantitative method proposed to standardise interpretation of liver biopsies, allowing comparison between subsequent biopsies in the same patient and between different patients in large studies.

HCV has been demonstrated in tissue sections by: immunohistochemistry; in situ hybridisation, and by electron microscopy.

Interferon-α is the mainstay of therapy in chronic HCV infection; interferons have both antiviral and immunomodulatory action. The rationale for therapy in HCV infection is to arrest activity, improve symptoms and prevent progression to complicated liver disease.
2.1 Diagnostic Laparoscopy and Liver Biopsy.

Serum and liver biopsy samples were obtained for the following studies (Chapters 3, 4, 5) at the time of diagnostic laparoscopy and liver biopsy. This was undertaken as previously described (Haydon et al. 1997).

Briefly, the procedure was carried out under local anaesthesia with mild sedation and analgesia. Following infiltration of the skin and subcutaneous tissue with 2% lignocaine, a veress needle was inserted 2cm below the umbilicus in the midline. The skin is first divided using a horizontal incision, and the muscle fibres bluntly dissected down to the peritoneum. Two litres of nitrous oxide were used to insufflate the peritoneal cavity, the veress needle was removed and a disposable trochar inserted through the skin incision.

The liver was viewed with a 5mm oblique viewing laparoscope: the falciform ligament was inspected for portal hypertension (the presence of dilated veins), followed by the left and right lobes of liver, gall bladder and peritoneum. Liver biopsies were taken with a “truecut” needle inserted at a site near the midline, inferior to the subcostal margin. Unless a focal lesion was demonstrated, biopsies were taken from segments II/III of the left lobe of liver, at an angle of 45°, away from the
anterior edge of the liver or the falciform ligament. Once haemostasis was achieved, the laparoscope was removed and a single silk suture applied to the insertion site.

2.2 Collection of Samples.

A small portion of the liver biopsy taken at laparoscopy was washed in ice cold normal saline, blotted on a sterile swab and immediately frozen in liquid nitrogen and stored at -70°C for future use. The remainder of the biopsy underwent histological analysis.

Serum samples were obtained, separated within 3 hours of collection on the day of laparoscopy and stored at -70°C. All samples were stored for 3-6 months maximum before analysis.

2.3 HCV RNA Extraction Techniques: Serum Samples.

RNA extraction from serum samples was carried out, as previously described (Jarvis et al. 1994), using a modification of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski et al. 1987). Briefly, viral RNA was extracted from 0.1 or 0.5ml of stored sera after pelleting virus by centrifuging at 100,000g for 90 min at 4°C and incubating at 37°C for 2 hours with 1mg/ml polyadenylic acid, 0.5% SDS, 0.1M NaCl, 50 mM TRIS HCl (pH 8.0), and 1mM EDTA. RNA was extracted with phenol; after centrifugation, the supernatant was re-extracted successively with phenol and chloroform-isoamyl alcohol (50:1). Nucleic acid was precipitated by the addition of one-tenth volume of
sodium acetate (pH 5.2) and 2 volumes of ethanol. The dried pellet was resuspended in 25μl of diethylpyrocarbonate treated water.

2.4 HCV RNA Extraction Techniques: Liver Biopsy Samples.

RNA extraction from liver biopsy samples was carried out using a commercial modification (RNAzol™, Biogenesis Ltd, Bournemouth, UK) of the single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski et al. 1987).

Tissue samples were homogenised with RNAzol solution (phenol derivative) and RNA was extracted by centrifugation at 12,000g for 15 minutes at 4°C with chloroform. RNA was precipitated by addition of isopropanol to the aqueous phase; the samples were stored for 15 minutes at 4°C and then centrifuged for 15 minutes at 12,000g (4°C); the RNA precipitates formed a white-yellow pellet at the bottom of the tube. The supernatant was removed and the RNA pellet washed once with 75% ethanol by vortexing and centrifugation for 8 minutes at 7,500g (4°C). The dried pellet was resuspended in 25μl diethylpyrocarbonate treated water.

2.5 HCV RNA Reverse Transcription Polymerase Chain Reaction.

HCV RNA was reverse transcribed by nested PCR using 5’noncoding region specific primers 939, 209 (outer sense and antisense respectively), 940 and 211 (inner sense and antisense respectively) as previously described (Chan et al. 1992). Briefly, first strand cDNA was synthesised from 5μl of RNA sample at 42°C for 30 minutes with MMV reverse transcriptase (Promega), reverse transcriptase buffer (Promega),
antisense primer for 5’NCR (primer 209) and nucleic acids (dATP, dCTP, dGTP and TTP). Further primers amplified a section of the housekeeping gene β-actin mRNA, which served as a reference to control for differences in cDNA concentration.

A primary PCR reaction was carried out using 5ul cDNA, Taq polymerase (promega), Taq buffer (Promega), nucleic acids and each of the outer nested primers. 1ul of the reaction mixture was then transferred to a second tube containing the same medium but with the inner pair of nested primers for a secondary PCR reaction.

The PCR assay has proved to be both sensitive and specific for the detection of HCV RNA in serum (Dow et al. 1993). To monitor possible contamination with exogenous sequences during extraction or setting up of the amplification reactions, RNAs from at least three negative and three positive control serum samples were extracted, reverse transcribed and amplified in each batch of donor samples examined by PCR.

The secondary PCR products were subjected to electrophoresis in 2% low melting point agarose gel and the fragments were detected by ethidium bromide staining and ultra violet illumination (Figure 2.1).

2.6 Restriction Fragment Polymorphism Analysis of Synthesised HCV cDNA

Previously it has been demonstrated that all six currently defined major genotypes can be distinguished on the basis of restriction fragment polymorphism analysis of RT-PCR products derived from the 5’NCR (Nakao et al. 1991; McOmish et al. 1993; McOmish et al. 1994; Simmonds et al. 1994).
Figure 2.1: Following the primary and secondary PCR reactions, the secondary PCR products were subjected to electrophoresis in 2% low melting point agarose gel and the fragments were detected by ethidium bromide staining and u-v illumination. This figure shows from left to right (3 negative controls; 3 positive controls; 1 negative sample; 5 positive samples).
Secondary PCR products were cleaved with pairs of restriction enzymes: Hae III/RsaI in one reaction and MvaI/Hinfl in the second (Figures 2.2 and 2.3). Restriction digests were carried out on 25μl of secondary PCR product for 4-16 hours after adjustment with 10 x enzyme reaction buffer as appropriate; reactions were carried out at 37°C. Digestion fragments were visualised under ultraviolet light after electrophoresis through 4% Metaphor agarose gel on x1 Tris borate buffer containing ethidium bromide.

The HCV genotype present is deduced by the combination of electropherotypes produced (McOmish et al. 1994). By using the combination HaeIII/RsaI, different banding patterns were produced for five of the six genotypes (genotypes 1/5;2;3;4;6); genotypes 1 and 5 were reliably differentiated using MvaI/Hinfl (Table 2.1). Phylogenetic comparison of sequences in the core, E1 and NS5 regions has confirmed that the 5’NCR can be used to reliably distinguish the six major genotypes (McOmish et al. 1994). The predicted accuracy of RFLP analysis of HCV cDNA for genotypes 1 to 6 is 96%.

Furthermore, it was possible to separate subtypes 1a and 1b and 2a and 2b by the cleavage patterns produced from digestion with MvnI/ BstU I and ScrFl/ Hinfl I respectively (Davidson et al. 1995).

2.7 Other Available Techniques for HCV Genotyping.

Several methods have been developed for typing HCV variants: details of their scope, accuracy and relative cost are given in Table 2.2. Types 1a, 1b, 2a, 2b and 3a can readily be distinguished by RT-PCR amplification of virus RNA, using type-specific primers designed to match only individual virus genotypes (Okamoto et al.
Figure 2.2: HCV genotyping. Secondary PCR products from 10 patients cleaved with the first pair of restriction enzymes Hae III/ Rsa I. Typical restriction digest patterns for genotypes 1 and 3 are demonstrated (From left to right: genotype 3 restriction digest pattern in the first well; genotype 1 restriction digest pattern in the second well). Size of PCR fragments is also labeled for reference.
Figure 2.3: HCV genotyping. Secondary PCR products from 10 patients cleaved with the second pair of restriction enzymes Mva I/ Hinf I. Typical restriction digest patterns for genotypes 1 and 3 are demonstrated (From left to right: genotype 1 restriction digest pattern in the first well; genotype 3 restriction digest pattern in the second well).
<table>
<thead>
<tr>
<th>Cleavage pattern</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>a 44 58</td>
<td>1; 3</td>
</tr>
<tr>
<td>b 102</td>
<td>1; 4; 5</td>
</tr>
<tr>
<td>c 44 46 58 56</td>
<td>2</td>
</tr>
<tr>
<td>d 44 46</td>
<td>2</td>
</tr>
<tr>
<td>e 56 46</td>
<td>2</td>
</tr>
<tr>
<td>f 33 69</td>
<td>3</td>
</tr>
<tr>
<td>g 33 46</td>
<td>3</td>
</tr>
<tr>
<td>h 44 46</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cleavage pattern</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 53 63 94</td>
<td>1</td>
</tr>
<tr>
<td>B 53 63 94</td>
<td>2</td>
</tr>
<tr>
<td>C 53 56 142/3</td>
<td>3; 4</td>
</tr>
<tr>
<td>D 53 198</td>
<td>2; 5</td>
</tr>
</tbody>
</table>

Table 2.1: Predicted association of different cleavage patterns of 5'NCR with sequences of HCV types 1 to 6 by cleavage with Hae III-Rsa I (Top) and Mva I- Hinf I (Bottom); few of these cleavage patterns were demonstrated in the Scottish populations studied. The expected sizes of restriction fragments are shown in the central columns. The numbers of the genotype associated with each pattern is given in the column on the right.
<table>
<thead>
<tr>
<th>Method</th>
<th>Region</th>
<th>Types Detected</th>
<th>Subtypes Detected</th>
<th>Associated Problems</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFLP</td>
<td>5’NCR</td>
<td>1-6</td>
<td>1a/1b</td>
<td>Misidentification of novel genotypes</td>
<td>Accurate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2a/2b</td>
<td>Limited detection of subtypes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3a/3b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFLP</td>
<td>Core</td>
<td>1</td>
<td>1a/1b</td>
<td></td>
<td>Detections novel variants of SE Asia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>plus novel variants of SE Asia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligonucleotide hybridisation</td>
<td>5’NCR</td>
<td>1-5</td>
<td>1a/1b</td>
<td>As 5’NCR RFLP</td>
<td>Reliable for identification of genotypes in Western countries</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2a/2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type-specific primers</td>
<td>Core</td>
<td>1-3</td>
<td>1a/1b</td>
<td>Difficult to extend to new genotypes</td>
<td>Rapid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2a/2b</td>
<td>Technically difficult</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3a</td>
<td>Misidentification of mixed infections</td>
<td></td>
</tr>
<tr>
<td>DNA enzyme immunoassay</td>
<td>Core</td>
<td>1-3</td>
<td>1a/1b</td>
<td>Limited in range of genotypes detected</td>
<td>Accurate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2a/2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slot-blot hybridisation</td>
<td>NS5</td>
<td>1,2</td>
<td>1a/1b</td>
<td>Limited in sensitivity and specificity</td>
<td>Uses more variable region of genome</td>
</tr>
<tr>
<td>Sequencing</td>
<td>E1/E2</td>
<td></td>
<td>2a/2b</td>
<td>Technically demanding</td>
<td>Definitive genotype identification</td>
</tr>
<tr>
<td></td>
<td>NS5</td>
<td></td>
<td>All</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Core</td>
<td></td>
<td>All</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. A comparison of different methods available for genotyping Hepatitis C virus.
This technique requires careful quality control however, as it can mistakenly identify some samples as being mixed infections.

An alternative method to RFLP analysis of HCV cDNA reverse transcribed and amplified from the 5'NCR is to detect type-specific substitutions in the amplified cDNA by hybridisation to type-specific oligonucleotides immobilised on nitrocellulose membrane (line probe assay (LiPA, Innogenetics)). The predicted accuracy of this method for determining the presence of genotypes 1 to 6 is 84% (Smith et al. 1995) (compared with 96% for RFLP analysis), although higher accuracy should be achieved with more recent versions of LiPA (Stuyver et al. 1995). LiPA is unable to distinguish type 1 viruses from some of the recently described type 6 related variants found in Southeast Asia, and it is unable to distinguish between the myriad of subtypes described for types 1, 2, 3 and 4. Both RFLP and LiPA methods are currently being extended for RT-PCR products derived from the more variable core region of the HCV genome (Mellor et al. 1996; Stuyver et al. 1995). RT-PCR products may also be analysed by hybridisation to cDNA probes specific to each genotype (Viazov et al. 1994; Enomoto et al. 1990). A major limitation to all assays is the large number of genotypes currently described, and the necessity to continually modify each system as new sequence variants are discovered.

An alternative method for genotyping is based on serological responses of the host to virus infection. Different virus genotypes encode proteins sufficiently divergent that serological responses to some antigens have a significant type-specific component. A genotyping assay capable of distinguishing between infection with types 1 to 6 has been developed using peptides from antigenic regions of NS4a
Similar assays, based on peptides derived from the core region (Machida et al. 1992) or recombinant NS4 polypeptides (Tanaka et al. 1994) have also been described. These last two assays, however, only distinguish between genotypes 1 and 2. None of the serological assays are able to distinguish between HCV subtypes. In addition, they are generally unsuitable for patients with impaired immune response, for example those co-infected with HIV (Table 2.3).

Direct sequencing and comparison with consensus sequences is certainly the most exact method for genotype and subtype analysis, but it is rather cumbersome and needs sophisticated facilities to manage a large number of clinical samples. These methods rely on analysis of more variable regions of the virus genome, such as the E1, E2 or NS5 genes (Simmonds et al. 1994). The role of sequencing is likely to be in epidemiological studies of virus transmission and medico-legal investigations. However, even here, caution is required in the interpretation of such studies. For example, the extent of variation between viruses in epidemiologically unrelated individuals within particular communities may have to be investigated specifically to generate appropriate control sequences that can determine the significance of sequence clusters in cases of suspected transmission.

2.8 Semi-Quantitation of Hepatitis C Virus Levels Using Limiting Dilution

Both liver and serum samples in the following studies were quantified by limiting dilution of cDNA reverse transcribed from HCV RNA. This has been described in detail elsewhere (Simmonds et al. 1990a; Simmonds et al 1990b).
<table>
<thead>
<tr>
<th>Method</th>
<th>Region</th>
<th>Types Detected</th>
<th>Subtypes Detected</th>
<th>Associated Problems</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>NS4 (peptides)</td>
<td>1-6</td>
<td>-</td>
<td>Does not identify subtypes. Problems with seronegative individuals and distinguishing past and current infections</td>
<td>Accurate, easy to carry out</td>
</tr>
<tr>
<td>ELISA</td>
<td>Core (peptides)</td>
<td>1,2</td>
<td>-</td>
<td>As above, plus difficult to identify coinfections</td>
<td>As above</td>
</tr>
<tr>
<td>ELISA</td>
<td>NS4 (recombinant proteins)</td>
<td>1,2</td>
<td>-</td>
<td>As Core ELISA</td>
<td>As above</td>
</tr>
</tbody>
</table>

Table 2.3 A comparison of different methods available for serotyping Hepatitis C virus.
Briefly, five microlitres of HCV cDNA were diluted in a series (5 in the case of serum samples and 7 in the case of liver biopsy samples) of 10 fold steps which allowed cDNA to be quantified to within 1 log of its actual concentration. Further refinement of the quantitation by adding a specific volume of cDNA to a number of replicate PCR reactions, thus giving a Poisson distribution of positive and negative samples and allowing the exact HCV concentration to be determined, has been demonstrated not to be necessary to improve the accuracy of the assay if the nearest whole log only, is required.

Positive controls (of known dilution e.g. $10^3$ or $10^5$ copies/ml) were run concurrently with each limiting dilution analysis to ensure reproducibility of the assay. Serum samples from healthy individuals without risk factors were examined for HCV RNA as negative controls (Figure 2.4).

The previously established efficiency of 5% for the reverse transcription reaction was assumed in this assay (Simmonds et al. 1990a; Simmonds et al. 1990b). Centrifugation of 0.1ml sera provided a detection sensitivity of approximately 4000 HCV copies/ml. To increase the sensitivity of the PCR method, samples that were negative at this level of detection (<4000 HCV/ml) were further analysed by centrifugation of 0.5ml (detection sensitivity: 800 HCV copies/ml) or if necessary 5.0ml (detection sensitivity: 80 HCV copies/ml) of sera.

The limiting dilution assay has been demonstrated to have significant reproducibility when multiple samples are tested in duplicate, using RNA extracted on separate days from separate aliquots of sample and different batches of reagents. Likewise a significant correlation has been demonstrated between limiting dilution and three commercial assays: bDNA1, bDNA2 (Chiron, Emeryville, Ca) and Roche.
<table>
<thead>
<tr>
<th>Serial Dilutions</th>
<th>Virus Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: 2 serial</td>
<td>$10^2$</td>
</tr>
<tr>
<td>dilutions</td>
<td></td>
</tr>
<tr>
<td>4: 3 serial</td>
<td>$10^5$</td>
</tr>
<tr>
<td>dilutions</td>
<td></td>
</tr>
<tr>
<td>7: Positive</td>
<td>$10^3$</td>
</tr>
<tr>
<td>control</td>
<td></td>
</tr>
<tr>
<td>2: 4 serial</td>
<td>$10^4$</td>
</tr>
<tr>
<td>dilutions</td>
<td></td>
</tr>
<tr>
<td>5: 5 serial</td>
<td>$10^5$</td>
</tr>
<tr>
<td>dilutions</td>
<td></td>
</tr>
<tr>
<td>8: Positive</td>
<td>$10^3$</td>
</tr>
<tr>
<td>control</td>
<td></td>
</tr>
<tr>
<td>3: 2 serial</td>
<td>$10^2$</td>
</tr>
<tr>
<td>dilutions</td>
<td></td>
</tr>
<tr>
<td>6: 5 serial</td>
<td>$10^5$</td>
</tr>
<tr>
<td>dilutions</td>
<td></td>
</tr>
<tr>
<td>9: Negative</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.4: Limiting dilution assay for semi-quantitation of HCV levels; 5ul of HCV cDNA were diluted in a series of ten fold steps which allowed cDNA to be quantified to within 1 log of its actual concentration. Positive and negative (not all shown) controls were run concurrently to ensure reproducibility of the assay.
Monitor. Also, when the quantity of transcripts of genotypes 1, 2 and 3 was compared by limiting dilution, there was a statistically similar distribution of the quantity of virus. This indicates that no correction factor for different genotypes (1, 2 and 3) is required when using this assay for quantitating hepatitis C virus levels (Hawkins et al. 1997).

2.9 Other Available Techniques for Quantitation of Hepatitis C Virus Levels.

Following the recognition that quantitation of HCV RNA sequences in plasma or serum is a valuable diagnostic tool for the pre-treatment evaluation of patients undergoing treatment with interferon and the subsequent monitoring of response, several methods have been developed for assessing HCV levels. These methods are summarised in Table 2.4 (Simmonds et al. 1990a; Simmonds et al. 1990b; Kaneko et al. 1992; Ishiyama et al. 1992; Kievits et al. 1991; Urdea et al. 1993) and many are now available commercially.

The most widely available assay is the branched DNA (bDNA) assay (Urdea et al. 1993). This assay may be applied directly to samples; it utilises the amplification of signal and can detect 350 000 genome equivalents per ml, making it 100-1000 times more sensitive than conventional molecular hybridisation methods for viral DNA or RNA. The bDNA assay however, is less sensitive than RT-PCR, giving positive results in approximately 70-80% of collected samples from patients with chronic HCV infection. The other major disadvantage of the bDNA assay (version 1: bDNA-1) is that, even though it is based on the hybridisation with the most conserved region (5'NCR), it may underestimate HCV RNA levels in patients with HCV type 2 by a factor of 3 and type 3 by a factor of 2, thus making the predictive value of the assay
## Table 2.4 A comparison of different methods for quantitating Hepatitis C virus.

<table>
<thead>
<tr>
<th>Method</th>
<th>Amplifies</th>
<th>Detection</th>
<th>Brief Description</th>
<th>Associated Problems</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limiting Dilution RT-PCR</td>
<td>Target</td>
<td>Semi-quantitative</td>
<td>Chapter 2</td>
<td>Time consuming; difficult technique</td>
<td>No correction for individual genotypes; reproducible</td>
</tr>
<tr>
<td>Competitive RT-PCR</td>
<td>Target</td>
<td>Semi-quantitative</td>
<td>A series of known concentrations of synthetic HCV mutant DNA is co-amplified with patient samples.</td>
<td>Time consuming; inaccurate; poor reproducibility; correct for genotypes</td>
<td>High sensitivity</td>
</tr>
<tr>
<td>Multicyclic RT-PCR</td>
<td>Target</td>
<td>Semi-quantitative-quantitative</td>
<td>Standards containing known concentrations of HCV RNA are co-amplified with unknowns. Products are dot-blot hybridised with oligonucleotide probes. Patient samples compared with standard series for semi-quantitation.</td>
<td>Time consuming; inaccurate; poor reproducibility; rarely used</td>
<td>No correction for genotypes; high sensitivity</td>
</tr>
<tr>
<td>NASBA (isothermal nucleic acid amplification)</td>
<td>Target</td>
<td>Semi-quantitative-quantitative</td>
<td>Multiple enzymes and primers are used to amplify target RNA sequence over one billion fold. Detection is by means of gel electrophoresis or oligonucleotide probe hybridisation</td>
<td>Time consuming; difficult technique; poor reproducibility; rarely used</td>
<td>No correction for genotypes; high sensitivity</td>
</tr>
<tr>
<td>bDNA</td>
<td>Signal</td>
<td>Quantitative</td>
<td>HCV RNA captured in a well by hybridisation; quantity assessed via chemilluminenscent signal amplification.</td>
<td>Correct for genotypes (bDNA-1); poor sensitivity</td>
<td>Good specificity (bDNA1); reproducible; extensive experience</td>
</tr>
<tr>
<td>Roche Monitor</td>
<td>Target</td>
<td>Quantitative</td>
<td>HCV RNA amplified with type specific primers in the presence of type specific competitor RNA. Both the competitor and the sample DNA were detected by hybridisation to a probe in a colorimetric assay.</td>
<td>Poor reproducibility; correct for genotypes; limited dynamic range</td>
<td>Easy technique; high sensitivity</td>
</tr>
</tbody>
</table>
dubious (Collins et al. 1995). Results from HCV types 1, 4, 5 and 6 are not affected. Using these corrected values for the bDNA-1 assay, no difference in median virus levels has been found between any of the 6 genotypes amongst HCV infected blood donors from different geographical origins (Smith et al. 1996). The implication of these findings is that the observed differences between genotypes in response to interferon treatment is independent of virus level, and thus virus level remains an independent predictor of response (Lau et al. 1995). Following the completion of these studies, a second version of the bDNA assay (version 2: bDNA-2) has been developed to allow equivalent quantitation of all genotypes.

Competitive RT-PCR methods of HCV RNA quantitation are also now available commercially (Amplicor™ Hepatitis C Monitor, Roche). In this assay, there is co-amplification of HCV RNA with a synthetic RNA quantification standard (IQS). The IQS differs from the viral sequence only by insertion of a specific probe sequence. the amplification is done by a single 5'NCR primer pair including a biotinylated downstream primer. After amplification, the products are serially diluted and hybridised to both HCV- and IQS-specific probes coated on microwells.

2.10 Summary

(1) Serum and liver biopsy samples were obtained for the studies in Chapters 3, 4 and 5 at the time of diagnostic laparoscopy and liver biopsy. All samples were stored immediately at -70°C for a maximum of 3-6 months.
(2) RNA extraction from serum samples was carried out using a modification of the single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.

(3) RNA extraction from liver biopsy samples was carried out using a commercial modification of the single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.

(4) HCV RNA was reverse transcribed by nested PCR using 5’NCR specific primers 939 and 209; 940 and 211. Primary and secondary PCR reactions were undertaken using 5μl of cDNA and standard reaction mixtures. The secondary PCR products were subjected to electrophoresis in 2% agarose gel.

(5) Secondary PCR products were cleaved with pairs of restriction enzymes: Hae III/RsaI in one reaction and MvaI/Hinfl in the second to distinguish genotypes 1, 2, 3, 4, 5 and 6. Subtypes 1a and 1b and 2a and 2b were separated by the cleavage patterns produced from digestion with MvnI and ScrFI.

(6) Both liver and serum samples were quantified by limiting dilution of cDNA reverse transcribed from HCV RNA.

(7) Multiple other techniques for genotyping and quantifying HCV are available; there is no universal agreement as to which technique is the most sensitive and reliable.
3.1 Introduction.

Many patients who are infected with HCV are asymptomatic for decades and remain undiagnosed until they present with symptomatic advanced liver disease (Seef et al. 1992; Seef et al. 1994). Clinical examination and serum aminotransferases (ALT) may also be normal in a patient with histologically active or severe disease (Stanley et al. 1996). Early diagnosis is important since treatment with α-interferon is more likely to be successful in the asymptomatic stage before progression to cirrhosis (Jouet et al. 1994; Camps et al. 1993). Thus a knowledge of the natural history of HCV, related to transmission epidemiology (host factors such as the age of the patient, mode of transmission and duration of infection) and the molecular virology of infection (HCV genotype and virus level) has important health care implications, since both α-interferon therapy and liver transplantation are expensive and a major factor in any health care budget.
Previous reports have suggested that HCV genotype 1b may be associated with more severe liver disease (in particular hepatic cirrhosis and hepatocellular carcinoma) and a more aggressive course of infection; however, these studies have commonly concentrated on single factors and have not concurrently considered host factors such as mode of infection and duration of infection. Likewise, the exact significance of a single assessment of circulating HCV RNA remains unclear. Reports have suggested a variety of significant relationships, including patient dependent factors such as sex and duration of infection (Gretch et al. 1994). Others have demonstrated that histologically severe disease is associated with high circulating levels of HCV RNA (Gretch et al. 1994; Kato et al. 1993). Attempts have also been made to correlate histological activity with serum HCV RNA level (Lau et al. 1993) and it has been suggested that the Knodell score reflects the replicative level of HCV in infected patients (Nakagawa et al. 1994). Additional reports using the branched chain DNA (b-DNA) signal amplification assay have indicated that serum HCV RNA levels are directly correlated with hepatic HCV RNA levels (Coelho-Little et al. 1994), although data have questioned the reliability of the b-DNA assay when assessing HCV quantitation in populations with differing HCV genotypes (Urdea 1993).

In this chapter, the clinical significance of molecular virological investigations (HCV genotype and serum virus level) and their relationship to host epidemiology (mode of infection and duration of infection) and liver histology is examined in a Scottish population at the time of staging investigations.
3.2 Patients and Methods

Patients

One hundred and thirty four patients (100 male; median age 38) presenting for diagnostic laparoscopy and liver biopsy between 01/01/1994 and 30/06/95 were included. All patients were positive for anti-HCV antibodies by second generation enzyme immunoassay (EIA-2, Abbot Laboratories, Weisbaden, Germany) and third generation recombinant immunoblot assay (RIBA-3, Chiron, Emeryville, Ca). Fifty one patients had acquired their infection through the abuse of intravenous drugs (IVDA), 17 from red cell concentrate transfusions, 35 from factor 8 concentrate transfusions and in 31 there were no obvious risk factors for infection.

At the time of admission to hospital, each patient was questioned about their geographical origin and likely duration of infection (in the case of the IVDA group, this was calculated from the year of first injection). Serum transaminase was measured on the day of diagnostic laparoscopy and an additional serum sample obtained which was stored at -70°C.

Methods

HCV RNA Extraction From Serum Samples.

HCV RNA was extracted from serum samples as described in section 2.3.
RT-PCR and Viral Genotyping.

RT-PCR for HCV RNA was carried out on serum samples according to the specifications in section 2.5. HCV genotyping was completed by RFLP analysis of secondary PCR products (section 2.6).

Quantitation of HCV Levels in Serum Samples.

This is as described in section 2.8.

Histological Analysis

All liver biopsy specimens were evaluated by a single pathologist who did not know the patient’s epidemiological status. In addition to confirming the features of infection with HCV the biopsies were classified as normal, chronic hepatitis (according to the criteria of Knodell) or cirrhosis.

Statistical Analysis

The results were analysed by nonparametric tests where appropriate: the chi-squared or Fisher’s exact test, the Mann-Whitney test or Kruskal-Wallis probability tests.
3.1 Results.

During an 18 month period, the liver histology of 134 patients was assessed by diagnostic laparoscopy and liver biopsy, as a prelude to interferon therapy (Table 3.1).

(1) Liver Histology.

Twenty nine patients had cirrhosis at diagnostic laparoscopy and biopsy. The remaining 105 patients had macroscopic and histological features of ongoing inflammatory activity in keeping with chronic HCV hepatitis.

(2) Virus Factors.

Qualitative HCV RNA RT-PCR.

Nineteen patients were RT-PCR negative for HCV RNA in serum; however, all had histological features of chronic hepatitis.

HCV Level.

There was no significant difference in virus level between patients with chronic hepatitis (median $4 \times 10^4$; range $8 \times 10^2$ to $4 \times 10^8$ copies/ml) or cirrhosis (median $4 \times 10^6$; range $4 \times 10^3$ to $4 \times 10^7$ copies/ml) ($p=0.3$) at the time of biopsy (Figure 3.1). There was also no significant difference in virus level between the four different subpopulations: transfusion (median $4 \times 10^5$; range $4 \times 10^3$ to $4 \times 10^6$ copies/ml), haemophiliac (median $4 \times 10^5$; range $4 \times 10^3$ to $4 \times 10^6$ copies/ml), IVDA (median $4 \times 10^4$; range $8 \times 10^2$ to $4 \times 10^7$ copies/ml) and sporadic (median $4 \times 10^5$; range $4 \times 10^3$ to $4 \times 10^7$ copies/ml) ($p=0.07$)(Figure 3.2). Likewise, there was no significant difference in virus level
between the six different genotypes: genotype 1a (median 4x10^5; range 8x10^2 to 4x10^8 copies/ml); genotype 1b (median 4x10^5; range 4x10^4 to 4x10^7 copies/ml); genotype 2a (median 4x10^6; range 4x10^5 to 4x10^7 copies/ml); genotype 2b (median 4x10^5; range 4x10^3 to 4x10^7 copies/ml); genotype 3 (median 4x10^5; range 4x10^3 to 4x10^8 copies/ml) and genotype 4 (median 4x10^4; range 4x10^3 to 4x10^6 copies/ml) (p=0.08) (Figure 3.3).

**HCV Genotype.**

There was a significant association between HCV genotype 1b (8 of 13 patients, 62%, had cirrhosis) and severe liver histology (p<0.01) (Figure 3.4). However, few 1b patients were infected under 20 years ago (4 of 13; 30%) and all the other genotypes (except for 2a) were represented in the cirrhosis group (Figure 3.5). Overall, most patients were infected by genotypes 1a (45 patients; 34%) or 3 (39 patients; 29%).

Figure 3.6 illustrates the distribution of HCV genotypes between the sub-populations.
<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Number</th>
<th>Sex (F:M)</th>
<th>Age*</th>
<th>Geographical Origin</th>
<th>Duration of Infection (years)*</th>
<th>Normal ALT</th>
<th>ALT (u/l)</th>
<th>RT PCR negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic</td>
<td>31</td>
<td>10:21</td>
<td>41(23-69)</td>
<td>4 Italian</td>
<td>Unknown</td>
<td>7</td>
<td>75(16-577)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 Egyptian</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemophiliac</td>
<td>35</td>
<td>3:32</td>
<td>34(30-68)</td>
<td>1 Maltese</td>
<td>20(8-30)</td>
<td>3</td>
<td>78(13-495)</td>
<td>4</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 Japanese</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVDA</td>
<td>51</td>
<td>16:35</td>
<td>35(19-51)</td>
<td>All British</td>
<td>13(5-30)</td>
<td>13</td>
<td>67(11-320)</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transfusion</td>
<td>17</td>
<td>5:12</td>
<td>55(35-88)</td>
<td>All British</td>
<td>9(1-40)</td>
<td>1</td>
<td>66(32-212)</td>
<td>1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>134</td>
<td>34:100</td>
<td>38(3-88)</td>
<td>14(1-40)</td>
<td>24</td>
<td>74(11-577)</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

*Median (Range).

Table 3.1: Demographic and virological data of the study population.
Disease state
1=Chronic hepatitis; 2=Cirrhosis.

Figure 3.1 Serum virus levels by disease state. There was no significant difference in serum levels between patients with chronic hepatitis and cirrhosis.
Figure 3.2 Serum virus levels according to mode of transmission of HCV. There was no significant difference in virus levels between the modes of transmission.
Figure 3.3 Serum virus levels by HCV genotype. There was no significant difference in virus levels according to genotype. 1=genotype 1a; 2=genotype 1b; 3=genotype 2a; 4=genotype 2b; 5=genotype 3; 6=genotype 4.
Figure 3.4 HCV Genotype according to disease state.
Figure 3.5 HCV genotype according to decade of infection.
Figure 3.6 HCV genotype according to mode of transmission.
3.4 Discussion.

These data have examined the clinical significance of molecular virological investigations and their relationship to host epidemiology and liver histology in a well defined population of 134 patients.

The results indicate that most patients with chronic HCV infection in Edinburgh are presently infected with genotypes 1a and 3; this genotype distribution is similar to that demonstrated in previous studies of Scottish blood donors and haemophiliacs (Davidson et al. 1994; Jarvis et al. 1994). Among the 6 HCV genotypes, no statistically significant difference was demonstrated in the mode of virus transmission. However, there was a significant association between HCV genotype 1b (8 out of 13 patients (62%) had cirrhosis) and liver histology; although these patients were mostly infected over 20 years ago (8 out of 13 (70%) and genotypes 1a, 2b, 3 and 4 were also represented in the cirrhotic subpopulation. These findings are similar to a recent study which demonstrated that the relative prevalence of genotype 1b was significantly higher in patients who were infected in the 1960s than in those who were infected in the 1980s (Nousbaum et al. 1995). These data may indicate a change in the epidemiology of HCV genotypes over time, which could explain the older mean age of patients with genotype 1b. Indeed, in this population, "waves" of HCV infection have affected Edinburgh over the past 40 years (Figure 5). The initial wave was probably caused by genotype 1b, followed by genotypes 1a and 3; unfortunately, these data are incomplete because only a very few patients infected 30-50 years ago are available for study compared with those infected in the 1970s to 1990s. A unique study investigating the pattern of diversity of hepatitis C virus in
relation to times of transmission supports this hypothesis (Smith et al. 1997). In the study, the rate of sequence change of HCV in vivo was used to date the spread of HCV genotype 1b in European, USA and Japanese populations. Silent substitution rates of 0.0011 and 0.0017 substitutions per site per year were observed in the NS5 and E1 regions by sequence comparisons from a cohort of individuals infected from a common source 17 years previously. Mean silent substitution frequencies of 0.169 and 0.224 in NS5 and E1, respectively were observed amongst type 1b variants infecting epidemiologically unrelated individuals from several countries. This predicted a time of divergence from a common ancestor of 64-69 years. The absence of country-specific groupings by phylogenetic analysis of these sequences suggested that the spread of this genotype occurred on a world-wide basis at a similar time. Dates for the spread of other genotypes varied from around 80 years (type 2a) to 54 years (type 3a), suggesting that different variants spread into communities at different times this century.

As discussed in Chapter 1, previous studies have reported significant associations between increased HAI and genotype 1b at the time of staging of chronic HCV infection and at reinfection of liver allograft after orthotopic liver transplantation (Konig et al. 1992; Feray et al. 1994; Feray et al. 1995). However, none have demonstrated that an increased HAI results in a severe outcome of liver disease. In the absence both of prospective studies suggesting a causal association between genotype 1b and severe liver disease, and of biological evidence indicating that genotype 1b is different to other genotypes, the association between genotype 1b and severe liver disease is probably a “cohort effect” in this population.
There is however, a role for genotyping in staging chronic HCV infection, prior to treatment with α-interferon therapy. Multivariate analyses of treated patients have identified genotype 1 as an independent factor for a poor response to therapy, compared with genotypes 2 and 3 (Yoshioka et al. 1992; Mita et al. 1994; Kasahara et al. 1995). Other studies have identified a higher mean viraemia associated with genotype 1 as the cause of this poor response (Collins et al. 1995), although no such difference was observed here. Whether apparent differences in viraemia as a function of genotype within populations is related to viral pathogenesis or epidemiological factors remains to be determined.

The exact significance of the quantity of circulating HCV RNA remains unclear. Reports have suggested a variety of significant relationships, including patient dependent factors such as sex, age and incubation of infection (Gretch et al. 1994). Others have suggested a significant correlation between disease severity and serum HCV level (Gretch et al. 1994; Kato et al. 1993; Gordon et al. 1994). Attempts have also been made to correlate disease activity with serum HCV RNA level and it has been suggested that the Knodell score reflects the replicative level of HCV in infected patients (Nakagawa et al. 1994). In our study, there was no correlation between virus level and mode of HCV transmission, HCV genotype or severity of infection. This is not a surprising finding; recent evidence has suggested that there is considerable flux in virus level, even over a relatively short time period such as three weeks (Bouliere et al. 1995; Giostra et al. 1995). Careful interpretation of single HCV RNA levels as part of a dynamic process is required. Undoubtedly the most useful application of quantitation is as a predictor of response to α-interferon therapy; a single low virus
level prior to therapy is more likely to be associated with a complete response to \(\alpha\)-interferon (Magrin et al. 1994; Shibata et al. 1993). Likewise, assessment of response during therapy may be followed by measurement of virus levels.

In conclusion these data suggest that development of severe and progressive liver disease in chronic hepatitis C infection is probably multifactorial in nature. In staging the disease, the temptation to infer an outcome from a virological parameter must be avoided. The full staging process requires a careful epidemiological history, including mode and duration of infection; a liver biopsy to assess severity of disease; and a molecular virological profile (HCV genotype and virus level) to predict response to \(\alpha\)-interferon therapy.

3.5 Summary

(1) In this chapter, the clinical significance of molecular virological investigations and their relationship to host epidemiology and liver histology was examined in a Scottish population.

(2) One hundred and thirty four patients were included. At the time of staging investigations, HCV genotypes were assessed by RFLP analysis of HCV cDNA; HCV levels were assessed by limiting dilution of HCV cDNA.

(3) There was no significant difference in virus level between patients with chronic hepatitis or cirrhosis; between the four different subpopulations or the six different genotypes.
(4) There was a significant association between HCV genotype 1b and severe liver histology, although these patients were predominantly infected over 20 years ago. Most patients were infected by genotypes 1a and 3.

(5) These data may indicate a change in the epidemiology of HCV over time; "waves" of HCV infection may have affected Edinburgh over the past 40 years.

(6) These data suggest that development of severe and progressive liver disease in chronic HCV infection is probably multifactorial in nature; individual prognoses should not be inferred from single virological parameters.

(7) The full staging process requires a careful epidemiological history; a liver biopsy to assess severity of disease and a molecular virological profile to perhaps predict response to interferon therapy.
4.1 Introduction.

Hepatitis C virus is the predominant cause of posttransfusion and sporadic non-A non-B hepatitis world-wide (Choo et al. 1989; Kuo et al. 1989; Kato et al. 1990; Alter et al. 1989; Esteban et al. 1989). Approximately 99.7% of HCV infected individuals can be identified by third generation anti-HCV testing (Courouce et al. 1994; Bousch et al. 1994)). However, this test does not indicate active infection, and there is considerable controversy as to the biochemical, virological and histological definitions of a “past infection”.

Initial, fundamental studies suggested that negative serum HCV cDNA PCR results in RIBA positive individuals correlated with the absence of inflammation in liver biopsy specimens (Alberti et al. 1992). Therefore, the serum HCV cDNA PCR result was a sensitive and specific marker of liver disease in anti-HCV positive subjects, independent of ALT values; true healthy carriers of HCV did not exist. Following these reports, it has been recommended that patients negative in serum for HCV cDNA do not undergo routine liver biopsy; further, that those with persisting
abnormal liver function tests should be screened for other liver diseases (e.g. autoimmune chronic active hepatitis or haemochromatosis), whilst those with normal liver function tests ought to be followed up annually until the natural history of the disease is better documented (Booth et al. 1995).

Many previous studies have examined the significance of serum HCV levels and have demonstrated a wide range of clinicopathological relationships (Kato et al. 1993; Yuki et al. 1993; Hu et al. 1992; Lau et al. 1993; Gunji et al. 1992; Kaneko et al. 1992; Kobayashi et al. 1993); however, there are fewer data examining intrahepatic HCV levels and their associations (Jeffers et al. 1993; Sakamoto et al. 1994; Nakagawa et al. 1994; Fong et al. 1991; Lau et al. 1994; Nouri-Aria et al. 1993; Haruna et al. 1993; Marrone et al. 1994; Di-Bisceglie et al. 1993; Hiramatsu et al. 1992; Blight et al. 1993; McGuiness et al. 1996; Lau et al. 1995). In general, previous data have not demonstrated any correlation between HCV levels in the liver and other demographic factors such as sex, age, duration of illness and risk factors. There is a suggestion that some genotypes, in particular genotype 1b may be associated with higher serum and liver virus levels (Lau et al. 1995; Yamada et al. 1994; Hayashi et al. 1995); but in contrast with serum studies, intrahepatic studies have not demonstrated any correlation between HCV RNA levels and liver injury (Jeffers et al. 1993; Sakamoto et al. 1994; Nakagawa et al. 1994; Fong et al. 1991; Lau et al. 1994; Nouri-Aria et al. 1993; Haruna et al. 1993; Marrone et al. 1994; Di-Bisceglie et al. 1993; Hiramatsu et al. 1992; Blight et al. 1993; McGuiness et al. 1996; Lau et al. 1995).
To address these issues further, the clinical, histological and intrahepatic virological profile of patients with serum repetitively negative for HCV RNA by RT-PCR was determined; this population was then compared with a control population of patients whose serum was repetitively positive for HCV by RT-PCR. Concurrently, the relationship between intrahepatic HCV levels and the clinicopathological characteristics of chronic HCV infection was investigated.

4.2 Patients and Methods

Patients

Ninety eight consecutive patients (69 male; mean age: 37.8+/−8 years) with chronic HCV infection were included in the study; none had received α-interferon therapy. All patients were positive for anti-HCV antibodies by second generation enzyme immunoassay (EIA-2, Abbot Laboratories, Weisbaden, Germany) and third generation recombinant immunoblot assay (RIBA-3, Chiron, Emeryville, Ca). Fifty eight patients had acquired their infection through abuse of intravenous drugs (IVDA), 10 from red cell concentrate transfusions, 4 were haemophiliacs transfused with infected blood products and in 26, there were no obvious risk factors for infection.

At the time of admission to hospital, each patient was questioned about the likely duration of their infection (in the case of the IVDA group, this was calculated from the year of first infection). Serum transaminase was measured on the day of diagnostic laparoscopy.

Liver biopsy and serum samples were collected as described in section 2.2.
Methods

HCV RNA Extraction From Serum Samples.

HCV RNA was extracted from serum samples as described in section 2.3.

HCV RNA Extraction From Liver Biopsy Samples.

HCV RNA was extracted from liver biopsy samples as described in section 2.4.

RT-PCR and Viral Genotyping.

RT-PCR for HCV RNA was carried out on serum and liver biopsy samples according to the specifications in section 2.5. HCV genotyping was completed by RFLP analysis of secondary PCR products (section 2.6).

Quantitation of HCV Levels in Serum Samples and Liver tissue

This is as described in section 2.8.

Histological Analysis

Liver biopsy specimens from all 98 patients were available for assessment by a single observer blinded to the clinical and serological data. Histological features were graded according to the classifications of Knodell (Knodell et al. 1980), assessing portal, periportal and lobular inflammation as well as fibrosis.

Statistical Analysis
The results were analysed by nonparametric tests where appropriate: the chi-
squared or Fisher’s exact test, the Mann-Whitney test or Kruskal-Wallis probability
tests.
4.3 Results.

During an eighteen month period, intrahepatic HCV levels of 12 patients repetitively negative for HCV RNA in serum by RT-PCR (on three separate occasions) were assessed and compared with 86 patients repetitively positive for HCV RNA in serum by RT-PCR.

The relationship between intrahepatic HCV levels and the clinicopathological characteristics of chronic HCV infection was then investigated.

(1) Virus Factors

Serum RT-PCR for HCV RNA

Ten out of twelve patients RT-PCR negative for HCV RNA in serum were RT-PCR positive in the liver; however, this group had a significantly lower intrahepatic HCV level than the remaining 86 patients ($p < 0.0001$) (Figure 4.1). Table 4.1 illustrates the demographic and virological similarity between the serum RT-PCR negative and positive patients.

HCV Genotype

Hepatitis C virus genotypes were assessed in 96 patients by RFLP analysis of HCV cDNA. There was no significant difference in intrahepatic virus levels between patients infected with genotype 1 ($n=41$); genotype 2 ($n=12$); genotype 3 ($n=36$) and genotype 4 ($n=7$) (Figure 4.2). The two patients RT-PCR negative in both liver and serum could not be genotyped.

HCV RNA Levels in Serum and Liver.
The virus level in liver tissue (median 7.7x10^7; range 1.38x10^4 to 8.9x10^9 copies/g.) was significantly greater than in serum (median 4x10^5; range 8x10^2 to 4x10^7 copies/ml) (p<0.00001). However, no significant correlation was demonstrated between the individual values of liver and serum HCV RNA in the 86 patients where this comparison was possible (Figure 4.3).

(2)Liver Histology

Ten patients had cirrhosis at diagnostic laparoscopy and liver biopsy. The remaining 88 patients had macroscopic and histological features of ongoing inflammatory activity in keeping with chronic HCV hepatitis. Neither histological severity or HAI affected the intrahepatic virus levels (Figure 4.4; Table 4.2).

Table 4.3 illustrates the descriptive histological findings in the ten patients serum RT-PCR negative for HCV RNA, but RT-PCR positive in the liver.

(3)Patient Factors

Mode and Duration of Infection

Intrahepatic HCV levels were unaffected by either the mode or duration of infection (Figure 4.5; Table 4.2).

Serum Transaminase

There was no significant correlation between serum ALT and intrahepatic HCV levels (Table 4.2).

Co-Factors

Likewise, Co-infection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV) or alcohol abuse did not significantly affect intrahepatic HCV levels.
Figure 4.1 Intrahepatic virus levels in patients repetitively HCV RNA positive (n=86) or negative (n=12) by RT-PCR. In 10 of 12 patients serum RT-PCR negative for HCV RNA, virus was present in the liver. However, the virus level was significantly higher in the serum RT-PCR positive population (p=0.0001) compared with the negative population. There were no other significant clinicopathological differences between these two subpopulations.
<table>
<thead>
<tr>
<th>Host or Virus Parameter</th>
<th>Serum RT-PCR Negative Population</th>
<th>Serum RT-PCR Positive Population</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>35.2+/-.6</td>
<td>37.8+/-.8</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>7:3</td>
<td>61:25</td>
<td>NS</td>
</tr>
<tr>
<td>Mode of Infection</td>
<td>6 IVDA; 1 Post-transfusion; 1 Haemophiliac; 2 Sporadic</td>
<td>51 IVDA; 9 Post-transfusion; 2 Haemophiliac; 24 Sporadic</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of infection (median (range))</td>
<td>16 (8-22)</td>
<td>12 (4-42)</td>
<td>NS</td>
</tr>
<tr>
<td>Alcohol</td>
<td>2</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>HIV</td>
<td>0</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>HBV</td>
<td>0</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Normal ALT</td>
<td>7</td>
<td>8</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Disease State</td>
<td>1 Cirrhosis; 9 Chronic Hepatitis</td>
<td>9 Cirrhosis; 77 Chronic Hepatitis</td>
<td>NS</td>
</tr>
<tr>
<td>HAI (median (range))</td>
<td>6 (1-13)</td>
<td>8 (1-14)</td>
<td>NS</td>
</tr>
<tr>
<td>Riba-3 Bands</td>
<td>9 present (&gt;2 bands); 1 Indeterminate</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>HCV Genotype</td>
<td>4 Genotype 1; 1 Genotype 2; 4 Genotype 3; 1 Genotype 4</td>
<td>37 Genotype 1; 11 Genotype 2; 32 Genotype 3; 6 Genotype 4</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4.1: This compares demographic and virological data of serum RT-PCR negative and positive patients, all of whom have persistent infection (111).
Figure 4.2 Intrahepatic virus levels by HCV genotype. There was no significant difference in virus levels between genotypes 1, 2, 3 and 4.

1 = genotype 1a (n=40); 2 = genotype 1b (n=1); 3 = genotype 2a (n=2); 4 = genotype 2b (n=10); 5 = genotype 3 (n=36); 6 = genotype 4 (n=7).
Figure 4.3 Serum hepatitis C virus levels by intrahepatic hepatitis C virus levels. There was no significant correlation between the levels demonstrated.
Figure 4.4 Intrahepatic virus levels by disease state. There was no significant difference in intrahepatic virus levels between patients with chronic hepatitis (n=88) and cirrhosis (n=10).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Population Median (Range)</th>
<th>Pearson Correlation Coefficient</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>37 (24 to 65)</td>
<td>0.0624</td>
<td>p=0.55</td>
</tr>
<tr>
<td>Serum Transaminase (u/l)</td>
<td>72 (13 to 577)</td>
<td>0.091</td>
<td>p=0.391</td>
</tr>
<tr>
<td>Duration of Infection (years)</td>
<td>12 (4 to 42)</td>
<td>-0.0661</td>
<td>p=0.592</td>
</tr>
<tr>
<td>Histological Activity Index</td>
<td>8 (1-14)</td>
<td>0.082</td>
<td>p=0.47</td>
</tr>
</tbody>
</table>

Table 4.2. Correlation coefficients between intrahepatic virus levels and the age of patient, duration of infection, serum transaminase level and histological activity index. There was no significant correlation between virus levels and any of these parameters.
<table>
<thead>
<tr>
<th>Patient Number</th>
<th>ALT (u/l)</th>
<th>log Liver Virus Level (copies/g)</th>
<th>Steatosis</th>
<th>Portal Inflammation</th>
<th>Lobular Inflammation</th>
<th>Periportal Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>4.72</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>5.42</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>4.10</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>6.57</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>7.68</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>4.44</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>9.28</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>4.49</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>105</td>
<td>4.22</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>5.58</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 4.3: This describes the qualitative histological findings, ALT and intrahepatic HCV level for serum RT-PCR HCV RNA negative patients with persistent hepatic infection.
Mode of transmission of HCV infection

Figure 4.5 Intrahepatic virus levels according to mode of transmission of HCV. There was no significant difference in virus levels between the modes of transmission. 
1 = IVDU (n=58); 2 = post-transfusion (n=10); 3 = haemophilic (n=4); 4 = sporadic (n=26).
4.4 Discussion.

The current pandemic of hepatitis C infection has affected 1-2% of the world’s population, and 0.02-0.2% of the population of the United Kingdom (Irving et al. 1994). However, it is unclear what proportion of patients who have a self-limiting illness successfully eliminate HCV and then become immune to re-infection; likewise, the host or virus factors increasing the likelihood of successful elimination, and the baseline markers of this process are unknown. In addition, the prognostic significance of individual clinical, epidemiological and virological parameters in this context has also not been clarified. For these reasons, the clinical, histological and virological profile of 98 consecutive patients, presenting for staging of their liver disease by diagnostic laparoscopy and liver biopsy was assessed; further, a unique serum HCV cDNA PCR negative population of 12 patients was compared with a larger subpopulation of HCV cDNA PCR positive patients. These data demonstrated that repetitive negative RT-PCR for HCV RNA did not indicate complete hepatic elimination of HCV in 10 of 12 (87.5%) patients; also that intrahepatic HCV levels were not determined by either host factors (age of patient, mode or duration of infection, concurrent alcohol abuse or concurrent HBV/HIV infection) or by virus factors (HCV genotype).

For confirmation of positive anti-HCV test results, recombinant immunoblot assays (RIBA) were developed; RIBA-3 results are interpreted as “positive” when one or more bands are positive, and “indeterminate” when only one band is positive. A high proportion (75-80%) of RIBA-3 positive patients have viraemia as detected by RT-PCR for HCV RNA. It has been suggested that RIBA-3 positive, but RT-PCR
negative patients may have cleared the virus from the circulation after a previous infection, may be viraemic below the RT-PCR detection level, or may represent false positive anti-HCV reactivity. Previous studies have indicated that RT-PCR negative / anti-HCV positive patients correlate with absence of inflammation on liver biopsy specimens and that this observation probably indicates clearance of the virus (Alberti et al. 1992). In this study 10 of 12 (87.5%) patients were RT-PCR positive for HCV RNA in liver tissue, but RT-PCR negative in serum and all had ongoing inflammation diagnosed at diagnostic laparoscopy and liver biopsy. This favours the hypothesis that these patients were viraemic below the RT-PCR detection level in serum (in this case, a detection sensitivity of 800 HCV copies/ml in 0.5ml of serum); further, patients RT-PCR positive for HCV RNA in serum had a significantly higher intrahepatic HCV level than those patients RT-PCR negative for HCV RNA in serum. Remarkably, comparison of the serum RT-PCR negative and positive subpopulations indicated that they were statistically similar in terms of demographic, histological and virological data; although the serum RT-PCR negative subpopulation did have a significantly lower serum transaminase than the RT-PCR positive patients despite the poor correlation between liver virus level and ALT overall. Possibly, the serum RT-PCR negative patients, with their concurrent lower intrahepatic HCV level and ALT may have a lower grade hepatic inflammation, however, there is no significant difference in HAI between the populations. Therefore, the prognostic importance of these data is that serum RT-PCR negative patients with chronic HCV infection should be followed up indefinitely and at present there is no indication that they are at a lower risk of severe liver disease in the future. Follow up studies are required, assessing these
patients for the presence of very low levels of viraemia by increasing the detection sensitivity to 80 copies HCV/ml in 5 ml of serum or even 8 copies HCV/ml in 50 ml of serum.

Secondly, in this study, there was an absence of a significant relationship between host or virus factors and intrahepatic HCV levels; thus the significance of a single assessment of intrahepatic HCV RNA in terms of diagnosis and prognosis remains unclear. Indeed, in contrast with similar serum studies, this finding corresponds to previous intrahepatic studies which have demonstrated no correlation between HCV RNA levels and liver injury (Jeffers et al. 1993; Sakamoto et al. 1994; Nakagawa et al. 1994; Fong et al. 1991; Lau et al. 1994; Nouri-Aria et al. 1993; Haruna et al. 1993; Marrone et al. 1994; Di-Bisceglie et al. 1993; Hiramatsu et al. 1992; Blight et al. 1993; McGuiness et al. 1996; Lau et al. 1995). The largest of these studies which utilised three different methodologies for quantitation of HCV RNA (Dot-Blot PRC, End-Point Titration and Roche Amplicor Monitor Assay), examined interassay and intraassay variability in detail and demonstrated no significant association with the degree of liver injury (Lau et al. 1995). There are at present no data measuring viral replication in addition to the total HCV load and its effect on liver injury. Recently, new techniques have been developed to overcome the methodological problems associated with detection of true replicative (negative or antisense) HCV RNA levels, but their ability to specifically quantitate HCV RNA over a wide HCV level is still limited (Gunji et al. 1994; Lanford et al. 1994).

Quantitation revealed that the liver contained 2.55x10^5-1.92x10^9 copies/g of HCV RNA and sera contained 8x10^7-4x10^7 copies/ml. 1g of infected liver tissue
contained $1-10^5$ (median $10^3$; $n=19$) times as many copies of HCV RNA as did 1ml of serum. Although, a significantly higher liver virus level compared with serum was found in our study, there was no correlation between individual values in tissue and serum. These results further suggest that it is too simplistic to expect that the serum virus level reflects a spillover of virions from the liver. Evidence is emerging of the importance of extra-hepatic viral replication; infection of peripheral blood mononuclear cells (B- and T-lymphocytes and monocytes) can lead to viral production from bone marrow and lymphoid organs which may contribute to virus level (Berenguer et al. 1995).

This contrasts with the findings of two previous studies, both of which have found a significant correlation between serum and liver HCV RNA levels (Nakagawa et al. 1994; Coelho-Little et al. 1995). The first used a competitive RT-PCR for quantitation and found that 1g of infected tissue contained $10^2-10^4$ (mean $10^3$; $n=23$) times as many copies as did 1ml of serum. The second used a branched DNA (b-DNA) signal amplification assay and found the mean ratio of liver to serum HCV RNA levels to be 36:1, with a range of 1:1 to 138:1. Both studies concluded that circulating HCV RNA levels reflect those in the liver at any point in time. However, results obtained using competitive RT-PCR may over or under estimate the quantity of viral RNA by a significant factor. The technique relies upon estimation of PCR product derived from liver tissue RNA against a known quantity of control mutant RNA. For example, if the quantity of PCR product is less than $10^7$ copies of mutant RNA but more than $10^5$ copies, it is estimated to be $10^6$ copies. Likewise, although the b-DNA assay is highly reproducible, it can only detect and quantitate HCV RNA
in 70-90% of serum samples from patients with chronic HCV infection (Collins et al. 1995). As discussed in Chapters 1 and 3, recent evidence has emerged that a correction factor for different HCV genotypes must be inserted into the results of this assay.

In addition, HCV genotype did not have a demonstrable effect on intrahepatic virus levels; however, genotypic analysis of these study data was limited by the presence of only one patient infected with genotype 1b, whilst forty patients were infected with genotype 1a.

There was also no correlation in this study between HCV RNA levels in liver and other demographic factors such as sex, age, duration of illness and mode of infection. These data confirm previous studies on liver virus levels but contrast with studies examining serum levels of HCV RNA where increased levels have been demonstrated in relation to age, sex, mode and duration of infection (Kato et al. 1993; Yuki et al. 1993; Hu et al. 1992; Lau et al. 1993; Gunji et al. 1992; Kaneko et al. 1992; Kobayashi et al. 1993; Chayami et al. 1993). This observed discrepancy may be explained by a combination of factors: contribution to serum HCV RNA levels from virus in other tissues (and from necrotic liver tissue), HCV RNA in serum immune complexes, technical variation between methods, different populations studied and time dependent fluctuation in serum HCV levels. Certainly, analysis based on serum studies is more difficult to interpret than that based on intrahepatic studies.

In conclusion, this study successfully demonstrates that repetitive negative RT-PCR for HCV RNA in serum does not indicate complete hepatic elimination of HCV. Indeed, these patients have a similar clinical, pathological and epidemiological
profile to serum RT-PCR positive patients; their prognosis and requirements for full staging of liver disease are thus likely to be similar, and further follow-up is mandatory. However, no significant associations were demonstrated between intrahepatic HCV levels and other clinicopathological parameters. Importantly, these data support the hypothesis that HCV does not cause liver disease by a cytopathic process; they also illustrate the limitations of a single assessment of HCV levels. Clearly, there is a requirement for sequential studies of chronic HCV infection in terms of molecular virological and clinical parameters, before the clinical significance of intrahepatic HCV levels is established.

4.5 Summary

(1) The clinical significance of a single assessment of circulating HCV RNA and its relationship to the level of intrahepatic HCV RNA is unclear.

(2) This chapter aimed to investigate the relationship between intrahepatic HCV levels and the clinicopathological characteristics of chronic HCV infection.

(3) Ninety eight consecutive patients (69 male; mean age 37.8+/-8 years) with chronic HCV infection were included. Of these, 12 patients were repetitively negative for HCV RNA in serum by RT-PCR.

(4) After diagnostic laparoscopy and liver biopsy, semi-quantitative analysis of intrahepatic HCV RNA levels was carried out by limiting dilution of HCV cDNA.
HCV genotypes were assessed in all patients. At the time of biopsy, routine clinical and biochemical parameters were also recorded.

(5) Ten of twelve patients RT-PCR negative for HCV RNA in serum were RT-PCR positive in liver; however, this group had a significantly lower intrahepatic HCV level and serum transaminase level than the remaining 86 patients.

(6) In this population, intrahepatic virus levels were not affected by host factors (age of patient, mode or duration of infection) or by virus factors (HCV genotype).

(7) These data also demonstrate that repetitive negative RT-PCR for HCV RNA in serum does not indicate absence of HCV from the liver.
CHAPTER 5: PREDICTION OF CIRRHOSIS IN PATIENTS WITH CHRONIC HEPATITIS C INFECTION BY ARTIFICIAL NEURAL NETWORK ANALYSIS OF VIRUS AND CLINICAL FACTORS.

5.1 Introduction.

At present, it is not possible to predict which patients will have cirrhosis at the time of staging HCV infection (Alter et al. 1995). This has major implications both for prognosis and the success of treatment regimens.

Previous reports have concentrated on single host (mode of transmission and duration of infection) (Gordon et al. 1993; Castells et al. 1995) or virus factors (HCV genotype or virus level) (Pozzato et al. 1991; Dusheiko et al. 1994; Kobayashi et al. 1996; Gretch et al. 1994; Kato et al. 1993; Gordon et al. 1994) and their relationship to progression of disease, all have been implicated in the progression to cirrhosis. Attempts to distinguish the relative contributions of these factors in determining pathogenicity by multivariate analysis have been inconclusive (Nousbaum et al. 1995; Watson et al. 1996).
Unlike multivariate analysis, artificial neural network (ANN) analysis is known to be particularly suitable for modelling complex multidimensional relationships (Cross et al. 1995; Baxt 1995; Bos et al. 1993). The ANNs consist of simple signal-processing units (neurons), the interactions of which are controlled by transfer functions and input associated weights. The construction allows for easy adjustment of the analysis tool in variable situations and may also provide an independent measure of the relative importance of individual inputs in characterising the output (Ala Korpela et al. 1995).

This chapter firstly examines the validation and training of ANNs with routine clinical host and viral parameters to predict the presence of cirrhosis in patients with chronic HCV infection and compares the performance of the ANN analysis with a conventional statistical model based on multiple logistic regression, and secondly, assesses and interprets the role of the different inputs on the ANN classification.
5.2 Patients and Methods.

Patients.

One hundred and thirty consecutive patients (100 male; mean age: 40.1±13.2 years) who presented between 01/01/94 and 30/06/95 and were positive for anti-HCV antibodies by second generation immunoassay (EIA-2, Abbot Laboratories, Weisbaden, Germany) and third generation recombinant immunoblot assay (RIBA-3, Chiron, Emeryville, Ca) were studied. Of these, one hundred and twelve patients were included, since 18 were repetitively negative for HCV RNA by RT-PCR. Risk factors for infection included: 39 intravenous drug abusers (IVDA), 31 haemophiliacs who had previously received non-virus inactivated factor VIII or factor IX concentrates and 15 patients who had received red cell concentrate transfusions. In 27 patients, there were no obvious risk factors for infection (sporadic population).

At the time of admission to hospital, the mode of infection was confirmed, and the duration of infection assessed (in the example of the IVDA group, this was calculated from the year of first injection). The disease severity was staged using diagnostic laparoscopy and liver biopsy. Liver biochemistry was measured on the day of diagnostic laparoscopy and an additional serum sample stored at -70°C. Patient details have been summarised in Table 5.1.

Methods

Histological Analysis

Liver biopsy specimens from all one hundred and twelve patients were available for assessment by a single observer blinded to the clinical and serological
data. According to the histopathological diagnosis, the liver disease was grouped as non-cirrhosis or established cirrhosis by the same pathologist.

RNA Preparation from Serum

HCV RNA was extracted from serum samples as described in section 2.3.

RT-PCR and Viral Genotyping

RT-PCR for HCV RNA was carried out on serum samples according to the specifications in section 2.5. HCV genotyping was completed by RFLP analysis of secondary PCR products (section 2.6).

Quantification of Serum Samples

This is as described in section 2.8.

Artificial Neural Network Analysis

General Description

An artificial neural network (ANN) consists of simple signal processing units, so-called neurons. Each neuron can have multiple inputs, but has only a single output. The input-output relationship is controlled by a transfer function (Cross et al. 1995; Baxt 1995). The inputs of a neuron are first multiplied by a weighting factor that determines the extent to which each input influences the output and the weighted inputs are summed to form a pre-neuron sum; this is then inserted through the transfer function resulting in the neuronal output. A complete network is
built up by organising individual neurons into a series of layers. In a feed-forward fully-connected network each neuron gets an input from neurons in the preceding layer and also gives the output for each neuron in the following layer. The layers between the input and output layer are called hidden layers. Within a given topology and transfer functions, the desired behaviour of an ANN can be approximated by adjustment of the neuronal connections. This is called training of the network and is carried out by using a data set for which the output of the corresponding input is available. An empirical model is obtained if the training process is able to reduce the errors in the outputs of the network for the training set to negligible values, i.e. the ANN has learned. The hypothesis is that the trained ANN also models the underlying process(es) that have generated the training data and that it can be used to estimate the output values for input data that were not used in the training. In practice, it has been demonstrated that properly trained ANNs do have this capability for generalisation (Cross et al. 1995; Baxt 1995; Bos et al. 1993; Ala-Korpela et al. 1995). In a successful case the resultant neuronal weights can also be used to assess the relative importance of the different inputs on the ANN outputs (Ala-Korpela et al. 1995).

**Details of the Application**

In this study, standard and so-called Ward-type feed-forward fully-connected three-layer ANNs were applied to predict the occurrence of hepatic cirrhosis in HCV infected patients. The general topology of the networks is illustrated in Fig. 5.1. The standard networks were structured as explained above and used
sigmoidal transfer functions. The constructed Ward-type ANNs differed from the standard ones in that the hidden layer neurons were divided into two parts with different transfer functions (Gaussian and Gaussian complement). The aim of using different functions in the hidden layer is to allow better detection of features in a pattern processed through a network: a Gaussian function in one part of the hidden layer to detect features in the mid-range of the data and a Gaussian complement in the other part to detect features from the upper and lower extremes of the data. The output layer is thus receiving information about the input data from different parts of the hidden layer. This may result in a better overall performance of the network.

All available data on the hepatitis C infected patients were used: age; sex; duration of infection (2 ANN inputs were used to enable coding of unknown duration); mode of infection (coded using 4 ANN inputs: IVDA; post-RCC transfusion; haemophilia and sporadic transmission of HCV); presence of ascites; serum bilirubin (μmol); serum albumin (g/l); platelet count; prothrombin time (PT-in secs); serum transaminase level (ALT-μ/l); HCV genotype (coded with 6 ANN inputs, namely: genotypes 1a, 1b, 2a, 2b, 3 and 4); log₁₀ hepatitis C virus level/ml; hepatitis B status (evidence of active HBV DNA replication: either patients had HBV DNA in their serum or were HBeAg positive); HIV status and concomitant alcohol abuse (>30 units/week) for > 10 years (Paton and Saunders 1981).

The ANN analysis was firstly performed using a training data set of 82 patients (Group I) and a test set of 30 patients (Group II) (Table 5.1). This allowed testing of both the ANN topologies and the importance of the number of inputs on the ANN performance. The best performance was obtained using an ANN with 24 input
neurons (all the clinical data available), 22 hidden neurons and 2 output neurons (i.e. either 0-1 or 1-0 for the presence or absence of cirrhosis) in the standard analysis and 24 input neurons, 12 plus 12 hidden neurons and 2 output neurons in the Ward-type analysis. The numbers of hidden neurons were actually the defaults given by the NeuroShell 2 program when training the nets with Group I (0.5 times the sum of input and output neurons plus the square root of the number of patterns in the training set. For the Ward-type nets the neurons were divided evenly between the hidden layer). When the number of inputs was varied (between 24 and 7 inputs; leaving out inputs that had the lowest correlation coefficients with the outputs and/or were given low relative weights in the ANN analysis when all 24 inputs were used) there was a decrease in the network performance; the worst result was that approximately 80 % of the cases in the test Group II were predicted correctly. Leave-one-out analysis for the whole patient data set was also performed for both types of networks.

All inputs were scaled between -1 and 1. A modified back propagation algorithm (that automatically controls the learning rate and momentum) was applied in the learning stage and a commercial NeuroShell 2 program utilised (Ward Systems Group, Inc. 1993). Using a 90 MHz Pentium PC, the training periods of the ANNs took 2-3 minutes depending upon the number of input and hidden neurons. The general ANN topology is shown in Fig. 5.1 and the use of the ANN weights (the Ward-type ANN analysis trained with Group I) as an aid to interpret the relevance of the different clinical inputs on the ANN classification is illustrated in Fig. 5.2.

*Multiple Logistic Regression*
Multiple logistic regression was used to test the multivariate significance of host and viral factors in the prediction of cirrhosis in patients with chronic HCV infection. To enable comparison of the ANN analysis with a statistical model, multiple logistic regression was firstly performed using the training data set of 82 patients (Group I). The model was then applied, using the significant variables obtained, to predict the presence or absence of cirrhosis in the test set of 30 patients (Group II).

Sensitivities, specificities, predictive values of a positive test and predictive values of a negative test were calculated for the ANN analyses and for the multiple logistic regression using standard formulae.
5.3 Results.

Of the one hundred and twelve patients who were studied, twenty five (22%) patients had laparoscopically and histologically confirmed cirrhosis: 17 (21%) in Group I and 8 (27%) in Group II. The molecular virological details of the patients are summarised in Table 5.2.

Artificial Neural Network Analysis

In the training set of 82 patients, two cirrhotic patients were incorrectly classified as non-cirrhotic when applying the standard ANN analysis and all the patients were classified correctly in the case of the Ward-type analysis. In the test set of 30 patients, two cirrhotic patients were incorrectly classified as non-cirrhotic in the standard and one in the Ward-type analysis. Thus, the standard ANN analysis correctly predicted the presence or absence of cirrhosis in 28 patients (93%) and the Ward-type analysis in 29 patients (96%) in Group II.

In the leave-one-out test for all the 112 patients, 4 (16%) of the cirrhotic patients were incorrectly classified as non-cirrhotic and 5 (5%) of the non-cirrhotic were incorrectly classified as cirrhotic in the standard analysis. The corresponding values in the Ward-type analysis were 2 (8%) and 1 (1%). Thus, in the case of all the patients the presence or absence of cirrhosis was correctly predicted for 103 patients (92%) using the standard and for 109 patients (97%) using the Ward-type analysis. The sensitivities, specificities and positive and negative predictive values in all the ANN analyses plus the multiple logistic regression analysis are given in Table 5.3.
The relative weights of the clinical input parameters for the Ward-type ANN analysis, trained with Group I, are shown in Fig. 5.2. A group of ten inputs that had greater weights than the rest of the inputs were notable. These were: patient age, serum albumin level, HCV level, HBV status, duration of infection, HIV status, platelets, infection by IVDA, alcohol abuse and HCV genotype 3.

**Multiple Logistic Regression**

Significant variables predicting the presence of cirrhosis in Group I using the multiple logistic regression model were: platelet count (regression coefficient ± standard error: -0.0374±0.0169; \( t = -2.22 \)); infection by RCC transfusion (-4.71±2.43; \( t = -1.94 \)); serum bilirubin level (0.1018±0.047; \( t = 2.16 \)); concomitant alcohol abuse (11.11±4.62; \( t = 2.40 \)) and HBV status (-8.79±3.76; \( t = -2.34 \)). The regression coefficient for the constant was 8.24±4.02 and the \( t \)-value 2.05.
<table>
<thead>
<tr>
<th></th>
<th>Group I (n=82)</th>
<th>Group II (n=30)</th>
<th>Total (n=112)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>40.7±14.5</td>
<td>40.6±12.7</td>
<td>40.7±15.9</td>
</tr>
<tr>
<td>Sex</td>
<td>59M : 23F</td>
<td>23M : 7F</td>
<td>82M : 30F</td>
</tr>
<tr>
<td>Duration of Infection (years)*</td>
<td>16±8</td>
<td>15.7±6.5</td>
<td>15.9±7.6</td>
</tr>
<tr>
<td>Mode of Infection</td>
<td></td>
<td>28 IVDA</td>
<td>39 IVDA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23 Haemophiliac</td>
<td>31 Haemophiliac</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11 RCC Transfusion</td>
<td>15 RCC Transfusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 Sporadic</td>
<td>27 Sporadic</td>
</tr>
<tr>
<td>HBV Co-infection</td>
<td>10</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>HIV Co-infection</td>
<td>10</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Alcohol Abuse</td>
<td>7</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)*</td>
<td>22±27</td>
<td>20±20</td>
<td>22±26</td>
</tr>
<tr>
<td>ALT (iu/l)*</td>
<td>104±88</td>
<td>100±46</td>
<td>103±79</td>
</tr>
<tr>
<td>Albumin (g/l)*</td>
<td>41±6</td>
<td>40±6</td>
<td>41±6</td>
</tr>
<tr>
<td>Platelets*</td>
<td>183±80</td>
<td>187±77</td>
<td>184±79</td>
</tr>
<tr>
<td>PT (seconds)*</td>
<td>12±1</td>
<td>14±7</td>
<td>13±4</td>
</tr>
</tbody>
</table>

*Mean±SD.

Table 5.1 The patient population characteristics. Group I is the subpopulation of patients used to train the ANNs and Group II the separate test subpopulation. In the leave-one-out tests the whole population could also be used as the test population.
Figure 5.1 The general topology of the feed-forward fully-connected artificial neural networks used in the study. In the standard networks all the hidden neurons (22 in the final version) had the same (sigmoidal) transfer function but in the Ward-type networks there were two parts in the hidden layer (12 + 12 neurons in the final version) which had different transfer functions (Gaussian and Gaussian complement). The four inputs for infection type were sporadic, haemophilia, intravenous drug abuse and from red cell concentrate transfusion. The two inputs for duration of infection were to enable coding of unknown duration.
Figure 5.2 The relative weights of all virus and host variables in a Ward-type feed-forward fully-connected network trained with patient Group I. The ten inputs that have greater weights than the rest of the inputs are: patient age, serum albumin level, HCV level, HBV status, duration of infection, HIV status, platelets, infection by Intravenous drug abuse (IVDA), alcohol abuse and HCV genotype 3.
<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR positive</td>
<td>82</td>
<td>30</td>
<td>112</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; HCV Level*</td>
<td>6±1.2</td>
<td>5.8±1.3</td>
<td>6±1.2</td>
</tr>
<tr>
<td>Genotype 1a</td>
<td>30</td>
<td>13</td>
<td>43</td>
</tr>
<tr>
<td>Genotype 1b</td>
<td>9</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Genotype 2a</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Genotype 2b</td>
<td>8</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Genotype 3</td>
<td>30</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td>Genotype 4</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.2 The molecular virology of the hepatitis C virus infection population (and subpopulations) studied (*Mean±SD).
<table>
<thead>
<tr>
<th>Method</th>
<th>Number of Patients Tested</th>
<th>Number of Patients with Hepatic Cirrhosis (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Predictive Value of a Positive Test (%)</th>
<th>Predictive Value of a Negative Test (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard type ANN</td>
<td>30</td>
<td>8 (27%)</td>
<td>75%</td>
<td>100%</td>
<td>100%</td>
<td>91.7%</td>
</tr>
<tr>
<td>Ward-type ANN</td>
<td>30</td>
<td>8 (27%)</td>
<td>87.5%</td>
<td>100%</td>
<td>100%</td>
<td>95.7%</td>
</tr>
<tr>
<td>Multiple logistic regression</td>
<td>30</td>
<td>8 (27%)</td>
<td>63.5%</td>
<td>72.7%</td>
<td>36.7%</td>
<td>88.8%</td>
</tr>
<tr>
<td>Leave-one-out Test</td>
<td>112</td>
<td>25 (22%)</td>
<td>84%</td>
<td>94.3%</td>
<td>80.8%</td>
<td>95.3%</td>
</tr>
<tr>
<td>(Standard type ANN)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leave-one-out Test</td>
<td>112</td>
<td>25 (22%)</td>
<td>92%</td>
<td>98.9%</td>
<td>95.8%</td>
<td>97.7%</td>
</tr>
<tr>
<td>(Ward-type ANN)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3 The sensitivities, specificities and predictive values of a positive and negative test for the various ANN analyses and the multiple logistic regression model.
5.4 Discussion.

The development of hepatic cirrhosis as a consequence of chronic hepatitis C infection is associated with a poor prognosis. A significant number of patients with cirrhosis will develop decompensated liver disease or hepatocellular carcinoma (Hasan et al. 1990; Kiyosawa et al. 1990). Multivariate analyses examining efficacy of standard regimens of interferon, have also identified cirrhosis as a highly significant factor associated with non-response to therapy (Jouet et al. 1994; Camps et al. 1993). Presently, there are no reliable non-invasive investigations predictive of cirrhosis and the presence or absence of cirrhosis must be assessed by a liver biopsy, a procedure associated with significant morbidity and mortality (Gilmore et al. 1995). Furthermore, the diagnosis of cirrhosis using only a liver biopsy may be missed in 1-60% of cases (Bruguera et al. 1974, Vido et al. 1969; Holund et al. 1980) (therefore in this study, the diagnosis of cirrhosis was made by a combination of laparoscopy and liver biopsy). Long-term follow up of patients with chronic hepatitis C also requires serial liver biopsies to repeatedly reassess the hepatic histology. For these reasons, HCV infected patients would greatly benefit from a reliable, non-invasive way of predicting cirrhosis.

Table 5.3 clearly demonstrates that the training and testing for the two types of feed-forward fully-connected ANNs were successful. In the first stage the ANN analysis was performed by training the networks with the patient Group I and testing their performance on the patient Group II. To allow direct comparison, the multiple logistic regression model was also constructed from Group I and its performance assessed in the case of Group II. It is evident from Table 5.3, that the sensitivities and
specificities, as well as the predictive values of a positive and negative test, were considerably lower for logistic regression. When limited data sets are handled by ANN analysis it is important to perform a leave-one-out test for the whole available data set to gain a better estimate for the generalisation power of the analysis than is obtained using only a separate test set. In this way the possible bias that may be introduced by the pre-selected test set and lead to either too positive or negative conclusions about the analysis performance, can be eliminated as the whole data set is used in the test. This is achieved by training and testing the networks as many times as there are cases in the whole data set, i.e. the network is trained with n-1 cases (111 in this example) and then the remaining one case entered and the network output assessed. This is repeated as many times as there are cases in the data set. As the ANN output for every available input pattern is independently assessed, this kind of testing gives an objective estimation of the analysis performance. It is interesting to note that for both ANN approaches, the leave-one-out testing methodology leads to sensitivities, specificities and predictive values that are almost at the same level as those obtained when tested only with Group II (Table 5.3). This result supports the conclusion that the information provided by all the clinical inputs contain a clearly resolvable pattern related to cirrhosis in the hepatitis C patients and it is not dramatically affected by the data size used. It is also noticeable that when the multiple logistic regression model was optimised for the whole data set of 112 patients, the resulting sensitivity was 44% and specificity 95.4%, together with the predictive value of a positive and negative test of 70% and 87.2%, respectively. These values, except the specificity, denote far lower performance than obtained for the Ward-type ANN analysis in the leave-one-
out test. This indicates that even when the information content of the whole patient data set was used for specifying the regression model, thus not enabling further independent testing, it could not reach the test performance of the Ward-type ANN analysis. This result is most likely related to the better description of non-linear relationships between variables in the ANN analysis compared with the multiple logistic regression. Thus, the ANN analysis seems to provide a superior method for the non-invasive prediction of cirrhosis in the HCV patients.

The performance of the feed-forward fully-connected ANNs was also assessed by running tests using different numbers of inputs, ranging from 25 to 7 (either by omitting clinical data with lowest correlation coefficients with the outcome and/or were given low relative contribution factors in the ANN analysis when all 24 inputs were used). There was a significant decrease in the performance of the networks, although at the worst, they were still able to predict the presence or absence of cirrhosis correctly in approximately 80% of the cases in test Group II.

The six most weighted inputs (for the best performing Ward-type analysis, trained with Group I) were patient age, serum albumin level, HCV level, HBV status, duration of infection and HIV status. Additionally, platelets, infection by IVDA, alcohol abuse and HCV genotype 3 had high weights. Of these, the least surprising parameter was the serum albumin level. This parameter is a component of the Child-Pugh score (Pugh et al. 1973); and a low serum albumin level indicates poor synthetic hepatic function resulting from decompensated liver disease. Of the others, increasing patient age has already been reported in association both with aggressive disease (rapid progression of cirrhosis to hepatocellular carcinoma) (Gordon et al. 1993;
Yano et al. 1993) and with non response to interferon therapy (Tsubota et al. 1994; Chamello et al. 1995), perhaps because of impaired immunity amongst older patients (Leveinson et al. 1991).

The clinical significance of circulating HCV RNA levels remains unclear; there have been many reports of significant relationships, perhaps most consistently with increasing disease severity (Gretch et al. 1994; Kato et al. 1993; Gordon et al. 1994); however, cautious interpretation of a single HCV RNA level as part of a dynamic process of rapidly fluctuating levels is required (Bourliere et al. 1995; Giostra et al. 1995). Univariate and multivariate analyses of modes of hepatocyte injury concomitant with HCV infection, for example concurrent HBV and/or HIV infection (Hanley et al. 1996; SanchezQuijano et al. 1995) and of course concomitant alcohol abuse (Farinati et al. 1992; Yamuchi et al. 1993) has indicated a synergistic interaction between these factors in the progression of liver disease. Finally, these data corroborate the clinical impression that the duration of HCV infection influences the histological course of this chronic viral disease. Prospective, multi-centre studies of populations infected by HCV contaminated blood transfusions have concluded that the prevalence of cirrhosis is about 20% after 20 years of infection, but significantly less after 10 years (Alter et al. 1992; Seef et al. 1992; Seef et al. 1994; Koretz et al. 1993).

The decrease in the ANN analysis performance when some of the clinical inputs were omitted and the low performance of the multiple logistic regression, suggests that non-linear combinations of all the factors are important for the prediction of cirrhosis. Thus, the most weighted inputs should not be interpreted as
providing all the necessary information for the classification of cirrhosis; instead, they are the parameters that have the most important effect on the ANN classification.

The presented ANN analysis performed well in the prediction of cirrhosis in the HCV infected patients and the resulting weights of the ANN inputs compared favourably with existing data assessing the factors affecting the development of cirrhosis in HCV patients. It should be emphasised, however, that ANN analysis does have its own inherent problems. The generalisation power of a network is dependent on the training data set and thus as extensive data sets as possible should be used in the training. Therefore, even though demanding leave-out-tests were performed in this study for the whole patient population available, the results need to be verified using other, preferably larger data sets. A useful feature in ANN analysis is that it is possible to continuously refine the capability of the network by re-training and testing when new data becomes available. It should also be remembered that the outcome of feed-forward, fully-connected ANNs relies on the accuracy of the clinical data used to train the networks. Thus, inaccuracies in the parameter values resulting from experimental errors (for example, when measuring the biochemical function of the liver) may mislead the ANN training and thus decrease the ANN performance.

In conclusion, in this chapter, ANN analysis was successfully applied to predict the presence or absence of cirrhosis based on a number of host and virus factors without the need for a liver biopsy and laparoscopy. The trained networks can be operated automatically using a personal computer. They can provide an instant answer with a high degree of confidence as to whether a patient with HCV infection is
cirrhotic or not. Further validation of the ANN analysis with additional data could thus potentially revolutionise the overall management of patients with HCV infection.

5.5 Summary

(1) Diagnosis of cirrhosis in patients with HCV infection is currently reached using a liver biopsy.

(2) In this chapter, artificial neural networks were trained and validated with routine clinical host and viral parameters, to predict the presence or absence of cirrhosis in patients with chronic HCV infection.

(3) Fifteen routine clinical and virological factors were collated from 112 patients. Standard and Ward-type feed-forward, fully-connected ANN analyses were carried out both by training the networks with data from 82 patients and subsequently testing with data from 30 patients.

(4) The performance of both ANN methods was superior compared with the logistic regression. The best performance was obtained with the Ward-type ANNs resulting in a sensitivity of 92% and a specificity of 98.9% together with a predictive value of a positive test of 95% and a predictive value of a negative test of 97%.

(5) Further validation of the ANN analysis is likely to provide a non-invasive test for diagnosing cirrhosis in HCV infected patients.
6.1 Introduction.

Liver function tests are an essential part of the assessment of hepatic function for diagnosis and monitoring of patients with liver disease (Hayes and Bouchier 1989). It is well recognised, however, that there is poor correlation between liver biochemistry and hepatic histology (Chopra and Griffin 1985; Hayes et al. 1990) and not infrequently patients with compensated cirrhosis have normal liver function. Once the diagnosis of cirrhosis has been established, the Child-Pugh classification based on measurement of serum bilirubin, albumin and prothrombin time combined with clinical parameters can provide information regarding the prognosis of the underlying liver disease (Albers et al. 1989). Dynamic tests of liver function such as indocyanine green clearance, galactose elimination capacity, mono-ethyl-glycine-xylidide (MEG-X) and antipyrine clearance, may provide information regarding functional liver mass, but do not provide information on aetiology or histology of liver disease (Jalan and Hayes 1995).
Hyaluronan (HA) is a high molecular weight polysaccharide which is widely distributed in connective tissue. It is mainly produced by mesenchymal cells, enters the circulation via lymph (Laurent and Laurent 1981) and is cleared by hepatic sinusoidal endothelial cells after binding specifically to a high affinity receptor (Eriksson et al. 1983). A minor pathway of excretion is via the kidneys which can excrete low molecular weight HA but accounts for less than 1% of turnover (Fraser et al. 1984). Increased HA levels have been found in patients with scleroderma, active rheumatoid arthritis and osteoarthritis and are thought to originate from the synovitis of the involved joints (Fraser et al. 1986; Engstrom et al. 1985).

Liver disease is associated with increased levels of HA (Engstrom-Laurent et al. 1985a). In primary biliary cirrhosis, significant correlations between serum HA concentration, the degree of liver fibrosis (Nyberg et al. 1988) and several biochemical tests of liver function (Engstrom-Laurent et al. 1985b) have been found. Serum Hyaluronan concentration is elevated in patients with fulminant hepatic failure (Bramley et al. 1991). In another study serum HA concentrations were increased when liver cirrhosis was present and a good correlation between the serum HA level and serum bilirubin was found (Simpson et al. 1993).

In this chapter, serum hyaluronan will be evaluated as a marker of liver cirrhosis in a population of patients with chronic hepatitis C infection.
6.2 Patients and Methods.

Patients.

Sixty nine patients were studied prospectively; all were clinically assessed for the presence of ascites or encephalopathy and conventional liver function tests including prothrombin time were measured. The decision to perform a liver biopsy was based on clinical grounds and the patients were included in the study after this decision was made; 10 ml of blood was obtained by venepuncture at the time of the liver biopsy, the serum separated by centrifugation and stored at \(-20^\circ\) C for measurement of hyaluronan levels. According to the histopathological diagnosis their liver disease was grouped as non-cirrhosis or established cirrhosis by the same pathologist who was unaware of the hyaluronan results; each patient was then classified according to the Knodell score (Knodell et al. 1981).

Methods

Hyaluronan assay

Serum Hyaluronan was measured using a radiometric assay (Pharmacia, Uppsala, Sweden). The principle of this test is based on the use of specific hyaluronan binding proteins (HABP) isolated from bovine cartilage. Hyaluronan in the patient samples reacts with I125 -labelled HABP in solution. The unbound I-125-HABP is then quantitated by incubating with HA covalently coupled to sephrose particles of small size and low density. Separation is performed by centrifugation followed by decanting. The radioactivity bound to the particles is measured in a gamma counter which is inversely proportional to the concentration of HA in the sample. The range of
HA levels in normals is 10-100 µg/l The limit of detection is less than 5 µg/l. There is no detectable interference with chondroitin sulphate at concentrations less than 100 µg/l, keratin sulphate at less than 50 µg/l or fibronectin at concentrations less than 500 µg/l. Serum HA is stable for 24 hrs at 4° C and can be stored for longer periods at -20° C (Simpson et al. 1993). For each patient HA was measured in paired samples of serum and the mean value of HA recorded. The variation coefficient of the assay was 6%.

**Statistical Analysis.**

When the data was not normally distributed, comparisons between two groups were made by using the Wilcoxon rank sum test. The Kruskall-Wallis one way analysis of variance method was used to make comparisons of HA levels across groups when more than two. The Pearson correlation coefficient was used to assess the degree of correlation between HA and other liver function tests, because the overall distribution of HA in our cohort was fairly normal. A p value <0.05 was considered statistically significant (two-tailed test of significance). The sensitivity of HA test over certain cut-off values was defined as the number of 'true positives' (cirrhotics with HA concentration above the cut-off value) divided by the total number of patients with cirrhosis; the specificity was defined as the number of 'true negatives' (non-cirrhotics with HA concentration below the cut-off value) divided by the total number of patients without cirrhosis. Receiver operator characteristic (ROC) curves were used to evaluate the cut-off points of different aetiologies of liver disease. All values were expressed as Mean ± SEM. All calculations were made using the
SPSS for Windows 6.1 (SPSS Inc, USA) statistical package on an IBM compatible computer.

...
6.3 Results.

In this study, serum hyaluronan was measured prospectively in 69 patients with chronic HCV infection at the time of liver biopsy.

Liver histology.

Serum hyaluronan was significantly higher in cirrhotic patients (n=15) compared with non-cirrhotics (n=54) (386.5±85.5 vs 44.5±6.01 (ug/l); p<0.0001) (Figure 6.1). No correlation was found between HA levels and histological activity index (Knodell score) in the whole population.

Sensitivity and Specificity of Serum Hyaluronan in the Diagnosis of Liver Cirrhosis.

An Ha value >100ug/l was sufficient to diagnose cirrhosis with a specificity of 93%. At a cut-off value of 200ug/l the specificity increased to 98%, and further to 100% at a value of 300ug/l (Table 6.1).

Liver Synthetic Function and Chemistry.

Serum HA levels correlated with platelet count (negative correlation) and bilirubin levels (positive correlation) both in cirrhotics (n=15) and overall (n=54). In contrast, no correlation was demonstrated between HA and serum transaminases, prothrombin time and gamma-GT (Table 6.2). Serum HA levels were significantly increased with severity of liver cirrhosis measured by Child-Pugh classification (Table 6.3).
Figure 6.1 Hyaluronan was significantly higher in cirrhotic patients (n=15) compared with non cirrhotics (n=54) (386.5 +/- 85.5 vs 44.5 +/- 6.01 (ug/l); p<0.0001). All values shown are mean +/- 95% confidence intervals.
<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Cut-off value</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis C</td>
<td>50</td>
<td>72.2%</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>87%</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>67%</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>47%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>7%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 6.1 Sensitivity and specificity at 5 cut-off levels of hyaluronan to diagnose liver cirrhosis in chronic hepatitis C infection.
<table>
<thead>
<tr>
<th></th>
<th>Serum hyaluronan</th>
<th></th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson correlation coefficient</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>All patients (n=68)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>-0.4</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Alk phosphatase</td>
<td>0.19</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>GGT</td>
<td>0.11</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.34</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Platelet count</td>
<td>-0.35</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Prothrombin time</td>
<td>0.15</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td><strong>Cirrhotics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>-0.4</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Alk phosphatase</td>
<td>0.02</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>GGT</td>
<td>0.02</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.24</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Platelet count</td>
<td>-0.31</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Prothrombin time</td>
<td>0.067</td>
<td>0.46</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2 Correlations between serum hyaluronan and liver function tests in all patients and in patients with liver cirrhosis. A p value < 0.05 is considered statistically significant.
<table>
<thead>
<tr>
<th>Child’s-Pugh class</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean+/-SE</td>
<td>353.4+/-51.2</td>
<td>506.7+/-60.7</td>
<td>783+/-95.7</td>
</tr>
<tr>
<td>95% CI</td>
<td>251.3-455.5</td>
<td>383.6-629.9</td>
<td>566.4-999.6</td>
</tr>
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<td></td>
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</tbody>
</table>

Kruskall-Wallis one way ANOVA p<0.0045

Table 6.3 Serum hyaluronan levels (ug/l) in cirrhotos according to severity of liver disease (Child-Pugh classification).
6.4 Discussion.

Hyaluronan (HA) is an unbranched high molecular weight polysaccharide which is widely distributed in connective tissue. It consists of repeating disaccharide units of glucuronate-\(\beta1,3\) N-acetylglucosamine linked together by \(\beta1,4\) bonds. It is mainly produced by mesenchymal cells and cleared by hepatic sinusoidal endothelial cells after binding specifically to a high affinity receptor (Chopra and Griffin 1985). Its normal half-life in serum is in the order of 2-5 minutes and the rapid turnover results in normal individuals in a concentration of about 20-40 \(\mu\)g/l at the age of 30 years with an upper limit of 100 \(\mu\)g/l at the age of 60-70 years.

In the liver, HA is primarily synthesised by the stellate (Ito) cells (Alston-Smith et al. 1993). Conditions such as severe endotoxinaemia and sepsis may increase HA production by several mechanisms which include (i) increasing the number of stellate cells, (ii) direct \textit{in vivo} activation of stellate cell population with subsequent increase of HA production; and (iii) activation of the reticuloendothelial system of the liver with subsequent production of a variety of mediators acting on cell proliferation of the stellate cell population and increased HA synthesis (Gressner et al. 1989).

The density of HA in cirrhotic livers is approximately 15 times higher compared with normal livers (Murata et al. 1985). The close proximity between the hepatic stellate cells and sinusoidal endothelial cells probably explains the close relationship between fibrogenesis and HA levels. Fibrogenesis is initiated by hepatocyte damage from a variety of causes (alcohol, viruses, autoimmune, inborn errors of metabolism) leading to recruitment and activation of inflammatory cells, Kupffer cells and platelets and the consequent release of cytokines and growth factors. In response, hepatic
stellate cells proliferate and synthesise components of connective tissue (Knittel and Ramadori 1995). When hepatic fibrosis is developing by increased transitional-type stellate cells, HA is produced in excess and is released into blood increasing its level. Ninety percent of HA is rapidly degraded by sinusoidal endothelial cells (SEC). Histopathological changes compatible with cirrhosis include reduction or disappearance of fenestrae in SEC, basement membrane formation on the basal side of these cells, as well as deposition of collagen fibres filling the Disse spaces, also known as hepatic sinusoidal capillarisation (Scaffner and Popper 1963). This coincides with the appearance of Weibel-Palade bodies which control the production of factor VIII-related antigen (FVIIIIRAg) (Taguchi and Asano 1988). In a study by Takato et al (Takato et al. 1993) it has been shown that during sinusoidal transformation of SECs to vascular endothelial cells, capillarisation coincides with a reduction in their capacity to clear HA, and that this was present in patients with HA values over 200 µg/ml. It therefore appears that the primary mechanism by which HA concentration is increased in liver cirrhosis is reduced clearance by SEC’s, while in inflammatory conditions such as rheumatoid arthritis it is due to increased production by the mesenchymal cells of the involved joints.

A recent study from Murawaki et al (Murawaki et al. 1996), demonstrated that in patients with chronic viral hepatitis HA levels were significantly higher in cirrhotic patients but no correlation was found between Child-Pugh grade and HA levels. The authors concluded that HA is a better test to diagnose cirrhosis compared with serum type IV collagen 7S domain. In a similar study by Guechot et al. (Guechot et al. 1996) in Hepatitis C patients, HA was found to be superior to aminoterminal peptide
of type III procollagen in diagnosing patients with cirrhosis using a cut-off value of 110µg/l with a sensitivity of 79.2% and specificity of 89.4%.

The results in this chapter are in agreement with these studies; they have illustrated that the measurement of serum HA can reliably differentiate cirrhosis from other histology. The specificity increases to 100% when the HA level is over 300µg/l in patients with chronic hepatitis C infection, which indicates that measurement of HA can very accurately diagnose liver cirrhosis.

The reason why patients with liver cirrhosis secondary to chronic hepatitis C infection have such high levels of serum HA is unclear, although the mechanism is more likely to be one of impaired clearance of HA rather than overproduction. Recently the presence of inclusion-containing endothelial cells which are sinusoidal cells with eosinophilic granules, has been described in patients with chronic active hepatitis secondary to hepatitis B and C infection (Iwamura et al. 1994). This provides some evidence that during hepatitis C infection SEC's are affected early, with possible subsequent impairment of HA clearance.

These data have also demonstrated that HA levels overall correlated well with those liver function tests which become chronically deranged as a result of the development of cirrhosis (low platelet count, low albumin, prolonged prothrombin time, raised bilirubin); however, once cirrhosis became established, these correlations became either less statistically significant or significance was lost. Although, there was a correlation between HA and the severity of liver disease as expressed by the Child-Pugh classification (Pugh et al. 1973) the most significant rise in HA level was seen from the non-cirrhotic to the cirrhotic state. These results support the hypothesis that
HA is primarily a marker of fibrosis and that the maximum changes in HA levels take place as the patient progresses from chronic hepatitis to cirrhosis and thereafter HA levels remain raised and correlate less well with liver biochemistry.

In conclusion, these data have demonstrated that measurement of serum Hyaluronan can reliably differentiate cirrhotic from non-cirrhotic liver disease in chronic hepatitis C infection. This is particularly important in those groups of patients where accurate knowledge of liver histology is crucial for their management and there is an absolute or relative contraindication to liver biopsy, for example in Hepatitis C infected haemophiliacs.

6.5 Summary

(1) Hyaluronan is a glucosaminoglycan synthesised by the mesenchymal cells and degraded by hepatic sinusoidal epithelial cells by a specific receptor mediated process. Elevated levels are associated with sinusoidal capillarisation as in cirrhosis.

(2) Serum HA was measured using a radiometric assay in 69 patients with chronic HCV infection. Liver function tests, full blood count, prothrombin time and Child Pugh score were also assessed.

(3) Serum hyaluronan was significantly higher in cirrhotic patients (n=15) compared with non-cirrhotics (n=54) (386.5+/−85.5 vs 44.5+/−6.01 (ug/l); p<0.0001). No correlation was found between HA levels and histological activity index (Knodell score) in the whole population.
(4) An Ha value $>$ 100ug/l was sufficient to diagnose cirrhosis with a specificity of 93%. At a cut-off value of 200ug/l the specificity increased to 98%, and further to 100% at a value of 300ug/l.

(5) There was a significant correlation between HA and albumin, platelet count and bilirubin but not with ALT.

(6) Measurement of HA levels reliably differentiates cirrhotic from non-cirrhotic liver disease; potentially, it is a useful test in the diagnosis of liver cirrhosis when a liver biopsy is contraindicated.
7.1 Introduction.

The rate of progression of clinical and immunological sequelae of human immunodeficiency virus (HIV) infection demonstrates considerable inter- and intra-population variability. Infection with other opportunistic viruses may affect the rate of disease progression, with members of the herpesvirus and human retrovirus families being among those implicated as cofactors (Nelson et al. 1990).

Both HIV and the hepatitis viruses (hepatitis B, C and D) are transmitted by parenteral routes, and coinfection with these viruses is common among patients with a history of intravenous drug abuse (IVDA). In the example of hepatitis B virus (HBV) infection, HIV infection has an adverse affect on survival of infected patients (Housset et al. 1992); conversely, actuarial survival in HIV-infected patients with or without AIDS does not appear to be altered by the presence of HBV coinfection in adults (Scharschmidt et al. 1992), although it may have an effect in children (Di Franco et al. 1994). The interactive effects between hepatitis C virus (HCV) infection and HIV infections are complex, in part because the mechanisms of pathogenicity in HCV...
infection are poorly understood (Horvath et al. 1994). Most studies examining dual infection with HCV/HIV in IVDA have been cross-sectional (Quan et al. 1993; Libre et al. 1993), comparing immunological or virological markers or mortality between anti-HCV-positive and anti-HCV-negative patients with HIV, without providing any longitudinal data. Likewise, reports of the effect of HIV on HCV-related liver disease have been conflicting, with some studies suggesting rapid progression of liver disease (Eyster et al. 1993; Telfer et al. 1994), while others have indicated no difference in hepatic pathology when comparing anti-HCV positive and negative individuals (Libre et al. 1993; Eyster et al. 1993; Yelfer et al. 1994; Areias et al. 1992).

This study examined the interaction between HCV and HIV using a cohort of IVDA who have been followed up in Edinburgh, as part of a long-term natural history study of HIV infection. The clinical and immunological progression of HIV infection and also the mortality from end stage liver disease in patients co-infected with HCV was compared with those infected with HIV alone.
7.2 Patients and Methods

Patients

The Edinburgh IVDA cohort has been characterised previously (Brettle et al. 1987). Recruitment into the cohort began in 1985, since when clinical and immunological data have been collected prospectively at 3-6 monthly intervals on 508 patients.

Serological testing for HIV and HCV

With the availability of serological testing for HCV from 1991, cohort members have been tested prospectively for evidence of chronic HCV infection. Individuals who had died prior to this date, or who had been lost to follow up were tested retrospectively using stored serum samples (when these were available). All samples were tested for anti-HCV by second generation enzyme immunoassay (EIA-2; Abbott, Illinois). Confirmatory testing was carried out in anti-HCV positive patients using a third generation recombinant immunoblot assay (RIBA-3, Chiron, California).

HIV seroconversion dates were determined by a method of interval estimates. Seroconversion was defined as the mid point between the last negative and the first positive test, if the interval between these times was less than 2 years. For those with no last negative sample, the earliest possible date of HIV acquisition was used as a surrogate first seronegative date marker (Jan 1983 for those indulging in risk activity (shared drug injection) prior to this time, or the historical date of initial risk activity.
for those who only started to inject drugs after this time). HIV-1/2 testing used a third
generation plus enzyme immunoassay (Abbott, Illinois), with confirmation by Biokit
ELISA (Biokit, Spain S.A.).

Using the above methods, a subset of 240 individuals who have estimated HIV
seroconversion dates and known HCV serostatus was defined.

Statistical Analysis

Comparison of demographic variables between groups was performed by Chi-
square analysis and student t tests. Progression from date of HIV seroconversion was
calculated by Kaplan Meier survival analysis. Clinical end points were: time of first
development of clinically-significant symptoms attributable to HIV (CDC stage IV),
time of development of AIDS (CDC 1993 criteria), and time of death. Immunological
end points were: time of CD4⁺ counts dropping below 200/mm³, 100/mm³ and
50/mm³ respectively (defined as the date at which the first of two consecutive values
below the respective CD4⁺ count were recorded no more than 3 months apart).

Survival analysis was performed for AIDS cases and those individuals with
CD4⁺ counts <200/mm³ for all causes of death and also for deaths from end stage
liver disease. Differences in progression were calculated by the log rank test. Cox’s
proportional hazards model was used for multivariate analysis, with covariates
including gender, age at HIV seroconversion, CMV status, HIV p24 antigen status
(positive or negative), continued drug injection, use of antiretroviral therapy (more
than 6 months continuous therapy with either zidovudine, didanosine, zalcitabine or a
combination), and opportunistic infection prophylaxis (more than 6 months
continuous therapy with anti-Pneumocystis/Toxoplasma agents). Age at HIV seroconversion was converted into a dichotomous variable for the analysis according to whether an individual's age was greater or less than the median.
7.3 Results.

The demographic characteristics for the 240 patients of known HCV serostatus in the HIV seroconverter cohort are listed in Table 7.1.

Progression to Clinical and Immunological Endpoints

Progression analysis assessing the time from HIV seroconversion to HIV-related clinical endpoints (symptoms or signs of CDC stage IV disease, AIDS or death) indicated that HCV co-infection was not a significant factor influencing the rate of clinical HIV progression (Table 7.2; Figures 7.1, 7.2). Progression analysis assessing the time from HIV seroconversion to immunological end points (CD4⁺ counts <200/mm³, <100/mm³ and <50/mm³ respectively) indicated that coinfection with HCV was not associated with differing rates of immunological progression (Table 7.3; Figures 7.3, 7.4). Likewise, survival following AIDS diagnosis or following a decrease in CD4⁺ count <200/mm³ was not influenced by coinfection with HCV (Table 7.2).

Mortality

A minority of the seroconversion cohort died from end stage liver failure; 11 were HCV seropositive, and one HCV seronegative (Table 7.1). This represented 19% of all medically-related deaths (those unrelated to HIV, and excluding drug overdose, suicide and trauma) in those who were HCV seropositive. In patients without AIDS, 8 (5.9%) of those who were HCV seropositive died of liver failure compared with none of the HCV seronegative group (p=0.216).
<table>
<thead>
<tr>
<th></th>
<th>HCV-infected</th>
<th>HCV-uninfected</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>202</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>138/64</td>
<td>25/13</td>
<td>NS</td>
</tr>
<tr>
<td>Median age at HIV seroconversion (years, range)</td>
<td>22.5</td>
<td>23</td>
<td>NS</td>
</tr>
<tr>
<td>Median date of HIV seroconversion</td>
<td>Dec 1983</td>
<td>Jan 1994</td>
<td>NS</td>
</tr>
<tr>
<td>Median duration of HIV infection (months, range)</td>
<td>96 (12-153)</td>
<td>95 (4-151)</td>
<td>NS</td>
</tr>
<tr>
<td>Median follow up (months)</td>
<td>77.5</td>
<td>82.0</td>
<td>NS</td>
</tr>
<tr>
<td>Year of first drug use (median)</td>
<td>1981</td>
<td>1982</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) with concurrent alcohol abuse</td>
<td>36 (18%)</td>
<td>9 (23%)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) with past/present HBV infection</td>
<td>58 (29%)</td>
<td>10 (25%)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) with CDC stage IV disease</td>
<td>143 (70.8%)</td>
<td>26 (68.4%)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) with AIDS</td>
<td>66 (32.7%)</td>
<td>10 (26.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) progressing to CD4+ &lt;200/mm³</td>
<td>111 (54.9%)</td>
<td>19 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) progressing to CD4+ &lt;100/mm³</td>
<td>75 (37.1%)</td>
<td>10 (26.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) progressing to CD4+ &lt;50/mm³</td>
<td>53 (26.2%)</td>
<td>9 (23.7%)</td>
<td>NS</td>
</tr>
<tr>
<td>Death resulting from AIDS-defining illness (%)</td>
<td>50 (24.8%)</td>
<td>8 (21.1%)</td>
<td>NS</td>
</tr>
<tr>
<td>Death resulting from end stage liver failure:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in patients without AIDS (%)</td>
<td>11 (5.4%)</td>
<td>1 (2.6%)</td>
<td>NS</td>
</tr>
<tr>
<td>in patients with AIDS (%)</td>
<td>8 (5.9%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Death from all causes (%)</td>
<td>53 (26.2%)</td>
<td>9 (23.7%)</td>
<td>NS</td>
</tr>
<tr>
<td>Death resulting from all causes (%)</td>
<td>85 (42.1%)</td>
<td>14 (36.8%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 7.1: Demographic characteristics of 240 HIV-infected IDUs with known dates of HIV seroconversion and with known HCV serostatus.
<table>
<thead>
<tr>
<th></th>
<th>HCV seropositive (n = 202)</th>
<th>HCV seronegative (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seroconversion to CDC IV</td>
<td>143/202 RR: 1.01 CI: 0.81-1.23</td>
<td>26/38 baseline</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>Seroconversion to AIDS</td>
<td>66/202 RR: 1.05 CI: 0.75-1.45</td>
<td>10/38 baseline</td>
</tr>
<tr>
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<td></td>
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<tr>
<td>Seroconversion to death</td>
<td>85/202 RR: 0.90 CI: 0.67-1.21</td>
<td>14/38 baseline</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>CD4+&lt;200 to death</td>
<td>56/110 RR: 0.81 CI: 0.58-1.14</td>
<td>11/18 baseline</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIDS to any death</td>
<td>47/66 RR: 0.85 CI: 0.59-1.22</td>
<td>9/10 baseline</td>
</tr>
</tbody>
</table>

Table 7.2. Proportional hazards analysis of the effect of HCV serostatus on progression to clinical HIV-related endpoints.
Fig 7.1. Progression analysis of time from HIV seroconversion to development of AIDS in 240 HIV-infected injection drug users; comparison by HCV serostatus
Fig 7.2. Progression analysis of time from HIV seroconversion to development of CDC stage IV disease in 240 HIV-infected injection drug users; comparison by HCV serostatus
<table>
<thead>
<tr>
<th></th>
<th>HCV seropositive (n = 202)</th>
<th>HCV seronegative (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seroconversion to CD4+ &lt;200</td>
<td>111/202</td>
<td>19/35</td>
</tr>
<tr>
<td>RR</td>
<td>1.04</td>
<td>baseline</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.82-1.33</td>
<td></td>
</tr>
<tr>
<td>Seroconversion to CD4+ &lt;100</td>
<td>75/201</td>
<td>10/34</td>
</tr>
<tr>
<td>RR</td>
<td>1.13</td>
<td>baseline</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.81-1.58</td>
<td></td>
</tr>
<tr>
<td>Seroconversion to CD4+ &lt;50</td>
<td>53/201</td>
<td>9/34</td>
</tr>
<tr>
<td>RR</td>
<td>0.97</td>
<td>baseline</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.68-1.38</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.3. Proportional hazards analysis of the effect of HCV serostatus on progression to immunological HIV-related endpoints.
Fig 7.3. Progression analysis of time from HIV seroconversion to dropping below CD4$^+$ lymphocyte count of 200 cells/mm$^3$ in 240 HIV-infected injection drug users; comparison by HCV serostatus
Fig 7.4. Progression analysis of time from HIV seroconversion to dropping below CD4⁺ lymphocyte count of 50 cells/mm³ in 240 HIV-infected injection drug users; comparison by HCV serostatus.
This prospective, longitudinal study has demonstrated both that HCV coinfection does not influence the clinical progression of HIV disease to AIDS and that it is not associated with a more rapid immunological decline.

In this cohort of 240 patients, 84% were confirmed HCV-infected by serological analysis. We did not examine serum samples for HCV RNA by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Using simple serological methods alone, it is possible to misclassify some individuals who may be falsely anti-HCV negative or have undergone HCV seroreversion as severe immunosuppression supervenes. However, this is unlikely to be the case in this cohort, as the majority of the patients were HCV-tested prospectively in the course of their HIV infection, before they became severely immunocompromised; HCV testing also included the use of 2nd generation EIA confirmed with 3rd generation RIBA which dramatically reduces chances of false negativity (Ragni et al. 1992). The most accurate method for assessing HCV infection retrospectively is serologically rather than through RT-PCR for HCV-RNA; since it is not always possible to determine how samples were initially processed and storage of samples for long periods exposes viral RNA to degradation by ribonucleases (Busch et al. 1992).

The findings on HIV progression are similar to those of previous cross-sectional and retrospective studies (Quan et al. 1993; Libre et al. 1993; Wright et al. 1994), and a smaller longitudinal study from Italy (Dorruci et al. 1995). In the Italian study, HCV co-infection also did not influence the rate of progression to either clinical (the development of AIDS) or immunological (CD4+ count <100 cells/mm³)
endpoints in HIV-infected IVDA. However, the length of follow up was considerably shorter than in the present study; 30 months compared with a median of 77.5 months (HCV-infected patients) and 82 months (HCV-uninfected patients) and it assessed a mixed population of HIV infected individuals (IDUs, homosexuals, heterosexuals, and those patients without any obvious risk factors for HIV transmission). The Italian study also did not attempt to assess individual seroconversion dates and progression, but instead assessed the clinical and immunological progression of the population over a defined 30 month period. Surprisingly, no deaths related to end stage liver disease were recorded, perhaps a finding also determined by the relatively short follow up period.

This study also considered the effect of chronic HIV infection on HCV-related liver disease in terms of mortality from end stage liver failure. Although there was no significant difference in mortality from liver disease between HCV antibody positive and HCV antibody negative patients, these deaths can be considered premature (in 8 of 11 patients) since they have occurred relatively early in the natural history of HIV disease, in individuals without AIDS and with relatively well maintained CD4+ counts. Interestingly, 3 of 8 patients were co-infected with hepatitis B virus (HBV) (and one with delta), perhaps an important cofactor in the progression of HCV-related liver failure. However, these data do not assess the prevalence or severity of clinically occult liver disease since liver biopsies were rarely performed, and mortality resulting from end stage liver failure alone was considered.

The interaction between immunosuppression and HCV-related liver disease is controversial. Reports have suggested that HIV may well accelerate the course of
HCV-related as well as HBV-related liver disease in selected patients (Eyster et al. 1993; Telfer et al. 1994). A large prospective study of haemophiliacs concluded that HIV accelerates the clinical course of HCV-related liver disease and that liver failure produces significant morbidity and mortality in patients coinfected with HCV and HIV; however, again, only the severe end of the spectrum of liver disease was considered in this study (Telfer et al. 1994). In the largest study of its kind, a recent multicentre cross-sectional study performed in 547 patients with chronically parenterally acquired hepatitis C infection with or without HIV infection (116 HIV positive and 431 HIV negative) demonstrated that HIV infection modifies the natural history of chronic HCV infection in this population with an unusually rapid progression to cirrhosis. For example, after a 10 year duration of infection, 13 of 87 (14.9%) HIV positive subjects developed cirrhosis compared with 7 of 272 (2.6%) in the HIV negative group (p<0.01); similar results were demonstrated at 5 and 15 years respectively. The authors calculated the mean interval from estimated time of infection to cirrhosis was significantly longer in the HIV negative compared with the HIV positive patients (23.2 v 6.9 years; p<0.001) (Soto et al. 1995).

The mechanism of potential interaction between immunosuppression and progression of HCV-related disease remains unclear. In vitro and in vivo studies have suggested that hepatitis C virus levels are increased in the presence of reduced numbers of CD4\(^+\) cells (Cribier et al. 1995; Soriano et al. 1995). However, the significance of this finding is uncertain; it is most unlikely that HCV is purely cytopathogenic, hepatic damage is thought to be at least partially immune mediated in nature (Krawcyzynski et al. 1992; Navas et al. 1994). Thus, severe
Immunosuppression in progressive HIV disease could even ameliorate the manifestations of immune mediated hepatic pathology, as is usually the case with HBV/HIV coinfection (Housset et al. 1992; Scharschmidt et al. 1992). There is also likely to be a complex interaction between other intrinsic and extrinsic factors in HIV/HCV coinfected patients. For example, in advanced HIV infection; there is evidence that in HCV-haemophiliacs some cases of liver failure may be precipitated by opportunistic infections or antiretroviral therapy (Eyster et al. 1993).

In conclusion, in this longitudinal study, co-infection with hepatitis C virus did not affect the clinical or immunological sequelae of chronic HIV infection. However, at least a proportion of premature deaths in HIV infection are likely to be due to progressive HCV-related liver disease; although the overall prevalence or severity of clinically occult HCV-related liver disease in this population is unknown because liver biopsies were rarely performed.

7.5 Summary

(1) It is unclear whether co-infection with HCV can influence HIV related morbidity or mortality, either by accelerating HIV related disease progression, or by contributing to end stage liver failure.

(2) This chapter examined the effect of HCV infection on the severity and progression of HIV disease in a cohort of Edinburgh intravenous drug abusers.
(3) In two hundred and forty patients (47%) of the Edinburgh cohort, both HIV seroconversion dates and anti-HCV serology were available.

(4) Parameters of HIV progression assessed included clinical end points (time of development of significant symptoms; time of development of AIDS and time of death) and immunological end points (CD4 counts < 200/mm³; 100/mm³ and 50/mm³ respectively).

(5) Two hundred and two of 240 patients (84%) had positive anti-HCV serology. There was no significant difference in the frequency of clinical and immunological endpoints between the anti-HCV positive and negative groups.

(6) Analysis from HIV seroconversion to HIV related clinical and immunological end points indicated that HCV serostatus did not significantly affect progression.

(7) Further data are required to assess the affect of HIV on the progression of HCV related liver disease.
CHAPTER 8: ASSOCIATION BETWEEN CHRONIC HEPATITIS C INFECTION AND HEPATOCELLULAR CARCINOMA IN A SCOTTISH POPULATION.

8.1 Introduction.

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, with an estimated annual incidence of 500,000 to 1,000,000 new cases (Moradpur and Wands 1994). Geographical areas carry different risk factors; the United Kingdom is regarded as an area of low risk for developing the disease (Haydon and Hayes 1995). It has been known for many years that hepatitis B virus (HBV) infection, aflatoxin B1 exposure, consumption of excess alcohol and some hereditary conditions (haemochromatosis, alpha-1-antitrypsin deficiency and hereditary tyrosinaemia) are important risk factors. Recently, there has been renewed interest in primary liver cancer following the recognition of the importance of chronic hepatitis C infection (HCV); epidemiological surveys have identified HCV in between 15-80% of patients with HCC depending on the population studied (Blum 1994). HCV appears to be the major cause of HCC in Japan, Italy and Spain, but is less
important in South Africa and Taiwan (Columbo et al. 1989; Simonetti et al. 1989; Bruix et al. 1989; Ruix et al. 1992; Kiyosawa et al. 1990; Saito et al. 1990; Kew et al. 1990; Bukh et al. 1993; Chen et al. 1990). There are no published data relating to HCV associated HCC in the United Kingdom.

The aim of this chapter was to assess the association between chronic HCV infection and HCC over a ten year period, in cases presenting to a single hospital in Scotland. Modes of transmission of HCV, incubation of infection and co-factors in the development of primary liver cancer were also considered.

8.2 Patients and Methods.

Patients.

One hundred and fourteen cases of histologically proven hepatocellular carcinoma (mean age: 69.2±11.4 years; 90 male; 109 (95%) of whom were ethnically Scottish), presenting to the Royal Infirmary, Edinburgh between 1/1/1985 and 31/12/1994 were included. The cases were obtained from examination of biopsy and autopsy data (stored in the University of Edinburgh Pathology Department) and also International Classification of Diseases hospital admissions data.

The records of these patients were examined to identify the aetiology of HCC. Documentation of the histology of liver tissue surrounding the cancer, together with possible sources of transmission and duration of blood-borne infectious hepatitis was made. The time of transmission of HCV infection was calculated from the year of first intravenous drug abuse or first blood transfusion; only these patients were used to
calculate the range / median duration of infection. Serum samples had been stored at -70°C, from the time of diagnosis of HCC.

A population of cirrhotic patients (n=29; 22 male; mean age: 52 +/- 14.5 years), presenting during the same time period (1985-1994), was also examined. All had chronic hepatitis C infection; a significant proportion had previously been diagnosed as having "cryptogenic" cirrhosis.

Methods

Anti-HBV and Anti-HCV Serology.

Serological markers of HBV infection were detected with standard assays (RIA, Abbott laboratories, Weisbaden, Germany).

Anti-HCV antibodies were detected by a second generation Enzyme Immunoassay (EIA, Abbot laboratories, Weisbaden, Germany) and also by third generation Recombinant Immunoblot Assays (RIBA-3, Chiron, Emeryville).

HCV RNA Extraction From Serum Samples.

HCV RNA was extracted from serum samples as described in section 2.3.

RT-PCR and Viral Genotyping.

RT-PCR for HCV RNA was carried out on serum and liver biopsy samples according to the specifications in section 2.5. HCV genotyping was completed by RFLP analysis of secondary PCR products (section 2.6).
8.3 Results.

Of 114 patients with hepatocellular carcinoma, presenting to the RIE over a 10 year period, 80 (70%) stored serum samples were available for HCV testing and assessment of HCC aetiology.

Aetiology of HCC

HCV Ab. serology was positive in 24 (21 male; mean age: 67+/−12 years; all ethnically Scottish) cases (30% of tested samples), of whom two also had markers of current HBV infection (HBsAg positive), and a further six had markers of past infection (HBsAg negative; Anti-HBs positive; Anti-HBc positive). An additional two had a history of alcohol abuse (>40 units/week) for more than ten years. 20 of 24 cases were positive for HCV RNA by RT-PCR.

Examination of cases with negative HCV serology indicated that 13 (16%) were chronic carriers of the HBV virus (HBsAg positive); alcohol abuse was the sole risk factor in 16 (20%) cases; haemochromatosis in 7 (9%); primary biliary cirrhosis in 2 (2.5%) and acute intermittent porphyria in 2 (2.5%). In 16 (20%) cases, the aetiology of liver cancer could not be determined either from the medical records or from serology (Figure 8.1).

HCV Genotyping in HCC and Cirrhotic Populations.

Genotyping of the virus was possible in 20 patients; of whom 16 were type 1b, 3 type 4 and 1 type 5. In the cirrhotic population, a broader genotype distribution was demonstrated: genotype 1a: n=7; genotype 1b: n=8; genotype 2b: n=3; genotype 3a:
n=8 and genotype 4; n=2. One cirrhotic patient was serum HCV RNA negative by RT-PCR (Table 8.1).

Risk Factors for HCV Transmission and Duration of Infection.

Examination of risk factors for HCV transmission identified 15 patients who had had blood transfusions between 1940 and 1972 in Scotland; two of these had had more than one transfusion. One had chronic HCV infection following injection of intravenous drugs between 1979 and 1989. A further 8 patients (one of whom was a Jehovah’s Witness) had no recognised risk factors for HCV infection. There were no data available concerning the acquisition of HCV infection by covert percutaneous exposure or non-parenteral routes. The time from presumed HCV transmission to development of HCC ranged from 10 to 50 years (median 30 years) in the posttransfusion / IVDA patients. The range in duration of infection in the cirrhotic population was 10 to 40 years (median: 19 years); this was a significantly shorter duration than in the HCC population (p=0.0009).

Histology of Non-Cancerous Liver Tissue in the HCC Population.

Cirrhosis preceded HCC in 20 out of 24 HCV infected cases (83%). In two cases, HCC developed from a hepatitic liver, and in one (the patient had a lobectomy), the background histology of the tumour was normal (Figure 8.2). In the whole HCC population, cirrhosis preceded HCC in 76 (82.5%) cases; other pathology was demonstrated in 8 (10%), and it was unclassified (because the tumour alone was biopsied) in 6 (7.5%).
Figure 8.1: Aetiology of Hepatocellular carcinoma 1985 - 1994.
<table>
<thead>
<tr>
<th></th>
<th>HCV Associated HCC</th>
<th>HCV Associated Cirrhosis</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>24</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Age (years)*</td>
<td>67 +/- 12</td>
<td>52 +/- 14.5</td>
<td>p = 0.0002</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>21:3</td>
<td>22:7</td>
<td></td>
</tr>
<tr>
<td>Years of Diagnosis</td>
<td>1986-1995</td>
<td>1986-1995</td>
<td></td>
</tr>
<tr>
<td>Mode of Infection</td>
<td>RCC Transfusion: 15</td>
<td>RCC Transfusion: 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IVDA: 1</td>
<td>IVDA: 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sporadic: 8</td>
<td>Sporadic: 6</td>
<td></td>
</tr>
<tr>
<td>Duration of Infection (years)#</td>
<td>30 (10-50)</td>
<td>19 (10-40)</td>
<td>p = 0.0009</td>
</tr>
<tr>
<td>Alcohol Abuse</td>
<td>2</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Current HBV Infection</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Past HBV Infection</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Serum RT-PCR positive (HCV RNA)</td>
<td>20</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>HCV Genotypes (%)</td>
<td>Genotype 1b: 16 (80)</td>
<td>Genotype 1a: 7 (25)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Genotype 4: 3 (15)</td>
<td>Genotype 1b: 8 (29)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Genotype 5: 1 (5)</td>
<td>Genotype 2b: 3 (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genotype 3a: 8 (29)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genotype 4: 2 (7)</td>
<td></td>
</tr>
</tbody>
</table>

Table 8.1 (*Mean +/- SD; #Median (Range)) Comparison between HCV-HCC population and HCV-cirrhotic population.
Histology of background liver tissue.

Figure 8.2: Histology of non-cancerous liver tissue.
8.4 Discussion.

In this study, we have identified chronic HCV infection as a major risk factor for the development of HCC in a Scottish population, an observation already made in high risk areas for HCC, such as Japan, Italy and Spain (Columbo et al. 1989; Simonetti et al. 1989; Bruix et al. 1989; Ruix et al. 1992; Kiyosawa et al. 1990; Saito et al. 1990).

There is a strong association between chronic HCV infection and hepatocarcinogenesis, through the pathway of chronic liver injury, regeneration and cirrhosis; cirrhosis preceded HCC in 20 out of 24 (83%) of our cases (Figure 4). In Western countries the cancer commonly occurs late in life, mostly in cirrhotic nodules (Kew and Popper 1984). These data support this observation; the patients were elderly and most were infected after receiving blood transfusions a median time of 30 years previously. This is a similar time period to that noted in a recent study from Spain, which found the interval between the date of blood transfusion and the diagnosis of HCC to be 26.8+/−12.4 years (Castells et al. 1995). However, in 3 cases, a tumour developed from a normal or hepatitis liver; further evidence for the persistence and replication of HCV genomes in primary liver cancer developing in the absence of cirrhosis or other risk factors (De Mitri et al. 1995). In these cases, the mode of hepatocarcinogenesis is unclear; HCV lacks reverse transcriptase activity and is not thought to be associated with genomic integration, whilst little is known about the biological properties of HCV proteins. Certainly the mechanism is different to hepatitis B virus (HBV). HBV is believed to be directly oncogenic either by HBV
integration causing increased genomic instability or by transactivation of cellular genes (Wang et al. 1990; Dejean et al. 1986; Feitelson 1992).

Co-factors to HCV seropositivity in the development of HCC were noted in four patients; either current HBV infection (2 patients) or alcohol abuse (2 patients). A further six patients had markers of past hepatitis B infection (HBsAg negative; Anti-HBs and Anti-HBc positive). HBV may still be implicated in these cancers since its DNA can persist in both serum and liver of patients with serology of past infection (Paterlini et al. 1990; Paterlini et al. 1995). The relationship between the two viruses is unclear. In vitro, HCV core protein can suppress HBV expression and encapsulation (Shih et al. 1993); whilst clinical studies have suggested a reciprocal and inverse relationship between HCV and HBV replication (Pontisso et al. 1993). Further studies are required to determine whether there is synergy between the HCV genome and HBV DNA in the process of hepatic oncogenesis.

HCV genotype 1b was predominant (16 out of 20 patients: 80%); this contrasts with the population of cirrhotics (only 8 out of 28 patients were infected with genotype 1b: 28%). As already discussed in Chapter 3 the difference in epidemiology may be partly explained by the prevalence of type 1b in older patients and in those with a longer duration of infection (our HCC population was significantly older, and had been infected significantly longer than the cirrhotic population). The distribution of HCV genotypes among blood donors in Scotland 30 to 50 years ago is unknown; identification of patients who received blood transfusions containing genotypes other than 1b, and their clinical course, would provide a control group to the patients who developed cancer in the present study. Unfortunately, it is not
possible to identify these patients and the suspicion remains that the prevalence of genotype 1b in this HCC population represents the most prevalent genotype a median time of 30 years ago.

Genotype 4 is commonly associated with HCV infection in Egypt, other African countries and the Middle East (Dusheiko et al. 1994). It has not been reported in Scottish or European blood donor populations. The three patients with genotype 4 and HCC had not visited any of these countries; however, all three were infected through blood transfusions in the 1940’s and 1960’s. Likewise, genotype 5 was also associated with HCC in one case, where a patient had received a blood transfusion in 1966, but had never travelled to the African continent. This genotype was originally found in South Africa; low frequencies have been reported in the Netherlands, Australia and Canada (Dusheiko et al. 1994). Currently, this is the only evidence for the association of genotypes 4/5 and primary liver cancer in the western world.

In conclusion, these observations indicate that, in a population of Scottish patients, there is a strong association between chronic HCV infection, cirrhosis and hepatocarcinogenesis. However, the study was unable to distinguish whether the high prevalence of genotype 1b in the HCC population reflects increased oncogenicity per se, or simply a background epidemiology of the most prevalent genotype, a median time of 30 years ago. Larger, population based studies are required to confirm that HCV genotypes vary in their propensity to produce clinically significant liver disease; and to establish the role of co-factors and the host response in these processes.

8.5 Summary
(1) Scotland is regarded as an area of low prevalence for hepatocellular carcinoma.

(2) This chapter assessed the association between HCV infection and HCC in 114 cases of histologically confirmed liver cancer presenting between 1985 and 1994.

(3) Samples positive for HCV Ab in this population were genotyped and compared with a population of 29 cirrhotic patients with chronic HCV infection, also diagnosed between 1985 and 1994.

(4) Chronic HCV infection was a major risk factor identified (30% of tested HCC patients. HCV genotype 1b was predominant (16 of 20 patients). The time from HCV transmission to development of cancer ranged from 10 to 50 years (median = 30 years).

(5) In the cirrhotic patient population, a broader range of genotypes was present. However, this population was significantly younger and demonstrated a significantly shorter duration of infection: range 10-40 years (median: 19 years).

(6) This chapter demonstrates a strong association between chronic HCV infection, cirrhosis and hepatocarcinogenesis in this Scottish population. However, it cannot distinguish whether the high prevalence of genotype 1b in the HCC population reflected increased oncogenicity per se, or whether 1b was simply the most prevalent genotype in Scotland when these patients were infected.
9.1 DISCUSSION

Hepatitis C virus infection is a global health problem, with an estimated 100 million people world-wide infected with the virus. In the UK, between 0.2 and 0.02% of the population are infected with the virus (Trent Regional Hepatitis C Virus Study Group 1994). The current healthcare cost of HCV-related illness is unknown, although in the USA, it adds up to approximately one billion dollars every year (Alter et al. 1995). The epidemiology and natural history of HCV are now well defined. Only interferon-alpha has been demonstrated to normalise serum alanine transferase in a sustained fashion, although this occurs in only a small proportion of patients with chronic HCV infection.

This thesis examined various aspects of the severity and activity of liver disease in patients chronically infected with the hepatitis C virus. Its aims were twofold; firstly to assess the clinical significance of staging investigations, in particular the significance of molecular virological investigations and the role of non-invasive investigations in terms of disease diagnosis and prognosis. Secondly, using these tools, the impact of chronic hepatitis infection was assessed in two populations:
patients diagnosed as having hepatocellular carcinoma and those immunocompromised by chronic HIV infection.

In Chapters 3 and 4, the clinical significance of HCV genotypes, serum and intrahepatic levels was established in the local Scottish population. Most patients with chronic HCV infection in Edinburgh are infected with genotypes 1a and 3; and although apparently there was a significant association between HCV genotype 1b and the development of cirrhosis, these patients were older and infected a significantly longer time ago. Likewise, in Chapter 8, the data were unable to distinguish whether the high prevalence of genotype 1b reflected increased oncogenicity per se, or the background epidemiology of the most prevalent genotype a median time of 30 years ago. These data may indicate a change in the epidemiology of HCV genotypes over time, which could explain the older mean age of patients with genotype 1b, and thus the association between genotype 1b and severe liver disease is probably a cohort effect. The model postulated to explain these changes describes “waves” of HCV infection affecting Edinburgh over the past 40 years; the initial wave was probably caused by genotype 1b, followed by genotypes 1a and 3.

Alternatively, it is possible that some HCV types or subtypes directly cause liver cell transformation. Recent in vitro findings have provided evidence for the clinical observations of the association between genotype 1b and the occurrence of HCC in non-cirrhotic livers (De Mitri et al. 1995), and the association between genotype 1b and hepatocellular carcinoma in a small prospective study examining a cohort of 163 consecutive HCV associated cirrhotic patients (Bruno et al. 1997). Stable expression in the NIH 3T3 cells of the N-terminal part of NS3, which encodes
one of the two serine proteases implicated in HCV polyprotein processing, can induce a transformed phenotype (Sakamuro et al. 1995). Further, a recent report has shown transformation of rat embryo fibroblasts upon cotransfection of the HCV capsid and the ras oncogene (Ray et al. 1996); this observation was made in the context of the multiple effects of the HCV capsid, which has been demonstrated to modulate the expression of several cellular genes such as c-myc, interferon beta and retinoblastoma (Ray et al. 1995; Wan Kim et al. 1994). The mechanisms for these observations are currently unknown, and it is also unknown whether the biological effects are genotype dependent, since 1b has served as the prototype and has been used in all these in vitro studies. Finally, concurrently, to this postulated mechanism of cellular transformation, it has also been noted that there is differing maturation of the capsid protein according to HCV genotype (Lo et al. 1995), although the functional relevance of these findings and in particular their impact on the transformation process are uncertain.

These data and others would suggest that clinical implications of HCV genotypes may be discussed in five broad areas: epidemiological distribution, diagnosis, prognosis, response to interferon and liver transplantation. As demonstrated in Chapter 3, the geographical distribution of HCV genotypes may provide information on viral transmission and even clues to viral origin; indeed, the study of HCV genotypes may provide important data on the pattern of population migration and transmission, but in practice, HCV genotyping provides little epidemiological information for use in individual cases. A high level of HCV heterogeneity has impact on both serological and molecular diagnostic assays for the quantification of HCV viraemia. As discussed in Chapters 1 and 4, previous studies
have demonstrated that patients with HCV genotypes 2 and 3 infection had a lower viraemia level compared with HCV genotype 1; although recent studies have disputed these data, and demonstrated no difference in HCV level between patients infected with HCV genotypes 1, 2 and 3 (Lau et al. 1996; Smith et al. 1996). Likewise, another recent study indicated that patients with genotypes 2 and 3 showed a lower level of antibody reactivity against HCV genotype 1 capture antigens in immunoassays (Dhaliwal et al. 1996). The authors suggested that this lower reactivity was probably caused by the different genotypic antigenicity related to the structure of the polypeptide sequence and structure. However, these data do not of course affect the current excellent specificity and sensitivity of the third generation immunoassays for HCV.

In Chapters 3, 4, 5 and 8, the individual role of HCV genotypes, and their role as part of several well defined host and viral factors in the severity and progression of chronic HCV infection was discussed. These studies highlighted three major obstacles to the clarification of these roles. Firstly, the evolution of chronic HCV infection is insidious and active disease develops in only a proportion of patients. Thus, these and other studies are retrospective or cross-sectional in nature, and in the future a large number of patients will need to be studied in a prospective fashion to determine the impact of HCV genotype on the natural history of HCV infection. Secondly, treatment with interferon may be more effective in certain HCV genotype infections, which will have an impact on the natural history if interferon is available to all participants. Thirdly, the changing epidemiology of HCV genotypes with time, well illustrated in Edinburgh, impacts on the interpretation of the role of
HCV genotype on the natural history of disease evolution. Certainly, the larger population based studies suggested above will be needed to confirm whether HCV genotypes vary in their propensity to produce clinically significant liver disease, and to confirm the co-factors in these processes; at present there is not enough evidence to recommend HCV genotypes as a prognostic indicator in disease progression.

It is now generally accepted that patients infected with genotypes 2 and 3 are more likely to respond to interferon compared with patients infected with genotype 1. The cause of the resistance of genotype 1 and particularly 1b infection to therapy with interferon is unknown. However, recent data have provided insights into a possible mechanism; studies have observed that HCV RNA persists in patients who do not respond to interferon, but that the distribution of quasispecies changes (Enomoto et al. 1996). The entire hepatitis C genome was sequenced in patients who were non-responders before and after interferon therapy, the authors hypothesising that therapy would clear interferon sensitive quasispecies, leaving behind the resistant ones. Side by side comparisons of the resulting predicted amino acid sequences showed that the predominant virus sequence in these patients changed and that the most consistent change occurred in the carboxy-terminal region of the NS5A gene, between amino acids 2209 and 2248: “the interferon sensitivity determining region” or ISDR. Isolates with an ISDR sequence that was the same as in the “wild type” genotype 1b HCV RNA were resistant to interferon, persisting in serum despite therapy. Isolates with multiple substitutions in the ISDR, referred to as the “mutant type”, disappeared during interferon therapy. The function of the ISDR enabling interferon resistance is currently unknown; indeed as stated in Chapter 1, the function of the NS5A region
has not been identified. However, the neighbouring NS5B region partially codes for viral RNA-dependent RNA polymerases, and therefore the NS5A component may also be important in viral replication. The NS5A region of the wild type genotype 1b virus might therefore encode a protein that helps HCV resist the inhibitory effects of interferon on protein synthesis or viral persistence in cells. These extraordinary data are preliminary, and yet have been confirmed in other studies in Japanese populations (Chayama et al. 1997; Kurosaki et al. 1997); interestingly, the frequency of the mutant genotype 1b has been too low in European populations for analysis (Zeuzem et al. 1997). Hence, differences in genomic diversity in the NS5A region may account for differences between response rates to interferon in Japan and Western countries.

Therefore, as stated in Chapters 3 and 4, HCV genotypes may be used for the selection of patients for therapy; however, for genotyping to become a predictive factor in the outcome of therapy, this factor has to be tested by well designed prospective studies that are analysed by multivariate analysis. In the absence of these studies, the relationship between HCV genotype and response to interferon remains an association, and all patients with chronic HCV infection, who have no contraindications, should be offered therapy.

Finally, the impact of HCV genotypes on liver transplantation also remains an unresolved issue. In Chapter 1, data were presented suggesting that HCV genotype 1b may be associated with a more aggressive course after liver transplantation (Feray et al. 1993; Gane et al. 1996). However, this was not confirmed by a large study based on patients in the USA (Zhou et al. 1996); therefore currently, HCV genotyping should have no impact in the management of transplant patients with chronic HCV
infection, especially since there are no 5 year follow up data on these patients and this heavily immunosuppressed population is an artificial one for studying the natural history of the disease.

The exact significance of the quantity of circulating HCV RNA remains unclear. Although previous reports have suggested a variety of significant relationships, no correlation was demonstrated in Chapter 3 between virus level and mode of transmission, HCV genotype or severity of infection. In Chapter 4, no correlation between serum and liver HCV RNA levels was demonstrated and currently, interpretation of serum virus levels as reflecting virus level in the liver or total virus level in the body of patients is invalid.

These data and others suggest that the clinical implications of serum HCV virus levels may be assessed in four broad areas. Firstly, although initial reports suggested that HCV RNA levels are higher in patients with more active disease (Gretch et al. 1995), recent studies have not demonstrated any relationship between HCV virus level and disease activity (Lau et al. 1996; Smith et al. 1996; Zeuzem et al. 1996) and therefore, these data do not have any prognostic implications. Secondly, it has been shown consistently that low pre-treatment serum HCV RNA levels are associated with a better subsequent response to interferon therapy (Davis et al. 1997). Multivariate analyses have indicated that low pre-treatment levels and HCV genotypes are independent factors associated with a more favourable response to therapy. In 652 patients in 11 reviewed studies, a complete and sustained response was more frequent in those patients who had HCV RNA levels less than 1x10^6 particles/ml than in subjects with higher levels (50.5% v 17.3%) (Davis et al. 1997).
However, the authors state that if a virus level $<1 \times 10^6$ particles/ml was used as a selection criteria for interferon, it would have low predictive value (71% for complete and sustained response), excluding a significant proportion of responders. Thirdly, there are now established preliminary data suggesting that serum virus levels will serve as an important factor in the design of an algorithm for monitoring therapy for patients with chronic hepatitis C; although the significance of the commonly observed discordance between biochemical response and change in virus levels remains unclear (Lau et al. 1993). Finally, in liver transplantation, studies have not yet addressed whether or not pretransplant HCV levels have any bearing on graft disease or survival; all patients with HCV infection who are transplanted have recurrence of HCV infection and there is a mean increase of one log in virus levels following liver transplantation (Chazouillerres et al. 1994; Lau et al. 1993). However, again there are no data demonstrating the positive diagnostic advantage of high HCV levels in transplant patients with recurrent HCV infection.

In Chapter 4, repetitive negative RT-PCR for HCV RNA in serum did not indicate complete hepatic elimination of HCV; future studies examining the natural history of HCV infection must include these patients, since currently, there is no evidence that these patients are at lower risk of severe disease in the future. These data also suggest that RT-PCR for HCV RNA in the liver is the “Gold Standard” for assessing the efficacy of modes of elimination of HCV from the liver such as new therapies. In parallel to the conclusions from Chapter 3, no significant associations were demonstrated between intrahepatic HCV levels and other clinicopathological parameters and again, future sequential studies of chronic HCV infection in terms of
molecular virological and clinical parameters are required before the clinical significance of intrahepatic levels is established.

In Scotland, there are likely to be over 5,000 patients infected with the hepatitis C virus, the logistics of undertaking a biopsy in each of these patients is considerable. Further, long-term follow up of these patients potentially requires serial liver biopsies to repeatedly reassess liver histology; for these reasons, HCV infected patients would benefit from a reliable, non-invasive method of predicting cirrhosis. Chapter 5 presents a pilot study using artificial neural network analysis and demonstrates it to be a superior method to multiple logistic regression as a non-invasive predictor of cirrhosis in HCV patients. Such a model could potentially revolutionise the overall management of patients with chronic HCV infection, particularly since the trained networks can be operated automatically using a personal computer. Indeed, they can provide an instant answer with a high degree of confidence as to whether a patient with HCV infection is cirrhotic or not.

Correlating these data with the results from Chapters 3 and 4, it is interesting that the six most weighted inputs (for the best performing Ward-type analysis) were patient age, serum albumin level, HCV level, HBV status, duration of infection and HIV status. However, when some of the clinical inputs were omitted, there was a consequent decrease in the ANN analysis performance; this suggests that the most weighted inputs should not be interpreted as providing all the necessary information for the classification of cirrhosis; instead they are parameters that have the most important effect on the ANN classification. It is thus hardly surprising that the prognostic importance of individual molecular virological variables as discussed above
was limited. Clearly, this type of analysis should now be applied to a large, well described population, for example, all those patients with chronic HCV infection in Scotland.

A further investigation of a non-invasive predictor of cirrhosis, uninfluenced by host and virus factors, is presented in Chapter 6; serum hyaluronan is demonstrated to also reliably differentiate cirrhotic from non-cirrhotic liver disease in chronic hepatitis C infection. An HA value > 100μg/l was sufficient to diagnose cirrhosis with a specificity of 93%; at a cut-off value of 200μg/l the specificity increased to 98%, and further to 100% at a value of 300μg/l. Although the raised levels of HA are primarily secondary to poor excretion by sinusoidal cells, HA is primarily a marker of fibrosis. As discussed, this is particularly important in those groups of patients where accurate knowledge of liver histology is crucial for their management and there is an absolute or relative contraindication to liver biopsy, for example in HCV infected haemophiliacs. This test could also be used to monitor the progression of fibrosis after a pilot biopsy, avoiding the need for multiple biopsies over the years of follow up of individual patients. These data also require to be confirmed in a larger population of patients, and could, for example, be incorporated into the neural network or, along with histological data used as a control for it.

The final population of patients studied were a group of intravenous drug abusers with chronic HIV infection (Chapter 7). HCV infection did not influence the clinical progression of HIV disease to AIDS and it was not associated with a more rapid immunological decline. Although a proportion of premature deaths in HIV infection were due to progressive HCV related liver disease, the reciprocal
relationship assessing the overall effect of HIV infection on the prevalence or severity of clinically occult liver disease could not be studied because liver biopsies were rarely performed. Clearly this issue needs to be addressed, particularly since immunosuppression per se affects host virus level. Because of the difficulty in obtaining liver biopsies in this large population, assessment of the incidence of cirrhosis in the entire population using non-invasive makers such as serum Hyaluronate, or prediction of the presence of cirrhosis using neural networks would be particularly relevant.

9.2 Suggestions for Future Work.

In this thesis it is clear that there are large numbers of patients in whom the mode of transmission of hepatitis C is unclear (labelled for convenience as “sporadic” transmission), hence continued monitoring of the epidemiology of acute and chronic hepatitis C is necessary. Additional studies of the specific mode of transmission in minority groups, low socio-economic groups, institutionalised individuals and intravenous drug abusers are needed as well as more information on sexual, household, occupational, nosocomial and perinatal transmission.

Secondly, large scale long-term studies are needed to define the natural history of hepatitis C both in large populations, and in subpopulations, such as ethnic minorities, children, those older than 60 years, HCV infected patients with normal ALT, HCV infected patients co-infected with HIV and intravenous drug abusers. Concurrent follow up studies assessing the role of ultrasonography and
alphafetoprotein monitoring for early detection of HCC in patients with chronic hepatitis C are required.

Thirdly, data are required on the recovery from and persistence of viral infection as well as the pathogenesis and mechanism of liver cell injury by HCV. The data from this thesis do not favour a cytopathic mechanism for liver disease, but whether it is wholly immunologically mediated is unclear. Likewise the mechanism of fibrosis is unknown, and as already discussed, it is also unclear whether fibrosis can be separated from inflammation / necrosis of the liver. Such studies would be greatly facilitated by the development of suitable animal and cell culture models.

Fourthly, there are a series of unresolved issues regarding the diagnostic tests for hepatitis C. The “gold standard” for HCV RNA assays remains to be established and the reliability, dynamic range and intra-assay variability of HCV RNA tests need to be improved. There are currently no criteria for selecting patients for, or withdrawing patients from treatment.

This discussion highlights the importance of clear and evidence based information being provided for both patients and physicians regarding chronic hepatitis C infection before a consensus over the natural history, means of prevention, management and therapy can be formulated for the next millennium.

9.3 Summary.

(1) The clinical implications of HCV genotypes may be discussed in five broad areas: epidemiological distribution; diagnosis; prognosis; response to interferon and liver transplantation.
(2) The clinical implications of serum HCV levels may be assessed in four broad areas: prognosis; response to interferon; designing algorithms for therapy and liver transplantation.

(3) The aims of future work in these areas should be as follows: firstly to examine further the large numbers of patients in whom the mode of transmission of the virus is unclear; secondly to undertake long-term studies to define the natural history of HCV in large populations and subpopulations; thirdly, to acquire data on the recovery from and persistence of viral infection as well as the pathogenesis and mechanism of liver cell injury and finally, to improve the reliability, dynamic range and intra-assay variability of HCV RNA tests.

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(i) The History of Epidemic Hepatitis.

Amongst the first recorded descriptions of disease of the liver are to be found in the Babylonian Talmud (5th Century BC); jaundice appears to have been common at this time. Hippocrates at about the same time, described epidemic jaundice as the “fourth kind of jaundice”. The first mention of the contagious nature of jaundice occurs in a letter from Pope Zacharias to St. Boniface, Archbishop of Mainz; Zacharias urged that patients with jaundice should be separated “lest others catch the contagion”. Sydenham (1624-1689) in London also recorded detailed observations of epidemic jaundice, although Herlitz introduced the term “icterus epidermicus” following the outbreak of an epidemic of jaundice amongst civilians in Gottingen in 1791. The concept of epidemic jaundice was not accepted however.

By the middle of the 19th century, there was considerable debate amongst physicians as to whether epidemic catarrhal jaundice was of hepatic origin or obstructive in nature. The principal proponents of the obstructive hypothesis were Bamberger (1855) and Virchow (1865) who believed that swelling or obstruction of the ostium of the common bile duct was the principle cause of jaundice. The onset of the disease was always associated with a gastrointestinal upset, and it was assumed that a microbial infection spread upwards from the intestine to block the bile duct by
catarrhal inflammation or cholangitis. Likewise, Eppinger (1908) taught that all jaundice was obstructive in origin, whether the obstruction occurred in the in the larger extrahepatic ducts, as in catarrhal jaundice, or in the small biliary capillaries in the case of cirrhosis.

However, these views were paradoxical since it was already widely known that pandemics of epidemic jaundice had been commonly associated with wars during the previous five centuries; in the Middle ages, such outbreaks were known as “Campaign Jaundice”. Jaundice has afflicted the British Army throughout history: in 1743, at the end of the seven years war a large outbreak of jaundice occurred amongst British troops in Flanders. Twenty years later, Monro (1764) documented a further episode affecting serving troops in Germany. During the Boer War (1897-1901), 5648 cases of jaundice were recorded; a decade later massive outbreaks occurred in Egypt, Gallipoli and Salonika during the First World War. The largest outbreak of all, affected the British and German Armies fighting at the Battle of El Alamain, where the fighting was so intense and the terrain so inhospitable that the two sides were unable to bury their dead, leading to a rapid spread of many infectious diseases.

Stokes (1839) was the first to propose that catarrhal jaundice was not due to obstruction of the bile ducts. Further, during the Gallipoli campaign, Martin (1918) reporting on cases of jaundice, demonstrated necrosis of the parenchymal cells of the liver, and considered jaundice to be due to hepatitis following a systemic infection. McDonald (1908) first predicted that infective jaundice was caused by an agent smaller than a bacterium, and postulated that it must be a virus. However, it was not
until the human volunteer studies during the Second World War that finally established the viral aetiology of infective hepatitis.

(i.ii) The History of Serum Hepatitis.

The history of serum hepatitis is much shorter; Lurman (1885) reported the earliest recognised epidemic of serum hepatitis among the shipyard workers of Bremen in 1883 following an extensive vaccination campaign against smallpox. 191 of 1289 vaccinated employees developed jaundice after intervals ranging from several weeks to six months. Workers who were inoculated with different batches of "lymph" were not affected.

The wide-scale introduction of large syringes and long needles in venereal disease clinics was soon followed by outbreaks of jaundice at the start of the 20th Century. However, it was treatment of these diseases such as arsenic and bismuth, which were suspected as hepatotoxins. In 1943, the incidence of syphilis in STD clinics was reported as increasing from 4.2% to 16.5% during the years of the Second World War. Concurrently, there were reports of outbreaks of jaundice in other clinics where needles were shared, especially diabetic clinics, where common needles were used not only for administering insulin, but also for blood sugar estimations (Flaum et al. 1926; Sherwood et al. 1950). MacCallum (1943) was the first to suggest that the common factor in all these outbreaks was the transmission of jaundice from patient to patient by means of unsterilised syringes. The term "serum hepatitis" followed, after a Ministry of Health memorandum described an outbreak of 41 case of jaundice following injection of measles convalescent serum into children.
The problem of jaundice amongst the armed forces following yellow fever vaccination was a considerable one. In 1942, 28,585 American soldiers inoculated against yellow fever developed jaundice, and of these, 62 died. There was considerable evidence that hepatitis and jaundice following yellow fever immunisation was due to a filterable agent present in the human serum incorporated in the Tyrode virus culture medium containing minced chick embryos. Thus it was recommended: "...that pools of apparently normal human serum should not be used for human inoculation, unless the medical history of all the donors can be followed over a considerable length of time, preferably at least 1 month, the probable incubation period of infective hepatitis.....". Following the large volumes of blood transfused during the Second World War, the development of serum hepatitis during convalescence after major trauma also became widespread.

(i.iv) The First Serological Tests for Viral Hepatitis.

Despite these observations, there was no breakthrough in determining the cause. A series of experimental animals were injected with presumed infective material; human studies were also carried out using volunteers, who were often conscientious objectors, prisoners or soldiers. Two studies demonstrated faecal transmission of infectious hepatitis; the first in the UK (MacCallum and Bradley 1943) produced hepatitis in volunteer patients suffering from rheumatoid arthritis by feeding them faecal material, and the second in the US (Havens et al. 1945) demonstrated faecal transmission to volunteer prisoners. Two virus types were postulated; one termed IH (infectious hepatitis, now hepatitis A) was faecally transmitted, and the

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other SH (serum hepatitis, now type B) was parenterally spread. Further distinction between the two types emerged from studies undertaken at the Willowbrook State School, an institution for the mentally retarded where viral hepatitis was an epidemic disease (Krugman et al. 1967). One type resembled “classical” infectious hepatitis and had an incubation period of 30 to 38 days, a relatively short period of abnormal serum transaminase activity (3 to 19 days), and a high degree of contagion. The other type resembled serum hepatitis and had a longer period of incubation (41 to 108 days) and a longer period of abnormal transaminase activity (35 to 200 days). This type was only moderately contagious. Patients with the “infectious” type were immune to the “serum” type, and patients with the serum type immune to the infectious type.

Concurrently, precipitating and complement fixing antibodies were demonstrated in convalescent sera which reacts with antigen in acute phase sera and in saline extracts of normal and human liver from patients with hepatitis (Gear 1948). In 1953, this experiment was repeated and distinguished between the types of hepatitis; a substance in the acute phase serum of a patient with serum hepatitis fixed complement with sera from patients convalescent from serum hepatitis, but not with sera from recovering from infectious hepatitis or other forms of jaundice.

However, it was not until the discovery of the Australia antigen (Blumberg et al. 1967) that a specific and reproducible test finally became available for the diagnosis of serum hepatitis. A significant gap followed until a serological test became available for infectious hepatitis (1973), paving the way for the discovery of further hepatitis viruses with the molecular biology revolution and the dawn of a exciting new era in the early 1990s.
### Recognition of the disease

- Earliest
- East Mediterranean Peoples
- Chinese People

<table>
<thead>
<tr>
<th>Disease Type</th>
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<tbody>
<tr>
<td>Pre-history</td>
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</tr>
<tr>
<td>Glimmers of contagion</td>
<td>200s AD</td>
</tr>
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### Glimmers of contagion

- Pope Zaccharius
- Epidemic jaundice

<table>
<thead>
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<th>Disease Type</th>
<th>Time Period</th>
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<tr>
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</tr>
<tr>
<td>Campaign jaundice</td>
<td>1700s AD</td>
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</table>

### Obstructive vs. Catarrhal jaundice

- Conventional wisdom
- Campaign jaundice

<table>
<thead>
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<th>Time Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obstructive</td>
<td>1700s-1900s</td>
</tr>
<tr>
<td>Catarrhal</td>
<td>1600s through WWII</td>
</tr>
</tbody>
</table>

### Viral aetiology

- Learned argumentation
- Serum hepatitis from yellow fever vaccine

<table>
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<th>Time Period</th>
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</thead>
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<tr>
<td>Viral aetiology</td>
<td>1800s</td>
</tr>
<tr>
<td>Serum hepatitis</td>
<td>1900-1950</td>
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### The human volunteer

- Definitive experimentation
- Hepatitis A and B named
- Discovery of Australia antigen
- Serological test for hepatitis A developed

<table>
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<td>The human volunteer</td>
<td>1942-1970</td>
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<td>Hepatitis A and B</td>
<td>1947</td>
</tr>
<tr>
<td>Australia antigen</td>
<td>1965</td>
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<tr>
<td>Serological test</td>
<td>1973</td>
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### The molecular biology revolution

- Hepatitis C and G named

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<th>Time Period</th>
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</thead>
<tbody>
<tr>
<td>The molecular biology revolution</td>
<td>1990s</td>
</tr>
<tr>
<td>Hepatitis C and G</td>
<td>1990s</td>
</tr>
</tbody>
</table>

Table i.i: A short history of viral hepatitis.
APPENDIX 2: PUBLICATIONS

The following papers are reproduced with kind permission of the respective joint authors and publishers.
Association between chronic hepatitis C infection and hepatocellular carcinoma in a Scottish population

G H Haydon, L M Jarvis, P Simmonds, D J Harrison, O J Garden, P C Hayes

Abstract

Background—Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. The geographical prevalence varies considerably in different countries and Scotland is regarded as an area of low risk for the disease.

Aims—To assess the association between chronic hepatitis C infection (HCV) and HCC in a population of patients presenting to a single hospital.

Patients—One hundred and fourteen cases of histologically confirmed liver cancer presenting to the Royal Infirmary of Edinburgh between 1985 and 1994 were examined.

Methods—Of 114 cases of HCC, 80 samples of stored sera were available. Samples positive for HCV Ab were genotyped by restriction fragment length polymorphism analysis of HCV c-DNA. A population of 29 cirrhotic patients (diagnosed between 1985 and 1994) with chronic HCV infection was also genotyped.

Results—Chronic HCV infection was a major risk factor (30% of tested HCC patients) identified. HCV genotype 1b was predominant (16 of 20 patients). The time from HCV transmission to development of cancer ranged from 10 to 50 years (median 30). In the cirrhotic patient population, a broader distribution of genotypes was present (genotype 1a: 7; genotype 1b: 8; genotype 2b: 3; genotype 3a: 8 and genotype 4: 2). However, this population was significantly younger. (Mean (SD) 52 (14±5) years (p=0.0002) and demonstrated a significantly shorter duration of infection: range 10–40 years (median: 19).

Conclusion—There is a strong association between chronic HCV infection, cirrhosis, and hepatocarcinogenesis in this Scottish population. The study was unable to distinguish whether the high prevalence of genotype 1b in the HCC population reflected increased oncogenicity in itself, or whether 1b was simply the most prevalent genotype in Scotland when these patients were infected.

(Gut 1997; 40: 128–132)

Keywords: hepatocellular carcinoma, hepatitis C virus, HCV genotypes, hepatitis B virus.

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, with an estimated annual incidence of 500 000 to 1 000 000 new cases.1 Geographical areas carry different risk factors; the United Kingdom is regarded as an area of low risk of developing the disease.2 It has been known for many years that hepatitis B virus (HBV) infection, aflatoxin B1 exposure, consumption of excess alcohol, and some hereditary conditions (haemochromatosis, α1-antitrypsin deficiency, and hereditary tyrosinaemia) are important risk factors. Recently, there has been renewed interest in primary liver cancer, following the recognition of the importance of chronic hepatitis C infection (HCV); epidemiological surveys have identified HCV in between 15–60% of patients with HCC depending on the population studied.3 HCV seems to be the major cause of HCC in Japan, Italy, and Spain, but is less important in South Africa and Taiwan.4–12 There are no published data relating to HCV associated HCC in the United Kingdom.

Examination of the epidemiology of HCV infection in different geographical regions and in different age, risk, and racial groups has become possible following the identification and classification of viral genotypes.13 The viral genome is divided into a structural and non-structural region. The structural region contains genes for a nucleocapsid protein and the envelope glycoproteins. The genes in the non-structural region code for functional proteins. In addition, a nucleotide sequence at the 5′ portion in the structural region does not encode a gene product. This 5′ non-coding (5′-NCR) sequence is the most highly conserved region; the greatest heterogeneity occurs in the envelope proteins. HCV has been classified into a total of six major genotypes, showing not more than 70% sequence homology, by the distinct nucleotide sequences in the 5′-NCR; many of these contain a number of more closely related subgroups (a, b, and c).14 Viruses may be typed by restriction fragment length polymorphism (RFLP) analysis of cDNA amplified from the 5′-NCR by the reverse transcription polymerase chain reaction (RT-PCR).15 Preliminary reports suggest major biological differences between the genotypes in response to treatment with interferon16–18 and perhaps, progression and severity of disease.19

In this study, we aimed to assess the association between chronic HCV infection and HCC over a 10 year period, in cases presenting to a single hospital in Scotland. Modes of transmission of HCV, incubation of infection, and cofactors in the development of primary liver cancer were also considered.
Methods

Patients
A total of 114 cases of histologically confirmed hepatocellular carcinoma (mean (SD) age: 69.2 (11.4) years; 90 male; 109 (95%) of whom were ethnically Scottish), presenting to the Royal Infirmary, Edinburgh between 1 January 1985 and 31 December 1994 were included. The cases were obtained from examination of biopsy and necropsy data (stored in the University of Edinburgh Pathology Department) and also International Classification of Diseases hospital admissions data.

The records of these patients were examined to identify the aetiology of HCC. Documentation of the histology of liver tissue surrounding the cancer, together with possible sources of transmission and duration of blood borne infectious hepatitis was made. The time of transmission of HCV infection was calculated from the year of first intravenous drug abuse or first blood transfusion; only these patients were used to calculate the range/median duration of infection. Serum samples had been stored at −70°C, from the time of diagnosis of HCC.

Serological markers of HBV infection were detected with standard assays (RIA, Abbott laboratories, Weisbaden, Germany). Anti-HCV antibodies were detected by a second generation enzyme immunoassay (EIA, Abbott laboratories, Weisbaden, Germany) and also by third generation recombinant immunoassay (RIBA-3, Chiron, Emeryville, CA) for antibody to non-structural proteins 5-1 (NS4), c-100-3 (NS4), c33c (NS3), and core-associated antigen c-22-3. A population of cirrhotic patients (n=29; 22 male; mean (SD) age: 52 (14.5) years), presenting during the same time period (1985–1994), was also examined. All had chronic hepatitis C infection; a significant proportion had previously been diagnosed as having 'cryptogenic' cirrhosis.

RNA extraction
Viral RNA was extracted from 0.5 ml of stored serum from each of the patients as previously described.20 Briefly, serum samples were incubated at 37°C for 1.5 hours with 1 mg/ml proteinase K in the presence of 40 µg/ml polyadenylic acid, 0.5% SDS, 0.1 M NaCl, 50 mM TRIS HCl (pH 8.0), and 1 mM EDTA. Nucleic acid was extracted successively with phenol and chloroform-isomyl alcohol (50:1) and precipitated by the addition of one tenth volume of sodium acetate (pH 5.2) and two volumes of ethanol. The dried pellet was resuspended in 25 µl of diethyl pyrocarbonate treated water.

HCV genotyping
RNA was reverse transcribed and amplified using nested primers matching conserved regions in the 5′-NCR.21 Product DNAs were cleaved with restriction enzymes Hae-111/Rsa-1 and Mva-1/HinF-1.22 The fragments were separated by agarose gel electrophoresis using 4% metaphor agarose (FMC BioProducts, Rockland, ME). Phylogenetic comparisons of sequences in the conserved region of the genome confirm that the 5′-NCR can be used to distinguish the six major genotypes of HCV.22 Separation of subtypes 1a and 1b was undertaken by the cleavage patterns resulting from digestion with Mva-1.23 In three cases direct nucleotide sequencing of PCR products was carried out.

Results
Of 114 patients with HCC, presenting to the Royal Infirmary, Edinburgh over a 10 year period, 80 (70%) stored serum samples were available for HCV testing and assessment of HCC aetiology.

Aetiology of HCC
HCV Ab serology was positive in 24 (21 male; mean (SD) age: 67 (12) years; all ethnically Scottish) cases (30% of tested samples); of whom two also had markers of current HBV infection (HBsAg positive), and a further six had markers of past infection (HBsAg negative; anti-HBs positive; anti-HBc positive). An additional two had a history of alcohol abuse (>40 units/week) for more than 10 years. Twenty of 24 cases were positive for HCV RNA by RT-PCR.

Examination of cases with negative HCV serology indicated that 13 (16%) were chronic carriers of the HBV virus (HBsAg positive); alcohol misuse was the sole risk factor in 16 (20%) cases; haemochromatosis in seven (9%); primary biliary cirrhosis in two (2.5%), and acute intermittent porphyria in two (2.5%). In 16 (20%) cases, the aetiology of liver cancer could not be determined either from the medical records or from serology (Fig 1).

HCV genotyping in HCC and cirrhotic populations
Genotyping of the virus was possible in 20 of 24 patients; it was 1b in 16; genotype 4 in three, and genotype 5 in one. In the cirrhotic population, a broader genotype distribution was demonstrated: genotype 1a: n=7; genotype 1b: n=8; genotype 2b: n=3; genotype 3a: n=8, and genotype 4: n=2. One cirrhotic patient was serum HCV RNA negative by RT-PCR (Table).

Risk factors for HCV transmission and duration of infection
Examination of risk factors for HCV transmission identified 15 patients who had had blood transfusions between 1940 and 1972 in Scotland; two of these had had more than one transfusion. One, had current infection with HCV, HBV, and HIV at diagnosis following injection of intravenous drugs between 1979 and 1989. A further eight patients (one of whom was a Jehovah's Witness) had no recognised risk factors for HCV infection. There
Figure 1: Aetiology of hepatocellular carcinoma (diagnosed between 1985 and 1994) in the 80 patients in whom stored samples of sera were available for anti-HCV testing. The bars are stacked to illustrate the background histology of the patients.

were no data available concerning the acquisition of HCV infection by covert percutaneous exposure of non-parenteral routes. The time from presumed HCV transmission to development of HCC ranged from 10 to 50 years (median 30) in the post-transfusion/intravenous drug misuse patients.

The range in duration of infection in the cirrhotic population was 10 to 40 years (median 19); this was a significantly shorter duration than in the HCC population (p=0.0009).

Histology of non-cancerous liver tissue in the HCC population
Cirrhosis preceded HCC in 20 of 24 HCV infected cases (83%). In two cases, HCC developed from a hepatic liver, and in one (the patient had a lobectomy), the background histology of the tumour was normal (Fig 2).

In the whole HCC population, cirrhosis preceded HCC in 66 (82.5%) cases; other pathology was demonstrated in eight (10%), and it was unclassified (because the tumour alone was biopsied) in six (7.5%).

Discussion
In this study, we have identified chronic HCV infection as a major risk factor for the development of HCC in a Scottish population, a finding already made in high risk areas of HCC, such as Japan, Italy, and Spain.4,5 There is a strong association between chronic HCV infection and hepatocarcinogenesis, through the pathway of chronic liver injury, regeneration, and cirrhosis; cirrhosis preceded HCC in 20 of 24 (83%) of our cases (Fig 2). In Western countries, the cancer commonly occurs late in life, mostly in cirrhotic nodules.6,24 Our study supports this finding; the patients were elderly and most were infected after receiving blood transfusions a median time of 30 years previously. This is a similar time period to that noted in a recent study from Spain, which found the interval between the date of blood transfusion and the diagnosis of HCC to be 26-8 (12-4) years.25 However, in three of our cases, a tumour developed from a normal or hepatic liver; further evidence for the persistence and replication of HCV genomes in primary liver cancer developing in the absence of cirrhosis or other risk factors.26 In these cases, the mode of hepatocarcinogenesis is unclear; HCV lacks reverse transcriptase activity and is not thought to be associated with genomic integration, while little is known about the biological properties of HCV proteins. Certainly, the mechanism is different to hepatitis B virus (HBV). HBV is believed to be directly onco-genic either by HBV integration causing increased genomic instability or by trans-activation of cellular genes.28-30

Cofactors to HCV seropositivity in the development of HCC were noted in four patients; either current HBV infection (two patients) or alcohol misuse (two patients). A further six patients had markers of past hepatitis B infection (HBsAg negative; anti-HBs, and anti-HBc positive). HBV may still be implicated in these cancers because its DNA can persist in both serum and liver of patients with serology of past infection.31,32 The relation

Comparison between HCV associated HCC population and HCV associated cirrhotic population

<table>
<thead>
<tr>
<th></th>
<th>HCV associated HCC</th>
<th>HCV associated cirrhosis</th>
<th>Significance</th>
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<tbody>
<tr>
<td>Number</td>
<td>24</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>67 (12)</td>
<td>62 (14-9)</td>
<td></td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>21:3</td>
<td>22:7</td>
<td></td>
</tr>
<tr>
<td>Mode of infection</td>
<td>RCC Transfusion: 15</td>
<td>HCC Transfusion: 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IVDA: 1</td>
<td>IVDA: 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sporadic: 8</td>
<td>Haemophiliac: 3</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td>Sporadic: 6</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Haemophiliac: 3</td>
<td></td>
</tr>
<tr>
<td>Duration of infection (y)†</td>
<td>30 (10-50)</td>
<td>19 (10-40)</td>
<td>p=0.0009</td>
</tr>
<tr>
<td>Alcohol misuse</td>
<td>2</td>
<td>9</td>
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</tr>
<tr>
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<td>9</td>
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</tr>
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<td>Past HBV infection</td>
<td>6</td>
<td>9</td>
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<tr>
<td>Serum RT-PCR positive (HCV RNA)</td>
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<tr>
<td>HCV genotypes (%)</td>
<td>Genotype 1b: 16 (80)</td>
<td>Genotype 1b: 9 (29)</td>
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<tr>
<td></td>
<td>Genotype 4: 3 (15)</td>
<td>Genotype 2b: 3 (10)</td>
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<td>Genotype 5: 1 (5)</td>
<td>Genotype 3a: 8 (29)</td>
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<tr>
<td></td>
<td></td>
<td>Genotype 4: 2 (7)</td>
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</tr>
</tbody>
</table>

(*mean (SD); †median (range)). In the cirrhotic population a wider distribution of genotypes was noted; however, this population was significantly younger and had a significantly shorter duration of HCV infection than the HCC population.
between the two viruses is unclear. In vitro, HCV core protein can suppress HBV expression and encapsidation; while clinical studies have suggested a reciprocal and inverse relation between HCV and HBV replication. Further studies are required to determine whether there is synergy between the HCV genome and HBV DNA in the process of hepatic oncogenesis.

HCV genotype 1b was predominant (16 of 20 patients: 80%) in this study; this genotype has previously been reported to be responsible for the development of severe liver disease and the failure of interferon therapy. The dominance of this type contrasts both with the genotype distribution in current Scottish blood donors, (where type 1a forms the majority: 70–80%), and in our population of cirrhotic patients (only eight of 28 patients were infected with genotype 1b: 28%). The difference in epidemiology may be partly explained by the prevalence of type 1b in older patients and in those with a longer duration of infection (our HCC population was significantly older, and had been infected significantly longer than the cirrhotic population). The distribution of HCV genotypes among blood donors in Scotland 30 to 50 years ago is unknown; identification of patients who received blood transfusions containing HCV other than 1b, and their clinical course, would provide a control group to the patients who developed cancer in this study. Unfortunately, it is not possible to identify these patients and the suspicion remains that the prevalence of genotype 1b in this HCC population represents the most prevalent genotype a median time of 30 years ago.

Genotype 4 is commonly associated with HCV infection in Egypt, other African countries, and the Middle East. It has not been reported in Scottish or European blood donor populations. The six patients with genotype 4 and HCC had not visited any of these countries; however, all six were infected through blood transfusions in the 1940s and 1960s. Likewise, genotype 5 was also associated with HCC in one case, where a patient had received a blood transfusion in 1966, but had never travelled to the African continent. This genotype was originally found in South Africa; low frequencies have been reported in the Netherlands, Australia, and Canada. Currently, this is the only evidence for the association of genotypes 4/5 and primary liver cancer in the Western world.

In conclusion, these findings indicate that, in a population of Scottish patients, there is a strong association between chronic HCV infection, cirrhosis, and hepatocarcinogenesis. However, the study was unable to distinguish whether the high prevalence of genotype 1b in the HCC population reflects increased oncogenicity in itself, or simply a background epidemiology of the most prevalent genotype, a median time of 30 years ago. Larger, population based studies are required to confirm that HCV genotypes vary in their propensity to produce clinically significant liver disease, and establish the role of cofactors and the host response in these processes.


The impact of chronic hepatitis C virus infection on HIV disease and progression in intravenous drug users
Geoffrey H. Haydon, Peter J. Flegg, Carol S. Blair, Ray P. Bettle, Sheila M. Burns and Peter C. Hayes

Objective It is unclear whether co-infection with hepatitis C virus (HCV) influences HIV-related morbidity or mortality, either by accelerating HIV-related disease progression, or by contributing to end-stage liver disease. The aim of this study was to examine the effect of HCV infection on the severity and progression of HIV disease in a cohort of Edinburgh intravenous drug users (IDUs).

Methods In 240 (47%) out of 508 patients in the Edinburgh IDU cohort both HIV seroconversion dates and anti-HCV serology were available. Demographic variables and HIV-related progression between anti-HCV positive and anti-HCV negative groups were compared. Parameters assessed included clinical endpoints (time of development of significant symptoms attributable to HIV (CDC stage IV), time of development of AIDS, and time of death) and immunological endpoints (time of CD4+ counts dropping below 200/mm³, 100/mm³ and 50/mm³).

Results Two hundred and two out of 240 patients (84%) had positive anti-HCV serology. There was no significant difference in the frequency of clinical and immunological endpoints between the anti-HCV positive and negative groups. Progression analysis from HIV seroconversion to HIV related clinical endpoints indicated that anti-HCV serology was not a significant factor influencing the rate of HIV progression (relative risks (RR) for anti-HCV positive group: seroconversion to CDC IV, 1.01; seroconversion to AIDS, 1.05; seroconversion to death, 0.90). Likewise, HCV serostatus did not significantly affect progression to immunological endpoints (RR for anti-HCV positive group: seroconversion to CD4+ < 200/mm³, 1.04; seroconversion to CD4+ < 100/mm³, 1.13; seroconversion to CD4+ < 50/mm³, 0.97). Overall mortality from end-stage liver failure was 4% in HCV-seropositive patients without AIDS. This suggests that HCV has had a clinically (though not statistically) significant impact on overall survival in this cohort.

Conclusions This study demonstrates that HCV co-infection does not influence the rate of progression to either clinical or immunological endpoints in our population of HIV-infected drug users. Further data are required to assess the effect of HIV on the progression of HCV-related liver disease.

Keywords: hepatitis C infection, HIV infection, intravenous drug users, chronic liver disease

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Introduction
The rate of progression of clinical and immunological sequelae of human immunodeficiency virus (HIV) infection demonstrates considerable inter- and intra-population variability. Infection with other opportunistic viruses may affect the rate of disease progression, with members of the herpes virus and human retrovirus families being among those implicated as cofactors [1].

Both HIV and the hepatitis viruses (hepatitis B, C and D) are transmitted by parenteral routes, and co-infection with these viruses is common among patients with a history of intravenous drug use. In the example of hepatitis B virus (HBV) infection, HIV infection has an adverse effect on survival of HBV-infected patients [2]; conversely, actuarial survival in HIV-infected patients with or without AIDS does not appear to be altered by the presence of HBV co-infection in adults [3], although it may have an effect in children [4]. The interactive effects between hepatitis C virus (HCV) infection and HIV infections are complex, in part because the mechanisms of pathogenicity in HCV infection are poorly understood [5]. Most studies examining dual infection with HCV/HIV in IDUs have been cross-sectional [6,7] comparing immunological or virological markers or mortality between anti-HCV positive and anti-HCV negative patients with HIV, without providing any longitudinal data. Likewise, reports of the effect of HIV on HCV-related liver disease have been conflicting, with some studies suggesting rapid progression of liver disease...
evidence With patients. colleted ously Patients Methods infected history To tive pathology [8,9], 486 Statistical ELISA HIV this indulging first years. test, in 1991, significant tests. -HCV by Edinburgh IDU European Journal of Gastroenterology and Hepatology [10]. mirial -seroconversion dates were determined by a method of interval estimates. Seroconversion was defined as the mid-point between the last negative and the first positive test, if the interval between these times was less than 2 years. For those with no last negative sample, the earliest possible date of HIV acquisition was used as a surrogate first seronegative date marker (January 1983 for those indulging in risk activity (shared drug injection) prior to this time, or the historical date of initial risk activity for those who only started to inject drugs after this time). HIV-1/HIV-2 testing used a third generation recombinant immunoblot assay (RIBA-3, Chiron, CA).

Using the above methods, we have defined a subset of 240 individuals who have estimated HIV seroconversion dates and known HCV serostatus.

Statistical analysis
Comparison of demographic variables between groups was performed by chi-square analysis and Student’s t-tests. Progression from date of HIV seroconversion was calculated by Kaplan–Meier survival analysis. Clinical endpoints were: time of first development of clinically significant symptoms attributable to HIV (CDC stage IV), time of development of AIDS (CDC 1993 criteria), and time of death. Immunological endpoints were: time of CD4⁺ counts dropping below 200/mm³, 100/mm³ and 50/mm³, respectively (defined as the date at which the first of two consecutive values below the respective CD4⁺ count were recorded no more than 3 months apart).

Survival analysis was performed for AIDS cases and those individuals with CD4⁺ counts < 200/mm³ for all causes of death and also for deaths from end stage liver disease. Differences in progression were calculated by log-rank test. Cox’s proportional hazards model was used for multivariate analysis, with covariates including gender, age at HIV seroconversion, CMV status, HIV p24 antigen status (positive or negative), continued drug injection, use of antiretroviral therapy (more than 6 months continuous therapy with either didovudine, didanosine, zalcitabine or a combination), and opportunistic infection prophylaxis (more than 6 months continuous therapy with anti-Pneumocystis/Toxoplasma agents). Age at HIV seroconversion was converted into a dichotomous variable for the analysis according to whether an individual’s age was greater or less than the median.

Results
The demographic characteristics for the 240 patients of known HCV serostatus in the HIV seroconverter cohort are listed in Table 1. Of the 240 patients, 202 (84%) were confirmed HCV-infected.

Progression to clinical and immunological endpoints
Progression analysis assessing the time from HIV seroconversion to HIV-related clinical endpoints (symptoms or signs of CDC stage IV disease, AIDS or death) indicated that HCV co-infection was not a significant factor influencing the rate of clinical HIV progression (Table 2; Figures 1 and 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>HCV-infected</th>
<th>HCV-uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>202</td>
<td>38</td>
</tr>
<tr>
<td>Male/female</td>
<td>138/64</td>
<td>25/13</td>
</tr>
<tr>
<td>Median age at HIV seroconversion (years, range)</td>
<td>22.5</td>
<td>23</td>
</tr>
<tr>
<td>Median date of HIV seroconversion</td>
<td>Dec 1983</td>
<td>Jan 1994</td>
</tr>
<tr>
<td>Median duration of HIV infection (months, range)</td>
<td>96 (12–153)</td>
<td>95 (4–151)</td>
</tr>
<tr>
<td>Median follow-up (months)</td>
<td>77.5</td>
<td>82.0</td>
</tr>
<tr>
<td>Year of first drug use (median)</td>
<td>1981</td>
<td>1992</td>
</tr>
<tr>
<td>No. (%) with concurrent alcohol abuse</td>
<td>35 (16%)</td>
<td>9 (23.7%)</td>
</tr>
<tr>
<td>No. (%) with past/current HBV infection</td>
<td>58 (29%)</td>
<td>10 (26.3%)</td>
</tr>
<tr>
<td>No. (%) with CDC stage IV disease</td>
<td>143 (70.8%)</td>
<td>25 (66.4%)</td>
</tr>
<tr>
<td>No. (%) with AIDS</td>
<td>66 (32.7%)</td>
<td>10 (26.3%)</td>
</tr>
<tr>
<td>No. (%) progressing to CD4⁺ &lt; 200/mm³</td>
<td>111 (54.9%)</td>
<td>19 (50%)</td>
</tr>
<tr>
<td>No. (%) progressing to CD4⁺ &lt; 100/mm³</td>
<td>75 (37.1%)</td>
<td>10 (26.3%)</td>
</tr>
<tr>
<td>No. (%) progressing to CD4⁺ ≤ 50/mm³</td>
<td>53 (26.2%)</td>
<td>9 (23.7%)</td>
</tr>
<tr>
<td>Death resulting from AIDS-defining illness (%)</td>
<td>50 (24.9%)</td>
<td>8 (21.1%)</td>
</tr>
<tr>
<td>Death resulting from end stage liver disease:</td>
<td>11 (5.4%)</td>
<td>1 (2.6%)</td>
</tr>
<tr>
<td>in patients without AIDS (%)</td>
<td>6 (2.9%)</td>
<td>0</td>
</tr>
<tr>
<td>in patients with AIDS (%)</td>
<td>3 (4.3%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Death from all causes (%)</td>
<td>85 (42.1%)</td>
<td>14 (36.8%)</td>
</tr>
</tbody>
</table>
Progression analysis assessing the time from HIV seroconversion to immunological endpoints (CD4+ counts <200/mm³, <100/mm³ and <50/mm³) indicated that co-infection with HCV was not associated with differing rates of immunological progression (Table 3; Figures 3 and 4).

Likewise, survival following AIDS diagnosis or following a decrease in CD4+ count <200/mm³ was not influenced by co-infection with HCV (Table 2).

**Mortality**
A minority of the seroconversion cohort died from end stage liver failure; 11 were HCV seropositive, and one HCV seronegative (Table 1). This represented 19% of all medically related deaths (those unrelated to HIV, and excluding drug overdose, suicide and trauma) in those who were HCV seropositive. In patients without AIDS, eight (5.9%) of those who were HCV seropositive died of liver failure compared with none of the HCV seronegative group \((P = 0.216, \text{Fisher’s exact test})\).

### Table 3: Proportional hazards analysis of the effect of HCV serostatus on progression to immunological HIV-related endpoints

<table>
<thead>
<tr>
<th>Event</th>
<th>HCV seropositive ((n = 202))</th>
<th>HCV seronegative ((n = 38))</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seroconversion to CD4+ &lt;200</td>
<td>111/202</td>
<td>19/35</td>
<td>5.91</td>
<td>1.33-21.99</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.04</td>
<td>1.04</td>
<td>1.04</td>
<td>1.04-1.04</td>
</tr>
<tr>
<td>Seroconversion to CD4+ &lt;100</td>
<td>75/201</td>
<td>10/34</td>
<td>7.37</td>
<td>2.18-24.55</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13-1.13</td>
</tr>
<tr>
<td>Seroconversion to CD4+ &lt;50</td>
<td>53/201</td>
<td>9/34</td>
<td>6.21</td>
<td>1.72-22.81</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97-0.97</td>
</tr>
</tbody>
</table>

Progression analysis of the effect of HCV serostatus on progression to clinical HIV-related endpoints:

<table>
<thead>
<tr>
<th>Event</th>
<th>HCV seropositive ((n = 202))</th>
<th>HCV seronegative ((n = 38))</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seroconversion to CDC IV</td>
<td>143/202</td>
<td>26/38</td>
<td>5.4</td>
<td>1.39-19.96</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01-1.01</td>
</tr>
<tr>
<td>Seroconversion to AIDS</td>
<td>66/202</td>
<td>10/38</td>
<td>6.1</td>
<td>1.69-23.19</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90-0.90</td>
</tr>
<tr>
<td>Seroconversion to death</td>
<td>65/202</td>
<td>14/38</td>
<td>4.6</td>
<td>1.24-16.86</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.87-1.21</td>
<td>0.87-1.21</td>
<td>0.87-1.21</td>
<td></td>
</tr>
<tr>
<td>CD4+ &lt;200 to death</td>
<td>56/110</td>
<td>11/18</td>
<td>4.9</td>
<td>1.37-16.75</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.81</td>
<td>0.81</td>
<td>0.81</td>
<td>0.81-0.81</td>
</tr>
<tr>
<td>AIDS to any death</td>
<td>47/66</td>
<td>9/10</td>
<td>5.4</td>
<td>1.39-23.19</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.85</td>
<td>0.85</td>
<td>0.85</td>
<td>0.85-0.85</td>
</tr>
</tbody>
</table>

### Table 2: Proportional hazards analysis of the effect of HCV serostatus on progression to clinical HIV-related endpoints

<table>
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<tr>
<th>Event</th>
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<th>95% CI</th>
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<tr>
<td>95% CI</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01-1.01</td>
</tr>
<tr>
<td>Seroconversion to AIDS</td>
<td>66/202</td>
<td>10/38</td>
<td>6.1</td>
<td>1.69-23.19</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90-0.90</td>
</tr>
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<tr>
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<tr>
<td>95% CI</td>
<td>0.81</td>
<td>0.81</td>
<td>0.81</td>
<td>0.81-0.81</td>
</tr>
<tr>
<td>AIDS to any death</td>
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<td>1.39-23.19</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.85</td>
<td>0.85</td>
<td>0.85</td>
<td>0.85-0.85</td>
</tr>
</tbody>
</table>
Our samples for long samples chances of included tively in the reversion (RT infected. We did not below Progression analysis of time from study: 30 follow rate using method However, this Discussion In this prospective, longitudinal study, we have demonstrated both that HCV co-infection does not influence the clinical progression of HIV disease to AIDS and that it is not associated with more rapid immunological decline.

In our cohort of 240 patients, 84% were confirmed HCV-infected. We did not examine serum samples for HCV RNA by reverse transcriptase polymerase chain reaction (RT-PCR). Using simple serological methods alone, it is possible to misclassify some individuals who may be falsely anti-HCV negative or have undergone HCV sero-reversion as severe immunosuppression supervenes. However, this is unlikely to be the case in our cohort, as the majority of our patients were HCV-tested prospectively in the course of their HIV infection, before they became severely immunocompromised; HCV testing also included the use of second generation EIA confirmed with third generation RIBA which dramatically reduces chances of false negativity [12]. The most accurate method for assessing HCV infection retrospectively is by using serology rather than RT-PCR for HCV-RNA, because it is not always possible to determine how samples were initially processed, and the storage of samples for long periods exposes viral RNA to degradation by ribonucleases [13].

Our findings on HIV progression are similar to those of previous cross-sectional and retrospective studies [6,7,14] and a smaller longitudinal study from Italy [15]. In the Italian study, HCV co-infection also did not influence the rate of progression to either clinical (the development of AIDS) or immunological (CD4+ count <100 cells/mm³) endpoints in HIV-infected IDUs. However, the length of follow-up was considerably shorter than in the present study: 30 months compared with a median of 77.5 months (HCV-infected patients) and 82 months (HCV-uninfected patients) and it assessed a mixed population of HIV infected individuals (IDUs, homosexuals, heterosexuals, and those patients without any obvious risk factors for HIV transmission). The study also did not attempt to assess individual seroconversion dates and progression, rather the clinical and immunological progression of the population over a defined 30 month period. Surprisingly, it found no deaths related to end stage liver disease, perhaps a finding also determined by the relatively short follow-up period.

Our study also considered the effect of chronic HIV infection on HCV-related liver disease in terms of mortality from end stage liver disease. Although there was no significant difference in mortality from liver disease between HCV antibody positive and HCV antibody negative patients, these deaths can be considered premature (in eight of 11 patients) because they have occurred relatively early in the natural history of HIV disease, in individuals without AIDS and with relatively well maintained CD4+ counts. Interestingly, three of eight patients were co-infected with hepatitis B virus (HBV) (and one with delta), perhaps an important co-factor in the progression of HCV-related liver disease. However, we are unable to comment on the prevalence or severity of clinically occult liver disease in this study because liver biopsies were rarely performed, and mortality resulting from end stage liver disease only was considered.

The interaction between immunosuppression and HCV-related liver disease is controversial. Reports have suggested that HIV may well accelerate the course of HCV-related as well as HBV-related liver disease in selected patients [8,9]. A large prospective study of haemophiliacs concluded that HIV accelerates the clinical course of HCV-related liver disease and that liver failure produces significant morbidity and mortality in patients co-infected with HCV and HIV; however, again, only the severe end of the spectrum of liver disease was considered in this study [9]. In the largest study of its kind, a recent multicentre cross-sectional study performed in 547 patients with chronic, parenterally acquired hepatitis C infection, with or without HIV infection (116 HIV positive and 431 HIV negative), demonstrated that HIV infection modifies the natural history of chronic HCV infection in this population with an unusually rapid progression to cirrhosis. For example, after a 10 year duration of infection, 13 of 87 (14.9%) HIV positive subjects developed cirrhosis compared with seven of 272 (2.6%) in the HIV negative group (P < 0.01); similar results were demonstrated at 5 and 15 years, respectively. The authors calculated the mean interval from estimated time of infection to cirrhosis was significantly longer in the HIV negative compared with the HIV positive patients (23.2 vs 6.9 years; P < 0.001) [16].
The mechanism of potential interaction between immunosuppression and progression of HCV-related disease remains unclear. In vitro and in vivo studies have suggested that HCV levels are increased in the presence of reduced numbers of CD4+ cells [17,18]. However, the significance of this finding is uncertain; it is most unlikely that HCV is purely cytopathogenic, hepatic damage is thought to be at least partially immune mediated in nature [9,20]. Thus, severe immunosuppression in progressive HCV disease could also ameliorate the manifestations of immune mediated hepatic pathology, as is usually the case with HBV/HIV co-infection [2,3]. There is also likely to be a complex interaction between other intrinsic and extrinsic factors in HIV/HCV co-infected patients. For example, in advanced HIV infection, there is evidence that in HCV haemophiliacs some cases of liver failure may be precipitated by opportunistic infections or anti-retroviral therapy [8].

In conclusion, in this longitudinal study, we have demonstrated that co-infection with HCV does not affect the clinical or immunological sequela of chronic HIV infection. We have also presented evidence that a proportion of premature deaths in HIV infection are likely to be due to progressive HCV-related liver disease. However, we cannot comment on the overall prevalence or severity of clinically occult HCV-related liver disease in our population, because liver biopsies were rarely performed, and therefore we are currently assessing this issue using non-invasive markers of liver disease.

References
Clinical significance of intrahepatic hepatitis C virus levels in patients with chronic HCV infection

G H Haydon, L M Jarvis, C S Blair, P Simmonds, D J Harrison, K J Simpson, P C Hayes

Abstract

Background—The clinical significance of a single assessment of circulating hepatitis C virus (HCV) RNA and its relation to the level of intrahepatic HCV RNA remains unclear.

Aims—To investigate the relation between intrahepatic HCV levels and clinicopathological characteristics of chronic HCV infection.

Patients—Ninety eight consecutive patients with chronic HCV infection were studied; none had received interferon therapy. Of these, 12 patients were repeatedly negative for HCV RNA in serum by reverse transcriptase polymerase chain reaction (RT-PCR).

Methods—After diagnostic laparoscopy and liver biopsy, semiquantitative analysis of intrahepatic HCV RNA levels was carried out by limiting dilution of HCV cDNA. HCV genotypes were assessed in 96 patients by restriction fragment length polymorphism analysis of HCV cDNA.

Results—Ten out of 12 patients who were RT-PCR negative for HCV RNA in serum were RT-PCR positive in liver; however, this group had a significantly lower intrahepatic HCV level and serum aminotransferase level than the remaining 86 patients. Histological severity (cirrhosis: n=10); histological activity index; HCV genotype (genotype 1: n=41; genotype 2: n=12; genotype 3: n=36; genotype 4: n=7); mode of infection (intravenous drug abuse: n=58; post-transfusion: n=10; haemophilia: n=4; sporadic: n=26) and alcohol abuse did not affect the intrahepatic virus level. There was no correlation between patient age, duration of infection, and intrahepatic HCV level.

Conclusions—Intrahepatic virus levels were not determined by host factors (age of patient, mode or duration of infection) or by virus factors (HCV genotype). Repeatedly negative RT-PCR for HCV RNA in serum does not indicate absence of HCV from the liver.

(Gut 1998;42:570–575)

Keywords: intrahepatic hepatitis C virus; chronic hepatitis C virus infection

Hepatitis C virus (HCV) is the predominant cause of post-transfusion and sporadic non-A non-B hepatitis worldwide. Approximately 95% of HCV infected individuals can be identified by third generation anti-HCV testing. However, this test does not indicate active infection, and there is considerable controversy as to the biochemical, virological, and histological definitions of a "past infection".

Initial, fundamental studies suggested that negative serum HCV cDNA polymerase chain reaction (PCR) results in recombinant immunoblot assay (RIBA) positive individuals correlated with the absence of inflammation in liver biopsy specimens. The serum HCV cDNA PCR result was therefore a sensitive and specific marker of liver disease in anti-HCV positive subjects, independent of serum alanine aminotransferase (ALT) values; true healthy carriers of HCV did not exist. Following these reports, it has been recommended that patients negative in serum for HCV cDNA do not undergo routine liver biopsy; furthermore, that those with persisting abnormal liver function tests should be screened for other liver diseases (for example, autoimmune chronic active hepatitis or haemochromatosis), while those with normal liver function tests ought to be followed up annually until the natural history of the disease is better documented.

Many previous studies have examined the significance of serum HCV levels and have shown a wide range of clinicopathological associations; however, there are fewer data examining intrahepatic HCV levels and their associations. In general, previous data have not shown any correlation between HCV levels in the liver and other demographic factors such as sex, age, duration of illness, and risk factors. There is a suggestion that some genotypes, in particular genotype 1b, may be associated with higher serum and liver virus levels but in contrast with serum studies, intrahepatic studies have not shown any correlation between HCV RNA levels and liver injury.

To address these issues further, we determined the clinical, histological, and intrahepatic virological profile of patients with serum repeatedly negative for HCV RNA by reverse transcriptase (RT) PCR; we then compared this population with a control population of patients whose serum was repeatedly positive for HCV by RT-PCR. Concurrently, we investigated the relation between intrahepatic HCV levels and the clinicopathological characteristics of chronic HCV infection.

Patients and methods

Patients

Ninety eight consecutive patients (69 male; mean age 37.8 ± 8 years) with chronic HCV infection were included in the study; none had received interferon therapy. All patients were positive for anti-HCV antibodies by second generation enzyme immunoassay (EIA-2,
Table 1: Comparison of demographic and virological data of serum RT-PCR positive and negative patients, all of whom have persistent infection

<table>
<thead>
<tr>
<th>Host or virus parameter</th>
<th>Serum RT-PCR negative</th>
<th>Serum RT-PCR positive</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>31.2 (6)</td>
<td>37.8 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>7/3</td>
<td>61/25</td>
<td>NS</td>
</tr>
<tr>
<td>Mode of infection</td>
<td>6 IVDA; 1 post-transfusion; 1 sporadic; 3 sporadic</td>
<td>51 IVDA; 9 post-transfusion; 2 sporadic; 2 sporadic</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of infection</td>
<td>(median (range))</td>
<td>(4-42)</td>
<td>NS</td>
</tr>
<tr>
<td>HIV</td>
<td>0</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>0</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Normal ALT</td>
<td>7</td>
<td>8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Disease state</td>
<td>1 Cirrhosis; 9 chronic hepatitis</td>
<td>9 Cirrhosis; 77 chronic hepatitis</td>
<td>NS</td>
</tr>
<tr>
<td>Histological activity index</td>
<td>6 (1-13)</td>
<td>8 (1-14)</td>
<td>NS</td>
</tr>
<tr>
<td>HCV genotype</td>
<td>4 genotype 1; 1 genotype 2; 4 genotype 3; 1 genotype 4</td>
<td>37 genotype 1; 11 genotype 2; 32 genotype 3; 6 genotype 4</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbot Laboratories, Weisbaden, Germany) and third generation recombinant immunoblot assay (RIBA-3, Chiron, Emeryville, California). Fifty eight patients had acquired their infection through abuse of intravenous drugs (IVDA), 10 had acquired infection from red cell concentrate transfusions, four were haemophiliacs transfused with infected blood products, and in 26 there were no obvious risk factors for infection.

At the time of admission to hospital, each patient was questioned about the likely duration of their infection (in the case of the IVDA group, this was calculated from the year of first injection). Serum aminotransferase was measured on the day of diagnostic laparoscopy.

A small portion of the liver biopsy specimen taken at laparoscopy was washed in ice cold normal saline, blotted on a sterile swab, and immediately frozen in liquid nitrogen and stored at −70°C for future use. The remainder of the biopsy specimen was used for histological analysis.

Serum samples were obtained, separated within three hours of collection on the day of laparoscopy, and stored at −70°C. All samples were stored for a maximum of three to six months before analysis.

HCV RNA extraction from serum samples
RNA was extracted from 0.1 or 0.5 ml of stored serum from each of the patients as previously described. Briefly, the RNA was pelleted by centrifugation at 100 000 g for 90 minutes at 4°C and incubation at 37°C for two hours with 1 mg/ml proteinase K in the presence of 40 μg/ml polyadenylic acid, 0.5% sodium dodecyl sulphate (SDS), 0.1 M NaCl, 50 mM Tris HCl (pH 8.0), and 1 mM EDTA. RNA was extracted with phenol; after centrifugation, the supernatant was re-extracted successively with phenol and chloroform-isooamyl alcohol (50:1). Nucleic acid was precipitated by the addition of one tenth volume of sodium acetate (pH 5.2) and two volumes of ethanol. The dried pellet was resuspended in 25 μl of diethylpyrocarbonate treated water.

HCV RNA extraction from liver biopsy samples
RNA extraction from liver biopsy samples was carried out using a commercial modification (RNAzol, Biogenesis Ltd, Bournemouth, UK) of the single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction.

Tissue samples were homogenised with RNAzol solution (phenol derivative); RNA was extracted by centrifugation at 12 000 g for 15 minutes at 4°C with chloroform. RNA was precipitated by addition of isopropanol to the aqueous phase; the samples were stored for 15 minutes at 4°C and then centrifuged for 15 minutes at 12 000 g (4°C); the RNA precipitates formed a white-yellow pellet at the bottom of the tube. The supernatant was removed, and the RNA pellet was washed once with 75% ethanol by vortexing and centrifuged for eight minutes at 7500 g (4°C). The dried pellet was resuspended in 25 μl diethylpyrocarbonate treated water.

RT-PCR and viral genotyping
RNA was reverse transcribed and amplified using nested primers matching the 5’-NCR. Product DNAs were cleaved with restriction enzymes Hae-111/Rsa-1 and Mva-1/Hinf-1. The fragments were
separated by agarose gel electrophoresis using 4% Metaphor agarose (FMC BioProducts). Phylogenetic comparisons of sequences in the conserved region of the genome confirm that 5'-NCR can be used to distinguish the six major genotypes.49

**Quantitation of HCV Levels in Serum Samples and Liver Tissue**

Liver tissue samples were quantified by limiting dilution of cDNA reverse transcribed from HCV RNA. This has been described in detail elsewhere.40 41 Briefly, 5 µl of HCV cDNA was diluted in a series (five for serum samples and seven for liver biopsy samples) of 10-fold steps which allowed cDNA to be quantified to within 1 log of its actual concentration. Further refinement of the quantitation by adding a specific volume of cDNA to a number of replicate PCR reactions, thus giving a Poisson distribution of positive and negative samples, and allowing the exact HCV concentration to be determined, has been shown not to be necessary to improve the accuracy of the assay if the nearest whole log only is required.

Positive controls (4 x 10^6 copies/ml) were run concurrently with each limiting dilution analysis to ensure reproducibility of the assay. Serum samples from healthy individuals without risk factors were examined for HCV RNA as negative controls.

The previously established efficiency of 5% for the reverse transcription reaction was assumed in this assay. Centrifugation of 0.1 ml serum provided a detection sensitivity of approximately 4000 HCV copies/ml. To increase the sensitivity of the PCR method, samples that were negative at this level of detection (less than 4000 HCV/ml) were further analysed by centrifugation of 0.5 ml (detection sensitivity: 800 HCV copies/ml) or if necessary 5.0 ml (detection sensitivity: 80 HCV copies/ml) of serum.

The limiting dilution assay has been shown to have significant reproducibility when multiple samples are tested in duplicate, using RNA extracted on separate days from separate aliquots of sample and different batches of reagents. Likewise a significant correlation has been shown between limiting dilution and three commercial assays: bDNA1, bDNA2 (Chiron, Emeryville, California), and Roche Monitor. Furthermore, when the quantity of transcripts of genotypes 1, 2, and 3 was compared by limiting dilution, there was a statistically similar distribution of the quantity of virus. This indicates that no correction factor for different genotypes (1, 2, and 3) is required when using this assay for quantitating hepatitis C virus levels.42

**Histological Analysis**

Liver biopsy specimens from all 98 patients were available for assessment by a single observer blinded to the clinical and serological data. Histological features were graded according to the classifications of Knodell et al,43 assessing portal, perportal, and lobular inflammation as well as fibrosis.

**Statistical Analysis**

The results were analysed by non-parametric tests where appropriate: the χ^2 or Fisher's exact test, the Mann-Whitney test, or Kruskal-Wallis probability tests.

**Results**

During an eighteen month period, intrahepatic HCV levels of 12 patients repeatedly negative for HCV RNA in serum by RT-PCR (on three separate occasions) were assessed and compared with 86 patients repeatedly positive for HCV RNA in serum by RT-PCR.

The relation between intrahepatic HCV levels and the clinicopathological characteristics of chronic HCV infection was then investigated.

**Virus Factors**

**Serum RT-PCR for HCV RNA**

Ten out of 12 patients RT-PCR negative for HCV RNA in serum were RT-PCR positive in the liver; however, this group had both a significantly lower intrahepatic HCV level (p<0.0001) (fig 1) and a significantly lower serum aminotransferase level (p<0.001) (table 1) than the remaining 86 patients. Table 1 illustrates the demographic and virological similarity between the serum RT-PCR negative and positive patients. There was no significant correlation between virus levels and any parameter.

**HCV genotype**

Hepatitis C virus genotypes were assessed in 96 patients by restriction fragment length polymorphism analysis of HCV cDNA. There was no significant difference in intrahepatic virus levels between patients infected with genotype 1 (n=41), genotype 2 (n=12), genotype 3 (n=36), and genotype 4 (n=7) (fig 2). The two patients who were RT-PCR negative in both liver and serum could not be genotyped.

**Liver Histology**

Ten patients had cirrhosis at diagnostic laparoscopy and liver biopsy. The remaining 88

---

**Table 2** Correlation coefficients between intrahepatic virus levels and patient age, duration of infection, serum aminotransferase level, and histological activity index.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Population median (range)</th>
<th>Pearson correlation coefficient</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>37 (24–65)</td>
<td>0.0624</td>
<td>0.55</td>
</tr>
<tr>
<td>Serum aminotransferase</td>
<td>72 (13–577)</td>
<td>0.091</td>
<td>0.391</td>
</tr>
<tr>
<td>Duration of infection (y)</td>
<td>12 (4–42)</td>
<td>-0.0661</td>
<td>0.592</td>
</tr>
<tr>
<td>Histological activity index</td>
<td>8 (1–14)</td>
<td>0.082</td>
<td>0.47</td>
</tr>
</tbody>
</table>

**Table 3** Qualitative histological findings and alanine aminotransferase (ALT) and intrahepatic HCV levels in serum RT-PCR HCV RNA negative patients with persistent hepatic infection.

<table>
<thead>
<tr>
<th>Patient no</th>
<th>ALT (UI)</th>
<th>Log liver virus level (copies/µl)</th>
<th>Steatosis</th>
<th>Portal inflammation</th>
<th>Lobular inflammation</th>
<th>Periportal inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>4.72</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>5.42</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>4.10</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>6.57</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>7.68</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>4.44</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>9.28</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>4.49</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>105</td>
<td>4.22</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>5.58</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
patients had macroscopic and histological features of ongoing inflammatory activity in keeping with chronic HCV hepatitis. Neither the histological severity nor the histological activity index (HAI) affected the intrahepatic virus levels (fig 3; table 2).

Table 3 illustrates the descriptive histological findings in the 10 patients who were serum RT-PCR negative for HCV RNA, but RT-PCR positive in the liver.

PATIENT FACTORS
Mode and duration of infection
Intrahepatic HCV levels were unaffected by either the mode or duration of infection (fig 4; table 2).

Serum aminotransferase
There was no significant correlation between serum ALT and intrahepatic HCV levels (table 2).

G cofactors
Coinfection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV), or alcohol abuse did not significantly affect intrahepatic HCV levels.

Discussion
The current pandemic of hepatitis C infection has affected 1–2% of the world’s population, and 0.02% of the population of the UK. However, it is unclear what proportion of patients have a self limiting illness, successfully eliminate HCV, and then become immune to reinfection; furthermore, the host or virus factors increasing the likelihood of successful elimination, and the baseline markers of this process are unknown. The prognostic significance of individual clinical, epidemiological, and virological parameters in this context has also not been clarified. For these reasons, we assessed the clinical, histological, and virological profile of 98 consecutive patients, presenting for staging of their liver disease by diagnostic laparoscopy and liver biopsy and further compared a unique serum HCV cDNA PCR negative population of 12 patients with a larger subpopulation of HCV cDNA PCR positive patients. Our data have shown that repeatedly negative RT-PCR for HCV RNA did not indicate complete hepatic elimination of HCV in 10 out of 12 (87.5%) of our patients; also that intrahepatic HCV levels were not determined by either host factors (age of patient, mode or duration of infection, concurrent alcohol abuse, or concurrent HBV/HIV infection) or by virus factors (HCV genotype).

For confirmation of positive anti-HCV test results, recombinant immunoblot assays were developed; RIBA-3 results are interpreted as "positive" when one or more bands are positive, and "indeterminate" when only one band is positive. A high proportion (75–80%) of RIBA-3 positive patients have viraemia as detected by RT-PCR for HCV RNA. It has been suggested that RIBA-3 positive but RT-PCR negative patients may have cleared the virus from the circulation after a previous infection, may be viraemic below the RT-PCR detection level, or may represent false positive anti-HCV reactivity. Previous studies have indicated that RT-PCR positive anti-HCV positive patients correlate with absence of inflammation on liver biopsy specimens, and this observation probably indicates clearance of the virus. In our study 10 out of 12 (87.5%) patients were RT-PCR positive for HCV RNA in liver tissue, but RT-PCR negative in serum and all had ongoing inflammation diagnosed at diagnostic laparoscopy and liver biopsy. This favours the hypothesis that these patients were viraemic below the RT-PCR detection level in serum (in our case, this was a detection sensitivity of 800 HCV copies/ml in 0.5 ml of serum); furthermore, patients RT-PCR positive for HCV RNA in serum had a significantly higher intrahepatic HCV level than patients RT-PCR negative for HCV RNA in serum. Remarkably, comparison of the serum RT-PCR negative and positive subpopulations indicated that they were statistically similar in terms of demographic, histological, and virological data; the serum RT-PCR negative subpopulation did however have a significantly lower serum ALT than the RT-PCR positive patients despite the poor correlation between liver virus level and ALT overall. Possibly, the serum RT-PCR negative patients, with their concurrent lower intrahepatic HCV level and ALT, may have a lower grade hepatic
inflammation; however, there is no significant difference in HAI between the populations. Therefore, the prognostic importance of these data is that serum RT-PCR negative patients with chronic HCV infection should be followed up indefinitely, and at present there is no indication that they are at a lower risk of severe liver disease in the future. We propose follow up studies assessing these patients for the presence of very low levels of viraemia by increasing the detection sensitivity to 80 copies HCV/ml in 5 ml of serum or even eight copies HCV/ml in 50 ml of serum.

The second important finding of this report was the absence of a significant relation between host or virus factors and intrahepatic HCV levels; thus the significance of a single assessment of intrahepatic HCV RNA in terms of diagnosis and prognosis remains unclear. Indeed, in contrast with similar serum studies, this finding corresponds to previous intrahepatic studies which have shown no correlation between HCV RNA levels and liver injury."

The largest of these studies involved three different methodologies for quantification of HCV RNA (Dot-Blot PCR, end point titration, and Roche Amplicor Monitor Assay), examined interassay and intra-assay variability in detail, and showed no significant association with the degree of liver injury. There are at present no data measuring viral replication in addition to the total HCV load and its effect on liver injury. Recently, new techniques have been developed to overcome the methodological problems associated with detection of true replicative (negative or antisense) HCV RNA levels, but their ability to quantitate specifically HCV RNA over a wide range of HCV levels is still limited.

There was no correlation in our study between HCV RNA levels in liver and other demographic factors such as sex, age, duration of illness, and mode of infection. These data confirm previous studies on liver virus levels but contrast with studies examining serum levels of HCV RNA where increased levels have been shown in relation to age, sex, mode, and duration of infection. This observed discrepancy may be explained by a combination of factors: contribution to serum HCV RNA levels from virus in other tissues (and from necrotic liver tissue), HCV RNA in serum immune complexes, technical variation between methods, different populations studied, and time dependent fluctuation in serum HCV levels. Certainly analysis based on serum studies is more difficult to interpret than that based on intrahepatic studies.

In addition, we did not show an effect of HCV genotype on intrahepatic virus levels. This conflicts with data indicating that genotype 1b generally has higher HCV RNA levels in serum and liver and therefore a higher viremic load than other genotypes. However, genotypic analysis of our data was limited by the presence of only one patient infected with genotype 1b, while 19 patients were infected with genotype 1a.

In conclusion, we have successfully shown that repeatedly negative RT-PCR for HCV RNA in serum does not indicate complete hepatic elimination of HCV. Indeed, these patients have a similar clinical, pathological, and epidemiological profile to serum RT-PCR positive patients; their prognosis and requirements for full staging of liver disease are thus likely to be similar, and further follow up is mandatory. However, we were unable to show any significant associations between intrahepatic HCV levels and other clinicopathological parameters. Importantly, these data support the hypothesis that HCV does not cause liver disease by a cytopathic process; they also illustrate the limitations of a single assessment of HCV levels. Clearly, there is a requirement for sequential studies of chronic HCV infection in terms of molecular virological and clinical parameters, before the clinical significance of intrahepatic HCV levels is established.
Intrahepatic chronic hepatitis


Prediction of cirrhosis in patients with chronic hepatitis C infection by artificial neural network analysis of virus and clinical factors

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SUMMARY

The diagnosis of cirrhosis in patients with hepatitis C virus (HCV) infection is currently made using a liver biopsy. In this study we have trained and validated artificial neural networks (ANN) with routine clinical host and viral parameters to predict the presence or absence of cirrhosis in patients with chronic HCV infection and assessed and interpreted the role of the different inputs on the ANN classification. Fifteen routine clinical and virological factors were collated from 112 patients who were HCV RNA positive by reverse transcriptase–polymerase chain reaction (RT–PCR). Standard and Ward-type feed-forward fully-connected ANN analyses were carried out both by training the networks with data from 82 patients and subsequently testing with data from 30 patients plus performing leave-one-out tests for the whole patient data set. The ANN results were also compared with those from multiple logistic regression. The performance of both ANN methods was superior compared with the logistic regression. The best performance was obtained with the Ward-type ANNs resulting in a sensitivity of 92% and a specificity of 98.9% together with a predictive value of a positive test of 95% and a predictive value of a negative test of 97% in the leave-one-out test. Hence, further validation of the ANN analysis is likely to provide a non-invasive test for diagnosing cirrhosis in HCV-infected patients.

Keywords: artificial neural networks, HCV genotypes, HCV infection, HCV quantification, hepatitis C cirrhosis

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is a major cause of liver disease worldwide [1]. Infected patients present a continuous spectrum of hepatic pathology from mild chronic HCV to end-stage cirrhosis and hepatocellular carcinoma (HCC). Prospective, multicentre studies of populations infected with HCV-contaminated blood transfusions have concluded that the prevalence of cirrhosis is about 20% after 20 years of injection [2–5]. However, at present, it is not possible to predict which patients have cirrhosis at the time of staging HCV infection [6]. This has major implications both for prognosis and for the success of treatment regimens.

Previous reports have concentrated on single host (mode of transmission and duration of infection) [7,8] or virus factors (HCV genotype or virus level) [9–14] and their relationship to progression of disease; all have been implicated in the progression to cirrhosis. Attempts to distinguish the relative contributions of these factors in determining pathogenicity by multivariate analysis have been inconclusive [15,16].

Unlike multivariate analysis, artificial neural network (ANN) analysis is known to be particularly suitable for modelling complex multidimensional relationships...
The ANNs consist of simple signal-processing units (neurons), the interactions of which are controlled by transfer functions and input-associated weights. The construction allows for easy adjustment of the analysis tool in variable situations and may also provide an independent measure of the relative importance of individual inputs in characterizing the output [20].

In this study we have, first, trained and validated ANNs with routine clinical host and viral parameters to predict the presence of cirrhosis in patients with chronic HCV infection and compared the performance of the ANN analysis with a conventional statistical model based on multiple logistic regression and, second, assessed and interpreted the role of the different inputs on the ANN classification.

PATIENTS AND METHODS

Patients

One hundred and thirty consecutive patients (100 male; mean age: 40.1 ± 13.2 years) who presented between 1 January 1994 and 30 June 1995, and were positive for antibodies to HCV (anti-HCV) by second-generation immunoassay (ELA-2, Abbot Laboratories, 23F) and third-generation recombinant immunoblot assay (RIBA-3, Chiron, Emeryville, CA), were studied. Of these patients, 112 were included in this work, as 18 were consistently negative for HCV RNA by reverse transcriptase–polymerase chain reaction (RT–PCR). Risk factors for infection included: 39 intravenous drug abusers (IVDA), 31 haemophiliacs who had previously received non-virus-inactivated Factor VIII or Factor IX concentrates and 15 patients who had received red cell concentrate (RCC) transfusions. In 27 patients, there were no obvious risk factors for infection (sporadic population).

At the time of admission to hospital, the mode of infection was confirmed and the duration of infection assessed (in the example of the IVDA group, this was calculated from the year of first injection). The disease severity was staged using diagnostic laparoscopy and liver biopsy. Liver biochemistry was measured on the day of diagnostic laparoscopy and an additional serum sample was stored at −70°C. Patient details are summarized in Table 1.

### Table 1 The patient population characteristics

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Group I (n = 82)</th>
<th>Group II (n = 30)</th>
<th>Total (n = 112)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>40.7 ± 14.5</td>
<td>40.6 ± 12.7</td>
<td>40.7 ± 15.9</td>
</tr>
<tr>
<td>Male/female</td>
<td>59M : 23F</td>
<td>23M : 7F</td>
<td>82M : 30F</td>
</tr>
<tr>
<td>Duration of infection (years)*</td>
<td>16 ± 8</td>
<td>15.7 ± 6.5</td>
<td>15.9 ± 7.6</td>
</tr>
<tr>
<td>Mode of infection</td>
<td>28 IVDA</td>
<td>11 IVDA</td>
<td>39 IVDA</td>
</tr>
<tr>
<td>HBV co-infection</td>
<td>23 haemophilic</td>
<td>8 haemophilic</td>
<td>31 haemophilic</td>
</tr>
<tr>
<td>HIV co-infection</td>
<td>11 RCC transfusion</td>
<td>4 RCC transfusion</td>
<td>15 RCC transfusion</td>
</tr>
<tr>
<td>Alcoholic abuse</td>
<td>20 sporadic</td>
<td>7 sporadic</td>
<td>27 sporadic</td>
</tr>
<tr>
<td>Bilirubin (μmolL⁻¹)</td>
<td>22 ± 27</td>
<td>20 ± 20</td>
<td>22 ± 26</td>
</tr>
<tr>
<td>ALT (IU L⁻¹)</td>
<td>104 ± 88</td>
<td>100 ± 46</td>
<td>103 ± 79</td>
</tr>
<tr>
<td>Albumin (g L⁻¹)</td>
<td>41 ± 6</td>
<td>40 ± 6</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>Platelet*</td>
<td>183 ± 80</td>
<td>187 ± 77</td>
<td>184 ± 79</td>
</tr>
<tr>
<td>PT (s)*</td>
<td>12 ± 1</td>
<td>14 ± 7</td>
<td>13 ± 4</td>
</tr>
</tbody>
</table>

*Mean ± SD.

Group I is the subpopulation of patients used to train the ANNs and Group II the separate test subpopulation. In the leave-one-out tests the whole population could also be used as the test population.

ALT: alanine aminotransferase; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IVDA, intravenous drug abuse; PT, prothrombin time; RCC, red cell concentrate.

observer blinded to the clinical and serological data. According to the histopathological diagnosis, the liver disease was grouped as non-cirrhosis or established cirrhosis by the same pathologist.

**RNA preparation from serum.** RNA was extracted from 0.1 ml of stored serum from each of the patients as previously described [21]. Briefly, serum samples were incubated at 37°C for 1.5 h with 1 mg ml⁻¹ proteinase K in the presence of 40 μg ml⁻¹ polyadenylic acid, 0.5% SDS, 0.1 M NaCl, 50 mM Tris HCl (pH 8.0), and 1 mM EDTA. Nucleic acid was extracted with phenol and chloroform-isooamyl alcohol (50:1), and precipitated by the addition of 0.1 volume of sodium acetate (pH 5.2) and two volumes of ethanol. The dried pellet was resuspended in 25 µl of diethyl pyrocarbonate-treated water.

**RT-PCR and viral genotyping.** RNA was reverse transcribed and amplified using nested primers matching the 5′- non-coding region (NCR) [22]. Product DNAs were cleaved with restriction enzymes HaeIII/Rsal and MvaI/HinFl [23,24]. The fragments were separated by agarose gel electrophoresis using 4% Metaphor agarose. Phylogenetic comparisons of sequences in the conserved region of the genome confirm that the 5′- NCR can be used to distinguish the six major genotypes of HCV [25].

**Quantification of serum samples.** HCV RNA levels in serum were measured semiquantitatively by limiting-dilution analysis of cDNA, which was reverse transcribed and amplified from RNA as described previously [26,27]. Amplified DNA was detected by 2% agarose gel electrophoresis and ethidium bromide staining.

PCR with nested primers detects single molecules of target DNA sequence. Tests on multiple replicates at a suitable limiting dilution give a Poisson distribution of positive and negative results that reflects the concentration of target DNA [27]. To obtain an RNA concentration from quantification of cDNA we have assumed an overall efficiency of 5% for the reverse transcription step. Centrifugation of 0.1 ml of sera provided a level of detection of approximately 4000 HCV copies ml⁻¹. To increase the sensitivity of the assay, samples that were negative at this level of detection (<4000 HCV copies ml⁻¹) were further analysed by centrifugation of 0.5 ml of sera, providing a cut-off point of approximately 800 HCV copies ml⁻¹.

Positive controls (4 x 10⁵ copies ml⁻¹) were run concurrently with each limiting dilution analysis to ensure reproducibility of the assay. Serum samples from healthy individuals without risk factors were examined for HCV RNA as negative controls.

**Artificial neural network analysis.** An artificial neural network (ANN) consists of simple signal processing units, so-called neurons. Each neuron can have multiple inputs, but has only a single output. The input–output relationship is controlled by a transfer function [17,18]. The inputs of a neuron are first multiplied by a weighting factor that determines the extent to which each input influences the output and the weighted inputs are summed to form a preneuron sum; this is then inserted through the transfer function resulting in the neuronal output. A complete network is built up by organizing individual neurons into a series of layers. In a feed-forward fully connected network, each neuron gets an input from neurons in the preceding layer and also gives the output for each neuron in the following layer. The layers between the input and output layer are called hidden layers. Within a given topology and transfer functions, the desired behaviour of an ANN can be approximated by adjustment of the neuronal connections. This is called training of the network and is carried out by using a data set for which the output of the corresponding input is available. An empirical model is obtained if the training process is able to reduce the errors in the outputs of the network for the training set to negligible values, i.e. the ANN has learned. The hypothesis is that the trained ANN also models the underlying process(es) that have generated the training data and that it can be used to estimate the output values for input data that were not used in the training. In practice, it has been demonstrated that properly trained ANNs do have this capability for generalization [17–19]. In a successful case the resultant neuronal weights can also be used to assess the relative importance of the different inputs on the ANN outputs [20].

In this study, standard and so-called Ward-type feed-forward fully-connected three-layer ANNs were applied to predict the occurrence of hepatic cirrhosis in HCV-infected patients. The general topology of the networks is illustrated in Fig. 1. The standard networks were structured as explained above and used sigmoidal transfer functions. The constructed Ward-type ANNs differed from the standard ones in that the
The ANN analysis was performed, first, by using a training data set of 82 patients (Group I) and then with a test set of 30 patients (Group II) (Table 1). This allowed testing of both the ANN topologies and the importance of the number of inputs on the ANN performance. The best performance was obtained using an ANN with 24 input neurons (all the clinical data available), 22 hidden neurons and two output neurons (i.e. either 0–1 or 1–0 for the presence or absence of cirrhosis) in the standard analysis and 24 input neurons, 12 plus 12 hidden neurons and two output neurons in the\textsuperscript{4} Ward-type analysis. The numbers of hidden neurons were actually the defaults given by the NeuroShell 2 program\textsuperscript{28} when training the nets with Group I (0.5 times the sum of input and output neurons plus the square root of the number of patterns in the training set. For the Ward-type nets the neurons were divided evenly between the hidden layer). When the number of inputs was varied (between 24 and 7 inputs; leaving out inputs that had the lowest correlation coefficients with the outputs and/or were given low relative weights in the ANN analysis when all 24 inputs were used) there was a decrease in the network performance; the worst result was that approximately 80% of the cases in the test Group II were predicted correctly. Leave-one-out analysis for the whole patient data set was also performed for both types of networks.

All inputs were scaled between -1 and 1. A modified back propagation algorithm (that automatically controls the learning rate and momentum) was applied in the learning stage and a commercial NeuroShell 2 program utilized [28]. Using a 90 MHz Pentium PC, the training periods of the ANNs took 2–3 min depending upon the number of input and hidden neurons. The general ANN topology is shown in Fig. 1 and the use of the ANN weights (the Ward-type ANN analysis trained with Group I) as an aid to interpret the relevance of the different clinical inputs on the ANN classification is illustrated in Fig. 2.

**Multiple logistic regression.** Multiple logistic regression was used to test the multivariate significance of host and viral factors in the prediction of cirrhosis in patients with chronic HCV infection. To enable comparison of the ANN analysis with a statistical model, multiple logistic regression was first performed using the training data set of 82 patients (Group I). The model was then applied, using the significant variables obtained, to predict the presence or absence of cirrhosis in the test set of 30 patients (Group II).

Sensitivities, specificities, predictive values of a positive test and predictive values of a negative test were calculated for the ANN analyses and for the multiple logistic regression using standard formulae.

**RESULTS**

Of the 112 patients who were studied, 25 (22%) patients had laparoscopically and histologically confirmed cirrhosis: 17 (21%) in Group I and eight (27%) in Group II. The molecular virological details of the patients are summarized in Table 2.

**Artificial neural network analysis**

In the training set of 82 patients, two cirrhotic patients were incorrectly classified as non-cirrhotic when applying the standard ANN analysis and all the patients were classified correctly in the case of the Ward-type analysis. In the test set of 30 patients, two cirrhotic patients were incorrectly classified as non-cirrhotic in the standard and one in the Ward-type analysis. Thus, the standard ANN analysis correctly predicted the presence or absence of cirrhosis in 28 patients (93%) and the Ward-type analysis in 29 patients (96%) in Group II.

**Table 2** The molecular virology of the hepatitis C virus (HCV) infection population (and subpopulations) studied

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR positive</td>
<td>82</td>
<td>30</td>
<td>112</td>
</tr>
<tr>
<td>Log$_{10}$ HCV level*</td>
<td>6 ± 12</td>
<td>5.8 ± 1.3</td>
<td>6 ± 1.2</td>
</tr>
<tr>
<td>Genotype 1a</td>
<td>30</td>
<td>13</td>
<td>43</td>
</tr>
<tr>
<td>Genotype 1b</td>
<td>9</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Genotype 2a</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Genotype 2b</td>
<td>8</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Genotype 3</td>
<td>30</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td>Genotype 4</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

*Mean ± SD.

RT-PCR, reverse transcriptase–polymerase chain reaction.

![Fig. 2 The relative weights of all virus and host variables in a Ward-type feed-forward fully connected network trained with patient Group I. The 10 inputs that have greater weights than the rest of the inputs are: patient age, serum albumin level, HCV level, HBV status, duration of infection, HIV status, platelets, infection by intravenous drug abuse (IVDA), alcohol abuse and HCV genotype 3.](image-url)
In the leave-one-out test for all the 112 patients, four (16%) of the cirrhotic patients were incorrectly classified as non-cirrhotic and five (5%) of the non-cirrhotic were incorrectly classified as cirrhotic in the standard analysis. The corresponding values in the Ward-type analysis were two (8%) and one (1%). Thus, in the case of all the patients, the presence or absence of cirrhosis was correctly predicted for 103 patients (92%) using the standard and for 109 patients (97%) using the Ward-type analysis. The sensitivities, specificities and positive and negative predictive values in all the ANN analyses plus the multiple logistic regression analysis are given in Table 3.

The relative weights of the clinical input parameters for the Ward-type ANN analysis, trained with Group I, are shown in Fig. 2. A group of 10 inputs that had greater weights than the rest of the inputs were notable. These were: patient age, serum albumin level, HCV level, HBV status, duration of infection, HIV status, platelets, infection by IVDA, alcohol abuse and HCV genotype 3.

**Multiple logistic regression**

Significant variables predicting the presence of cirrhosis in Group I using the multiple logistic regression model were: platelet count (regression coefficient ± standard error: \(-0.0374 \pm 0.0169; t = -2.22\)); infection by RCC transfusion (\(-4.71 \pm 2.43; t = -1.94\)); serum bilirubin level (\(0.1018 \pm 0.047; t = 2.16\)); concomitant alcohol abuse (\(11.11 \pm 4.62; t = 2.40\)) and HBV status (\(-8.79 \pm 3.76; t = -2.34\). The regression coefficient for the constant was \(8.24 \pm 4.02\) and the \(t\)-value 2.05.

**Table 3** The sensitivities, specificities and predictive values of a positive and negative test for the various artificial neural network (ANN) analyses and the multiple logistic regression model

<table>
<thead>
<tr>
<th>Analysis Type</th>
<th>Number of patients tested</th>
<th>Number of patients with hepatic cirrhosis (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Predictive value of a positive test (%)</th>
<th>Predictive value of a negative test (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard type ANN</td>
<td>30</td>
<td>8 (27%)</td>
<td>75%</td>
<td>100%</td>
<td>100%</td>
<td>91.7%</td>
</tr>
<tr>
<td>Ward-type ANN</td>
<td>30</td>
<td>8 (27%)</td>
<td>87.5%</td>
<td>100%</td>
<td>100%</td>
<td>95.7%</td>
</tr>
<tr>
<td>Multiple logistic regression</td>
<td>30</td>
<td>8 (27%)</td>
<td>63.5%</td>
<td>72.7%</td>
<td>36.7%</td>
<td>88.8%</td>
</tr>
<tr>
<td>Leave-one-out test (standard type ANN)</td>
<td>112</td>
<td>25 (22%)</td>
<td>84%</td>
<td>94.3%</td>
<td>80.8%</td>
<td>95.3%</td>
</tr>
<tr>
<td>Leave-one-out test (Ward-type ANN)</td>
<td>112</td>
<td>25 (22%)</td>
<td>92%</td>
<td>98.9%</td>
<td>95.8%</td>
<td>97.7%</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The development of hepatic cirrhosis as a consequence of chronic hepatitis C infection is associated with a poor prognosis. A significant number of patients with cirrhosis will develop decompensated liver disease or HCC [2,5,29,30]. Multivariate analyses, examining the efficacy of standard regimens of interferon, have also identified cirrhosis as a highly significant factor associated with non-response to therapy [31,32]. Presently, there are no reliable non-invasive investigations predictive of cirrhosis and the presence or absence of cirrhosis must be assessed by liver biopsy, a procedure associated with significant morbidity and mortality [33]. Furthermore, the diagnosis of cirrhosis using only a liver biopsy may be missed in 1–60% of cases [34–36] (therefore in this study, the diagnosis of cirrhosis was made by a combination of laparoscopy and liver biopsy). Long-term follow-up of patients with chronic hepatitis C also requires serial liver biopsies to reassess repeatedly the hepatic histology. For these reasons, HCV-infected patients would greatly benefit from a reliable, non-invasive method of predicting cirrhosis.

One hundred and thirty patients were originally assessed for inclusion in this study. Of these, 18 were repeatedly HCV RNA negative in serum by RT–PCR and could not therefore, by definition, be assessed for HCV genotype and virus level and thus undergo ANN analysis. To measure HCV RNA levels in serum, we chose a semiquantitative method of limiting-dilution analysis of HCV cDNA.

Our results given in Table 3 clearly demonstrate that the training and testing for the two types of feed-forward fully-connected ANNs were successful. In
the first stage the ANN analysis was performed by training the networks with the patient Group I and testing their performance on the patient Group II. To allow direct comparison, the multiple logistic regression model was also constructed from Group I and its performance assessed in the case of Group II. It is evident from Table 3 that the sensitivities and specificities, as well as the predictive values of a positive and negative test, were considerably lower for logistic regression. When limited data sets are handled by ANN analysis it is important to perform a leave-one-out test for the whole available data set to gain a better estimate for the generalization power of the analysis than is obtained using only a separate test set. In this way the possible bias, which may be introduced by the presellected test set and lead to either too positive or negative conclusions about the analysis performance, can be eliminated as the whole data set is used in the test. This is achieved by training and testing the networks as many times as there are cases in the whole data set, i.e. the network is trained with (n - 1) cases (111 in this example) and then the remaining one case put in and the network output assessed. This is repeated as many times as there are cases in the data set. As the ANN output for every available input pattern is independently assessed, this kind of testing gives an objective estimation of the analysis performance. It is interesting to note that for both ANN approaches the leave-one-out testing methodology leads to sensitivities, specificities and predictive values that are almost at the same level as those obtained when tested only with Group II (Table 3). This result supports the conclusion that the information provided by all the clinical inputs contains a clearly resolvable pattern related to cirrhosis in the hepatitis C patients and it is not dramatically affected by the data size used. It is also noticeable that when the multiple logistic regression model was optimized for the whole data set of 112 patients, the resulting sensitivity was 44% and specificity 95.4% together with the predictive value of a positive and negative test of 70% and 87.2%, respectively. These values, except the specificity, denote far lower performance than obtained for the Ward-type ANN analysis in the leave-one-out test. This indicates that even when the information content of the whole patient data set was used for specifying the regression model, thus not enabling further independent testing, it could not achieve the test performance of the Ward-type ANN analysis. This result is probably related to the better description of non-linear relationships between variables in the ANN analysis compared with the multiple logistic regression. Thus, the ANN analysis seems to provide a superior method for the non-invasive prediction of cirrhosis in the HCV patients.

The performance of the feed-forward fully-connected ANNs was also assessed by running tests using different numbers of inputs, ranging from 25 to seven (either by omitting clinical data with lowest correlation coefficients with the outcome and/or were given low relative contribution factors in the ANN analysis when all 24 inputs were used). There was a significant decrease in the performance of the networks although, at worst, they were still able to predict the presence or absence of cirrhosis correctly in approximately 80% of the cases in test Group II.

The six most weighted inputs (for the best-performing Ward-type analysis, trained with Group I) were patient age, serum albumin level, HCV level, HBV status, duration of infection and HIV status. Additionally, platelets, infection by IVDA, alcohol abuse and HCV genotype 3 had high weights. Of these, the least surprising parameter was the serum albumin level. This parameter is a component of the Child-Pugh score [37], and a low serum albumin level indicates poor synthetic hepatic function resulting from decompensated liver disease. Of the others, increasing patient age has already been reported in association both with aggressive disease (rapid progression of cirrhosis to HCC) [7,38] and with a non-response to interferon therapy [32,39,40], perhaps because of impaired immunity amongst older patients [41].

The clinical significance of circulating HCV RNA levels remains unclear; there have been many reports of significant relationships, perhaps most consistently with increasing disease severity [12-14]; however, cautious interpretation of a single HCV RNA level as part of a dynamic process of rapidly fluctuating levels is required [42,43]. Univariate and multivariate analyses of modes of hepatocyte injury concomitant with HCV infection, for example concurrent HBV [16] and/or HIV infection [44,45] (and, of course, concomitant alcohol abuse [46,47]), has indicated a synergistic interaction between these factors in the progression of liver disease. Finally, our data corroborate the clinical impression that the
duration of HCV infection influences the histological course of this chronic viral disease. Prospective, multicentre studies of populations infected with HCV-contaminated blood transfusions have concluded that the prevalence of cirrhosis is about 20% after 20 years of infection, but significantly less after 10 years [2–5].

The decrease in the ANN analysis performance when some of the clinical inputs were omitted and the low performance of the multiple logistic regression, suggests that non-linear combinations of all the factors are important for the prediction of cirrhosis. Thus, the most weighted inputs should not be interpreted as providing all the necessary information for the classification of cirrhosis; instead, they are the parameters that have the most important effect on the ANN classification.

The ANN analysis presented performed well in the prediction of cirrhosis in the HCV-infected patients, and the resulting weights of the ANN inputs compared favourably with existing data assessing the factors affecting the development of cirrhosis in HCV patients. It should be emphasized, however, that ANN analysis does have its own inherent problems. The generalization power of a network is dependent on the training data set and thus as extensive data sets as possible should be used in the training. Therefore, even though demanding leave-out-tests were performed in this study for the whole patient population available, the results need to be verified using other, preferably larger, data sets. A useful feature in ANN analysis is that it is possible to continuously refine the capability of the network by retraining and testing when new data become available. It should also be remembered that the outcome of feed-forward fully-connected ANNs relies on the accuracy of the clinical data used to train the networks. Thus, inaccuracies in the parameter values resulting from experimental errors (for example, when measuring the biochemical function of the liver) may mislead the ANN training and thus decrease the ANN performance.

In conclusion, we have successfully applied ANN analysis to predict the presence or absence of cirrhosis, based on a number of host and virus factors, without the need for a liver biopsy and laparoscopy. The trained networks can be operated automatically using a personal computer. They can provide an instant answer with a high degree of confidence as to whether a patient with HCV infection is cirrhotic or not. Further validation of the ANN analysis with additional data could thus potentially revolutionize the overall management of patients with HCV infection.

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