MD THESIS

HEPATITIS C INFECTION IN THE WEST OF SCOTLAND: EPIDEMIOLOGY, TREATMENT AND DISEASE PROGRESSION

Dr Douglas Thorburn

MB ChB (Glasgow 1990)

MRCP (UK) (1993)

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from the:

Institute of Virology,
University of Glasgow,
Church Street,
Glasgow,
G11 5JR.

Gastroenterology Unit,
Level 8, Gartnavel
General Hospital,
1053 Great Western Road,
Glasgow, G12 OYN.

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ABSTRACT

After the discovery of and development of diagnostic tests for hepatitis A and B it became apparent that a parenterally transmissible agent was responsible for cases of non-A non-B hepatitis. It was not until 1989, a further fifteen years later, that the agent responsible for most cases, the hepatitis C virus (HCV), was identified. This virus was initially thought to cause a mild self-limiting hepatitis. With the introduction of serological screening tests for HCV, it was soon apparent that it caused a chronic asymptomatic hepatitis which could be accompanied by significant fibrosis and sometimes cirrhosis and hepatocellular carcinoma.

Epidemiological studies have since revealed that up to eighty percent of those infected develop chronic infection and that in the developed world hepatitis C virus infection is widespread. An estimated 0.5% of the UK population and 1.8% of the population in the USA are infected. It is now accepted that HCV can cause an asymptomatic indolent infection that can progress over decades with the development of cirrhosis. Hepatitis C infection is now established as the single most common condition referred to hepatologists and the leading indication for hepatic transplantation in Europe and the USA.

Since the discovery of the hepatitis C virus much research has focussed on the epidemiology, natural history and treatment of the condition. The first chapter provides an overview of HCV and places in context the research contained in this thesis. In western countries the role of the healthcare setting in transmitting hepatitis C virus is poorly understood. We performed a large retrospective serological survey of hepatitis C virus infection in healthcare workers from the

West of Scotland. This revealed the overall prevalence of HCV infection in healthcare workers to be low regardless of involvement in exposure-prone procedures. This indicates that the risk of acquisition of hepatitis C virus infection by healthcare workers in an area with a large HCV infected intravenous drug using population is small and that the risk posed to patients by contact with the HCV infected healthcare workers is also low.

Liver biopsy is the gold standard for assessing the extent of liver injury and determining prognosis in chronic hepatitis C. Non-invasive markers of liver injury have proved disappointing such that serial liver biopsies are required to monitor disease progression. In this thesis the hepatocellular enzyme α–glutathione s-transferase is studied as a non-invasive marker of liver injury and as a means of assessing response to treatment with α - interferon. Disappointingly α–glutathione s-transferase performed poorly as a non-invasive marker of liver injury but showed some promise as a marker of response to interferon therapy.

Three chapters of this thesis then focus on factors that may influence the natural history of chronic hepatitis C virus infection and in particular account for the variable rates of progression of liver fibrosis observed in chronic HCV. The role of iron and polymorphisms in the haemochromatosis gene (HFE) were studied. Carriage of HFE mutations was not related to the serum and liver markers of iron accumulation or the progression of liver fibrosis. Elevated liver iron concentrations were rarely observed, occurring in patients with more severe liver disease, and whether this was the cause or result of hepatocellular injury was unclear. Carriage of genetic polymorphisms in the renin-angiotensin system, which are associated with increased systemic renin-angiotensin system activity, were studied. This novel

study was designed to explore whether these polymorphisms, known to influence the progression of renal and cardiac fibrosis in a number of cardiovascular diseases, influenced the progression of liver fibrosis in chronic HCV. In this study no association between these functional renin-angiotensin polymorphisms and the progression of liver fibrosis was observed. Hepatitis G virus infection was sought in a cohort of hepatitis C virus infected blood donors to investigate whether co-infection with this virus influenced the severity of hepatitis C virus related liver injury. Although hepatitis G virus co-infection was frequently observed it did not effect the severity of liver injury assessed biochemically and histologically. The factors that account for the variable rates of progression of chronic hepatitis C virus infection remain to be elucidated.

Finally in this thesis the role of α - interferon therapy in the management of asymptomatic blood donors found to have hepatitis C virus infection at blood donation is studied. Most patients detected in this manner have only mild hepatitis with minimal fibrosis and little data exists as to whether they are appropriate candidates for treatment. In a randomised crossover study these patients were observed to tolerate α - interferon therapy and have comparable response rates to other patient groups with chronic hepatitis C infection. These patients appear to be suitable candidates for treatment, although more data are required to establish what the prognosis is for these individuals if chronic HCV is left untreated.

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ABBREVIATIONS

α - GST	α - Glutathione S-transferase
ACE	Angiotensin converting enzyme
ALT	Alanine aminotransferase
Ang II	Angiotensin II
Anti-HCV	Antibodies to HCV
AT 1 R	Angiotensin 1 receptor
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunoabsorbent assay
E1	Putative envelope protein 1 of HCV
E2	Putative envelope protein 2 of HCV
GBV-C	GB virus - C
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HFE gene	Haemochromatosis gene
HGV	Hepatitis G virus
HII	Hepatic iron index
ISDR	Interferon sensitivity determining region
LIC	Liver iron concentration
NS	Non-structural proteins of hepatitis C virus
NANBH	Non-A, non-B hepatitis
NHS	National Health Service
NS	Non-structural HCV protein
PCR	Polymerase chain reaction

RAS	Renin angiotensin system
RFLP	Restriction fragment length polymorphism
RIBA	Radio-immunoblot assay
RNA	Ribonucleic acid
RT	Reverse transcription
TGF - β1	Transforming growth factor β1
UTR	Untranslated region

PUBLICATIONS AND COMMUNICATIONS ARISING FROM THIS THESIS

PAPERS

1. Thorburn, D., Bird, G.L.A., Spence, E., MacSween, R.N.M. & Mills, P.R. (1996) α-Glutathione S-transferase levels in chronic hepatitis C infection and the effect of α-interferon therapy. *Clinica Chimica Acta*; **253**, 171-180.

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CHAPTER 1

HEPATITIS C VIRUS INFECTION: AN OVERVIEW

1.1 CONTENTS **Page**

1.2 VIRAL HEPATITIS AND THE IDENTIFICATION OF HEPATITIS C VIRUS

1.2.1 Recognition and Characterisation of the Non-A, Non-B Hepatitis Virus

As long ago as the 1950s it was recognised that at least two types of hepatitis agent existed. Definitive evidence for two distinct agents was provided by the work of Krugman and colleagues in the 1960s who recognised and categorised MS1 (formerly "infectious" and now type A) and MS2 (formerly "serum" and now type B) hepatitis (Krugman, S., et al. 1967).

About the same time the work of Blumberg et al. led to the identification of the hepatitis B virus (Blumberg, B.S., et al. 1965, Blumberg, B.S., et al. 1967). They discovered that an antigen first detected in the serum of an Australian aboriginal reacted with antibodies from multiply transfused patients and was associated with viral hepatitis. Prince proceeded to demonstrate that this antigen was specifically associated with transfusion associated, hepatitis B (Prince, A.M. 1968). Shortly thereafter the Australia antigen was shown to be the surface antigen of a virus like particle (the Dane particle) detected in patients with hepatitis B. This antigen was renamed hepatitis B surface antigen (HBsAg) and became the basis of the test employed in blood donor screening. The hepatitis A virus was identified in 1973 when Feinstone and colleagues found a 27-nm virus-like particles in the faeces of volunteers infected with the MS1 strain and diagnostic tests for the hepatitis A virus soon followed (Feinstone, S.M., et al. 1973).

When the new tests for hepatitis A virus were applied to the serum of patients with non-B post-transfusion hepatitis none of the cases were found to have been caused

by hepatitis A and were termed non-A, non-B hepatitis (NANBH) (Feinstone, S.M., et al. 1975). At that time three-quarters of cases of post-transfusion hepatitis were NANBH. Successful passage of serum from patients with NANBH to chimpanzees established this to be caused by one or more transmissible agents (Tabor, E., et al. 1978).

In the early 1980s, Feinstone and colleagues established that one type of NANBH virus contained essential lipids (and hence was enveloped) by establishing that the lipid solvent chloroform destroyed it's infectivity (Feinstone, S.M., et al. 1983). Subsequently He et al. determined that the main strain of NANBH was 30 to 60 nm in diameter by filtering it through various sized polycarbonate filters (He, L-I., et al. 1987). Thus prior to the identification of the hepatitis C virus (HCV) as the causative agent of NANBH some of its physical characteristics had been established. These studies were essential preliminaries to the later molecular characterisation of HCV.

1.2.2 Cloning of the hepatitis C virus.

In a collaboration between Chiron Corporation (Emeryville, California) and the Centre for Disease Control and Prevention (CDC) the virus responsible for most cases of NANBH was finally identified in 1989. The RNA sequence of this viral genome was determined by an elegant combination of molecular biological techniques and detection of the proteins encoded by the RNA by sera from "pedigreed" NANBH cases (Choo, Q.L., et al. 1989). Serological assays were then developed to detect antibodies to the protein product of these clones (Kuo, G., et al. 1989).

The initial step in this process was the extraction of nucleic acid from a pellet produced by ultracentrifugation of chimpanzee plasma that had previously been shown to have a high infectivity titre. Prior to the synthesis of complementary DNA (cDNA), the extracted nucleic acid was denatured so that both DNA and RNA could act as a template. The cDNA was then inserted into a cloning vector that allowed the viral proteins to be expressed in *Escherichia Coli.* After lysis of the bacteria the expressed proteins were screened initially in a radio-immunoassay with serum from a patient who had had NANBH. Using this method, a single positive clone (C100) was identified after testing 1×10^6 specimens and further RNA sequence was assembled using the C100 clone to screen for overlapping clones.

This C100 clone became the capture antigen in the original imunoassays for HCV. This assay was tested on a reference panel of serum from patients with known NANBH and revealed that 70% to 90% of cases were related to this agent. Within a year the whole genome was sequenced and named the hepatitis C virus (HCV). Subsequently the hepatotropism of HCV has been confirmed by the demonstration of this spherical enveloped virus 50 nm in diameter within human hepatocytes by electron microscopy (Shimizu, Y.K., et al. 1996).

1.3 STRUCTURE AND FUNCTION OF THE HCV GENOME.

1.3.1 Genome organisation.

The genome of HCV consists of a positive sensed single–stranded RNA molecule approximately 9.5 kilobases in length. A large open reading frame, flanked by 5' and 3' untranslated regions encodes a precursor viral protein of 3008 to 3037 amino acids in length (Choo, Q.L., et al. 1989). Comparative analysis of the full-length sequences of HCV isolates suggest that the virus is closely related to the pesti- and flavivirus families within the genus Flaviviridae (Miller, R.H. & Purcell, R.H., 1990). HCV has been assigned to a separate genus within this family (hepacivirus, which also contains GB virus-C / hepatitis G virus) as similarity with pestiviruses and flaviviruses is limited. The organisation of the HCV genome is schematically represented in Figure 1.1. The HCV polyprotein undergoes post-translational cleavage mediated by both cellular and viral proteases to generate both structural (S) and non-structural (NS) proteins (Grakoui, A., et al. 1993).

1.3.1.1 5' Untranslated region (UTR).

The 5' untranslated region of HCV is highly conserved and around 319 to 340 nucleotides in length (Okamoto, H. & Mishoro, S. 1994). It contains an internal ribosome entry site which directs ribosomes to bind internally on the HCV genome and initiate translation at one of the AUG start codons contained within this region. This essential function of the 5'UTR was confirmed by Wang and colleagues and explains the highly conserved nature of this region (Wang C., et al. 1993). Modification to this region limits the translation of the HCV genome and is a site of potential therapeutic importance (Tang, S., et al. 1999).

1.3.1.2 HCV Structural Proteins.

The structural proteins of HCV are located at the N terminus of the HCV polyprotein and are processed by host-encoded proteases (Ralston, R., et al. 1993). Three putatitive structural proteins have been identified; core, E1 and E2.

Core

The first 191 amino acids of the polyprotein precursor encode a 21 kilo Dalton protein which is the putative nucleocapsid of the HCV virion (Grakoui, A., et al. 1993). Expression of core protein in a variety of human and non-human cell lines reveals that full length core protein is associated with the endoplasmic reticulum; however after core has undergone cleavage it is associated with the cell nucleus. Whether this cleavage event occurs in-vivo is controversial. Core protein has been reported as having a wide variety of functions including a role in HCV persistence by suppressing apoptotic cell death (Ray, R.B., et al. 1996) and disease pathogenesis by binding to immunoregulatory cytokines (Matsumoto, M., et al. 1997). How many of its purported functions are relevant in vivo is debatable (McLaughlin, J. 2000).

Envelope Proteins

Downstream of the core protein are located the two putative envelope proteins named E1 (31 - 35 kiloDalton in size) and E2 (68 – 72 kiloDalton in size) respectively. There is evidence that E1 and E2 form complexes which are either non-covalently associated (Ralston, R., et al. 1993) or have disulphide links (Grakoui, A., et al. 1993). The predominant complex is formed non-covalently within the endoplasmic reticulum. The E2 envelope protein contains the most variable region of the HCV genome, named the hypervariable region. This region

is further discussed in Section 1.3.2.2. The E2 envelope protein also contains a binding site for CD81, a tetraspanin expressed on hepatocytes, B lymphocytes and many other tissues, which may function as a cellular receptor or co-receptor for the virus (Pileri, P., et al. 1998).

1.3.1.3 HCV Non-Structural Proteins

The remainder of the HCV polyprotein encodes six non-structural (NS) proteins (review: Neddermann, P., et al. 1997). These proteins have a critical function in the life cycle of hepatitis C virus and represent attractive targets for antiviral therapy (Kolykhalov, A.A., et al. 2000).

NS2

This gene encodes a 21-23 kiloDalton protein which is closely associated with the structural proteins (Grakoui, A., et al. 1993). The biological function of NS2 remains unclear, however a potential zinc-dependant metalloproteinase which is encoded by nucleotides which bridge the NS2 and NS3 regions has been identified (Grakoui, A., et al. 1993).

NS3

The NS3 (70 kiloDaltons in size) has several diverse biochemical functions. In addition to the putative zinc-dependant metalloproteinase function with NS2, it has a serine-like protease domain and a RNA-helicase domain (Miller, R.H. et al. 1990, Houghton, M., et al. 1991).

NS4A and NS4B

The function of these proteins remains unclear. The NS4A (8 kilo Daltons in size) appears to act as a NS3 protease cofactor and its presence is required for the efficient post-translational cleavage of the non-structural proteins (Failla, C., et al. 1994). The role of NS4B has not been established.

NS5A

The full length NS5A interacts with the NS3 RNA-helicase within cytoplasm but the mechanism of this interaction is unclear. NS5A has been demonstrated as a potential transcription activator (Kato, N., et al. 1997). Many carcinogenic viruses, such as human papillomavirus, encode transcription activators and it is possible that the NS5A may play a role in the generation of hepatocellular carcinoma by HCV. A region within the NS5A, the so-called interferon sensitivity determining region (ISDR), has been identified. In studies from Japan the ISDR has been proposed to mediate interferon resistance (Herion, D. & Hoofnagle, J.H. 1997). A putative mechanism for this resistance to interferon has been proposed (Gale, M. Jr. & Katze, M.G. 1998). Studies of HCV isolates from the UK and USA do not support this role for the ISDR (McKechnie, V.M., et al. 2000, Chung, R.T., et al. 1999).

NS5B

The role of NS5B as an RNA-dependent RNA-polymerase was predicted by Miller and Purcell in 1990 and this has subsequently been confirmed (Miller, R.H. & Purcell, R.H. 1990). NS5B protein has recently been shown to locate in the nuclear periplasmic membrane along with NS5A and may participate in a membrane bound replication complex (Hwang, S.B., et al. 1997).

1.3.1.4 3' Untranslated Region (UTR).

Downstream of the stop codon of the open reading frame of the HCV genome is the 3' untranslated region (UTR). This has three distinct regions with the 3' terminus 98 nucleotide sequence highly conserved even between genotypes (Kolykhalov, A.A., et al. 1996). Forty-six bases of this highly conserved region forms a stem loop structure (Kolykhalov, A.A., et al. 1996) which may play a role in the initiation of viral replication (Clarke, B. 1997). Modification to the 3' untranslated region impairs virus replication in vivo and again may be a site for targeting therapy against hepatitis C virus infection (Kolykhalov, A.A., et al. 2000).

1.3.2 Genetic variability of the HCV Genome

1.3.2.1 Genotypes of HCV.

After the full-length sequence of the hepatitis C virus was cloned it became clear that there was significant viral heterogeneity at the nucleotide level. Phylogenetic analyses have allowed the classification of the majority of HCV isolates into 6 major groups (Genotypes 1 to 6) and these genotypes are further subdivided into more than 80 subtypes (Simmonds, P., et al. 1994). The evolution of HCV genotypes is the result of random nucleotide errors by the RNA-dependant RNA polymerase. This produces mutations which may be viable or non-viable depending on the site. The high rate of random nucleotide substitutions results in slow genetic evolution accounting for the great genetic heterogeneity of HCV. Differences between genotypes are relatively large with nucleotide differences in more than 30% of the viral sequence, while subtypes differ by up to 20%. Isolates within a specific subtype have more than 90% homology in genome nucleotide sequence (Smith D.B. 1995). Within an individual patient, the virus exists as a

quasispecies, a group of related variants which may have 1 - 2% nucleotide heterogeneity depending on the region of the genome examined.

Direct comparison of full length HCV sequences is laborious and time consuming. Analysis of the 5' untranslated region by restriction fragment length polymorphism (RFLP) has consequently found favour as a more rapid and cheaper method of genotyping HCV isolates for clinical purposes (Davidson, F., et al. 1995). To allow further subtyping of HCV isolates, sequence analysis of additional regions of the genome such as core, E1 or NS5 is required.

Throughout the world HCV genotypes differ with geographic region. In the USA genotype 1 predominates, accounting for over two-thirds of cases (Alter, M.J., et al. 1999). In Europe genotype 1 remains most common however genotype 3 can be found in up to 50% of cases (McOmish, F., et al. 1994). Genotype 4 is found almost exclusively in the Middle East, genotype 5 in South Africa and genotype 6 largely in Hong Kong (Mellor, J., et al. 1995). The relative frequency of genotypes varies within populations depending on population migration and route of transmission. In Western Europe genotype 1b is more common in patients over fifty years of age, who largely acquired HCV through blood transfusion, while genotypes 1a and 3a predominate in younger age groups, who are more likely to have acquired HCV through intravenous drug use (Webster, G., et al. 2000). With the dramatic reduction in HCV transmission by blood transfusion as the result of blood donor screening the relative frequency of infections with genotypes 1a and 3a will increase.

The role of HCV genotype on the clinical course of HCV infection and response to treatment is discussed later in Sections 1.6.3.1 and 1.7.3 respectively.

1.3.2.2 Variability in the HCV genome

Variation is found throughout the whole length of the HCV genome although it is not uniformly spread. Areas of the genome with crucial functions tend to be most highly conserved as mutations in these regions usually result in an alteration in structure which impairs function. The 3' UTR is the most conserved region of the genome with the 5' UTR also highly conserved (Bukh, J., et al. 1995). Core is the most highly conserved of the HCV proteins followed by conserved regions in NS3 and NS5B proteins (Okamoto, H., et al. 1992). Comparison of full length HCV nucleotide and deduced amino-acid sequences have identified two distinct hypervariable regions lying within the putative envelope 2 (E2) region termed hypervariable regions 1 (HVR1) and 2 (HVR2).

The hypervariable regions account for 30 – 47% of all nucleotide differences and 40 – 63% of all amino acid differences observed between HCV isolates (Weiner, A.J., et al. 1991). Despite the high degree of variability in the hypervariable regions, conserved structural elements are found in the envelope region. Twenty-six cysteine residues are conserved within the envelope region of all HCV isolates. These may be essential for the conformation of the envelope proteins through the formation of disulphide bonds. In contrast no consistent sequences are found in the hypervariable regions between isolates such that there can be no consistency to the secondary structure of these regions. This variability in the putative envelope protein of HCV may contribute to the persistence of HCV through evasion of the host immune response.

1.3.2.3 Viral quasispecies

The term quasispecies is defined as a swarm of related genetic variants within an individual with only minor sequence differences of less than 5% of the viral genome. This occurs as the result of the aforementioned error-prone process of HCV replication generating a heterogeneous population of distinct but closely related genomic sequences. The circulation of HCV as a population of different but related variants may be an important factor in the ability of HCV to cause persistent infection and evade the host immune response. Even if the host mounts a neutralizing immune response to the dominant strain any of the other variants present already can continue to replicate and become the predominant strain. Studies of chimpanzee inoculated with human sera at various time points after infection has shown that humans develop neutralizing antibodies to HCV but that these antibodies are highly strain specific and incapable of preventing the emergence of viral variants that can maintain the infection (Farci, P., et al. 1992).

Quasispecies diversity may have further clinical relevance as some studies have suggested that increased quasispecies complexity is correlated with more severe liver disease. This is discussed further in Sections 1.6.3.1. In addition patients with greater quasispecies diversity may respond poorly to interferon therapy (Gonzalez-Peralta, R.P., et al. 1996).

1.4 DIAGNOSIS OF HEPATITIS C

1.4.1 Diagnostic testing for HCV

1.4.1.1 General considerations

Serological assays are generally robust and can be performed on any form of blood sample although poor storage conditions may influence the sensitivity and specificity of these tests. In contrast, molecular biological tests are easily influenced by conditions of sample collection and storage (Busch, M.P., et al. 1992). Serum and plasma are acceptable for these assays however samples should ideally be separated and frozen within three hours of sampling. It is mandatory that samples are stored for prolonged periods at -70°C or -80°C and to avoid freeze thaw cycles to avoid degradation of RNA (Busch, M.P., et al. 1992, Damen, M., et al. 1998).

1.4.1.2 HCV antibody detection

These tests are based on the use of enzyme linked immunoabsorbent assays (ELISA) and use synthetic peptides or recombinant HCV antigens to capture antibodies to HCV (anti-HCV) onto the wells of a microtitre plate. The presence of anti-HCV is detected by the addition of anti-IgG antibodies labelled with an enzyme that catalyses the conversion of a colourless substrate into a coloured compound. Changes in the optical density in the well can be assessed by an automated reader and is proportional to the amount of anti-HCV antibody in the sample. The newer third generation ELISAs have superseded the second generation tests by having an improved sensitivity for detecting antibodies to the core, NS3 and NS4 proteins and including NS5 as an antigen (Huber, K.R., et al. 1996). In high prevalence immunocompetent populations sensitivities of 98.8 -

100% and specificities of 99.3 – 100% have been observed (Vrielink, H., et al. 1995). False negative results can be observed in immunocompromised patients, while false positives can be observed in patients with immunological conditions and hypergammaglobulinaemia. With these assays anti-HCV can be detected in 50 –70% of patients with acute HCV infection at presentation and in 90% within 6 months. (National Institute of Health. 1997).

Due to the lack of specificity of the first generation ELISA assays recombinant immuno-blot assays (RIBA) were introduced as confirmatory tests for anti-HCV. These assays are based on the detection of anti-HCV by means of HCV antigens coated as parallel bands onto nitrocellulose strips. With the improved sensitivity of the newer third-generation ELISAs these assays have little additional to offer (Pawlotsky, J.M., et al. 1997). They may however retain a role in confirming that patients who are reactive by ELISA and persistently RT PCR negative do have anti-HCV.

1.4.1.3 Detection of HCV RNA

These assays are based on the amplification of HCV RNA using a cyclic enzymatic reaction (polymerase chain reaction, PCR) to generate many copies after the extraction of viral RNA and a reverse transcription (RT) step to produce double stranded complementary DNA (cDNA). The product of this reaction can then be identified by comparison with known positive samples and molecular markers run on agarose gels or by hybridisation with specific probes. In order to detect the low circulating levels of HCV RNA in body fluids this amplification process is required and can detect as few as 100 to 1,000 copies of HCV RNA per millilitre.

Many different assays for HCV RT PCR have been described and there is poor standardisation between laboratories with respect to methods, specimen handling and storage. These problems have been addressed by the introduction of international HCV RNA reference panels (Damen, M., et al. 1996) and lead to the development of standardised qualitative RT PCR assays such as the Amplicor test from Roche Molecular Diagnostics (Young, K., et al. 1995).

From these initial qualitative molecular biological tests for HCV RNA there have developed tests for quantifying HCV RNA in blood and hence estimates of the patients 'viral load'. A number of quantitative assays are available and the best evaluated is the branched DNA–based signal amplification assay from Bayer Diagnostics. This assay is less sensitive than qualitative RT PCR assays with a cut-off of 200,000 copies per ml and should not be used in the place of qualitative HCV RT PCR to confirm viral clearance.

1.4.1.4 HCV Genotype determination

The reference method for HCV genotype determination is genome sequence analysis and alignment followed by phylogenetic analysis. This process is however laborious and not suitable for diagnostic virology. Restriction fragment length polymorphism (RFLP) of the highly conserved 5' untranslated region has consequently found favour as a more rapid and cheaper method with satisfactory sensitivity (Davidson, F., et al. 1995). The main disadvantages of this technique are its lack of standardisation and inability to detect mixed genotype infections in most cases.

1.4.1.5 Local Protocol for HCV diagnosis

The protocol for HCV testing now employed by the West of Scotland Regional Virus Laboratory is outlined in Appendix 1. Serum samples are initially screened for anti-HCV using a third generation ELISA (Ortho Diagnostics, Raritan, NJ). Confirmatory testing of reactive samples is by RT PCR using the second generation Amplicor test (Roche Diagnostics). Supplemental third generation RIBA testing (Ortho Diagnostics, Raritan, NJ) is reserved for patients who are RT PCR negative. HCV genotyping is performed only on those patients RT PCR positive who are being considered for treatment.

1.4.2 Evaluation of disease severity in chronic HCV infection

1.4.2.1 Non-invasive assessment of disease severity

Most patients detected with HCV infection are asymptomatic and hence history and clinical examination are not useful for assessing the severity of liver disease unless there is evidence of hepatic decompensation. Unfortunately the use of standard non-invasive serum tests of liver function has proved unreliable in determining the activity of liver disease. Histological evidence of chronic hepatitis can be seen in up to 70% of RT PCR positive patients with normal serum transaminases. However patients with persistently normal alanine aminotransferase (ALT) levels are less likely to have significant liver disease on liver biopsy than patients whose ALT levels are persistently elevated (Silini, E., et al. 1995). At best the correlation between the ALT and liver grade and stage of chronic hepatitis is poor (Haber, M.M., et al. 1995). This is discussed further in Chapter 4.

Numerous studies have attempted to correlate HCV genotype and RNA load with liver histology and have found no consistent correlation (review: Fried, M.W.,

1997). This is discussed further in Section 1.6.3.1. Thus in the absence of signs of hepatic decompensation liver biopsy is the gold standard to determine the extent of HCV-related liver injury.

1.4.2.2 Pathology of HCV

The histological features of chronic HCV are diverse but commonly include portal inflammation with lymphoid aggregates, macrovesicular steatosis, lobular inflammation (rarely amounting to confluent necrosis) and portal and septal fibrosis. A number of scoring systems for the quantitative assessment of liver histology in chronic HCV have been proposed (Scheur, P.J., et al. 1991, Ishak, K., et al. 1995, Bedossa, P. & Poynard T. 1996) and each allows the classification of chronic hepatitis into mild, moderate and severe disease as initially proposed (Knodell, R.G., et al. 1981). By histologically grading the severity of necroinflammatory activity and staging the degree of fibrosis on liver biopsy of patients infected with HCV, it is almost possible to predict those patients at risk of progressing to cirrhosis (Yano, M., et al. 1996). Histological assessment is therefore important to assess the current severity of HCV-related liver injury and also the interval until the development of cirrhosis such that appropriate patients can be offered treatment.

The grading and staging systems employed in this thesis are those of Scheuer (Scheuer, P.J. 1991) (Chapter 4) and Ishak et al. (Ishak, K., et al. 1995) (Chapters 5 to 8) and are summarised in Table 1.1

1.5 EPIDEMIOLOGY OF HCV

1.5.1 Prevalence of HCV infection

As chronic HCV is largely asymptomatic, its prevalence in a population is difficult to estimate without an extensive programme of screening. The World Health Organisation. estimates that 3% of the world's population are chronically infected (World Health Organisation, 1997). This figure is an average with reported rates worldwide showing great geographical variation. Egypt has the highest reported prevalence worldwide with an estimated prevalence of 24% (Cohen, J. 1999). Within Europe the prevalence in blood donors ranges from approximately 0.04% in northern countries to 2% in southern Mediterranean areas (Hepatitis C European network for Co-operative Research. 1998). A prevalence as high as 11% has been reported in areas of southern Italy (Guadagnino, V., et al. 1997). In the United Kingdom, as with the rest of the world, there is a lack of population based serological studies of HCV prevalence. It is possible to estimate a minimum population prevalence of HCV by centralised recording of all reported HCV infections. From 1991 to 1997 there were 2161 individuals who tested positive for HCV in the Greater Glasgow Health Board catchment area (prevalence 0.42% [3807 / 906,000]) (SCIEH Weekly Report, 2000). This compares with a 0.05% (2546 of 5.12 million) prevalence of known HCV antibody positivity in the Trent region of England (Mohsen, A.H. et al. 2001). These figures are likely to be an underestimate of the true population prevalence of HCV, as many asymptomatic patients remain undiagnosed.

1.5.2 Transmission of HCV

On the introduction of screening tests for HCV the commonest risk factors for acquisition of the infection were a history of blood transfusion or intravenous drug use, which were present in from 60% to 80% of cases (Serfaty, L., et al. 1993, Zeuzem, S., et al. 1996). With the increased sensitivity of third generation screening tests, the risk of transfusion associated HCV infection is estimated at 0.01% to 0.001% per unit transfused (Schreiber, G.B., et al. 1996). No recipients of blood in Scotland are known to have been exposed to HCV contaminated transfusions since screening of donors was introduced in 1991. As a result of donor screening, blood products and transfusion are no longer a major route of hepatitis C transmission.

Intravenous drug use is currently the main risk factor for HCV transmission. The prevalence of anti-HCV among intravenous drug users in the West of Scotland is estimated to be 72% and is related to the duration of drug injection (Taylor, A., et al. 2000). With the introduction of needle-exchange programmes and methadone maintenance therapy in the early 1990s there has been a reduction in the frequency with which injectors share injecting equipment (Bath, G.E., et al. 1993). There is evidence that, even when duration of injection is controlled for, the incidence of HCV among injectors has decreased over the same period (Taylor, A., et al. 2000, Goldberg, D., et al. 1998). Despite these measures, new cases of HCV are still occurring and this likely reflects the ongoing sharing of injecting equipment among users despite the free availability of sterile needles and syringes (Information and Statistics Division of the Common Services Agency, National Health Service in Scotland. 1998).

Sexual transmission of HCV is recognised but the frequency with which it occurs is uncertain. In a recent study high-risk sexual behaviour, defined as an early age at first intercourse or more than fifty lifetime sexual partners, was an independent risk factor for the acquisition of HCV (Alter, M.J., et al. 1999). A study of non-intravenous drug using genito-urinary clinic attenders in Scotland found a low prevalence of anti-HCV in heterosexual males (0.8%) and females (0.3%) and homosexual males (0.6%) compared with attenders that used intravenous drugs (49%) (Goldberg, D., et al. 2001). In studies from the western world, the average anti-HCV positivity rate among spouses of HCV infected cases who report no other risk factors for infection is 1.3% again suggesting that HCV can be acquired by sexual intercourse but the probability is low (Wasley, A. & Alter, M.J. 2000). Vertical transmission is recognised from mothers HCV RNA positive at the time of delivery and occurs in 6% of deliveries increasing to 17% if the mother is co-infected with HIV (Wasley, A., et al. 2000).

Transmission of HCV in the healthcare setting may be from patient to patient, from patient to healthcare worker or vice versa. In developing countries where the provision of sterile medical equipment is inadequate, this is likely to be a major route of transmission. This may account for the high prevalence of anti-HCV observed in Egypt where the injection of anti-schistosomal treatment with reusable equipment is widespread (Cohen, J. 1999). In western countries, outbreaks of HCV have been observed among patients within haemodialysis units presumably as the result of incorrect implementation of infection control policies by staff (Wasley, A. et al. 2000). There have been two reports of HCV transmission from infected healthcare workers who perform invasive procedures to their patients when infection control policies have been contravened (Bosch, X. 1998, Ross, R.S., et al.

2000). In addition there have been two reports of transmission of HCV from health care workers (both cardiothoracic surgeons) performing invasive procedures when adequate infection control had been observed (Esteban, J.I., et al. 1996, Duckworth, G.J et al. 1999). As few patients develop symptoms following infection the significance of the healthcare setting as a mechanism of HCV transmission in western countries is not established.

In the USA only 10% of patients with acute or chronic hepatitis C infection do not have a commonly identified risk factor for the acquisition of HCV (Wasley, A. et al. 2000). Other plausible routes of HCV transmission have been proposed to account for these cases however there is no evidence from studies that body piercing, tattooing or commercial barbering transmit HCV.

1.5.3 Prevention of HCV

No vaccine or effective post-exposure prophylaxis exists for hepatitis C virus infection hence the emphasis is on primary prevention for patients that are at high risk of acquiring HCV infection and the counselling of infected persons so that they can reduce the risk of transmitting to others. This includes education to reduce the risk of HCV transmission in the healthcare setting by emphasizing the importance of infection control and the avoidance of unsafe practices.

In developing countries, where programmes of blood donor screening and inactivation of blood products have not been implemented, these routes remain the major source of transmission and hence improving the safety of the blood supply remains the greatest priority (World Health Organisation. 1999). It is recommended that programs should also be instituted for the sterilization or

disinfection of medical supplies and the education of all healthcare practitioners in the use of appropriate infection control practices.

In developed countries, primary prevention of intravenous drug use, which is the single most common route of infection, should be the highest priority. In addition although the relative importance of sexual transmission of HCV is unclear, behavioural modification and appropriate barrier contraception should be endorsed to reduce the risk of transmission of HCV and all other sexually transmitted diseases. Healthcare professionals should be vigilant for patients with a history of intravenous drug use or other high risk practices to identify individuals for appropriate testing and counselling. Counselling and education to prevent initiation of drug injecting and high-risk sexual practices and to modify behaviour in those that already indulge in these practices but are not infected is necessary. The establishment of needle exchange clinics has already been shown to prevent the transmission of HCV infection (Taylor, A., et al. 2000, Goldberg, D., et al. 1998).

Those persons known to be infected with HCV should be counselled on ways to reduce their risk for transmitting HCV to others (Centre for Disease Control and Prevention 1998). They should be advised not to donate blood, organs, tissue or semen; not to share toothbrushes and razors and to cover cuts and sores. Those in stable relationships need not change their behaviour however they should discuss the need for counselling and testing with their partner and the couple should be allowed to make an informed decision regarding the need for change in their sexual practices. Infected individuals should be provided with information on ways of preventing further harm to the liver through the avoidance of alcohol and immunisation against hepatitis A and B where appropriate.

1.6 NATURAL HISTORY OF HCV INFECTION

HCV infection is infrequently diagnosed during the acute phase of the infection and is usually diagnosed in individuals who already have chronic infection (persistent HCV viraemia for > 6 months). The infection is indolent and the outcome of infection may not be known for many decades. At present, few studies provide sufficient follow-up to conclusively record the natural history of chronic HCV such that extrapolation is required beyond twenty years of infection. It appears that for most HCV infected individuals the infection has a low morbidity and mortality. The problem is that at present we cannot predict those individuals who will develop progressive liver disease and hence a more dire outcome.

1.6.1 Acute HCV infection

This is an uncommon presentation of HCV infection. Clinical manifestations occur in between 25% and 35% of cases, usually within 7 to 8 weeks (range 2 to 26 weeks) after exposure to HCV (National Institute of Health 1997). In those patients that develop symptoms, they are usually mild and consist of nausea, malaise and occasionally jaundice. Fulminant hepatitis at this stage has been described but is very rare (Farci, P., et al. 1996). HCV infection is self-limited in the minority of cases, as evidenced by clearance of HCV RNA from serum and return of liver enzymes to normal within six months of infection. Initial studies reported progression to chronicity and persistent viraemia in 74% to 86% of infections (Alter, M.J., et al. 1999, Conry-Catalina, C., et al. 1996). Later studies with follow-up over decades report rates of clearance of HCV RNA from serum of up to 45% (Kenny-Walsh, E., et al. 1999).

1.6.2 Chronic HCV infection

As already stated the natural history of chronic hepatitis C infection has largely been documented in studies over the first 20 years of infection. Variable rates of disease progression have been reported and these are often dependent on the study design employed.

The most severe outcomes of HCV infection have been documented in retrospective studies of patients already established to have chronic hepatitis, where duration of infection has been estimated retrospectively (studies summarised in Table 1.2). These studies do not include patients with subclinical, asymptomatic disease or those that recovered from the infection. As a result it is clear that serious liver disease can be anticipated in some patients with HCV infection however it is not possible to determine what proportion of the total number infected this represents.

Prospective studies originally designed to investigate the incidence and causes of transfusion associated hepatitis were extended when it became apparent that many patients developed persistent elevation of serum transaminases. Most of these patients were subsequently identified as having HCV infection and this study design allowed prospective follow-up on these patients. The results of these studies are summarised in Table 1.3. These studies were however flawed in that some patients received treatment, which may have modified outcomes, liver biopsy data was only available on those individuals who had more severe biochemical hepatitis and follow-up was documented only for the first couple of decades of the infection.

Combined retrospective-prospective studies have also been performed when patients with HCV infection have been identified retrospectively through the testing of serum available at or soon after the time of development of hepatitis. Prospective follow-up of these individuals was then possible, circumventing the problems of retrospective and prospective studies. These studies include 'outbreaks' of HCV infection in groups of patients who have an identifiable common source for HCV acquisition and are summarised in Table 1.4.

The rate of fibrosis progression to cirrhosis in HCV has been evaluated in a large multicentre study in France, which recruited 2,235 patients who had biopsy proven chronic HCV (Poynard, T, et al. 1997). Fibrosis progression was determined as a ratio between the fibrosis stage (scale of 0 – 4 METAVIR units) and the duration of infection in years. Most patients had a single biopsy however 70 patients had paired samples. The median rate of fibrosis progression was 0.133 units/year. Thus, if fibrosis progression were linear it would take a median of thirty years to progress from no fibrosis to cirrhosis. The rate of fibrosis progression was not normally distributed and they concluded that there were three distinct groups of patients with one third developing cirrhosis within 20 years, one third not developing cirrhosis by fifty years and the final third in between. Males who drank more than 50g of alcohol per day and were infected after the age of forty had the worst prognosis. These findings are consistent with prospective studies of transfusion associated hepatitis (Di Bisceglie, A.M., et al. 1991, Koretz, R.L., et al. 1993).

The reported prognosis of HCV-related cirrhosis has been found to be unexpectedly good. In their study of 384 patients with HCV-related cirrhosis

Fattovich et al. demonstrated a 9% five year mortality for liver related deaths, rising to 21% at 10 years (Fattovich, G., et al. 1997b). If however patients had evidence of hepatic decompensation the five year mortality was 50%. The annual incidence of hepatocellular carcinoma was 1.4% and hepatic decompensation developed in 18% of those that did not develop hepatocellular carcinoma over the course of the study. Overall most patients survived without evidence of significant liver complications. Subsequently this relatively benign prognosis has been questioned by a similar study of 103 patients with HCV-related cirrhosis where the four year survival was 84% (Serfaty, L., et al. 1998).

HCV can cause hepatocellular carcinoma usually through the development of cirrhosis. Because HCV does not integrate into the host genome, the prevailing concept is that malignant transformation is the result of numerous mitotic events that accompany hepatic regeneration as the result of viral or immune mediated hepatocellular destruction. Initial studies from Japan revealed that 74% to 94% of cases of HBV-negative hepatocellular carcinomas have anti-HCV (Kiyosawa, K., et al. 1990, Nishioka, K., et al. 1991) In contrast, studies from western countries have not identified such a strong link between serological markers of HCV and hepatocellular carcinoma. The most compelling evidence for a role for HCV was provided by Liang et al. who used serological and molecular methods to detect HCV in the serum and liver tissue of patients from the USA with hepatocellular carcinoma (Liang, T.J., et al. 1993). They found evidence of HCV infection in 55% of HBV-negative cases. The less significant role for HCV observed in the west may reflect differing genetic susceptibilities in these populations. In addition it is possible that the HCV epidemic began 10 – 20 years earlier in Japan, such that the

role of HCV in hepatocellular carcinoma pathogenesis will increase in the west over time.

1.6.3 Factors influencing the progression of fibrosis in chronic HCV infection

Based on the evidence outlined above it is speculated that of 100 persons acutely infected with HCV, 20% (20 patients) will recover spontaneously and 80% (80 patients) will develop persistent infection. Of the 80 persons with persistent infection (chronic HCV), it would appear that 30% (24 patients) will develop progressive liver disease culminating in cirrhosis and / or hepatocellular carcinoma and 30% (24 patients) will have stable liver disease that will not show progression. The remaining 40% (32 patients) will develop slowly progressive liver disease whose outcome cannot be predicted. Factors which alter the rate of progression of liver fibrosis in this cohort are poorly understood. It would clearly be useful to identify the 70% (56 patients) who will develop progressive liver disease such that treatment can be targeted to this group before they develop significant symptomatic liver disease. A number of factors which might influence the rate of progression of hepatitis C related liver fibrosis have been studied. These include viral, host and environmental factors and are summarised in Table 1.5. The evidence for the statements in this table is given in the next few pages. Many studies exploring the roles of these factors on the progression of fibrosis in chronic HCV are hampered by the failure to exclude confounding variables already known to influence disease progression in chronic HCV infection.

1.6.3.1 Viral factors

Viral factors including HCV genotype, viral load, quasispecies complexity and size of inoculum have been studied. Studies exploring the role of HCV genotype on

disease progression have been conflicting and inconsistent. Genotype 1b has been proposed to be associated with the development of more significant liver disease (Pozzato, G., et al. 1991). Further studies have not confirmed this role for genotype 1b (Poynard, T., et al. 1997, Serfaty, L., et al. 1998). The explanation for these inconsistencies may be that genotype 1b is more common in older patients who acquired HCV through blood transfusion and have longer durations of infection (see Section 1.3.2.1). HCV viral load has not consistently been shown to be associated with disease outcome (Gretch, D., et al. 1994). Initial evidence suggested that increased quasispecies complexity was associated with progression of liver fibrosis and the putative mechanism for this was proposed to be the presence of an increased number of targets for cytotoxic T-lymphocytes on infected hepatocytes (Yuki, N., et al. 1997, Hayashi, J., et al. 1997). More recently this has been challenged and currently quasispecies diversity is not believed to be associated with disease progression (Leone, F., et al. 1998, Lopez-Labrador, F.X., et al. 1999). Gordon et al. compared the long-term outcome in patients who acquired HCV by blood transfusion with persons who had acquired HCV through intravenous drug use (see Table 1.2) and found the outcome related to the mode of transmission (Gordon, S.C., et al. 1993). This may be the result of a larger inoculating viral load in those patients acquiring HCV by blood transfusion.

1.6.3.2 Host factors

A number of host factors which may influence the progression of HCV-related liver injury have been studied (see Table 1.5). Age at acquisition of infection seems to be an important determinant of disease progression. Children and adults infected under the age of forty have the mildest outcomes (Vogt, M., et al. 1999, Kenny-Walsh, E., et al. 1999, Seeff, L.B., et al. 1998b). In addition older age has

been shown on multivariate analysis to be associated with more rapid progression of fibrosis and decreased survival in chronic HCV (Poynard, T., et al. 1997, Niederau, C., et al. 1998). It is important however to distinguish age at infection from age at diagnosis as duration of infection, which correlates with disease progression, is likely to be longer in those diagnosed with HCV when older. As a result it is unclear whether older age, and its effects on hepatic blood flow and the immune system, directly influences the progression of HCV. Male gender is associated with more rapid progression of hepatic fibrosis (Poynard, T., et al. 1997). Why females are protected against progression of hepatic fibrosis is unclear but may be the result of oestrogens modulating fibrogenesis (Yasuda, M., et al. 1999). Host immunodeficiency is associated with a more rapid progression of fibrosis in chronic HCV. Studies report that approximately a third of patients with hypogammaglobulinaemia who acquired HCV through contaminated immunoglobulin infusions develop cirrhosis within 10 years (Bjoro, K., et al. 1994). About 30-40% of HIV infected individuals also carry HCV, often reflecting a common mode of transmission through intravenous drugs or transfusion (Soriano, V., et al. 1999, Dieterich, D.T. 1999). Studies of patients co-infected with the human immunodeficiency virus and HCV have revealed a more rapid rate of fibrosis than HCV infected controls (Garcia-Samaniego, J., et al. 1997, Benhamou, Y., et al. 1999). The mechanism for this association is not well elucidated however there appears to be an association between low CD4 count and rate of progression (Benhamou, Y., et al. 1999). The role of co-infection with hepatitis G virus on the progression of chronic HCV infection is discussed in Chapter 7.

The role of host genetic factors on the outcome of HCV infection has been studied. Specific human leukocyte antigen alleles have been identified to be linked with

disease progression however studies are inconsistent (Thurz, M., et al. 1999, Aikawa, T., et al. 1996). The role of functional polymorphisms in the genes for pro-fibrotic cytokines which influence the activity of hepatic stellate cells have been studied and are discussed further in Chapter 6. The role of hepatic iron and haemochromatosis mutations on progression of fibrosis are discussed in Chapter 5.

Some histological features of chronic HCV infection have been shown to be associated with disease progression. As already discussed, macrovesicular steatosis is a common finding on liver biopsy in chronic HCV infection (see Section 1.4.2.2). The presence and severity of this steatosis has been found to be associated with more rapid progression of HCV-related liver fibrosis (Adinolfini, L.E., et al. 2001). In addition by histologically grading the necroinflammatory activity and staging the fibrosis on liver biopsy it is possible to predict the rate of ongoing fibrosis. The assessment of necroinflammatory activity is not a good predictor of fibrosis progression alone and the fibrosis score alone is the best predictor of ongoing fibrogenesis (Paradis, V., et al. 1996). Thus far no studies have demonstrated that activity grades are predictive of fibrosis progression independently of fibrosis stage (Yano, M., et al. 1996).

1.6.3.3 Environmental factors

Excess alcohol consumption (> 50g / day) is the one environmental factor which has consistently been shown to be associated with more rapid progression of fibrosis in chronic HCV infection (Poynard, T. et al. 1997). Further evidence for its role has been supplied by Corrao and Arico (Corrao, G. & Arico, S. 1998). They compared the rates of cirrhosis in tee-totalers and alcohol abusers (175g / day) with and without HCV infection. Persons who did not drink and were HCV negative

were the reference population. The relative risk of cirrhosis for HCV negative patients who did drank excessively, HCV positive patients who were tee-total and HCV infected alcohol abusers were 15 x, 9 x and 147 x respectively. It is therefore generally accepted that HCV infected individuals should abstain or limit their alcohol consumption to less than one drink per day.

1.7 TREATMENT OF HCV INFECTION.

1.7.1 General measures

All HCV infected patients should be counselled to avoid alcohol and with advice to minimise the risk of HCV transmission to others (see Section 1.5.3).

1.7.2 Antiviral Therapy

1.7.2.1 Interferon monotherapy

Even before HCV was identified as the chief etiologic agent in non-A, non-B hepatitis, α - interferon therapy was associated with normalization of ALT in some patients with this condition (Hoofnagle, J.H., et al. 1986). In 1989 the first cases of successful interferon treatment of HCV infection were documented with normalisation of liver biochemistry in approximately 30% of patients after six months of therapy (Davis, G.L., et al. 1989). High biochemical relapse rates were observed and frequently necessitated retreatment, which was invariably unsuccessful. Over time a number of different interferons were studied and all demonstrate similar efficacy (National Institute of Health. 1997).

Later clinical trials have assessed response to treatment in terms of biochemical response (normalisation of ALT), virological response (as defined by a negative qualitative RT PCR result for HCV RNA in serum) and in some cases histological response, however in the clinical setting liver biopsy post-treatment is rarely indicated. Since responses to therapy may not be maintained after treatment is stopped, the response in clinical trials has been evaluated in terms of response at the end of therapy (end of treatment response) and six months after the completion of therapy (sustained treatment response). Patients with sustained virological

response have a high probability of having a durable biochemical, virological and histological response (Reichard, O., et al. 1999). This has been shown to improve quality of life, halt progression of the liver disease and to hopefully reduce risk of hepatocellular carcinoma. Increase in patient survival has not yet been demonstrated but awaits larger-scale studies with longer follow-up.

Using these more robust markers of response to treatment has proven that initial studies overestimated the proportion of patients that eradicated the virus with only 6% of patients having been shown to have a sustained virological response after six months of α - interferon therapy (McHutchison, J.G., et al. 1998). These poor results with interferon monotherapy prompted studies employing longer durations of therapy and higher doses of interferon. Increasing the duration of therapy led to sustained response rates of up to 25% such that 12 months treatment is now recommended (Poynard, T., et al. 1996). Increasing the dose of interferon also led to improved response rates however this was offset by the number of patients who were unable to tolerate the higher doses such that optimal dose and interval between doses remains to be determined (Poynard, T., et al. 1996).

1.7.2.2 Interferon / Ribavirin combination therapy

Given the disappointing response rates with interferon monotherapy a variety of other drugs were studied for the treatment of HCV infection. Ribavirin is an orally active synthetic guanosine nucleoside analogue that has a broad spectrum of anti-viral activity. It inhibits the replication of many viruses by inhibiting the initiation of translation, RNA replication and viral RNA packaging (Smith, R. 1984). Initial studies of ribavirin monotherapy in chronic hepatitis C showed that it improved liver function tests without clearing HCV from serum. Studies of ribavirin in

addition to interferon confirmed this combination to be superior to interferon monotherapy with sustained virological response rates of 38% and 43% after one year's treatment reported in the initial studies (McHutchison, J.G. & Poynard, T. 1999). The findings of these studies have since been reproduced such that combination therapy has now been accepted as first line therapy for patients with chronic HCV. The main benefit of combination therapy appears to be a 60% reduction in the proportion of end of treatment responders that relapse compared with interferon monotherapy.

1.7.2.3 Pegylated Interferon

Despite the documented improvement in response rates with combination therapy, sustained response rates remain suboptimal. The most recent advance in the treatment of chronic HCV has been the development of depot preparations of interferon, produced by combining interferon with polyethylene glycol (so called pegylated interferon). This can be administered once weekly and has the advantage of avoiding fluctuations in the serum levels of α - interferon, minimising side-effects and improving the tolerability of treatment. Two randomised controlled trials of pegylated α - interferon monotherapy have now been published and report sustained virological response rates of 39% in patients with moderate histological changes and 30% in patients with severe fibrosis and cirrhosis (Zeuzem, S., et al. 2000, Heathcote, E.J., et al. 2000). In both studies response rates were superior to standard α - interferon monotherapy with fewer side effects. Studies of combination therapy with pegylated α - interferon and ribavirin have so far only been published in abstract form but sustained virological response rates in excess of 60% have been observed.

Pegylated interferon alone and in combination with ribavirin is likely to become the gold standard for treatment of interferon naïve patients with chronic HCV, however at present it is unavailable to NHS patients in the UK.

1.7.3 Predictors of response

From clinical trials a number of pre-treatment factors have now been identified which are associated with a successful response to interferon therapy (see Table 1.6) (Davis, G.L. & Lau, J.Y.N. 1997). This knowledge helps to target those patients most likely to gain a sustained response to treatment to maximise the limited resources for HCV treatment in the UK. The most clinically important of these factors are HCV genotype, the level of HCV RNA and the histology of pre-treatment liver biopsy. The role of viral factors are considered in more detail here and the role of histological severity of liver disease is considered in Section 1.7.6.

HCV genotype

Studies comparing response rates according to viral genotype have consistently shown that infection with HCV genotypes 1a and 1b are associated with a lower probability of achieving a sustained virological response to interferon In their detailed review, Davis and Lau analysed 15 studies of interferon monotherapy and reported a sustained response rate of 18% for patients infected with genotype 1 and 54% for patients with non-genotype 1. (Davis, G.L. & Lau, J.Y.N. 1997). This improvement in response rate according to HCV genotype is also observed in studies of combination therapy (McHutchison, J.G., et al. 1999).

Viral load

Despite efforts to standardise assays for the quantification of HCV RNA no gold standard exists and what is considered to be a high or low viral titre is

consequently somewhat arbitrary. The cut-off between low and high viral load is accepted to be 3.5 million copies / millilitre (Poynard, T. et al. 2000). The impact of viral load on sustained virological response rate appears to be less for combination therapy than for interferon monotherapy, particularly if treatment is continued for 48 weeks (McHutchison, J.G., et al. 1999).

In an analysis of the two large studies comparing the combination of interferon and ribavirin with interferon monotherapy the importance of pre-treatment viral factors and choice and duration of therapy can be observed (McHutchison, J.G., et al. 1999). Patients infected with non-genotype one with low viral load who received combination therapy for 48 weeks experienced a sustained virological response rate of 64% compared with patients infected with genotype 1 and a high viral load treated with interferon monotherapy for 24 weeks, where only 1% experienced a sustained virological response.

1.7.4 Side effects of interferon and ribavirin treatment

α - Interferon and ribavirin both have significant side-effects which are important when selecting patients for treatment (see Table 1.7). Almost all patients receiving α - interferon develop some adverse effects. Common problems include influenza-like reactions 6-8 hours after the initial injections, fatigue, malaise, headache and mood changes (Dusheiko, G. 1997). Ribavirin is well tolerated, but causes a dose-dependent haemolytic anaemia, skin rashes and risk of teratogenicity (Glue, P. 1999). In combination with interferon the only adverse effects occurring more frequently than with interferon monotherapy alone are anorexia, dyspnoea, pruritus and rash.

Patients admitted to controlled clinical trials are generally fit and highly selected from the pool of HCV patients. In daily clinical practice adverse reactions to combination therapy may be more frequent, with withdrawals as high as 20% and compliance with therapy less than optimal (Heathcote, E. J. 2000). Contra-indications to combination therapy are listed in Table 1.8.

1.7.5 Management of untreated (naïve) patients with chronic HCV

The indications for treatment of chronic HCV have gradually been clarified over many years. Unfortunately, those patients most responsive to treatment also tend to have the best untreated prognosis. The corollary to this is that the more the patient is in need of treatment, because of risk of progressive liver disease, the less likely one is to obtain a response. In addition, the published randomised clinical trials of therapy have often been very selective in their patient entry criteria, such that it is not always possible to reproduce the reported response rates (Reichard, O., et al. 1999, Poynard, T., et al. 1998, McHutchison J.G., et al. 1998).

As a consequence several organisations have published consensus conference recommendations concerning indications for treatment (Booth, J.C.L., et al. 2001, Dhumeaux, D., et al. 1997, National Institute of Health. 1997, Canadian Association for the Study of the Liver. 2000, European Association for the Study of the Liver. 1999). The recommendations for naive (untreated) patients are outlined in Table 1.9. The consensus is remarkably consistent in recommending that treatment is restricted to patients with elevated serum transaminases for more than six months, active viral replication as indicated by a positive RT PCR test for serum HCV-RNA, and moderate or severe inflammation or fibrosis on liver biopsy. Patients with mild change on liver biopsy should not be treated yet until

there is a better understanding of their natural history and patients with compensated cirrhosis may be treated at the discretion of the clinician.

The selection of patients for treatment is strongly influenced by the efficacy of current therapy. Figure 1.2 illustrates the rapidly improving efficacy of therapy in recent years with the promise of further advances to come. Improvement in treatment responses comes at a cost, but the cost-effectiveness has been demonstrated when compared to other routine medical interventions (Wong, J.B. 1999). Nevertheless the resources available to pay for treatment of hepatitis C within the NHS in the UK are limited and clinicians need to tailor their treatment program to match local budgets. The Greater Glasgow Health Board has just published a hepatitis C strategy review (Greater Glasgow Health Board. 2000). One section of the review is a practical protocol for treatment and monitoring of chronic HCV infection and extracts are shown in Appendix 2 and 3. The inclusion and exclusion criteria match broadly those shown in Tale 1.9 but the upper age limit is increased to 70 years and children are included.

1.7.6 Treatment of specific patient groups

The recommendations for treatment outlined above have to be interpreted pragmatically as in clinical practice many patients do not fit neatly into these categories. For example the long natural history of HCV mitigates against treating older patients for whom the cost-benefit ratio rises rapidly above 60 years (Bennett, W.G. et al. 1997). There are few data on the treatment of children and hence treatment is recommended only within the confines of controlled clinical trials (Schwimmer, J.B. et al. 2000). There is little evidence for the treatment of asymptomatic blood donors with chronic HCV but expectations for treatment may

be high as they have become patients as a consequence of their altruistic action (see Chapter 8). Other categories of patient are considered in more detail:

Acute hepatitis C

Presentation with acute HCV infection is uncommon (see Section 1.6.1) hence data are limited. The evidence is that acute infection responds well to interferon monotherapy (Quin, J.W. 1997) and patients should probably all now be considered for combination therapy although no data on efficacy are yet available.

Normal serum transaminases

HCV is known to be associated with fluctuation in serum ALT levels so this condition needs to be defined. It is suggested that both serum transaminases should be within the normal range on several occasions at least one month apart over a total period of at least 12 months (Di Bisceglie, A.M. 1999). Small numbers of such patients have been treated with interferon monotherapy and have tended to show similar response rates to patients with abnormal ALT and more severe histological changes. In general, this group of patients have a favourable prognosis and they should probably not be treated at present unless they have significant histological liver disease.

Mild disease

Mild chronic hepatitis C is defined by liver biopsy criteria as scoring both < 4 for grade of necro-inflammatory activity and < 3 for stage of fibrosis (Ishak, K., et al. 1995) or Metavir activity score < A2 (Bedossa, P., et al. 1996). There are few data on the natural history and treatment of such patients (see Chapter 8). At present it is recommended that this group should remain under observation with liver

biopsies performed every three to five years such that treatment can be considered if they develop evidence of progression.

Cirrhosis

Patients with compensated cirrhosis are clearly a priority group to consider for treatment but, unfortunately, sustained virological responses to interferon monotherapy and combination therapy with ribavirin are lower than in pre-cirrhotic patients (Davis, G.L., et al. 1997, Bonino, F., et al. 1999). However, useful sustained response rates of 36% can now be obtained in patients with bridging fibrosis or cirrhosis given combination therapy for 12 months (Davis, G.L. 2000) or 30% when treated with pegylated interferon monotherapy for 12 months (Heathcote, E.J., et al. 2000). In addition, therapy may delay progression to liver failure and hepatocellular carcinoma in cirrhotic patients even when viral clearance has not been achieved (Nishiguchi, S., et al. 1995, Fattovich, G., et al. 1997a).

Relapse after interferon monotherapy

Approximately 50% of HCV patients will have a successful end of treatment response to interferon monotherapy with normal serum ALT level and negative HCV-RNA assay. Relapse is defined as the re-appearance of serum HCV-RNA within 6 months of an end of treatment response. These patients are clearly responsive to treatment and are good candidates for additional measures.

Initially, re-treatment with interferon alone at increased dose or for longer duration produced further sustained responses (Buti, M., et al. 1999). However, the real advance came from combination therapy with ribavirin. In a randomised study of

345 patients assigned to re-treatment with interferon alone or combination therapy for 6 months, sustained responses were found in 5% and 49% of patients respectively (Davis, G.L. et al. 1998). Therefore combination therapy is indicated for the re-treatment of patients who have relapsed after a course of interferon monotherapy. Combination therapy has now become the recommended initial therapy for naive patients. The management of patients who relapse after combination therapy remains uncertain.

Interferon monotherapy non-responders

Non-response to interferon monotherapy is defined as being serum HCV-RNA positive at the end of treatment. Usually this is established when patients show no response at 12 weeks into therapy and treatment is stopped. However, it also includes those who show breakthrough by becoming serum HCV-RNA positive again prior to the end of treatment despite having an initial response.

Possible options for these patients include dose escalation, changing the interferon, using combination therapy, phlebotomy to reduce iron stores, long-term therapy or observation (Bacon, B.R. 1999a). None of these measures is of proven benefit and at present this group of patients is probably best managed by continuing observation until more successful therapy is found.

HIV co-infection

The use of highly active anti-retroviral therapy (HAART) has improved the prognosis of HIV infection. As a consequence HCV co-infection is assuming more importance and liver failure becoming an increasing cause for mortality.

Interferon monotherapy and combination therapy can produce similar sustained responses for HCV infection in HIV positive and negative patients (Soriano, V., et al. 1999, Landau, A., et al. 2000). However, caution is required as there is a theoretical interaction with ribavirin leading to reduced phosphorylation of zidovudine and more data are required. In addition 10-15% of HIV positive subjects experience a transient decrease in the CD4 count between the 6th to 14th week of treatment and in a few cases this decline is irreversible.

HBV co-infection

Dual infection with HCV and HBV is frequent because of shared routes of transmission. Co-infection with HBV leads to more severe liver disease and a higher prevalence of cirrhosis and appear to have a synergistic effect leading to a higher risk of hepatocellular carcinoma. Therefore dual chronic infection is a strong indication to consider therapy however there are few data to guide us in the most appropriate regime and duration of treatment.

Haemophilia

HCV infection is almost universal in the haemophiliac population who were treated with clotting factor concentrates before the availability of heat-treatment in the mid-1980s (Fried, M.W. 1999) Co-infection with HIV is common. Studies of interferon monotherapy for 12 months in HIV negative patients reveal a sustained response in 13% of patients (Rumi, M.G. et al. 1997). Results of combination therapy studies are awaited. For HIV-negative patients with end-stage liver disease, liver transplantation is effective and also cures the underlying coagulation disorder.

Renal failure

HCV infection is common in patients on renal dialysis and several small studies have demonstrated the efficacy of interferon monotherapy for these patients (Carithers, R.L. 1999). It is possible that reduced renal clearance of interferon leads to improved efficacy but also a high incidence of side-effects. Interferon is best avoided in renal transplant recipients, as there are several reports of allograft rejection. Ribavirin should be avoided in patients with renal failure as reduced renal excretion may produce uncontrollable haemolysis (Glue, P. 1999).

1.7.7 Developments in the treatment of chronic hepatitis C

The high prevalence of this chronic infection with serious long-term consequences has stimulated the pharmaceutical industry to make substantial investments in research into anti-viral therapy. Significant progress is being made with the prospect that amantadine, rimantadine, and protease and helicase inhibitors may become part of the therapeutic regime in the future (Clarke, B.E. 2000). However much the treatment of HCV infection improves, the major long-term priorities are to develop an effective vaccine and improve public health measures to prevent initial infection.

1.7.8 Liver transplantation for chronic HCV

Liver transplantation is the only available treatment option for patients with decompensated HCV-related cirrhosis and is also indicated for some patients with early stages of hepatocellular carcinoma. Reinfection of the graft with HCV is nearly inevitable, and the majority of patients will have histological evidence of hepatitis and even cirrhosis (Feray, C., et al. 1999). Despite these drawbacks the five year survival (72%) after liver transplantation is comparable to those with

other common indications for liver transplantation (Feray, C., et al. 1999). The main concern relates to the long-term survival post-transplantation. For that reason many therapeutic modalities have been tried in an attempt to prevent HCV recurrence or reduce the severity of liver disease once recurrent disease has developed. Studies of interferon monotherapy have reported at best a delay in the development of recurrent HCV without evidence of increased graft rejection. In initial studies the combination of ribavirin and interferon has proved to be safe with promising reports of sustained clearance of HCV RNA from serum (Bizollon, T., et al. 1997). However further long-term controlled studies are required to determine which agents are most effective and least harmful and for how long therapy is required.

Table 1.1 Histological scoring systems for the grading and staging of HCV employed in this thesis.

	Qualitative Assessment	Quantitative Assessment
(a) Scheuer, P.J. (1991)		
Necroinflammatory activity		
Portal / periportal activity	None – Severe piecemeal necrosis	**0 - 4**
Lobular activity	None – Damage with bridging necrosis	
Fibrosis score	None – Probable or definite cirrhosis	**0 - 4**
(b) Ishak, K., et al. (1995) – Modified histological activity index		
A. Periportal interface hepatitis	Absent - Severe	0 - 4
B. Confluent necrosis	Absent – Panacinar necrosis	0 - 6
C. Focal (spotty) lytic necrosis	Absent – More than ten foci per 10x field	0 - 4
D. Portal inflammation	None – Marked, all portal areas	0 - 4
Necroinflammatory scores	**A + B + C +D**	**0 - 18**
Fibrosis scores	No fibrosis - Cirrhosis	**0 - 6**

Table 1.2 Long-term outcome of HCV infection from retrospective studies. Persons with established liver disease referred to tertiary referral centres for investigation.

Author, Year	Country	No. of patients	Interval from Exposure (mean or range, yrs) *	Cirrhosis (%)	Hepatocellular Carcinoma (%)	Liver Death (%)
Kiyosawa, 1990	Japan	231	10 – 29	35.1	23.4	Not reported
Tong, 1995	USA	131	14 – 28	51	10.6	15.3
Yano, 1996	Japan	70	Not reported	50	Not reported	Not reported
Niederau, 1998	Germany	838	9 – 22	16.8	2.0	3.7
Gordon, 1993	USA	215 ‡	19	55.0	3.7	Not reported
Gordon, 1993	USA	195 §	20	21.0	1.0	Not reported

* Based on interval from transfusion or initial use of intravenous drugs.

‡ Exposure through transfusion

§ Exposure through intravenous drug use

Table 1.3 Long-term outcome of HCV infection from prospective studies. Persons studied from the time of acute infection.

Author, Year	Country	No. of patients	Interval from Exposure (mean or range, yrs) *	Cirrhosis (%)	Hepatocellular Carcinoma (%)	Liver Death (%)
Di Bisceglie, 1991	USA	65	9.7	12.3	0	3.7
Koretz, 1993	USA	80	16.0	7.0	1.3	1.3
Mattson, 1993	Sweden	61	13.0	8.0	Not reported	1.6
Tremolada, 1992	Italy	135	7.6	15.6	0.7	3.7

* Based on interval from transfusion or initial use of intravenous drugs.

Table 1.4 Long-term outcome of HCV infection from combined retrospective-prospective studies. Studies of persons diagnosed with acute hepatitis in prior prospective transfusion studies called back for renewed prospective follow-up with non-hepatitis controls.

Author, Year	Country	No. of patients	Interval from Exposure (mean or range, yrs) *	Cirrhosis (%)	Hepatocellular Carcinoma (%)	Liver Death (%)
Seeff, 1998b	USA	103	20	15	1.9	2.7
Seeff, 2000	USA	17	45 – 50	5.9	0	5.9
Kenny-Walsh, 1999	Ireland	376	17	2.0	0	0
Wiese, 2000	Germany	1018	20	0.4	0	0.2
Vogt, 1999†	Germany	458	17	0.3	0	0

* Based on interval from transfusion or initial use of intravenous drugs.

† Paediatric population

Table 1.5 Factors associated with progression of hepatic fibrosis in chronic hepatitis C infection.

	Associated	Possibly Associated	Not Associated
Viral		Size of inoculum	Viral Load
		Genotype	Quasispecies complexity
Host	Age at infection	Genetic susceptibility	
	Duration of infection	Haemochromatosis heterozygotes	
	Male gender	Steatosis	
	Immune deficiency	Necroinflammatory activity on biopsy	
	Co-infection with HIV		
	Comorbid conditions e.g. HBV*		
	Initial fibrosis score on biopsy		
Environmental	Alcohol consumption		

*HBV Chronic Hepatitis B virus co-infection

Table 1.6 Factors reported to be predictive of a biochemical response to interferon monotherapy.

Drug	Dose > 3 million units
Demographics	Female sex
	Younger age
	History of injecting drug use
	Unknown source of infection
	Short duration of infection
Histology	Mild chronic hepatitis
	Absence of fibrosis / cirrhosis
Biochemical	Low serum AST
	Low gamma glutamyl transpeptidase
	Low serum iron or ferritin
Virological	Low HCV RNA level
	Genotype 2 or 3
	Low number of quasispecies
	Multiple amino acid differences from the resistant type interferon sensitivity determining region of NS5A.
Interferon response	Early normalization of serum ALT
	Early loss of serum HCV RNA

Table 1.7 Side-effects of α - interferon and ribavirin therapy for chronic hepatitis C.

Frequency of side effect	α - Interferon	Ribavirin
>30% (very common)	Influenza-like	Haemolysis
	Headache	Nausea
	Myalgia	
	Fatigue	
	Fever	
	Rigors	
	Myalgia	
	Thrombocytopenia	
	Induction of antibodies	
1 – 30% (common)	Anorexia	Anaemia
	Reaction at injection site	Nasal congestion
	Insomnia	Pruritus
	Partial alopecia	
	Lack of motivation	
	Inability to concentrate	
	Irritability	
	Emotional lability	
	Depression	
	Diarrhoea	
	Induction of autoimmune disease	
	Leukocytopenia	
	Taste perversion	
<1% (rare)	Polyneuropathy	Gout
	Paranoia / Suicidal ideation	
	Diabetes mellitus	
	Retinopathy	
	Optic neuritis	
	Hearing impairment	
	Seizures	
	Loss of libido	
	Cardiotoxicity	

Table 1.8 Contra-indications to α - interferon and ribavirin therapy for chronic hepatitis C.

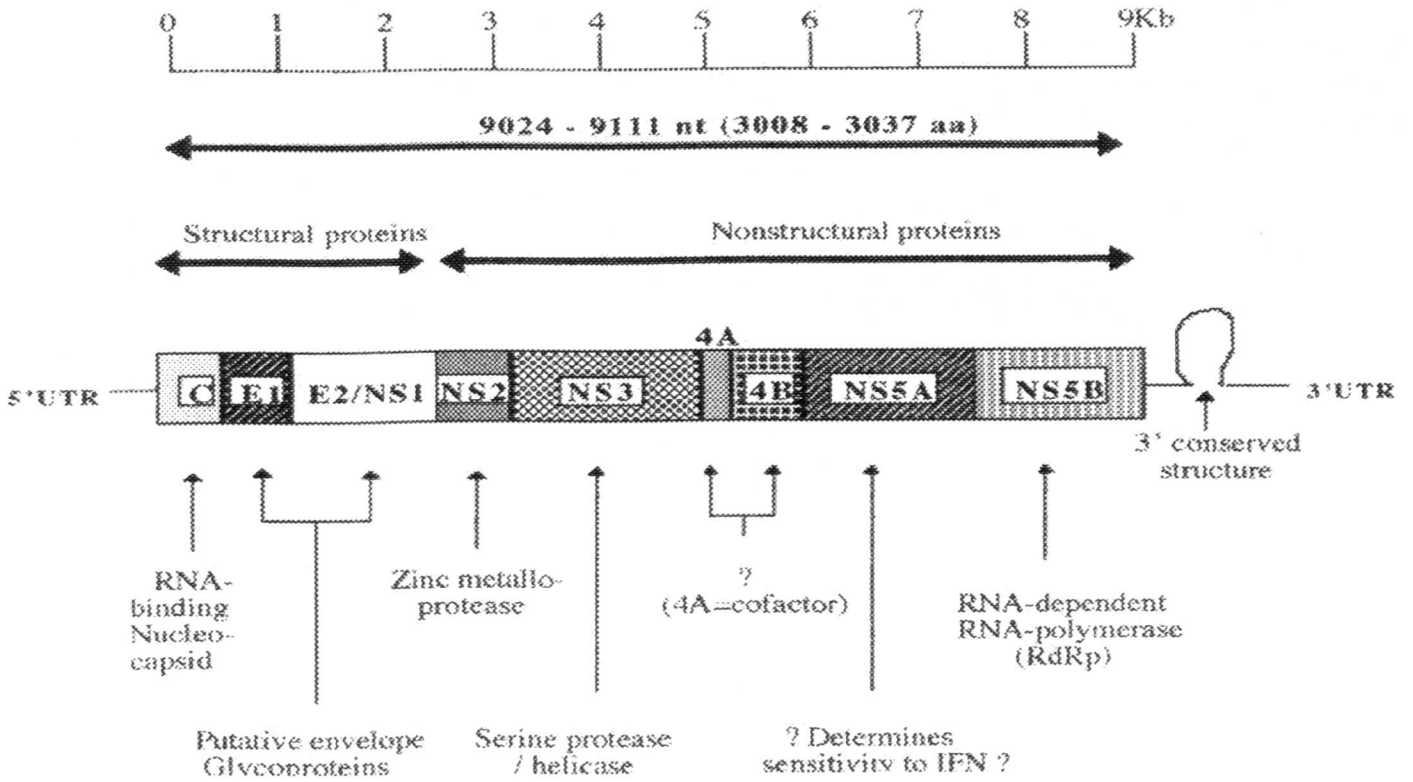
Absolute	Relative
Pregnancy	Severe depression
Inadequate contraception for both sexes	Decompensated cirrhosis
Renal failure	Auto-immune hepatitis
Continued intravenous drug use	Hyperthyroidism
Continued alcohol excess	Cardiac disease
	Renal transplant
	Seizures
	Psoriasis
	Retinopathy - diabetes or hypertension
	Hypersplenism
	Thrombocytopenia
	Leucopenia
	Anaemia

Table 1.9 National and international groups recommended indications for treatment of chronic hepatitis C

	United Kingdom	USA	French Consensus	Canada	Europe
Author	Booth, J.C.L. et al.	National Institute of Health	Dhumeaux, D., et al.	Canadian Association for the Study of the Liver	European Association for the Study of the Liver
Year	2001	1997	1999	2000	1999
Age	No recommendation	18-60 years	< 65 years	No restriction	No restriction
Transaminases	No recommendation	Elevated	Elevated	Elevated ≥ 1.5 upper limit of normal	Elevated
HCV PCR	Positive	Positive	Positive	Positive	Positive
Liver biopsy	Moderate/severe changes	Moderate/severe changes	Metavir score ≥ A2*	Moderate/severe changes	Moderate/severe changes
Cirrhosis	All offered	Uncertain	Only in clinical trials	No mention	Optional
Genotype	No restriction	No restriction	No restriction	No restriction	No restriction
Tailoring of therapy	Genotypes 2, 3 + 1 with low viral load 24 weeks therapy			Genotype 1 – 48 weeks therapy Genotypes 2,3 – 24 weeks therapy	Genotype 1 + high viral load – 48 weeks therapy

* Hepatology 1996; 24: 289-293.

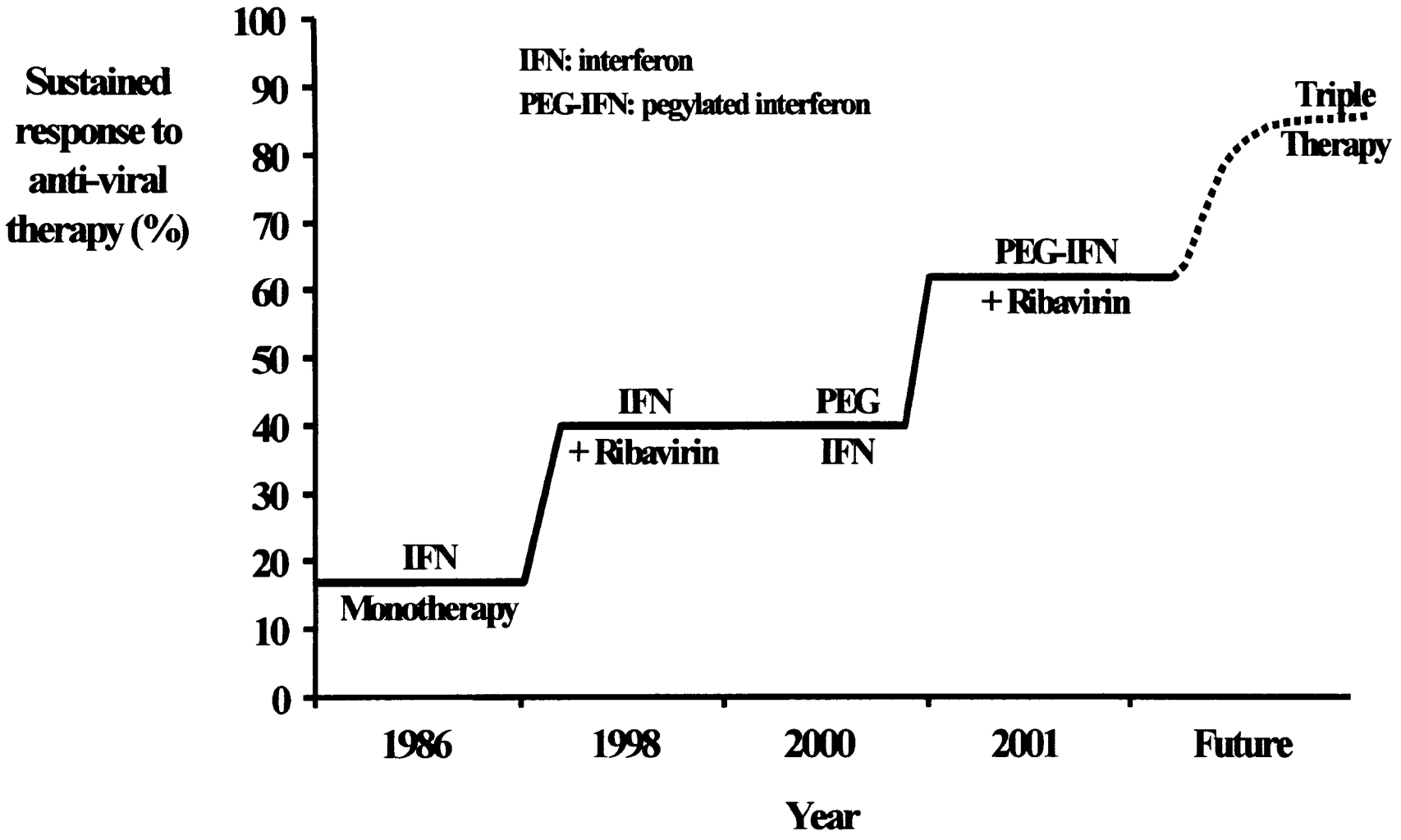
Figure 1.1 :- Schematic representation of the HCV genome. The putative function of each gene is indicated.



Legend

UTR	Untranslated region	E	Envelope
C	Core	NS	Non-structural region

Figure 1.2 Improvement in the efficacy of anti-viral therapy for chronic hepatitis C over time in treatment naïve patients.



CHAPTER 2

BACKGROUND TO THIS THESIS

As outlined in Chapter 1 there has been much progress in our understanding of hepatitis C virus (HCV) over the twelve years that it has been recognised. There remain many areas in which knowledge is lacking. The virus is blood borne and transmitted in most cases by parenteral exposure although up to one third of those infected have no clear risk factor. Many individuals infected with the hepatitis C virus are asymptomatic or have only mild symptoms and as a result the population prevalence and natural history of the infection is poorly documented. Why some patients develop progressive liver disease and others do not is poorly understood. Currently α - interferon in combination with ribavirin is the treatment of choice and a sustained virological response to treatment is seen in approximately fifty percent of patients. With limited resources for therapy in the UK, treatment should be targeted to those who will experience the greatest benefit. The factors that predict a successful response to treatment remain poorly understood.

The work contained in this thesis addresses a number of these questions. Intravenous drug use is the most important mechanism for transmission of HCV in western countries, yet up to one third of patients have sporadic HCV infection with no identifiable route of infection. Whether the transmission of HCV from healthcare workers to patients accounts for many of these sporadic cases is unknown. We performed an anonymous retrospective cohort study of hepatitis C infection in over 10,000 healthcare workers in the West of Scotland which is reported in Chapter 3.

Prevalence of hepatitis C antibodies was reported according to age and occupational category such that the risk of transmission to those performing invasive procedures and the risks posed to patients from those infected could be estimated.

Liver biopsy is the gold standard for assessing the severity of HCV-related liver injury. The prolonged natural history of chronic HCV dictates that follow-up over decades is required with serial liver biopsies necessary to monitor the condition. However liver biopsy is associated with significant morbidity and mortality and consequently non-invasive means of assessing the severity of liver injury would be advantageous. In Chapter 4 we studied the hepatocellular enzyme α - glutathione s-transferase in the grading of HCV-related chronic hepatitis and assessing the response to α-interferon treatment.

The natural history of chronic HCV infection is controversial with variable rates of progression observed. The mechanisms which account for these variations are poorly defined. Hence it is not possible to predict in advance which patients with chronic HCV will develop progressive liver disease with associated morbidity and mortality. As a result it is not possible to target those patients with the worst prognosis before they develop advanced liver disease, when they are most likely to respond to therapy. In Chapters 5, 6 and 7 we studied a number of factors which might influence the rate of progression in chronic HCV. In Chapter 5 the role of iron and haemochromatosis gene mutations on the progression of chronic HCV was assessed. Patients with chronic HCV are recognised to have elevated serum iron studies and liver iron concentrations. This has been reported to be associated with the progression of liver fibrosis. In

addition carriage of mutations in the HFE gene has been reported to be associated with more advanced hepatic fibrosis in chronic HCV. In Chapter 5 we studied the association between serum iron studies, liver iron concentration and carriage of mutations in the HFE gene and the development of hepatic fibrosis in chronic HCV. In Chapter 6 we studied the role of genetic polymorphisms in the renin-angiotensin system on the development of hepatic fibrosis in chronic HCV. These functional polymorphisms are recognised to influence the progression of cardiac and renal fibrosis in a number of cardiovascular diseases and a role in the development of hepatic fibrosis is untested. In Chapter 7 the influence of co-infection with the hepatitis G virus on the severity of HCV related liver injury is studied.

As HCV infected patients with advanced liver fibrosis have the lowest sustained virological response rates to treatment, treating patients with mild liver disease before they develop significant liver fibrosis might be expected to result in improved response rates to treatment. There is little evidence to support this hypothesis. In Chapter 8 we assessed the tolerability and efficacy of treatment with α - interferon alone in a cohort of asymptomatic patients with chronic HCV detected at blood donor screening and with mild liver disease on liver biopsy.

Work for this thesis was largely performed between April 1996 and August 1998 when I was employed as the Kinnear-Brown Lecturer in Medicine and Gastroenterology in the Department of Medicine at the Western Infirmary in Glasgow. Dr Peter R Mills, Consultant Physician and Gastroenterologist supervised the work and the patients with hepatitis C infection studied attended the viral hepatitis clinics at

Gartnavel General Hospital. I performed the hepatitis G virus PCR and HCV genotyping under the supervision of Dr. Liz McCruden at the Institute of Virology, University of Glasgow. Ethics approval for each study was obtained from the Research Ethics Committee at the Western Infirmary. Ethics approvals for the multicentre epidemiological study of healthcare workers was sought and obtained from each of the NHS Trusts in the West of Scotland included in the study.

CHAPTER 3

EPIDEMIOLOGY OF HEPATITIS C VIRUS INFECTION: A STUDY OF THE PREVALENCE IN HEALTHCARE WORKERS IN THE WEST OF SCOTLAND.

3.1 CONTENTS

3.2 SUMMARY

Whether healthcare workers have an increased prevalence of hepatitis C virus infection as a result of exposure to patient's blood and body fluids is controversial. This study assesses the prevalence of hepatitis C virus infection in healthcare workers, relates it to the performance of exposure-prone procedures and duration of occupational exposure, allowing an estimate to be made of the incidence of occupationally acquired hepatitis C infection among medical staff. This anonymous retrospective cohort study estimates prevalence of hepatitis C infection in 10,654 healthcare workers. ELISA-3 testing was performed on pools of five sera collected during immunisation against hepatitis B. Healthcare workers were arranged in five occupational groups, according to degree of patient exposure, and three age bands (<30 years, 30-39 years, >40 years). Prevalence of antibodies to hepatitis C is 0.28% (30 / 10,654), comparable in all occupational groups ($p = 0.34$) and unrelated to duration of potential exposure. Assuming that all detected infections had been occupationally acquired, the maximum estimated risk of hepatitis C infection in exposure-prone medical staff is low: 1.4% for surgeons and 1.0% for physicians over a thirty-five year professional career.

Hepatitis C infection is infrequent in the healthcare workers in Glasgow. Those conducting exposure-prone procedures do not seem to be at higher risk than other healthcare staff.

3.3 INTRODUCTION

The hepatitis C virus (HCV) was first identified in 1989 but has been prevalent for many decades. In western countries, HCV is most commonly transmitted among injecting drug users who share injecting equipment. Once infected most patients develop chronic HCV infection, a disease with associated morbidity and mortality (Niederau, C., et al. 1998). The incidence of HCV seroconversion after accidental needlestick exposure is uncertain with reports ranging from 0 to 10% (Kiyosawa, K., et al. 1991, Mitsui, T., et al. 1992, Lanphear, B.P., et al. 1994, Puro, V., et al. 1995). Whether healthcare workers have a higher prevalence of hepatitis C virus infection than the general population through percutaneous occupational exposure is unclear (Zuckerman, J., et al. 1994, Neal, K.R., et al. 1997, Thomas, D.L., et al. 1993, Cooper, B.W., et al. 1992, Panlilio, A.L., et al. 1995, Shapiro, C.N., et al. 1996, Thomas, D.L., et al. 1996). The aim of this study was to assess the prevalence of HCV antibodies in healthcare workers in Glasgow, a city where injecting drug use is common, and to analyse rates in relation to specific occupational groupings and duration of potential exposure to HCV. The study particularly focussed on staff performing exposure-prone procedures, where injury to the worker may result in the exposure of the patient's open tissues to the blood of the worker. These rates could then be compared with existing prevalence data in other local populations to determine if certain health care workers might be at increased risk of occupationally acquired HCV.

3.4 PATIENTS, MATERIALS AND METHODS

3.4.1 Setting

This study was performed in the Greater Glasgow Health Board area, which incorporates the largest city in Scotland and had a population of 909,000 in 1996. It is estimated that around 80% of Glasgow's large injecting drug user population have been infected with HCV (Frischer, M., et al. 1993, Goldberg, D., et al. 1998). Glasgow is served by eight NHS Hospital Trusts, providing a full range of clinical services, a single diagnostic virology laboratory service (Regional Virus Laboratory) and a coordinated occupational health service integrated into each NHS Trust.

3.4.2 Study Population

All healthcare workers in Glasgow, who presented for hepatitis B virus (HBV) immunisation between October 1994 and October 1997 and had serum samples stored for anti-HBs assay were identified. Employees from one NHS Trust, which denied ethics approval, were excluded.

Of the 24,077 serum samples received by the Regional Virus Laboratory from healthcare staff there were 11,577 individuals that had occupational health casenotes available after duplicate samples had been removed. Samples from 10,654 (92%) of these healthcare workers were located in the virology serum archive. According to 1997 Scottish Health Statistics payroll data, this sample represents 44% of the 24,150 healthcare workers employed in the seven Glasgow NHS Hospital Trusts studied (personal communication). Included are: 1430 of 1972 (73%) hospital medical staff,

203 of 547 (37%) dentists, 5,913 of 11,803 (50%) nursing and midwifery staff, and 3108 of 9828 (32%) others.

3.4.3.Study Procedure

This study was necessarily performed in an anonymous manner. Serum samples were initially linked to the corresponding employee's immunisation records at the occupational health clinics using a database consisting of employee's name, gender, date of birth, location of employment and occupational group. When data collection was complete, the age of each employee at the time of sample collection was derived. Individuals were then grouped into one of five occupational categories by their degree of patient exposure and three age bands (Table 3.1) to ensure that any combination of the two contained more than 50 individuals, thus protecting any infected individual against identification. All employee identifiers were then deleted from the database leaving only non-identifying details of occupational category and age band, linked to the stored serum laboratory number.

A unique non-identifying code was generated for each database entry and printed on labels adjacent to the corresponding laboratory serum number. The appropriate sera were then labelled with their unique code and transferred to a separate laboratory for testing. By then deleting the laboratory serum numbers from the database, the HCV antibody results could only be linked with the anonymised data (occupational category and age band) via the unique non-identifying code.

3.4.4 Samples for testing

Sera were initially stored by sequential laboratory number at either 4°C or –20°C on the day of collection in the virology department. The study sera were then identified, aliquoted into 2.5ml Sarstedt vials, labelled with the unique non-identifying code and stored at –20°C for testing.

3.4.5 Hepatitis C Tests

Sera were screened for antibodies to the hepatitis C virus by ELISA-3 (Ortho Diagnostics, Raritan, NJ, USA). Reactive samples were only considered positive if they were confirmed by RIBA-3 testing (Ortho Diagnostics, Raritan, NJ, USA). All samples confirmed positive were further tested for the presence of HCV RNA by reverse transcriptase polymerase chain reaction (RT PCR) using an in-house method (Dow, B.C., et al. 1993). RT PCR positive samples were genotyped by restriction fragment length polymorphism (McOmish, F., et al. 1993).

In view of the large number of sera to be tested a pooling protocol was developed. Pools of five sera were tested by third generation ELISA. Twenty microlitres of each of the five sera to be tested were pooled and mixed thoroughly. Fifty microlitres of this pool was transferred into the test plate (the equivalent of 10 µl of each serum), diluent was added to a total volume of 200 µl and thereafter testing followed the Ortho ELISA-3 protocol. The final dilution of each individual serum in the pool was 1 in 20 compared with individual testing with a 1 in 11 dilution. The five sera in any reactive pool were then tested individually by ELISA-3 to identify the reactive samples requiring confirmatory testing by RIBA-3.

3.4.6 Statistics

The data were analysed as a prevalence of antibodies to HCV in each age band and occupational group. The prevalence in each of these categories was compared using the Chi-square test or Fisher's exact test.

By assuming that 24 years is the median age at which doctors qualify in the UK the probability of acquiring HCV per 100 person-years of occupational exposure for the medical staff was estimated to be:

$$\text{Probability of HCV infection / 100 person-years exposure} = \frac{\text{No. positive for HCV antibodies} \times 100}{\text{No. tested} \times (\text{Median age in occupational group} - 24)}$$

This estimate is a maximum because it is possible that infections among HCV antibody positive individuals were acquired before healthcare work was commenced or through a non-occupational route. The median age for each occupational group was available from the dates of birth on the original database.

3.4.7 Ethics

Ethics approval for this study was obtained from 7 of the 8 NHS Trust research ethics committees in Glasgow. Employees of Yorkhill NHS University Trust, which did not grant approval, were excluded.

3.5 RESULTS

3.5.1 Evaluation of Pooling

The pooling method was evaluated on the first 2190 serum samples from employees in exposure-prone occupations. They were initially tested individually and then arranged into 438 pools and tested by a single operator blinded to the location of the known positive samples (Table 3.2). Nine of these pools contained a single serum sample known to be reactive when tested individually by ELISA-3. On pooled testing, all five sera confirmed positive by RIBA-3 individual testing and one serum considered indeterminate by RIBA-3 testing (C33 2+ band only) were detected. Two pools were reactive when individual ELISA-3 and RIBA-3 testing confirmed all ten sera to be unreactive (false positive pools). Three pools containing sera known to be reactive by individual ELISA-3 testing were unreactive. Two of these pools contained single samples which were RIBA-3 negative and considered false positives for antibodies to HCV and one was of indeterminate RIBA-3 reactivity (C33 2+ band only) and hence of doubtful significance. With confirmatory testing by RIBA-3, our method of pooling therefore had a sensitivity of 100% (5 / 5) and a specificity of 100% (433 / 433) for samples that were confirmed positive for antibodies to HCV.

3.5.2 HCV Prevalence

Thirty-eight (1.8%) of the 2131 pools tested were reactive by ELISA-3 and the individual sera were analysed further (Figure 3.1). The overall prevalence of HCV antibodies was 0.28% (30 / 10,654, 95% C.I. 0.19 – 0.41%). The occupational category and age band of the employees tested are summarised in Table 3.1. There

was no statistically significant difference in the prevalence of HCV antibodies between the five occupational categories ($p = 0.34$) nor between exposure-prone staff and all non-exposure-prone staff (0.23% v 0.30%, $p = 0.59$). The prevalence of antibodies to HCV was significantly higher in the 30 to 39 years age band ($p = 0.012$).

Twelve (40%) of the thirty sera confirmed to contain antibodies to HCV had viral RNA detectable in serum by RT PCR. These were typed as follows: 6 type 1, 4 type 3, 1 type 5 and 1 could not be typed.

3.5.3 Probability of HCV infection

Within the exposure-prone category the median age and age ranges for each occupational group were: surgeons 30 years (22 – 62 years), physicians 34 years (20 – 63 years), dental staff 29 years (16 – 72 years) and nurses 34 years (17 – 64 years).

The maximum estimated probability of occupationally acquired HCV infection per 100 person years in the exposure-prone group was 0.04 for surgeons and 0.03 for physicians, which would equate to a 1.4% and 1% risk respectively over a professional career of thirty-five years.

3.6 DISCUSSION

Hepatitis C is a blood borne virus transmitted by the parenteral route. Infection frequently results in a chronic asymptomatic carrier state for many years before the development of symptomatic liver disease. HCV infected healthcare workers may therefore be unaware of their condition and their potential to infect patients. Healthcare workers, who perform exposure-prone procedures, where injury to the worker may result in the exposure of the patient's open tissues to the blood of the worker, are theoretically at increased risk of infection with blood borne viruses. According to UK health departments' guidelines, these occupations include surgeons, interventional physicians, intensive care unit and accident and emergency staff. If occupational transmission of HCV was common, staff performing exposure-prone procedures might be expected to have a higher prevalence of hepatitis C antibodies compared with staff in less exposed occupations. Our results reveal the overall prevalence of hepatitis C antibodies in healthcare workers in Glasgow to be low (0.28%) and comparable to those reported in two smaller studies from England (Zuckerman, J., et al. 1994, Neal, K.R., et al. 1997). The data indicate that the performance of exposure-prone procedures does not frequently result in the acquisition of HCV by healthcare workers (exposure-prone 0.23% v non-exposure-prone 0.30%, $p = 0.59$). The prevalence of HCV antibodies among surgeons [only 1 (0.23%) of 438 tested], the group perceived to be at greatest risk of occupational exposure, was comparable with that observed among healthcare workers who have little or no patient contact, including student nurses. However the similar prevalence may not reflect similar routes of infection and we were unable to explore the presence

of other risk factors for HCV transmission with this study design. Needlestick injuries to surgeons in operating theatres are common. In a study from one of the hospitals included in this study Glasgow the incidence of percutaneous exposure was 15.5 per operator per 1000 operations (Camilleri, A.E., et al. 1991). Even this is likely to underrepresent the rate of needlestick injury as it is recognised that surgeons report only one in 20 of the needlestick injuries that they receive (Nash, G.F. & Goon, P. 2000). Even if we assume that all the infections occurring in exposure-prone staff were occupationally acquired, the estimated maximum probability of occupationally acquired HCV infection in surgeons is 1.4% (1 in 70) over a surgical career of thirty-five years (0.04 per 100 person years). This figure agrees with a model-based estimate of professional lifetime risk of HCV infection for a French surgeon of between 0.3% and 3.1% (Yazdanpanah, Y., et al. 1999).

Studies have reported a higher prevalence of antibodies to HCV in healthcare workers compared with blood donors, however they are a non-representative self-selected group and exclude persons with high risk behaviour (Zuckerman, J., et al. 1994, Neal, K.R., et al. 1997). The population prevalence of antibodies to HCV in Glasgow is unknown. From 1991 to 1997 there were 2161 individuals testing positive for HCV in the Greater Glasgow Health Board catchment area (prevalence 0.24% [2161 / 905,100]) (SCIEH Weekly Report. 1999). This is likely to be an underestimate of the true population prevalence, yet is comparable with the prevalence recorded in healthcare workers in this study (0.28% v 0.24%). A mixed population of hospital attenders (male outpatients or in-patients, aged 16 to 49 years) at two of the general hospitals in our study were screened for HCV antibodies from July to December 1997

using the same pooling protocol (manuscript in preparation). The prevalence of antibodies to HCV in this population was 4.0% (107 / 2702 individuals tested) significantly higher than the healthcare worker population prevalence (0.28% v 4.0%, $p < 0.0001$). Thus healthcare workers, including those performing exposure-prone procedures, have an estimated prevalence of antibodies to HCV which does not exceed that already known in the local population and is less than that in a cohort of their male hospital patients.

Pooling of sera allowed us to increase the number of third generation ELISA tests performed without loosing sensitivity or generating an unacceptable number of false positives requiring further testing. This is in keeping with earlier reports employing similar pooling methods with second generation ELISA kits for epidemiological studies in low prevalence populations (Garcia, Z., et al. 1996, Liu, P., et al. 1997). Of the 30 healthcare workers with antibodies to HCV, only 12 were found to be viraemic. This may reflect the sub-optimal storage condition of the sera for RNA analysis. Alternatively it may reflect the high rate of clearance of HCV RNA from the serum of healthy individuals exposed to HCV many years previously (Grellier, L., et al. 1997). Most viraemic patients were infected with genotype 1 (6, 50%) or genotype 3 (4, 33%), similar to our local clinic population where genotypes 1 and 3 occur with equal frequency. One individual was infected with genotype 5 suggesting acquisition of infection abroad.

The risk posed to patients by an HCV infected healthcare worker is not known and there are no guidelines to assist in their management. There have been two published

reports of HCV transmission to patients from infected anaesthetists as a result of failure to observe infection control policies (Ross, R.S., et al. 2000) and negligent medical practices (Bosch, X. 1998). Whether HCV infected healthcare workers performing exposure-prone procedures should have their practice restricted is controversial. There have been two published reports of HCV transmission from surgeons to patients on whom they operated, despite universal precautions having been observed. Esteban et al. from Spain first reported a transmission rate of 2.2% (5 of 222 operations) from an HCV-infected cardiac surgeon (Esteban, J.I., et al. 1996). In a report from the UK, the reported HCV transmission rate was 0.36% (1 of 278 operations) from an HCV infected cardiac surgeon (Duckworth, G.J., et al. 1999). In the absence of guidelines regarding HCV infected healthcare workers, patients and healthcare managers should be reassured by the low reported prevalence of HCV infected healthcare workers in the health service, provided that appropriate infection control policies are followed.

Healthcare personnel and patients can both be reassured that the reservoir of healthcare personnel in the West of Scotland who are infected with HCV is extremely small (2.8 per 1000). It appears that working in the healthcare environment and performing exposure-prone procedures, in an area with a large HCV infected injecting drug using population, only rarely leads to the acquisition of HCV by healthcare workers. The risk that a patient may become infected by contact with the health service is probably low. However these findings should not lead to any complacency in the implementation of universal precautions and the reporting and follow-up of needlestick injuries.

Table 3.1 Occupational categories and age bands of healthcare workers tested for antibodies to hepatitis C.

Occupational Category	No. confirmed positive / No. tested (Prevalence %)			
	<30 years	30-39 years	>39 years	Total
Staff conducting exposure-prone procedures				
Surgeons	0 / 196	0 / 154	1 / 88	**1/438 (0.23%)**
Physicians	0 / 84	0 / 156	1 / 69	**1 / 309 (0.32%)**
Dental Staff	0 / 296	0 / 160	1 / 120	**1 / 576 (0.17%)**
Nurses *	0 / 262	2 / 366	0 / 254	**2 / 882 (0.23%)**
Subtotal	**0 / 838**	**2 / 836 (0.24%)**	**3 / 531 (0.56%)**	**5 / 2205 (0.23%)**

* Includes midwifery staff, theatre, intensive care unit, accident and emergency and renal unit nursing staff.

Table 3.1 (Continued) Occupational categories and age bands of healthcare workers tested for antibodies to hepatitis C.

Occupational Category	No. confirmed positive / No. tested (Prevalence %)			
	<30 years	30-39 years	>39 years	Total
Staff in contact with patients and body fluids				
Medical staff	0 / 323	0 / 232	0 / 129	**0 / 684**
Nurses	3 / 1072	3 / 1086	0 / 1176	**6 / 3334 (0.18%)**
Psychiatric Nurses	2 / 184	1 / 279	1 / 217	**4 / 680 (0.59%)**
Student Nurses	1 / 530	1 / 78	0 / 35	**2 / 643 (0.31%)**
Paramedical Staff †	0 / 273	2 / 139	2 / 171	**4 / 583 (0.69%)**
Portering Staff	0 / 78	1 / 78	0 / 127	**1 / 283 (0.35%)**
Subtotal	**6 / 2460 (0.24%)**	**8 / 1892 (0.42%)**	**3 / 1855 (0.16%)**	**17 / 6207 (0.27%)**

† Includes physiotherapists, chiropodists, radiographers, cardiology staff, medical illustration, speech and occupational therapists.

Table 3.1 (Continued): Occupational categories and age bands of healthcare workers tested for antibodies to hepatitis C.

Occupational Category	No. confirmed positive / No. tested (Prevalence %)			
	<30 years	30-39 years	>39 years	Total
Laboratory Medical and Technical Staff				
	0 / 156	3 / 139	0 / 238	**3 / 533 (0.56%)**
Staff with patient contact only ‡				
	1 / 299	1 / 347	0 / 592	**2 / 1238 (0.16%)**
Staff with no patient contact §				
	0 / 116	3 / 136	0 / 219	**3 / 471 (0.64%)**
Total All Categories	**7 / 3869 (0.18%)**	**17 / 3350 (0.51%)**	**6 / 3435 (0.17%)**	**30 / 10654 (0.28%)**

‡ Includes staff in audiology, dietetics, optics, pharmacy, physics, psychology, social work and ward clerical staff.

§ Includes administrative, catering, clerical, security and catering staff.

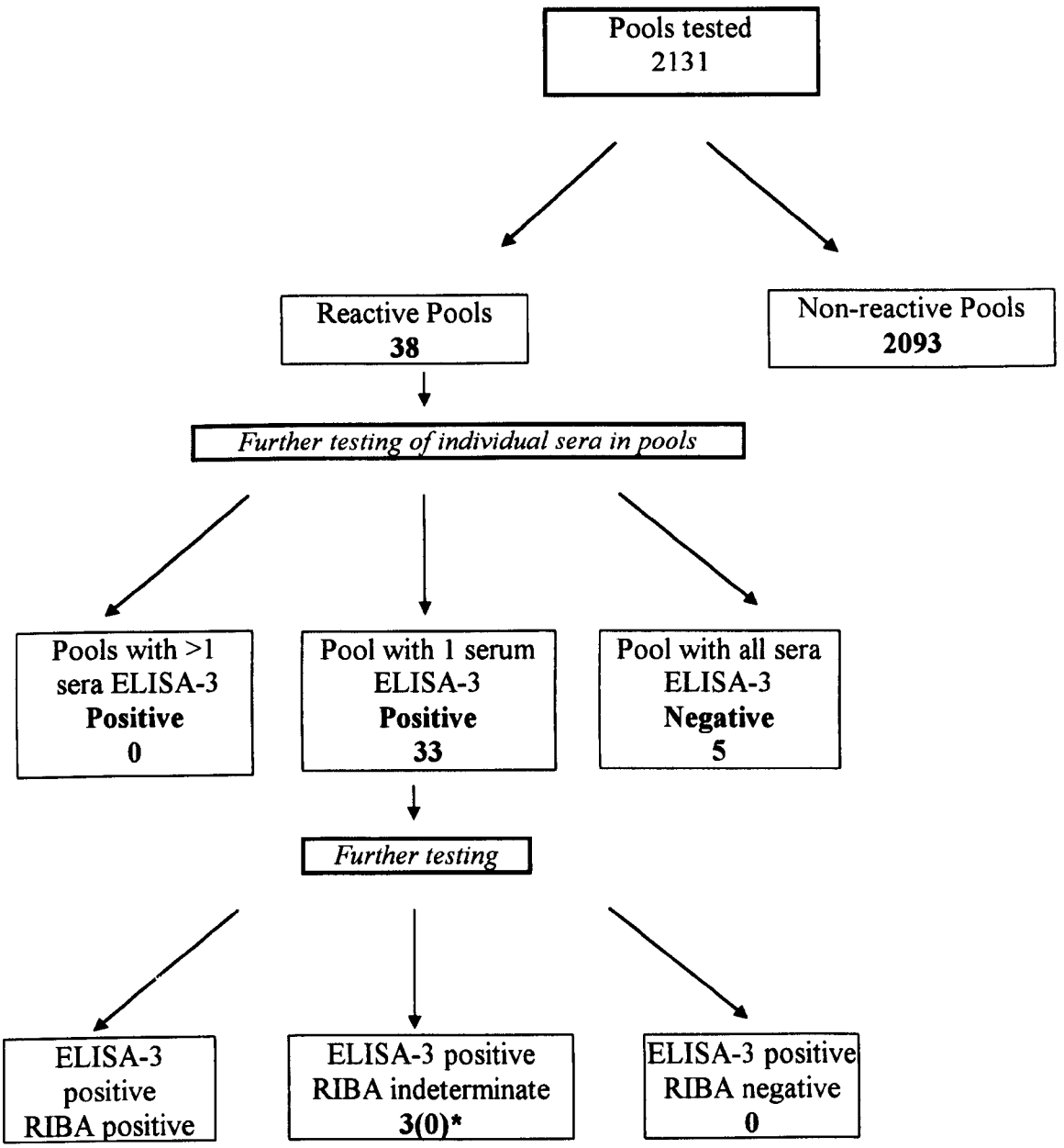
Table 3.2 Comparison of ELISA-3 HCV antibody test on individual and pooled sera from 2,190 healthcare workers who conduct exposure-prone procedures.

Individual sera	Pooled sera*		
	ELISA positive	ELISA negative	Total
ELISA positive	5	0	5
RIBA positive			
ELISA positive	1	1	2
RIBA indeterminate			
ELISA positive	0	2	2
RIBA negative			
ELISA negative	2**	427	429
Total	8	430	438

* 438 pools containing 5 sera each.

**i.e. 10 sera tested in which no individual serum was ELISA or RIBA positive

Figure 3.1 Results of testing 10,654 healthcare worker sera for antibodies to HCV by ELISA-3 in pools of 5.



* indicates numbers of sera positive by RT-PCR for HCV RNA

CHAPTER 4

THE UTILITY OF α-GLUTATHIONE S-TRANSFERASE FOR NON-INVASIVE ASSESSMENT OF HEPATITIS C RELATED LIVER INJURY

4.1 CONTENTS **Page**

4.2 SUMMARY

Serum α–glutathione s-transferase (α - GST) has been shown to be a sensitive marker of liver injury. We compared the relationship of both serum α–GST and alanine transaminase (ALT) with liver biopsy inflammatory activity in patients who had chronic hepatitis C infection (HCV) and examined the effects of α – interferon therapy on serum α–GST and ALT concentrations. Of thirty-two patients with chronic HCV infection studied, seventeen patients received α – interferon 4.5MU thrice weekly for three months and 15 patients acted as controls. Liver biopsy just prior to treatment was scored for the grade of inflammation (Scheuer histological activity index). Serum α–GST and ALT were assayed just prior to biopsy and three months later.

Neither serum α–GST nor ALT levels showed any correlation with baseline inflammation on liver biopsy. α – Interferon significantly reduced serum α–GST concentration at three months ($p = 0.01$). ALT fell with treatment but not significantly ($p = 0.05$). Small falls in α–GST and ALT were noted in the control group and when these were considered the significance of the changes in α–GST and ALT with treatment were lost ($p = 0.35$ and $p = 0.09$ respectively).

This study shows that serum α–GST is not a useful marker of the degree of liver inflammation in chronic HCV infection, though it may be of more value than ALT in monitoring response to treatment with α – interferon.

4.3 INTRODUCTION

The hepatitis C virus (HCV) is the most common cause of parenterally-acquired hepatitis in the western world. Following infection up to 80% of patients go on to develop a chronic carrier state with mild chronic hepatitis and between 10 to 20% of this group will develop cirrhosis, usually within 30 years (Seeff, L.B., et al. 1992, Alter, M.J., et al. 1992). This has significant health and economic implications as an estimated 0.5% of the British population are chronic carriers.

It is known that standard liver function tests (serum aspartate and alanine aminotransferase activity) do not accurately reflect the degree of inflammation seen on liver biopsy in chronic HCV infection (Alberti, A., et al. 1992, Persico, M. & Romano, M., et al. 1993). At best the correlation between the ALT and liver grade and stage of chronic hepatitis is poor (Haber, M.M., et al. 1995). Serum concentrations of the hepatocyte-derived enzyme α–glutathione s-transferase (α - GST) have been shown as a more sensitive and specific marker of hepatocellular damage, when compared to serum aminotransferases in a number of liver disorders (Beckett, G.J., et al. 1989, Hayes, P.C., et al. 1988, Trull, A.K., et al. 1994). The first aim of this study was to compare serum α - GST levels with alanine aminotransferase (ALT) as a means of grading the severity of inflammation in chronic hepatitis C infection as assessed by liver histology.

α–Interferon remains the mainstay of antiviral therapy against HCV whether used alone or in combination with ribavirin. Large controlled studies of interferon monotherapy have shown that although 50% of patients show an complete initial biochemical response, in only 25% of cases is this response sustained, as defined

by a persistently normal serum ALT six months after completing α – interferon therapy (Tine, F., et al. 1991). ALT remains the generally accepted method of monitoring response to α – interferon therapy, although normal serum transaminases do not exclude continuing hepatic inflammation (Alberti, A., et al. 1992, Persico, M. & Romano, M. 1993). The second aim of this study was to compare serum α - GST with ALT as a means of monitoring response to α – interferon therapy in chronic hepatitis C infection.

4.4 PATIENTS, MATERIALS AND METHODS

The study was approved by the West Glasgow Hospitals University NHS Trust ethics committee and consent was obtained from each patient prior to recruitment.

4.4.1 Patients

Thirty-two consecutive asymptomatic blood donors were studied. Each had serological evidence of HCV infection and had been referred to the regional specialist Gastroenterology clinic by the blood transfusion service. Other causes of chronic liver disease were sought and excluded. Each patient underwent percutaneous needle liver biopsy at entry to the study and all showed evidence of chronic hepatitis. A single pathologist (R.N.M MacSween) reviewed liver histology and graded the degree of necroinflammatory activity on a scale of 0 to 4 (Scheuer, P.J. 1991).

Thereafter the patients were randomised to receive either α – interferon (Roferon, Roche) at a dose of 4.5 MU three times a week subcutaneously or no treatment (observation) for 3 months. There were 17 and 15 patients in each group respectively.

4.4.2 HCV determination

Hepatitis C infection was confirmed by positive anti-HCV serology, using both ELISA-3 (Ortho Diagnostic Systems, Raritan, NJ, USA) and RIBA-3 (Chiron Corp., Emeryville, CA, USA) followed by detection of HCV-RNA using a reverse transcription polymerase chain reaction (RT-PCR). Prior to entry into the study each patient was confirmed to have chronic HCV infection by testing positive for

anti-HCV and HCV RNA in serum on two occasions six months apart. The qualitative RT-PCR used in the study was an in-house technique (Dow, B.C., et al. 1993). This assay performed well in the second EUROHEP HCV-RNA reference panel study and had a lower limit of detection of at most 2000 genomes / ml (Damen, M., et al. 1996). Twenty-six patients had virus genotype assessed by restriction fragment length polymorphism analysis of the 5' non-coding region RT-PCR products using a well established method (Davidson, F., et al. 1995).

4.4.3 Assessment of ALT and α - GST levels

Both serum α - GST and ALT were assayed at entry into the study, just prior to liver biopsy, and again three months later. The analysis of specimens for ALT took place immediately on an Olympus AU 5200 analyser (Olympus Optical Company Ltd., Eastliegh, UK) using the International Federation of Clinical Chemistry coupled enzyme reference method with reagents produced by Bio-stat Ltd. (Stockport, UK). The reference range for ALT is 10-50 units/l.

Samples for α - GST analysis were centrifuged immediately and serum stored at -20°C until the study was completed when all the specimens were assayed as a batch. Although the effects of retrospective testing were not assessed in this study, sample storage has already been shown not to influence testing (Nelson, D.R., et al. 1996, Loguercio, C., et al. 1998b). α - GST was measured using an enzyme immunometric assay (Hepkit, Biotrin, Dublin, Ireland). Serum samples and calibrators were appropriately diluted (usually 1 in 5) in phosphate buffered saline with 0.05%(v/v) Tween-20 (PBST). 100μl was added in duplicate to Nunc Maxisorp microtiter plates previously coated with affinity-purified polyclonal anti-

human α–GST IgG, shaken and incubated at 20-25°C for 1 hour. After extensive washing with PBST, a conjugate IgG (anti α–GST-horseradish peroxidase) was added to the microtiter plate (100μl/well) for 45 minutes and incubated as described above. The assay plate was then washed with PBST and 100μl of liquid tetramethylbenzidine substrate (TMB) was added to each well and colour allowed to develop for 15 minutes at 20-25°C. The reaction was terminated by addition of 1N H_2SO_4 (100μl) and the absorbance measured at 450nm using a Dynatech MR5000 plate reader (Dynatech, UK). Sample α–GST concentrations were determined from a plot of absorbance versus concentration for assay calibrators. The manufacturers reference range is 0-8 ng/ml in serum based on a population of 219 healthy blood donors.

4.4.4 Statistical Analysis

Analysis of the data produced was performed on Minitab for Windows 10 (Minitab Inc., State College, PA, USA). The Kruskal-Wallis test was used to detect any relation between levels of ALT and α - GST and liver inflammation on biopsy. The correlation between ALT and α - GST was assessed using Pearson product-moment correlation coefficient. The Wilcoxon signed rank paired test was employed to look for changes over the treatment period in the ALT and α - GST levels in both control and treatment groups. The comparison of these changes between the treatment and control group was made using the Mann-Whitney test.

4.5 RESULTS

4.5.1 Utility of ALT and α - GST in grading the necroinflammatory activity

In figures 4.1 and 4.2 the baseline serum ALT and α - GST levels are compared with the severity of hepatic inflammation in the thirty-two patients. All of the patients had evidence of chronic hepatitis on liver biopsy. Seven of the 32 patients had serum ALT levels within the reference range whereas 6 had serum α - GST levels within the reference range. There was no significant difference in α - GST or ALT levels between the four liver inflammation groups ($p = 0.19$ and $p = 0.31$ respectively).

4.5.2 ALT response to α – interferon

In Figure 4.3 the changes in the serum ALT levels over the three month period are demonstrated. In the treatment group thirteen of 17 patients had elevated ALT levels at entry into the study and 7 (54%) of these had a complete initial response to α – interferon with ALT levels within the reference range at three months. Three patients (23%) had a partial response to α – interferon with ALT falling but not to within the reference range. The three remaining patients (23%) who had elevated ALT levels at entry had no response to α – interferon. Despite evidence of inflammation on their liver biopsies, 4 patients had ALT's within the reference range throughout the 3 months of the study. There was a small but not statistically significant fall in the ALT levels in the observation group over three months ($p = 0.90$).

4.5.3 Utility of α - GST in assessing the response to α – interferon

In Figure 4.4 the changes in serum α - GST levels over the three months are demonstrated. α - GST concentrations were raised in 12 of 17 patients in the treatment group at the start of the study. After treatment only 4 (33%) subsequently had α - GST levels within the reference range, 6 (50%) had shown a fall but outwith the reference range, and 2 (16%) had a rise in α - GST. The 5 remaining patients had serum α - GST levels within the reference range throughout the study. There was a fall in serum α - GST levels in the control group over the three month period of observation but this did not achieve statistical significance ($p = 0.22$). Of the seven patients who had a complete response to α – interferon as monitored by ALT, five had a fall in their serum α - GST levels, although in two patients the α - GST levels did not return to the reference range. The remaining two patients had α - GST levels within the reference range throughout.

The overall change in ALT levels in the treatment group over the three month period of observation did not quite achieve statistical significance ($p = 0.05$) and when this was considered in relation to the small change witnessed in the control group ($p = 0.90$), there was less evidence of a significant change ($p = 0.09$). While treatment lead to a fall in serum α - GST which appeared significant ($p = 0.01$) this was lost ($p = 0.35$) when the fall in α - GST in the observation group ($p = 0.22$) was taken into account.

4.5.4 Correlation between ALT and α - GST

Figure 4.5 is a plot of ALT levels against concentrations in the study patients. There was a significant correlation between serum α - GST concentrations and ALT levels ($p < 0.01$).

4.5.5 Relation between HCV genotype and necroinflammatory activity

Of the 32 patients studied we were able to determine the HCV on 26 patients. Table 4.1 compares genotype with the necroinflammatory activity in the liver biopsy. The majority of patients were either genotype 1 or 3 (10 and 13 patients respectively). There did not appear to be any relation between the histological activity index on liver biopsy and the HCV genotype, but the numbers are small and difficult to assess formally.

4.6 DISCUSSION

α – GST is known to be a useful serum marker of acute hepatocellular damage in autoimmune hepatitis, following paracetamol overdose, in identifying acute liver transplant rejection and more recently in hypertensive disorders of pregnancy (Beckett, G.J. & Hayes, J.D. 1989, Hayes, P.C., et al. 1988, Trull, A.K., et al. 1994, Knapen, M.F.C.M., et al. 1998). α - GST is uniformly distributed throughout the hepatic acinus, is found in high concentrations within the cytosol of hepatocytes, and is of low molecular weight, all of which favour its release following any hepatic insult (Beckett, G.J., et al. 1989). The half-life of α – GST is less than 1 hour so plasma levels rapidly fall back to normal when hepatic injury ceases. This contrasts with ALT which has a long half-life (47 hours) and is predominantly distributed within the periportal area (zone 1) of the hepatic acinus.

ALT levels are known to fluctuate over time in chronic HCV infection and patients may have long periods (>12 months) when ALT levels are normal despite histological evidence of chronic hepatitis (Alter, M.J., et al. 1992, Alberti, A., et al. 1992, Persico, M. et al. 1993, Healey, C.J., et al. 1995). In our study there was no relationship between serum ALT levels and the severity of inflammation on liver biopsy, indeed six patients had normal serum ALT levels despite active hepatitis on liver biopsy. The reason why liver function tests do not correlate with histological features in chronic hepatitis C is not clearly understood. It is possible that confounding variables not considered here could influence serum transaminases without altering the necroinflammatory activity seen on biopsy. Two studies of patients with chronic HCV infection have shown concomitant excess alcohol ingestion may be associated with elevation of serum transaminases without

evidence of alcohol related liver damage on liver biopsy (Zignego, A.L. et al. 1994, Fog, T., et al 1994). In this series of patients none were drinking at the time of biopsy and there was no histological evidence of alcohol-induced liver disease in any of the liver biopsies.

This study demonstrated no evidence of a relationship between serum α–GST levels and severity of inflammation on liver biopsy in chronic hepatitis C. This is in agreement with the only previously published data on this subject (Nelson, D.R., et al. 1995). In our study, 6 patients had normal α - GST levels in the presence of chronic hepatitis on liver biopsy indicating that serum α - GST is not a sensitive marker of hepatic inflammation in chronic hepatitis C. Nothing is known about the fluctuations in α–GST with time in chronic HCV infection. It is possible that the short half-life of α–GST (approximately 1 hour) results in significant changes in serum level over short periods of time and unless liver histology and serum α–GST are checked simultaneously, true comparison cannot be made between them. Nelson and colleagues assessed liver histology and serum α–GST levels in 89 patients with hepatitis C and found no correlation between them (Nelson, D.R., et al. 1995). Unfortunately only 67 patients had histological assessment of their liver using the Knodell activity index and the time relationship of the serum collection and biopsy were within three months (Knodell, R.G., et al. 1981). The Knodell score is known to be of limited value in assessing the severity hepatic inflammation because it includes a numerical score of the degree of fibrosis as well as necroinflammatory activity on liver biopsy (Desmet, V.J., et al. 1994).

Several similar studies assessing α–GST as a non-invasive marker of liver injury in HCV have now been reported. Again the Knodell score was used for grading the severity of chronic hepatitis in these studies (Namour, F., et al. 1999, Loguercio, C., et al. 1998b, Vabourdelle, M., et al. 1995). Although there was a significant correlation between ALT and α–GST reported in each, only one study reported a significant association between α–GST and liver histology (Vabourdelle, M., et al. 1995). In all of these studies the interval between blood sampling and liver biopsy was not reported. In conclusion therefore serum α–GST does not appear to be useful in grading the severity of hepatitis caused by HCV. This is in keeping with the evidence that α–GST is not a useful marker of hepatocellular damage in a number of chronic liver disorders including primary biliary cirrhosis, chronic hepatitis B and alcoholic liver cirrhosis (Mulder, T.P.J., et al. 1997). This is likely to reflect the rapid clearance of α–GST from plasma after hepatocellular damage.

When this study was designed, serum transaminases were widely used to assess response to α – interferon therapy in chronic HCV infection. Detection of HCV-RNA in serum is now the gold standard for monitoring treatment and the discrepancy between transaminases and virological response to interferon therapy is well documented (Lau, J.Y.N., et al. 1993). This study showed a promising response of α–GST to interferon but reductions in both enzymes during the observation period were sufficient to diminish the statistical significance of these changes. In their series Nelson et al. treated 16 patients with α – interferon and considered that α–GST may have some use in monitoring the response to treatment and our study supports this suggestion (Nelson, D.R., et al. 1995). Subsequently Loguercio et al. performed a larger study with longer follow-up and

reported both biochemical and virological responses to interferon (Loguercio, C. et al. 1998a). They found pre-treatment α–GST levels to be higher in patients that did not have a sustained virological response to interferon. In addition they noted that α–GST remained elevated during treatment in patients who experienced an end of treatment biochemical response but subsequently relapsed after completing therapy. This suggests that measurement of α–GST prior to and during treatment can help predict those patients who will experience a sustained virological response to interferon.

This study fails to show an advantage for α–GST in grading severity of liver inflammation in chronic HCV infection but α - GST may have a role in monitoring response to α – interferon therapy. Liver biopsy remains the only good marker for the degree of hepatic inflammation in chronic hepatitis C.

Table 4.1 HCV genotype and Scheuer histological activity index on liver biopsy.

HCV genotype	Histological activity index			
	1	**2**	**3**	**4**
1	2	4	2	2
2	1	0	1	1
3	2	3	3	5

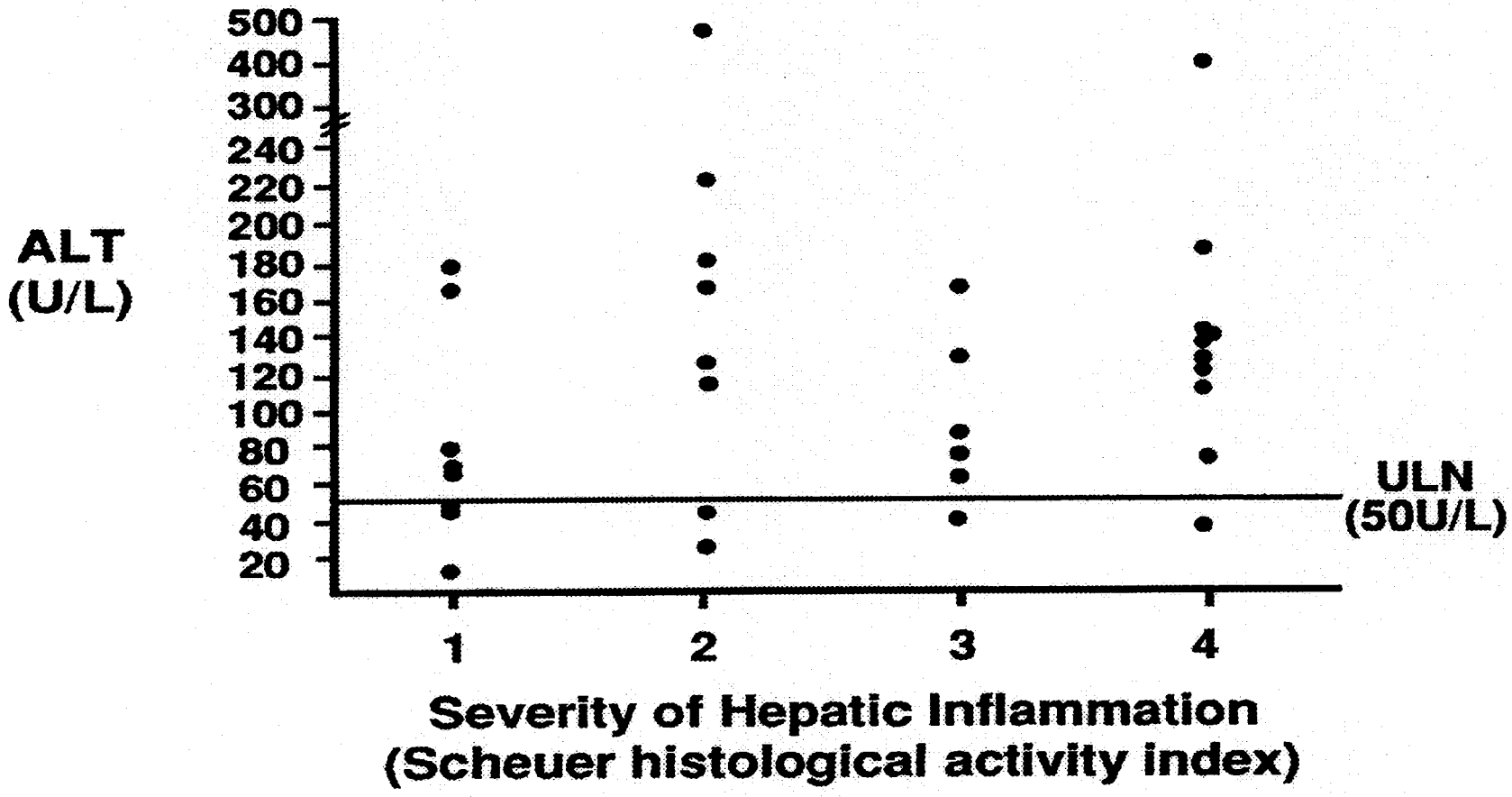


Figure 4.1 Serum ALT levels compared with severity of liver inflammation on liver biopsy in patients with chronic HCV infection. ULN= upper limit of normal.

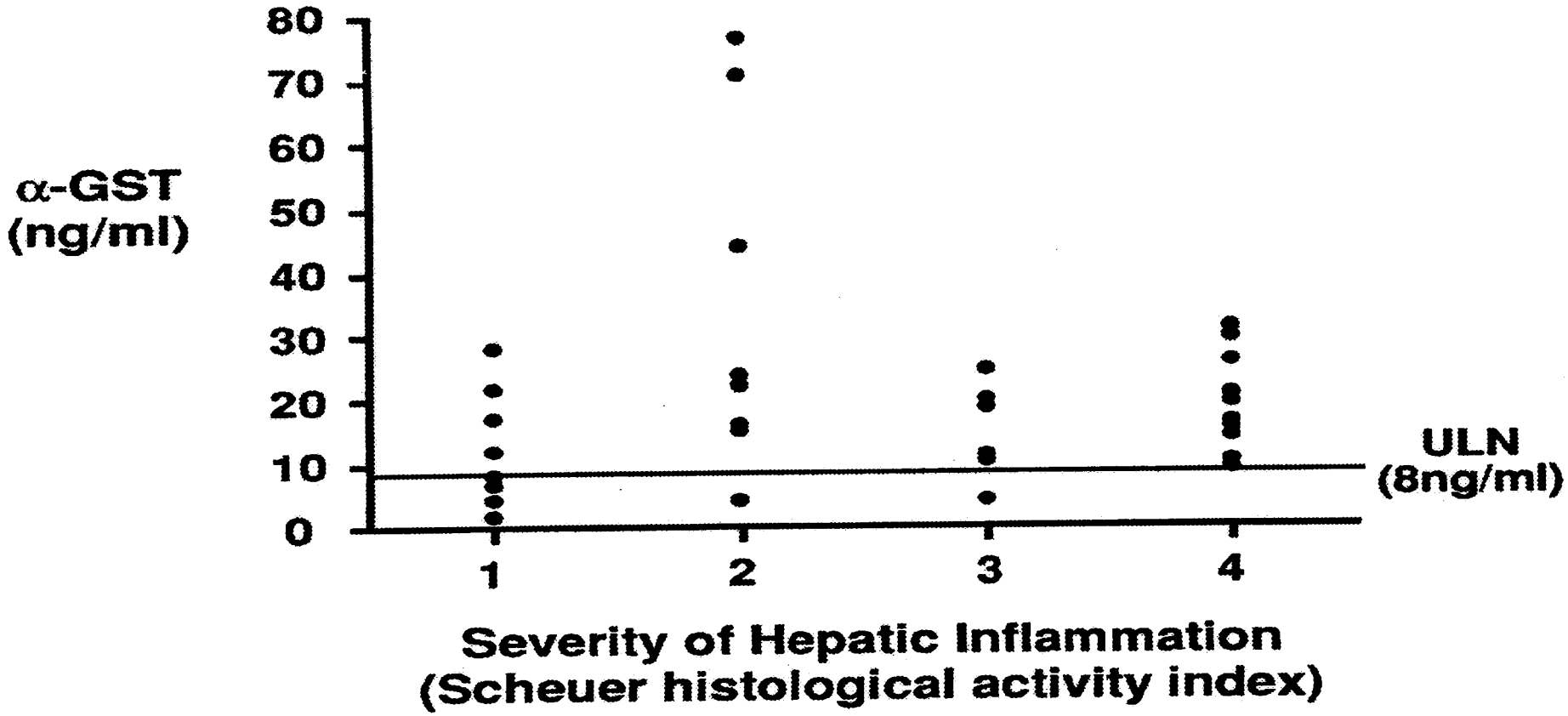


Figure 4.2 Serum α - GST levels compared with severity of liver inflammation on liver biopsy in patients with chronic HCV infection.

ULN= upper limit of normal.

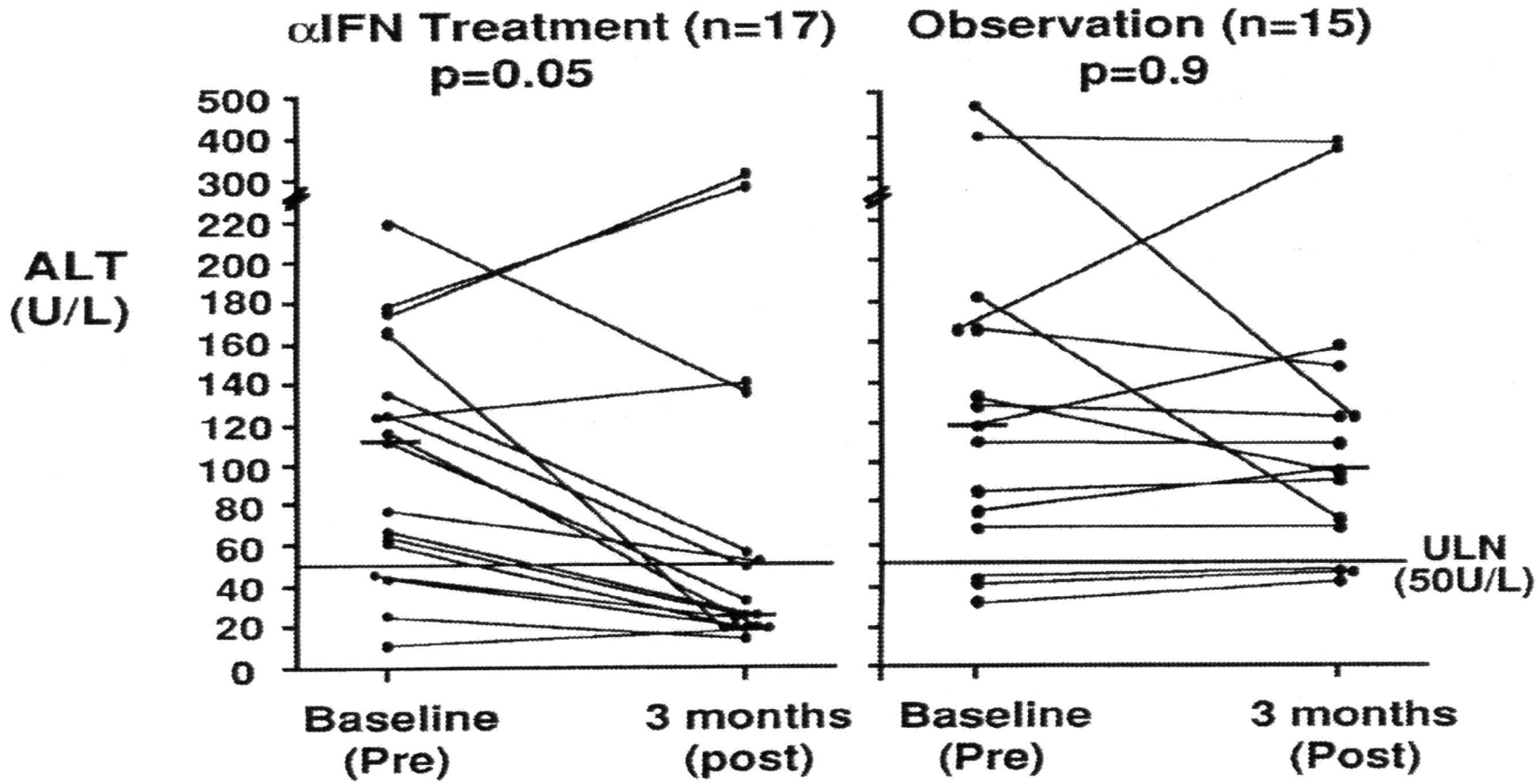


Figure 4.3 Individual changes in serum ALT levels in patients with chronic HCV infection over three months in (a) those treated with α - interferon and (b) the observation group. ULN= upper limit of normal. Medians marked with horizontal line.

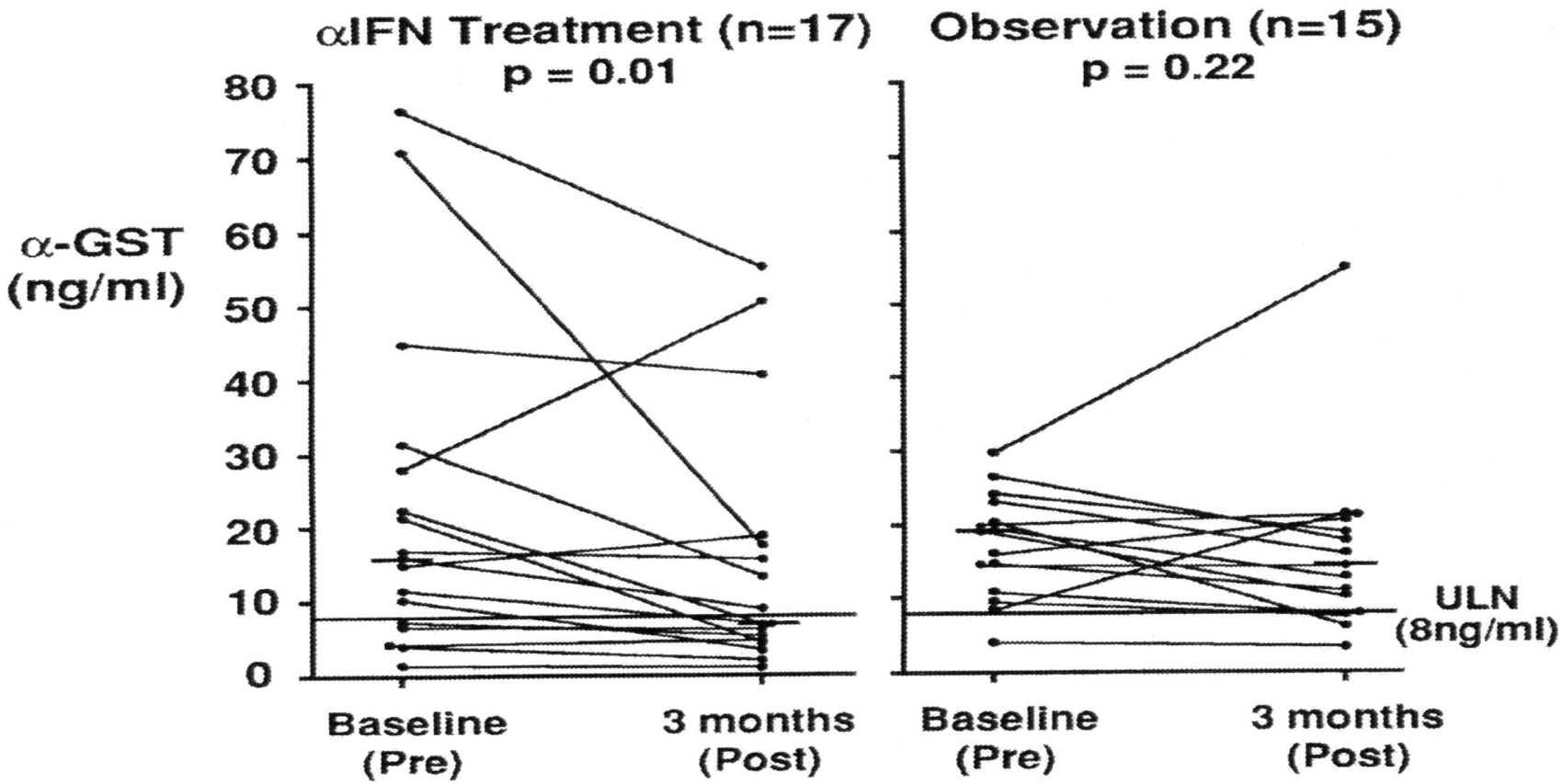


Figure 4.4 Individual changes in serum α - GST in patients with chronic HCV infection over three months in (a) those treated with α - interferon and (b) the observation group. ULN= upper limit of normal. Medians marked with horizontal line.

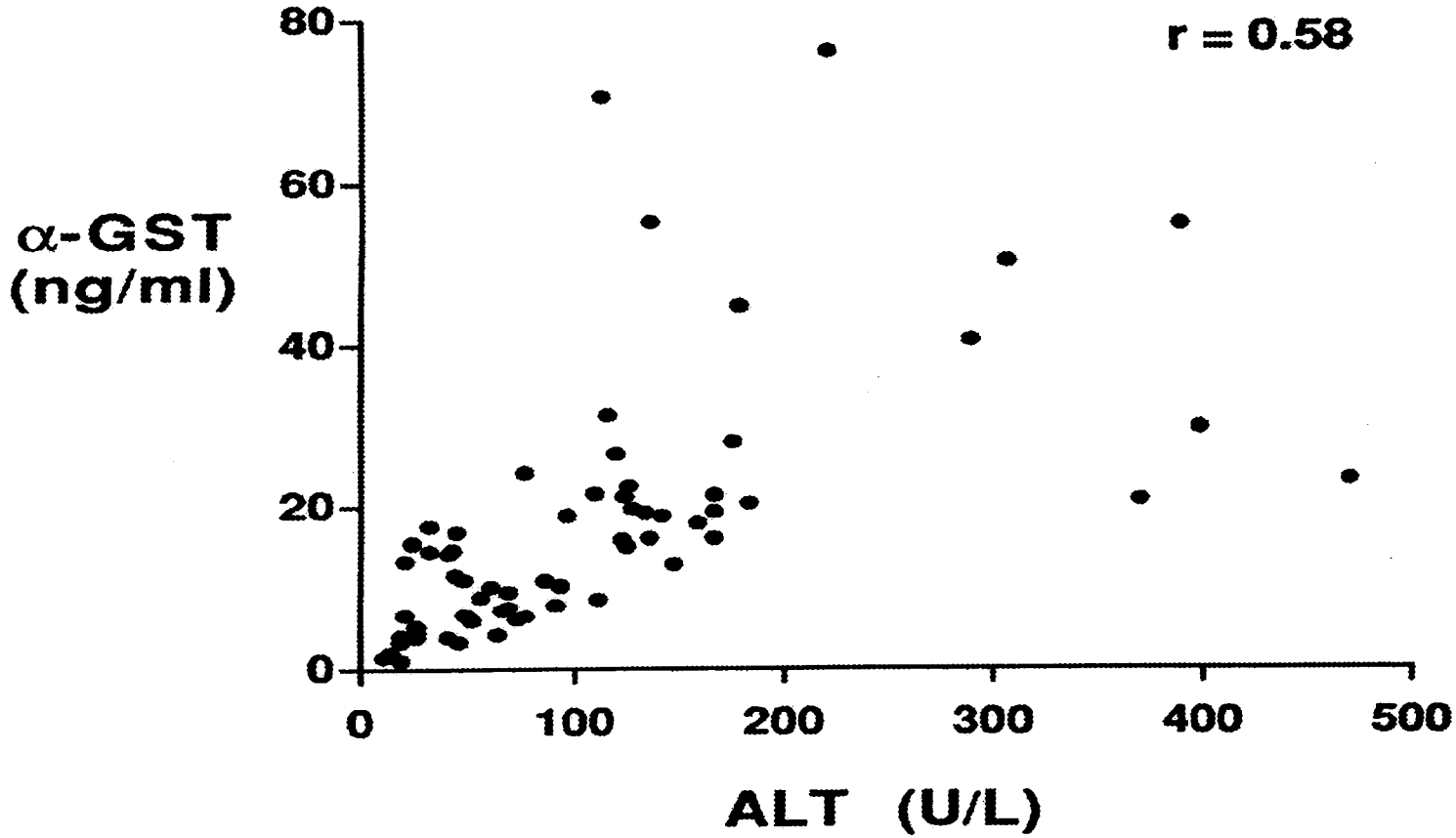


Figure 4.5 Plot of serum α - GST against serum ALT in 32 patients with chronic hepatitis C infection (measurement at baseline and three months recorded for each patient).

CHAPTER 5

THE ROLE OF IRON AND HAEMOCHROMATOSIS GENE MUTATIONS IN THE PROGRESSION OF LIVER DISEASE IN CHRONIC HEPATITIS C

5. 1 CONTENTS

5.2 SUMMARY

Chronic hepatitis C virus (HCV) infection is frequently associated with elevated markers of iron stores. Recessively inherited mutations in the HFE gene are responsible for iron accumulation in most cases of hereditary haemochromatosis and may have a role in HCV infection. They may also be associated with progressive liver fibrosis although this remains controversial. The aim of this study was to assess the prevalence of HFE mutations in HCV infected patients in the West of Scotland and to explore the effect of the carrier state on serum and liver iron stores and the severity of liver disease. One hundred and sixty-four patients with antibodies to HCV who underwent liver biopsy were assessed prospectively. Each patient was screened for HFE mutations (Cys282Tyr and His63Asp). Iron markers were assessed in serum (ferritin, transferrin saturation) and on liver biopsy (stainable iron, liver iron concentration (LIC) and Hepatic Iron index). There were 67 (41%, 26 Cys282Tyr, 33 His63Asp, 8 compound) heterozygotes. Forty-four (28%) patients had elevated serum iron markers, 24 (15%) had stainable liver iron and 5 (3%) had elevated LICs. Carriage of HFE mutations was not associated with any clinical, biochemical, virological or pathological feature, including accumulation of liver iron. Elevated serum iron markers were associated with male gender, increased alcohol consumption and increased liver inflammation and fibrosis. Patients with elevated LICs were older, acquired HCV infection earlier and had more liver inflammation. Patients with chronic HCV infection frequently have elevated serum iron markers although elevated LICs are uncommon. Elevated serum iron studies and LICs occur in patients with more severe liver disease. Carriage of HFE mutations, although frequently observed in these HCV infected

patients, does not have a role in the accumulation of iron or the progression of liver disease in HCV infection.

5.3 INTRODUCTION

Approximately eighty percent of those who acquire acute hepatitis C virus (HCV) infection develop a chronic low-grade, slowly progressive hepatitis which may result in cirrhosis and hepatocellular carcinoma. The natural history of the chronic liver disease caused by HCV remains controversial with varying rates of progression to cirrhosis reported (Seeff, L.B. 1998a).

Several factors have been proposed to account for the variation observed, including excess alcohol consumption, HCV genotype 1b, older age at acquisition of infection and male gender (Seef, L.B. 1997). Of recent interest has been the role of iron in chronic HCV infection. It has been long recognized that hepatic iron overload promotes hepatic fibrosis in hereditary haemochromatosis (Bacon, B.R. & Tavill, A.S. 1996). Serum iron stores are frequently increased in patients with chronic hepatitis C (Riggio, O., et al. 1997). Enhanced liver fibrosis has been reported in HCV infected patients with stainable iron on liver biopsy compared to controls with no detectable liver iron (Beinker, N.K., et al. 1996). The mechanism by which iron accumulates in chronic HCV is not established but may in part be the result of iron release from damaged hepatocytes (Bonkovsky, H.L., et al. 1997).

With the discovery that mutations in the HFE gene account for most cases of hereditary haemochromatosis, there has much interest in their role in the development and progression of other liver diseases. The two recognized recessively inherited mis-sense mutations of the HFE gene result in amino acid substitutions at position 282 (cysteine to tyrosine, Cys282Tyr) and at position 63

(histidine to aspartic acid, His63Asp) (Feder, J.N., et al. 1996, Feder, J.N., et al. 1998). Over 90% of British patients with hereditary haemochromatosis are homozygous for at least one of these mutations (The UK haemochromatosis consortium. 1997). The significance of heterozygosity for these recessively inherited HFE mutations is unclear, however there have been reports of an association with liver iron accumulation (Piperno, A., et al. 1998) and increased liver fibrosis in HCV associated liver disease (Smith, B.C., et al. 1998, Pirisi, M., et al. 2000, Martinelli, A.L.C., et al. 1999).

The aim of this study was to assess the prevalence of HFE mutations and increased serum and liver iron markers in Scottish patients infected with HCV and to then explore any association between heterozygosity for the HFE gene mutations, serum and liver iron stores and the severity of HCV related liver disease.

5.4 PATIENTS , MATERIALS AND METHODS

5.4.1 Patients

One hundred and sixty four consecutive patients with evidence of hepatitis C virus infection were prospectively studied. All were anti-HCV positive with abnormal liver function tests and underwent needle biopsy of the liver. History of alcohol abuse, alcohol consumption at the time of biopsy and risk factors for HCV acquisition were established.

One hundred and three patients were male and fifty-three had a history of excess alcohol consumption, defined as alcohol consumption of >24g / d (>3U / d) for males and >16g / d (>2 U / d) for females. Ten (6.3%) patients were non-Caucasian. Risk factors for acquisition of HCV infection were intravenous drug use (77 patients, 47%), blood products (39 patients, 24%), sexual transmission (8 patients), needlestick injury (4 patients), tattooing (3 patients), human bite (1 patient) and thirty-two (19.5%) patients had no identifiable risk factors for the acquisition of HCV infection. Twenty five patients had received previous anti-viral therapy with alpha-interferon. The proportion of interferon treated patients was not significantly different among the four groups. Duration of infection at the time of liver biopsy was recorded for those patients who had an identifiable year of infection. For those patients who acquired HCV through intravenous drug use, the year of infection was assumed to be the year that injecting commenced. Local data indicates that approximately half of those intravenous drug users who acquire HCV infection are infected within one year of commencing injecting and almost all are infected within three to five years (personal communication Avril Taylor, Scottish Centre for Infection and Environmental Health).

5.4.2 HCV Determination

Anti-HCV was assayed by third generation enzyme linked immunoabsorbent assay (Ortho Diagnostics, Raritan, NJ, USA). Supplemental testing was by RIBA-3 (Ortho Diagnostics, Raritan, NJ, USA). Reverse transcription PCR was performed on patient sera using an in-house method (Dow, B.C., et al. 1993). Genotyping of HCV was performed by restriction fragment length polymorphism on RT PCR positive samples (McKechnie, V.M., et al. 2000).

5.4.3 Iron Parameters

Iron status was assessed for each patient using biochemical tests, histological grading of liver iron content and by measurement of liver iron concentration. Serum iron (normal: males 10 – 40 μmol/l, females 8 – 40 μmol/l) was measured by the ferrozine method, ferritin (normal: 15 – 300 ng/ml) was measured by turbidimetry using a Chiron ACS180 automatic analyzer (Bayer, USA) and transferrin (normal: 2 – 4 g/l) was measured by nephelometry (Beckmann Array Analyser, USA). Transferrin saturation was calculated as follows: Serum Iron x 70.9 / Serum Transferrin (normal: 16 - 45%).

5.4.4 Histological evaluation

All liver biopsies were performed under ultrasound guidance. As is our practice, two cores of liver tissue were obtained from each patient: one formalin fixed for histological analysis and one stored immediately at -20°C for liver iron concentration estimation. Formalin fixed specimens were paraffin embedded and sections stained with Haematoxylin and Eosin and Perl's Prussian blue stain. Slides were then evaluated by two experienced pathologists (R.N.M. MacSween and K. Oien) blinded to the clinical and laboratory information. All biopsy

specimens were graded for the degree of necroinflammatory activity and staged for the extent of fibrosis using the criteria of Ishak et al (Ishak, K., et al. 1995).

5.4.5 Determination of Hepatic Iron

Histological assessment of hepatocyte and macrophage iron stores were graded on a scale of 0 to 4 on Perl's Prussian blue stained liver sections as previously described (Searle, J., et al. 1994). In addition one hundred and twenty patients had liver iron concentrations (LICs) measured by atomic absorption spectrometry with results expressed as micrograms per gram of dry weight of liver tissue (normal range 170 - 1400 μg/g dry weight liver tissue). The hepatic iron index was calculated by dividing the LIC (in μmol / g dry wt) by the age of the patient (in years) (Summers, et al. 1990). Forty four patients did not have a second liver biopsy specimen for hepatic iron assay for the following reasons: lack of patient consent or reluctance of the radiologist to take a second biopsy specimen for clinical reasons, inadequacy of specimens obtained for LIC estimation and occasional omissions at the time of biopsy.

5.4.6 HFE mutation analysis

The presence of HFE gene mutations was verified by means of restriction fragment length polymorphism on the PCR products of genomic DNA extracted from peripheral blood mononuclear cells (Jazwinska, E.C., et al. 1996, Feder, J.N., et al. 1996). Extracted DNA fragments were amplified using oligonucleotide primers for the Cys282Tyr and His63Asp loci synthesized according to previously reported sequences (Feder, J.N., et al. 1996). Amplification, digestion and visualization of the PCR products were performed as previously reported (Jazwinska, E.C. et al. 1996).

5.4.7 Statistical analysis

Statistical analysis of the data was performed using SigmaStat (SPSS Science Software UK Ltd, Birmingham, UK). Differences between nominal variables were analysed by Chi-square tests. Differences among continuous variables were evaluated using the Kruskal - Wallis test. Logistic regression analysis with a stepwise approach was applied to determine those variables independently associated with the presence of liver iron and the grade and stage of liver disease. Consent to carry out this study was provided by the West Glasgow Hospitals University NHS Trust ethics committee.

5.5 RESULTS

5.5.1 HFE gene data

Table 5.1 shows the results of analysis for the Cys282Tyr and His63Asp mutations in 164 patients with chronic hepatitis C. Seventy-four patients (45%) carried at least one of the mutations. The allele frequencies for the Cys282Tyr and His63Asp mutations were 12.2% and 14.9%.

Seven (4.2%) patients were homozygous for one of the mutations (3 Cys282Tyr, 4 His63Asp) and were excluded from further analysis (Table 5.2). All three patients homozygous for the Cys282Tyr mutation had stainable iron on liver biopsy. LICs and transferrin saturations were elevated in two (66.7%) of these patients but there was little evidence of associated hepatic inflammation or fibrosis on liver biopsy. Three of the patients homozygous for the His63Asp mutation had elevated serum iron studies (2 transferrin saturation, 1 ferritin) but none had stainable iron on liver biopsy. LIC was not increased in either of the His63Asp homozygotes in whom it was estimated. One (25%) patient homozygous for the His63Asp mutation was cirrhotic.

5.5.2 Clinical data

To ensure that the role of gender and menstruation on iron metabolism did not bias the study, data for male and female patients in each of the four groups were assessed separately. The proportion and ages of male and female patients in each of the four groups were comparable. Analysis excluding female patients, and hence the effects of menstruation, did not affect the results for any clinical, serum or liver biopsy parameter. Analysis excluding the ten non-Caucasian patients, two of

whom were heterozygous for the His63Asp mutation, did not influence the results and all analysis discussed included these patients. In comparison with interferon naïve patients, those previously treated with interferon therapy had comparable serum and liver iron studies and severity of HCV related liver injury and all analysis included these patients.

In Table 5.3, the remaining 157 patients including 67 with heterozygous HFE mutations and 90 without were compared with regard to demographic, clinical and virological data. No differences with respect to any of these features were found. One hundred and fifty four patients were RT PCR positive for HCV RNA of whom 64 were infected with genotype 1 and 72 with genotype 3.

5.5.3 Serum iron markers

The association between serum iron markers and HFE gene status indices is shown in Table 5.4. No significant differences were observed. Sixteen (10%) patients had elevated serum ferritin levels, six (9%) carrying an HFE mutation (1 His63Asp/Cys282Tyr, 1 Cys282Tyr/WT, 4 His63Asp/WT) and ten (11%) without ($p = 0.84$). Transferrin saturation was elevated in thirty-six (23%) patients, eighteen (27%) with (3 Cys282Tyr/His63Asp, 3 Cys282Tyr/WT, 12 His63Asp/WT), and 18 (20%) without an HFE mutation ($p = 0.67$). Either serum ferritin or transferrin saturation was elevated in forty-four (28%) patients, twenty-one (31%) of the 67 patients with and twenty-three (26%) of the 90 patients without HFE gene mutations ($p = 0.54$). Patients with elevated serum iron markers were more commonly male (37 / 44 (84%) v 62 / 113 (55%), $p = 0.001$), drank significantly more alcohol (median 12.5 v 4 u/wk $p < 0.001$), more frequently had stainable iron on liver biopsy (13 (29%) v 11 (10%), $p = 0.004$), had higher LICs

(median 0.540 v 0.375, p = 0.009), and had more active chronic hepatitis (median necroinflammatory scores 5 v 4, p = 0.017) with more fibrosis (median fibrosis scores 2 v 1, p = 0.025) than patients with normal iron studies.

5.5.4 Liver biopsy indices

The association between necroinflammatory and fibrosis scores, stainable iron, liver iron concentrations and HFE gene status is also shown in Table 5.4. No significant differences were observed.

Eleven (7%) patients had scores of four or more for stage of fibrosis, two (3%) of the sixty-seven patients carrying an HFE mutation compared with nine (10%) of the ninety without an HFE mutation (p = 0.16). Six (4%) patients had cirrhosis, one (1%) with and five (6%) without HFE mutations (p = 0.37). By stepwise regression using a backwards approach, fibrosis scores could be predicted by a combination of age, alcohol consumption and liver biopsy scores for interface hepatitis and confluent necrosis (all p < 0.001).

Stainable iron was detected on 24 (15%) biopsies, with hepatocyte staining alone in eleven, macrophage staining in five and staining of both in eight. No patient had an iron staining grade of 3 or 4 for hepatocyte iron and one only one patient had macrophage iron staining of grade three. Hepatocyte or macrophage iron staining was present on the liver biopsy of 13 (19%) of the 67 patients with an HFE mutation (3 Cys282Tyr/His63Asp, 6 Cys282Tyr/WT, 4 His63Asp/WT) and 11 (12%) of the 90 patients without (p = 0.31). Patients with stainable iron on liver biopsy were older (p = 0.007), with higher serum ferritins (p < 0.001) and had

higher LICs ($p < 0.001$). There was no significant difference in the grade and stage of liver disease between patients with and without iron staining on liver biopsy.

One hundred and fifteen out of the 157 non-homozygous patients had liver iron concentrations measured, five (3%) had LICs >1400μg/g dry wt (median 1650; range 1480 - 3400) and only one (His63Asp/WT) carried an HFE mutation. Three (60%) patients with an elevated LIC had stainable iron on liver biopsy (1 hepatocyte staining alone, 2 with hepatocyte and macrophage staining) compared with 21 (14%) of the one hundred and fifty two patients with a normal LIC ($p = 0.03$). Patients with elevated LICs were older ($p = 0.05$), acquired HCV infection earlier ($p = 0.02$), had higher serum ferritins ($p=0.003$), and had more severe necroinflammatory activity on liver biopsy ($p = 0.02$). There was a trend towards higher fibrosis scores in those patients with elevated LICs although this did not reach statistical significance (median 2 v 1, $p = 0.06$). By stepwise regression analysis with a backward approach, LICs could be predicted by a combination of serum ferritin, transferrin saturation and hepatocyte staining with iron on liver biopsy (all $p< 0.001$). No patient had an elevated HII diagnostic of hereditary haemochromatosis (HII >1.9) and there was no significant difference in HII between patients with and those without HFE mutations.

5.6 DISCUSSION

The prevalence of both HFE gene mutations in this population is higher than that reported in studies of HCV infected populations from Europe, England and Brazil (Kazemi-Shirazi, L., et al. 1999). This may reflect the high prevalence of HFE mutations in the local population, which is of largely Nordic and Celtic extraction, rather than a susceptibility to HCV infection in carriers of HFE mutations (Bacon, B.R., et al. 1999b).

There was no evidence in this study that heterozygosity for either HFE mutation was associated with more severe grade or stage of HCV related liver disease. This contrasts with the study by Smith et al. which first proposed an association between the carriage of HFE mutations and increased hepatic fibrosis scores (Smith, B.C., et al. 1998). In that study ten Cys282Tyr heterozygotes of whom 4 (40%) had cirrhosis were compared with 127 normal controls of whom 11 (8.7%) were cirrhotic ($p = 0.01$). Heterozygotes had more advanced liver disease ($p = 0.01$) and more commonly had iron staining on Perl's stained liver sections ($p = 0.02$). Further studies by other groups have supported (Pirisi, M., et al. 2000, Martinelli, A.L.C., et al. 1999) and refuted (Kazemi-Shirazi, L., et al. 1999, Knoll, A., et al. 1998, Hezode, C., et al. 1999) these findings. Some studies included small numbers of homozygous patients (Pirisi, M., et al. 2000, Martinelli, A.L.C., et al. 1999, Kazemi-Shirazi, L., et al. 1999, Hezode, C., et al. 1999). Factors, which are known to be associated with the progression of HCV related liver disease, were controlled for inconsistently in these studies. The lack of association between carriage of HFE mutations and the stage of fibrosis in this study may have resulted from excluding all homozygous patients from the analysis and controlling for

many possible confounding variables including gender, age at HCV acquisition, duration of HCV infection, route of HCV infection, HCV genotype, current alcohol consumption and past history of alcohol excess. In this study factors associated with stage of fibrosis were patient age and alcohol consumption at time of biopsy, which is compatible with current understanding (Poynard, T., et al. 1997, Wiley, T.E., et al. 1998). In addition the grade of hepatitis, in particular scores for interface hepatitis and the presence of confluent necrosis, were predictors of fibrosis. This is in keeping with studies that have shown these features on liver biopsy to be associated with progressive disease and a worse prognosis in chronic hepatitis (MacSween, R.N.M. 1980, Cooksley, W.G.E. 1986).

Patients carrying an HFE mutation had no evidence of increased serum iron studies or intrahepatic iron deposition assessed both directly and indirectly. The prevalence in this study of increased serum iron stores in patients with chronic HCV infection is comparable to that reported elsewhere, with twenty-eight percent of patients having an elevated ferritin or transferrin saturation (Bonkovsky, H.L., et al. 1997). Patients with elevated serum iron markers were more commonly male, drank more alcohol and had more active chronic hepatitis with more liver fibrosis. This suggests that elevated serum iron markers in patients with chronic HCV infection are the result of active hepatitis, either through leakage from damaged hepatocytes or as part of the systemic inflammatory response (Bonkovsky, H.L. et al. 1997)

Despite frequently elevated serum iron studies few patients had direct or indirect evidence of increased liver iron with only 3% of patients having an elevated LIC and 15% of biopsies having stainable iron almost exclusively of grades 1 or 2. The

prevalence of elevated LIC in this study is lower than that reported in earlier studies of patients with chronic hepatitis C, where a prevalence of 10 - 36% has been recorded (Piperno, A., et al. 1998, Tung, B.Y. & Kowdley, K.V. 1999, Kazemi-Shirazi, L., et al. 1999). There was no evidence that carriage of the HFE mutations was associated with liver iron accumulation. This finding is consistent with earlier studies exploring the role of HFE mutations in HCV that assessed liver iron histologically (Smith, B.C., et al. 1998, Pirisi, M., et al. 2000, Knoll, A., et al. 1998, Hezode, C., et al. 1999) and the only other study to have assessed liver iron concentration (Kazemi-Shirazi, L., et al. 1999).

Significant iron deposition in the liver was uncommon and overall the quantity of iron that was detectable histologically and biochemically was unrelated to the grade and stage of HCV related liver injury. The mechanism by which liver iron accumulates in a small number of patients is unclear. These patients have significantly more liver inflammation and a trend towards increased fibrosis compared with those with normal liver iron concentrations, however there is considerable overlap between the two groups. Whether this iron accumulation is the cause or result of liver injury is unclear.

In summary we found that although many patients with chronic HCV infection have elevated serum iron studies, few have significant iron deposition within the liver. Carriage of the recognized mutations in the HFE gene, although frequently observed, do not account for the elevated serum iron studies and liver iron deposition that is present nor are they associated with any clinical, biochemical, virological or pathological feature. The severity of HCV related liver injury could be predicted by patient age, alcohol consumption, and histological grades of

interface hepatitis and confluent necrosis as previously described and overall the concentration of liver iron did not have a significant role in the progression of HCV related liver injury. These findings do not support a role for iron depletion by venesection in patients with chronic HCV infection, including those with elevated serum iron studies.

Table 5.1 Distribution of HFE gene mutations in patients with chronic hepatitis C infection.

Mutation Status	Number (%)
Cys282Tyr / Cys282Tyr	3 (1.8)
His63Asp / His63Asp	4 (2.4)
Cys282Tyr / His63Asp	8 (4.9)
Cys282Tyr / Wild type	26 (15.8)
His63Asp / Wild type	33 (20.1)
Wild type / Wild type	90 (54.9)
Total	164 (100)

Table 5.2 Patient demographics, biochemical, and liver biopsy data for patients homozygous for Cys282Tyr and His63Asp HFE mutations. Two patients homozygous for the His63Asp mutation did not have Liver Iron Concentration assessed.

Homozygous	Age (Years), Gender	Serum Ferritin (ng/mll)	Transferrin saturation (%)	Grade of inflammation (0-18)	Stage of fibrosis (0-6)	Stainable Iron Hepatocyte (0 – 4)	Stainable Iron Macrophage (0 – 4)	Liver Iron Concentration (μg/g)	Hepatic Iron Index
Cys282Tyr	22, female	177	108.8	4	1	2.5	0.5	3240	2.52
Cys282Tyr	30, female	130	24.3	4	1	0.5	0	1250	0.75
Cys282Tyr	32, male	492	108.6	2	1	2	1	1780	1
His63Asp	35, female	6	12.5	3	0	0	0	70	0.04
His63Asp	29, male	323	19.7	6	2	0	0	840	0.52
His63Asp	45, male	93	56.1	4	1	0	0	--	--
His63Asp	46, male	213	45.6	6	6	0	0	--	--

Table 5.3 Demographic and clinical characteristics for the 157 HCV infected patients studied grouped by HFE genotype.

Figures quoted as Median (Range) unless otherwise stated. * WT – Wild type

Variable		His63Asp/Cys282Tyr (n=8)	Cys282Tyr/WT* (n=26)	His63Asp/WT* (n=33)	WT/WT* (n=90)	p value
Age (Years)		38 (25 – 54)	34 (23 – 61)	35 (23 – 61)	36.5 (42 – 70)	0.53
Gender (Male:Female)		6 : 2	19 : 7	18 : 15	56 : 34	0.45
Age at acquisition of HCV (Years)		23 (14 – 35)	20 (13 – 37)	24 (14 – 54)	21 (13 – 51)	0.31
Duration of HCV infection at time of biopsy (Years)		8 (3 – 25)	15 (5 – 39)	12 (6 – 23)	15 (5 – 28)	0.26
Alcohol consumption at biopsy (Units/wk) (Mean ± SD)		12 ± 13.7	12 ± 16	8 ± 11	15 ± 35	0.70
Past history of excess alcohol consumption [no. (%)]		3 (37)	14 (54)	10 (30)	25 (34)	0.11
Previous interferon therapy [no. (%)]		3 (37)	4 (15)	3 (9)	15 (17)	0.26
Route of HCV infection (no.)	IVDU	4	20	15	39	0.19
	Blood products	3	2	9	23	
	Other	1	4	9	28	
Serum HCV RNA positive (no.)		8	25	32	89	
Genotype	1	3	13	15	33	0.94
	2	1	3	4	9	
	3	4	9	13	46	
	4	0	0	0	1	

Table 5.4 Relationship between HFE genotype, serum iron indices, liver biopsy findings, stainable iron and hepatic iron indices are shown.

		His63Asp/Cys282Tyr (n=8)	Cys282Tyr/WT (n=26)	His63Asp/WT (n=33)	WT/WT (n=90)	P Value
Serum Ferritin (ng/ml) (Mean ± SD)		138 ± 145	106 ± 115	171 ± 270	162 ± 369	0.94
Serum Iron (μmol/l) (Mean ± SD)		25.1 ± 10.1	17.8 ± 7.1	26.1 ± 12.4	22.7 ± 11.7	0.06
Serum Transferrin (g/l) (Mean ± SD)		2.4 ± 0.3	2.8 ± 0.6	2.8 ± 0.3	3.1 ± 1.6	0.01
Transferrin saturation (%) (Mean ± SD)		45.9 ± 18.1	32.2 ± 15.4	41.0 ± 17.6	34.8 ± 20.9	0.21
Grade of Inflammation (0 - 18)		5 (1 – 6)	4 (1 – 9)	4 (1 – 9)	5 (0 – 13)	0.22
Interface hepatitis (0-4)		1 (0 – 2)	1 (0 – 3)	1 (0 – 2)	1 (0 - 4)	0.05
Confluent Necrosis (0-6)		0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 6)	0.20
Parenchymal Inflammation (0-4)		2 (1 – 3)	2 (0 – 3)	2 (0 – 4)	2 (0 – 4)	0.14
Portal Inflammation (0-4)		1 (0 – 2)	1 (1 – 4)	1 (0 – 3)	1 (0 – 3)	0.59
Stage of fibrosis	0	3	5	8	10	0.09
	1	3	11	14	38	
	2	2	6	7	23	
	3	0	2	4	10	
	4	0	1	0	3	
	5	0	0	0	1	
	6	0	1	0	5	

Table 5.4 (contd.) Relationship between HFE genotype, serum iron indices, liver biopsy findings, stainable iron and hepatic iron indices are shown.

		His63Asp/Cys282Tyr (n=8)	Cys282Tyr/WT (n=26)	His63Asp/WT (n=33)	WT/WT (n=90)	P Value
Stainable iron - Hepatocyte	0	5	22	29	82	0.06
	1	2	4	3	5	
	2	1	0	1	3	
Stainable iron - Macrophage	0	6	24	31	83	0.28
	1	1	2	1	5	
	2	1	0	1	1	
	3	0	0	0	1	
No. with hepatic iron estimation		5	22	24	64	
Liver Iron Concentration (μg/g) (Mean ± SD)		462 ± 286	535 ± 321	508 ± 320	544 ± 568	0.82
Hepatic Iron index (Mean ± SD)		0.23 ± 0.10	0.26 ± 0.15	0.24 ± 0.14	0.25 ± 0.22	0.66

CHAPTER 6

THE ROLE OF POLYMORPHISMS IN THE RENIN-ANGIOTENSIN SYSTEM ON THE DEVELOPMENT OF HEPATIC FIBROSIS IN CHRONIC HEPATITIS C INFECTION

6. 1 CONTENTS

6.2 SUMMARY

The natural history of chronic HCV remains uncertain with variable rates of progression of hepatic fibrosis observed. The mechanisms that account for these variations are poorly understood. Activated human hepatic stellate cells are the main effector cells in the development of hepatic fibrosis and have recently been demonstrated to possess angiotensin II receptors. Binding of angiotensin II to these receptors has been shown to induce further stellate cell activation and may contribute to the generation of hepatic fibrosis. A number of polymorphisms in the genes of the renin-angiotensin system are described and are recognised to contribute to the variable outcomes observed in a number of cardiac and renal diseases. The aim of this study was to explore the role of three renin-angiotensin system polymorphisms (M235T angiotensinogen, D/I angiotensin I converting enzyme and A1166C AT1 angiotensin II receptor polymorphisms), known to influence renin-angiotensin phenotype, on the progression of hepatic fibrosis in chronic HCV.

One hundred and ninety one patients with chronic HCV were grouped by liver biopsy Ishak fibrosis stage [97 group 1 (stage 0 or 1), 70 group 2 (stage 2 or 3), 24 group 3 (stage 4 to 6)]. Renin-angiotensin polymorphisms were assessed using PCR based methods. The allele frequencies of the T235 variant of angiotensinogen gene, the D variant of the angiotensin I converting enzyme gene and the C1166 AT1 angiotensin II receptor gene were 58.9%, 50.8% and 24.3%. The prevalence of these gene polymorphisms associated with increased renin-angiotensin activity were comparable in each of the fibrosis groups [T235 angiotensinogen ($p = 0.68$), D angiotensin I converting enzyme ($p = 0.08$), C1166 AT1 angiotensin II receptor

($p = 0.68$)]. Patients homozygous for one or more of the renin-angiotensin system polymorphisms associated with increased renin-angiotensin activity did not exhibit more advanced liver fibrosis on liver biopsy ($p= 0.89$).

In summary, renin-angiotensin polymorphisms resulting in phenotypes with functional increases in the activity of the systemic renin-angiotensin system, and that are already recognised to influence the progression of cardiac and renal diseases, are not associated with accelerated progression of hepatic fibrosis in chronic HCV infection.

6.3 INTRODUCTION

Approximately eighty percent of patients who acquire hepatitis C virus (HCV) infection develop a chronic low-grade, slowly progressive hepatitis, which may result in cirrhosis and hepatocellular carcinoma. The natural history of the chronic liver disease caused by HCV remains controversial with varying rates of progression to cirrhosis reported (Seeff, L.B. 1998a).

Several factors have been proposed to account for the variation observed, including excess alcohol consumption, HCV genotype 1b, older age at acquisition of infection and male gender (Seeff, L.B. 1997). Despite this the exact mechanisms that predispose to a poor prognosis in chronic HCV infection remain elusive. Activated hepatic stellate cells are the main effector cells in the development of hepatic fibrosis by producing excess extracellular matrix (review: Friedman, S.L. 2000). Initiation of stellate cell activation results from the release of a number of factors from injured hepatocytes and the neighbouring Kupffer and endothelial cells. The response is enhanced by the recruitment of further stellate cells by autocrine and paracrine expression of cytokines by activated stellate cells. The most influential of these profibrotic cytokines is the autocrine expression of transforming growth factor-β1 (TGF-β1). Several polymorphisms in the transforming growth factor-β1 gene are recognised. Individuals homozygous for a proline (Pro) to arginine (Arg) substitution at codon 25 of the TGF-β1 gene have a phenotype with increased TGF-β1 activity. A recent study found an association between inheritance of the Arg / Arg TGF-β1 genotype (high TGF-β1 activity phenotype) and more severe liver fibrosis in chronic HCV infection (Powell, E.E.,

et al. 2000). Thus the variable course observed in chronic HCV infection may be accounted for by host genetic factors which promote hepatic fibrosis.

Angiotensin II is the principal effector molecule of the renin-angiotensin system. It exerts its effects by binding to two cognate receptors, known as AT1 and AT2 receptors. The primary effect of its action is mediated via the AT1 angiotensin II receptor resulting in an increase in blood pressure by increasing vascular tone and decreasing renal salt excretion. In addition activation of the renin-angiotensin system and generation of angiotensin II has been implicated in the pathogenesis of hypertension and cardiac and renal fibrosis and pharmacological angiotensin blockade is recognised to abrogate these effects (review: Border, W.A. & Noble, N.A. 1998). Studies reveal that angiotensin II strongly induces transforming growth factor-β1 production by mesangial cells, the renal equivalent of hepatic stellate cells in tissue culture and in-vivo (Kagami, S., et al. 1994). This has led investigators to conclude that the fibrogenic effects in the kidney that have been attributed to angiotensin II are actually mediated by TGF-β1 (review: Border, W.A. & Noble, N.A. 1998). Whether this process is relevant in the progression of hepatic fibrosis is unclear. A recent study by Bataller et al. demonstrated that angiotensin II binds to AT1 angiotensin II receptors on activated human hepatic stellate cells and stimulates their proliferation and contraction (Bataller, R., et al. 2000). Angiotensin II may therefore stimulate fibrogenesis in the human liver by perpetuating stellate cell activation directly or through the autocrine expression of TGF-β1 by activated hepatic stellate cells.

Many functional polymorphisms in the genes of the renin-angiotensin system have been described and these polymorphisms have been postulated to contribute to the

interindividual variability in the outcome of several renal and cardiovascular diseases including hypertension, coronary artery disease and diabetic nephropathy. Nine polymorphisms in the gene for angiotensinogen, which is cleaved by renin to produce angiotensin I, have been identified (Sato, N., et al. 2000). These include a threonine (T) for methionine (M) amino acid substitution at position 235 of the amino-acid sequence (M235T angiotensinogen polymorphism), which is associated with an increase in plasma angiotensinogen levels (Bloem, L.J. et al. 1997, Sato, N., et al. 2000). A polymorphism in the angiotensin I converting enzyme (ACE) gene, which converts angiotensin I to angiotensin II, is recognised. The absence of an insertion (D) within intron 16 in this gene is associated with increased ACE and angiotensin II levels (Rigat, B. et al. 1990). A polymorphism in the AT1 angiotensin II receptor (AT 1 R) gene is also recognised. A C to A nucleotide substitution at position 1166 within the 3 prime untranslated region of the AT 1 R (A1166C polymorphism) has been shown to be associated with increased sensitivity to physiological levels of angiotensin II (Spiering, W., et al. 2000).

The aim of the current study was to assess the prevalence of these renin-angiotensin system variants in Scottish patients with chronic HCV infection and to then explore whether those variants associated with increased renin-angiotensin activity contributed to the development of hepatic fibrosis in chronic HCV.

6.4 PATIENTS, MATERIALS AND METHODS

6.4.1 Patients

One-hundred and ninety-one patients with evidence of hepatitis C virus infection were retrospectively studied. All were anti-HCV positive with abnormal liver function tests and underwent needle biopsy of the liver. History of alcohol abuse, alcohol consumption at the time of biopsy and risk factors for HCV acquisition were established.

One hundred and nineteen (62%) were male and sixty (31%) had a history of excess alcohol consumption, defined as alcohol consumption of >24g / d (>3U / d) for males and >16g / d (>2 U / d) for females. Fifteen (8%) patients were non-Caucasian. Risk factors for acquisition of HCV infection were intravenous drug use (91 patients, 48%), blood products (47 patients, 25%), sexual transmission (8 patients), needlestick injury (5 patients), human bite (1 patient) and thirty-four (18%) patients had no identifiable risk factors for the acquisition of HCV infection. Thirty-one patients (16%) had received previous anti-viral therapy with alpha-interferon. The analysis of the role of renin-angiotensin polymorphisms on hepatic fibrosis was not influenced when patients who had previously received interferon therapy were excluded and all analysis discussed included all 191 patients. Duration of infection at the time of liver biopsy was recorded for those patients who had an identifiable year of infection. For those patients who acquired HCV through intravenous drug use, the year of infection was assumed to be the year that injecting commenced. Local data indicates that approximately half of those intravenous drug users who acquire HCV infection are infected within one year of commencing injecting and almost all are infected within three to five years

(personal communication Avril Taylor, Scottish Centre for Infection and Environmental Health).

6.4.2 HCV Determination

Anti-HCV was tested for by third generation enzyme linked immunoabsorbent assay (Ortho Diagnostics, Raritan, NJ, USA). Reactive samples were confirmed positive for anti-HCV by RIBA-3 testing assay (Ortho Diagnostics, Raritan, NJ, USA). Reverse transcription PCR was performed on patient sera using an in-house method (Dow, B.C., et al. 1993). Genotyping of HCV was performed by restriction fragment length polymorphism on RT PCR positive samples (McOmish, F., et al. 1993).

6.4.3 Histological evaluation

All liver biopsies were performed under ultrasound guidance. Formalin fixed specimens were paraffin embedded and sections stained with Haematoxylin and Eosin. Slides were then evaluated by two experienced pathologists (R. N. M. MacSween. and K. Oien) blinded to the clinical and laboratory information. All biopsy specimens were graded for the degree of necroinflammatory activity and staged for the extent of fibrosis according to the criteria of Ishak and colleagues (Ishak, K., et al. 1995). Patients were grouped according to the stage of liver fibrosis on liver biopsy: group 1 (fibrosis score 0 or 1), group 2 (fibrosis score 2 or 3) and group 3 (fibrosis score 4 to 6).

6.4.4 Testing for renin-angiotensin system polymorphisms

Polymorphisms in the renin-angiotensin system were assessed by means of polymerase chain reaction based methods. Initially genomic DNA was extracted

from 10 millilitres of whole blood collected in tubes containing potassium EDTA. After cell and nuclear lysis DNA was extracted using a standard method for salting out proteins (Miller, S.A., et al. 1988).

The mis-sense M235T amino acid substitution in the angiotensinogen gene was screened for using standard methods (Russ A.P., et al. 1993). This involves restriction enzyme digestion of the product (165 base pair) of a single round of PCR using angiotensinogen specific primers. The products of this digestion were then visualised against 1 kilobase molecular markers (Gibco-BRL) run on a 3% MetaPhor agarose gel.

The D/I polymorphism in the angiotensin I converting enzyme gene was screened for as previously described (Rigat, B., et al. 1992). Briefly the product of a single round of PCR reaction utilising angiotensin I converting enzyme gene specific primers was visualised on 1% agarose gel run against 1 kilobase molecular marker (Gibco-BRL). The PCR product is a 190 base pair fragment in the absence of the insertion (D) and a 490 base pair fragment in the presence of the 287 kilobase insertion (I) within the angiotensin I converting enzyme gene.

The AT1 angiotensin II receptor A1166C polymorphism was assessed by Dde I restriction enzyme digestion of the product of a single round of PCR using previously published primer sequences (Tiret, L., et al. 1994). The C1166 allele is detected if digestion of the PCR product is observed after visualisation against 1 kilobase molecular marker run on 3% MetaPhor agarose gel.

6.4.5 Statistical analysis

Statistical analysis of the data was performed using SigmaStat (SPSS Science Software UK Ltd, Birmingham, UK). Differences between nominal variables were analysed by Chi-square tests. Differences among continuous variables were evaluated using the Kruskal - Wallis test. Multiple linear regression analysis was applied to determine those variables independently associated with the grade and stage of liver disease. Consent to carry out this study was provided by the West Glasgow Hospitals University NHS Trust ethics committee.

6.5 RESULTS

5.5.1 Fibrosis scores

Ninety-seven patients (51%) with chronic hepatitis C virus infection had liver biopsy fibrosis scores of 0 or 1 (group 1), 70 patients (37%) had fibrosis scores of 2 or 3 (group 2) and 24 patients (13%) had fibrosis scores of 4, 5 or 6 (group 3).

6.5.2 Distribution of polymorphisms in the renin-angiotensin system

Table 6.1 summarises the distribution of the polymorphisms in the renin-angiotensin system that were studied. One hundred and eighty-seven patients (98%) carried at least one of the alleles associated with increased activity in the renin-angiotensin system. Eighty-two patients (43%) were heterozygous for at least one of these alleles, eighty-one (42%) were homozygous for one abnormal allele, 23 (12%) were homozygous for two abnormal alleles and one patient was homozygous for the three abnormal alleles studied. The allele frequencies of the T235 variant of angiotensinogen gene, the D variant of the angiotensin I converting enzyme gene and the C1166 AT1 angiotensin II receptor gene were 58.9%, 50.8% and 24.3%.

The distribution of renin-angiotensin polymorphisms in each of the fibrosis groups is shown in Figures 6.1 (M235T angiotensinogen polymorphisms), Figure 6.2 (D/I angiotensin I converting enzyme polymorphisms) and Figure 6.3 (A1166C AT 1 angiotensin II receptor polymorphisms). The distribution of the gene polymorphisms associated with increased renin-angiotensin system activity (T235, D, C1166 alleles) were comparable in each of the fibrosis groups. We examined the effect of being homozygotes for none, one or more than one of the

polymorphisms associated with increased activity of the renin-angiotensin (see Figure 6.4). There was no significant difference in the distribution of RAS polymorphisms among the fibrosis groups ($p = 0.89$).

6.5.3 Univariate and multivariate analysis of factors associated with HCV-related fibrosis

Table 6.2 summarises the demographic, clinical, virological and liver biopsy data for the 191 patients with chronic HCV who were studied. On univariate analysis the factors associated with liver fibrosis were the severity of necroinflammatory activity on liver biopsy (<0.001), older age at the time of acquisition of infection ($p = 0.04$) and liver biopsy ($p = 0.002$), non-Caucasian ethnic origin ($p = 0.002$) and a past history of excess alcohol consumption ($p = 0.014$).

Multivariate analysis was performed to identify factors predictive of the Ishak fibrosis score on liver biopsy. Multiple linear regression analysis was performed using a model that included the presence of the three gene polymorphisms associated with increased renin-angiotensin activity, the patients ethnic origin, gender, age at the time of liver biopsy, age and route of acquisition of HCV infection, duration of HCV infection, alcohol consumption at the time of liver biopsy, past history of excess alcohol consumption, HCV genotype and necroinflammatory score on liver biopsy. The factors significantly associated with fibrosis were the necroinflammatory activity on liver biopsy ($p < 0.001$) and a history of excess alcohol consumption ($p = 0.01$).

6.6 DISCUSSION

The natural history of chronic HCV-related liver injury remains uncertain and the mechanisms accounting for the variable rate of fibrosis progression observed are poorly understood. This study was designed specifically to explore the role of functional polymorphisms in the renin-angiotensin system on the rate of hepatic fibrogenesis in chronic HCV. It is well recognised that patients with chronic liver disease, even in the absence of cirrhosis and ascites, have activation of the systemic renin-angiotensin system (Schweisfurth, H. & Wernze, H. 1979, Wernze, E., et al. 1978). This is widely accepted to represent a homeostatic response to counterbalance the vasodilation, arterial hypotension, and renal hypoperfusion observed in portal hypertension. More recently Bataller and colleagues demonstrated that activated hepatic stellate cells possess AT1 angiotensin II receptors and that the binding of angiotensin II to these receptors induces contraction and proliferation of hepatic stellate cells (Bataller, R., et al. 2000). This has led to speculation that the activated renin-angiotensin system in chronic liver disease could play a role in the response to liver injury by amplifying stellate cell proliferation and activation directly. This process has been observed in cardiac and renal tissues where angiotensin II induced production of transforming growth factor-β1 has been shown to be associated with the progression of fibrotic disease (Border, W.A. & Noble, N.A. 1998).

Currently there is no data from human studies to support a fibrogenic role for angiotensin II in the liver, however there is increasing evidence for this role in animal studies. Wei and colleagues have demonstrated that primary cultured rat hepatic stellate cells possess AT1 angiotensin II receptors but only when they are

activated (Wei, H.S., et al. 2000). In a model of hepatic fibrosis induced by carbon tetrachloride, they proceeded to demonstrate that angiotensin II induced rat hepatic stellate cell proliferation and collagen synthesis and that this effect was abrogated by the angiotensin I converting enzyme inhibitor enalapril and the AT1 angiotensin II receptor antagonist losartan. In a similar study, Jonsson and colleagues demonstrated that four weeks of treatment with the angiotensin I converting enzyme inhibitor captopril significantly reduced the intrahepatic production of transforming growth factor-β1 and liver fibrosis in a rat bile duct ligation model of hepatic fibrosis (Jonsson, J.R., et al. 2001). These findings are similar to studies of renal fibrosis, with angiotensin II binding to activated stellate cells stimulating the production of extracellular matrix and that this effect can be abrogated by pharmacological angiotensin blockade with reduced production of TGF-β1 and collagen. This contrasts with an earlier study where no fibrogenic response was observed in rat liver after a continuous systemic infusion of angiotensin II for one week, while significant cardiac and renal fibrosis were detected (Yoo, K.H., et al. 1998). These conflicting results can be explained by the finding from both human (Bataller, R., et al. 2000) and rat studies (Wei, H.S., et al. 2000) that AT1 angiotensin II receptors are only expressed on activated hepatic stellate cells. In the absence of initial liver cell injury, hepatic stellate cell activation will not occur and angiotensin II receptors will not be expressed.

If the evidence from these animal studies can be extrapolated to human disease, it can be speculated that systemic activation of the renin-angiotensin system influences the progression of liver fibrosis in chronic liver disease and pharmacological inhibition of this system may prevent progression of fibrosis. Studies of the effects of angiotensin I converting enzyme inhibitors and AT1

angiotensin II receptor antagonists on hepatic fibrogenesis in chronic liver disease are required. However it will take a number of years before the results of prospective studies with prolonged follow-up are available.

This study was designed to study the role of the renin-angiotensin system in the progression of chronic liver disease using a different approach. Many genetic polymorphisms in the renin-angiotensin system exist and several of these are recognised to account for interindividual variation in renin-angiotensin system activity (Bloem, L.J. et al. 1997, Sato, N., et al. 2000, Rigat, B., et al. 1990, Spiering, W., et al. 2000) and, probably as a result, the development and outcomes of hypertension (Winkelman, B.R., et al. 1999), coronary artery disease (Tiret, L., et al. 1994) and renal diseases (Kennon, B., et al. 1999). We hypothesised that if the renin-angiotensin system played a significant role in the progression of hepatic fibrosis in chronic liver disease, those patients possessing renin-angiotensin genotypes associated with increased renin-angiotensin system activity would develop more severe hepatic fibrosis and that this mechanism might account for the variable rate of progression observed in chronic HCV infection. We studied three alleles already recognised to be associated with an increased renin-angiotensin activity phenotype (T235 angiotensinogen allele, D angiotensin I converting enzyme allele, C1166 AT1 angiotensin II receptor allele) and found they had no effect on hepatic fibrosis scores either alone or in combination. In the only other similar study to be performed Powell et al. examined the role of the D angiotensin I converting enzyme allele and a nucleotide substitution (G to A) 6 base pairs from the transcription start site in the promoter of angiotensinogen (AT-6) on the progression of chronic HCV (Powell, E.E., et al. 2000). They found homozygosity for the A substitution at position 6 of the angiotensinogen gene to be

significantly associated with progressive hepatic fibrosis while no association with the D allele of the angiotensin I converting enzyme was detected.

The lack of association between renin-angiotensin genotypes and hepatic fibrosis in our study does not rule out a role for the renin-angiotensin system in the progression of hepatic fibrosis in humans. Prospective studies of angiotensin I converting enzyme inhibitors and AT1 angiotensin II receptor antagonists in patients with chronic liver disease are required to establish whether the mechanisms speculated on in this study are clinically relevant. Recent publications have highlighted the hazards of these therapies in patients who have established cirrhosis and portal hypertension, where pharmacological inhibition of the renin-angiotensin system resulted in arterial hypotension and reduced glomerular filtration rates (Gonzalez-Abraldes, J., et al. 2001, Schepke, M., et al. 2001). As a result these treatments should be studied in patients with mild chronic liver disease without evidence of portal hypertension and long-term follow-up would be required to establish an effect on fibrosis progression.

On multivariate analysis the only factors observed to be associated with fibrosis progression in chronic HCV infection were a history of excess alcohol consumption and the severity of necroinflammatory activity on liver biopsy. The role of excess alcohol consumption on fibrogenesis in chronic HCV is already well established (Poynard, T., et al. 1997, Corrao, G. & Arico, S. 1998). The association between necroinflammatory activity and fibrosis on liver biopsy has been observed previously in chronic hepatitis (MacSween, R.N.M. 1980, Cooksley, W.G.E. 1986). However the severity of necroinflammatory activity on biopsy is not

predictive of the subsequent development of cirrhosis in chronic HCV (Paradis, V., et al. 1996, Yano, M., et al. 1996).

In summary genetic polymorphisms in the renin-angiotensin system do not account for the variable rate of progression observed in chronic HCV infection and an effect of the renin-angiotensin system on hepatic fibrogenesis is not established. Further study is required to identify the mechanisms of hepatic fibrogenesis in chronic HCV infection and to establish if host genetic variability contributes to the variable rates of progression observed.

Table 6.1 Distribution of polymorphisms in the renin-angiotensin system in one hundred and ninety-one patients with chronic hepatitis C virus infection who underwent liver biopsy staging.

Renin-Angiotensin System Genotypes	Renin-Angiotensin System Phenotype			
	Low Activity	Intermediate Activity	High Activity	Total
M235T Angiotensinogen polymorphism	MM	MT	TT	
No. of patients (%)	30 (16%)	96 (51%)	64 (34%)	190 *
D/I Angiotensin I converting enzyme polymorphism	II	DI	DD	
No. of patients (%)	47 (25%)	90 (47%)	54 (26%)	191
A1166C AT1 Angiotensin II Receptor polymorphism	AA	AC	CC	
No. of patients (%)	110 (58%)	69 (36%)	12 (6%)	191

* It was not possible to assess the angiotensinogen genotype of one of the patients

Table 6.2 Demographic, clinical and virological data for one hundred and ninety-one patients with chronic hepatitis C virus infection grouped according to liver biopsy fibrosis score. All figures median and range unless otherwise stated.

Fibrosis score	Fibrosis Group			
	Group 1 (n = 97)	Group 2 (n = 70)	Group 3 (n = 24)	p value
Stage of fibrosis	0 1	2 3	4 5 6	
No. of patients	27 70	40 30	7 5 12	
Grade of Inflammation (0 - 18)	4 (0 – 7)	6 (2 – 10)	7 (1 – 15)	<0.001
Age at the time of liver biopsy (Years)	34 (22 – 60)	38 (23 – 70)	43 (30 – 61)	0.002
Gender (Male:Female)	56 : 41	49 : 21	14 : 10	0.25
Ethnic origin (No. Non-Caucasian, %)	1 (1%)	10 (14%)	4 (17%)	0.002
Age at acquisition of HCV (Years)	20 (13 – 51)	23 (3 – 64)	24 (19 – 47)	0.04
Duration of HCV infection at time of biopsy (Years)	13 (1 – 32)	15 (3 – 42)	14 (3 – 39)	0.46
Alcohol consumption at biopsy (Units/wk)	4 (0 – 112)	6 (0 – 280)	0 (0 – 140)	0.19
Past history of excess alcohol consumption [no. (%)]	24 (25%)	23 (33%)	13 (54%)	0.014
Previous interferon therapy [no. (%)]	18 (19%)	9 (13%)	4 (17%)	0.50

Table 6.2 (Continued) Demographic, clinical and virological data for all 191 patients with chronic hepatitis C virus infection grouped according to liver biopsy fibrosis score. All figures median and range unless otherwise stated.

Fibrosis score		Fibrosis Group			
		Group 1 (n = 97)	Group 2 (n = 70)	Group 3 (n = 24)	p value
Route of HCV infection (no.)					0.52
	IVDU / Needlestick injury	45	39	12	
	Blood products	29	13	5	
	Other	23	18	7	
Genotype (no.)	1	45	25	6	0.27
	2	8	9	1	
	3	39	32	15	
	4	1	0	0	
	Untyped	4	4	2	

Figure 6.1 Distribution of the M235T angiotensinogen polymorphism in one hundred and ninety patients with chronic hepatitis C virus infection grouped by liver biopsy Ishak fibrosis scores [Ishak fibrosis score 0 or 1 (Group 1), 2 or 3 (Group 2) and 4 to 6 (Group 3)]. The prevalence of the T235 angiotensinogen variant was comparable in each of the three fibrosis groups (p = 0.68).

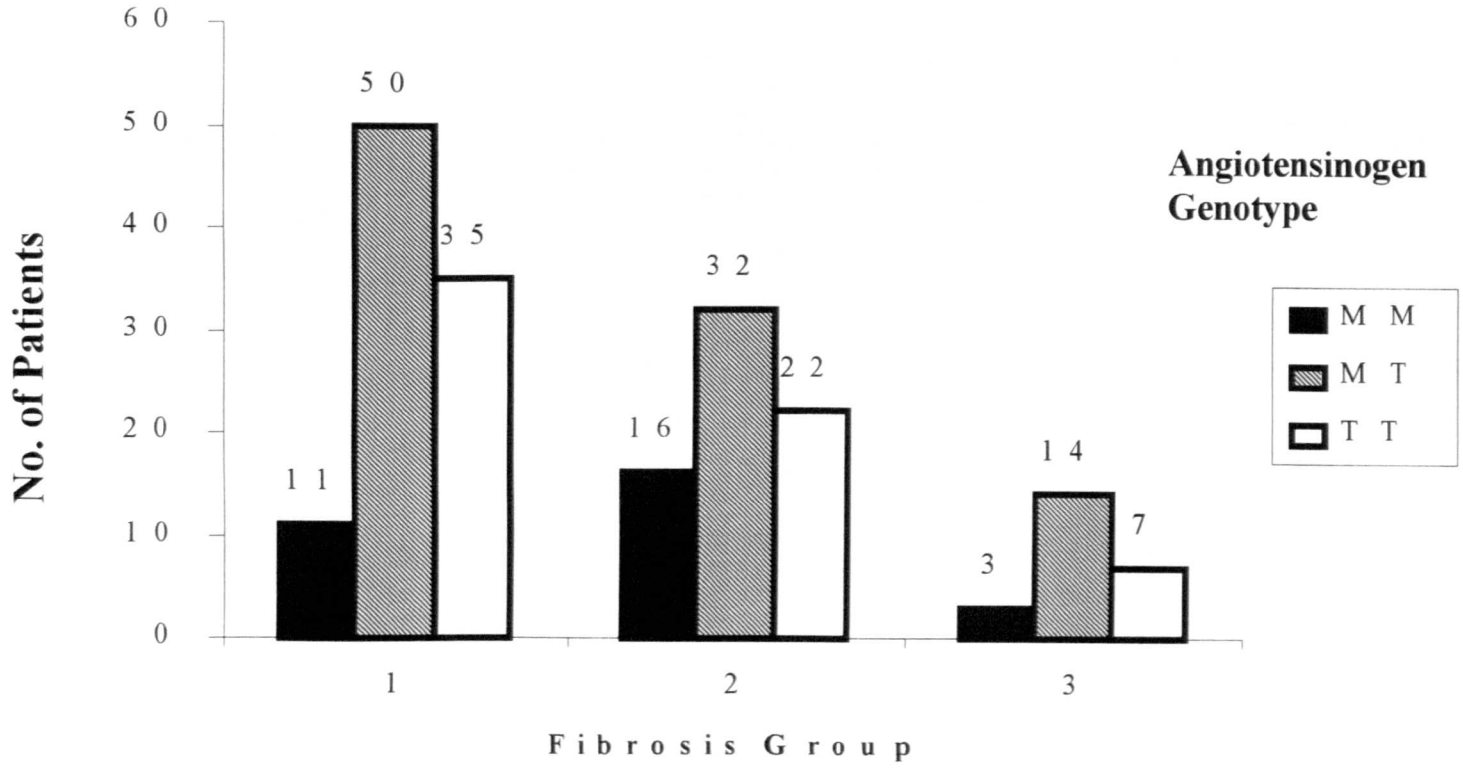


Figure 6.2 Distribution of the deletion (D) or insertion (I) variants of the angiotensin I converting enzyme (ACE) gene in one hundred and ninety-one patients with chronic hepatitis C virus infection grouped by liver biopsy Ishak fibrosis score [Ishak fibrosis score 0 or 1 (Group 1), 2 or 3 (Group 2) and 4 to 6 (Group 3)]. The prevalence of the D allele was comparable in each of the three fibrosis groups (p = 0.08).

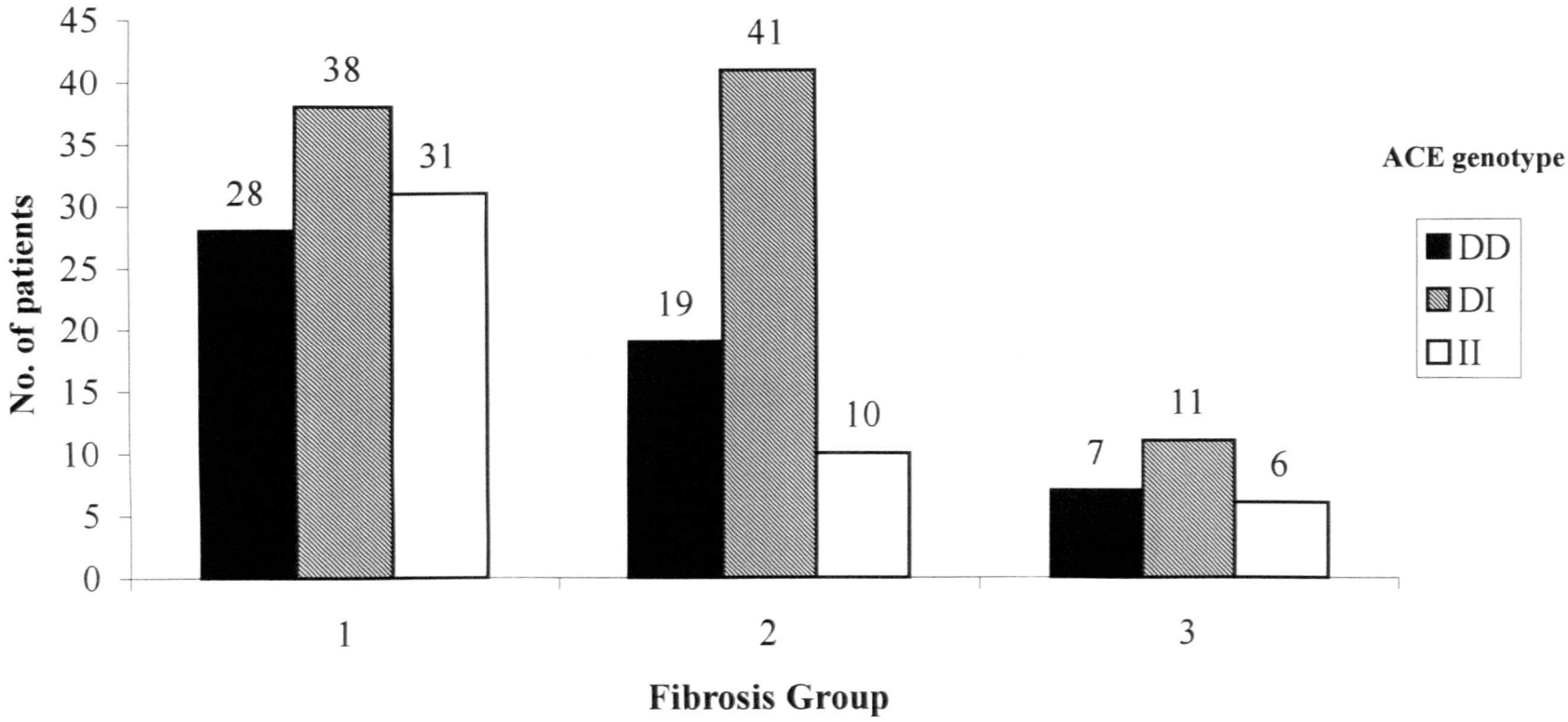


Figure 6.3 Distribution of the A1166C AT1 angiotensin II receptor (AT 1 R) polymorphism in one hundred and ninety-one patients with chronic hepatitis C virus infection grouped by liver biopsy Ishak fibrosis score [Ishak fibrosis score 0 or 1 (Group 1), 2 or 3 (Group 2) and 4 to 6 (Group 3)]. The prevalence of the C1166 allele was comparable in each of the three fibrosis groups (p = 0.68).

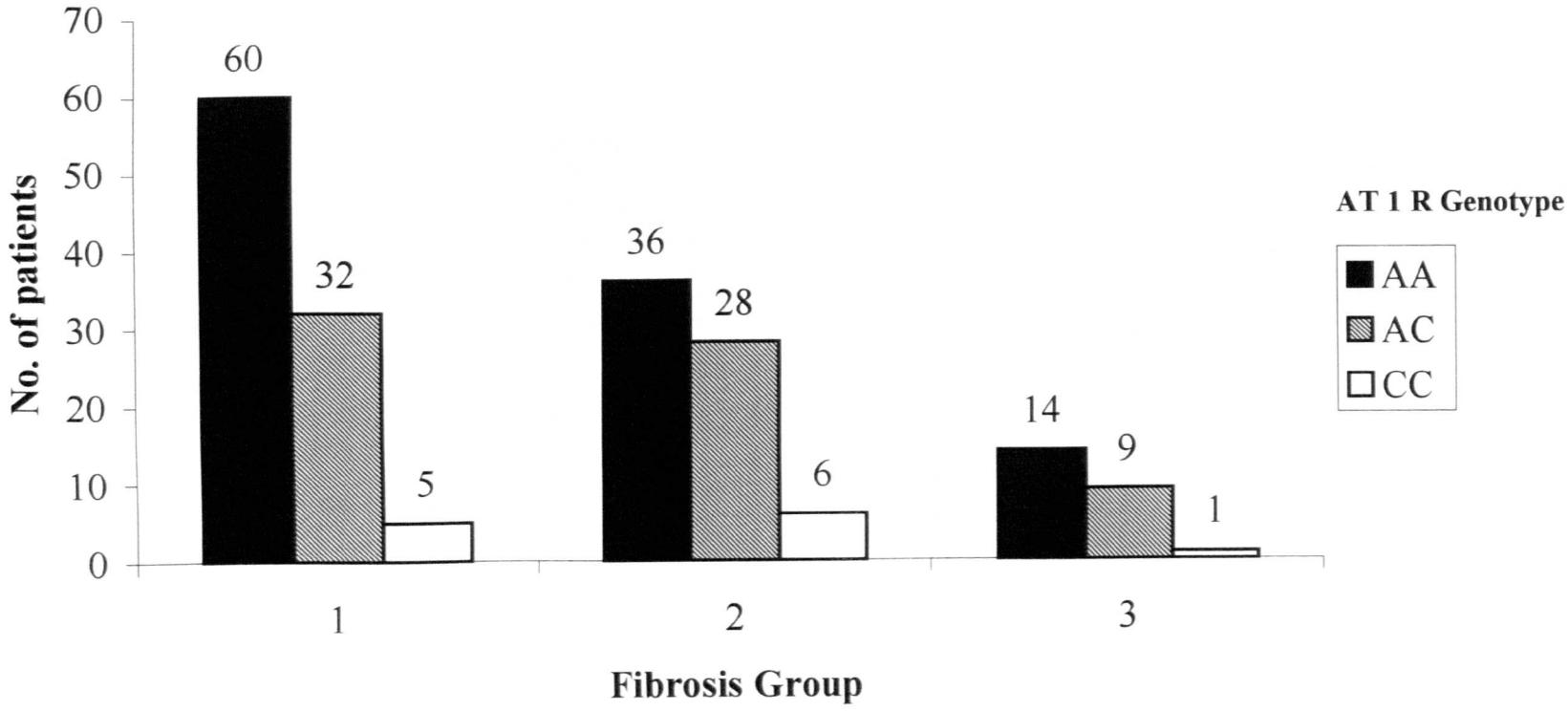
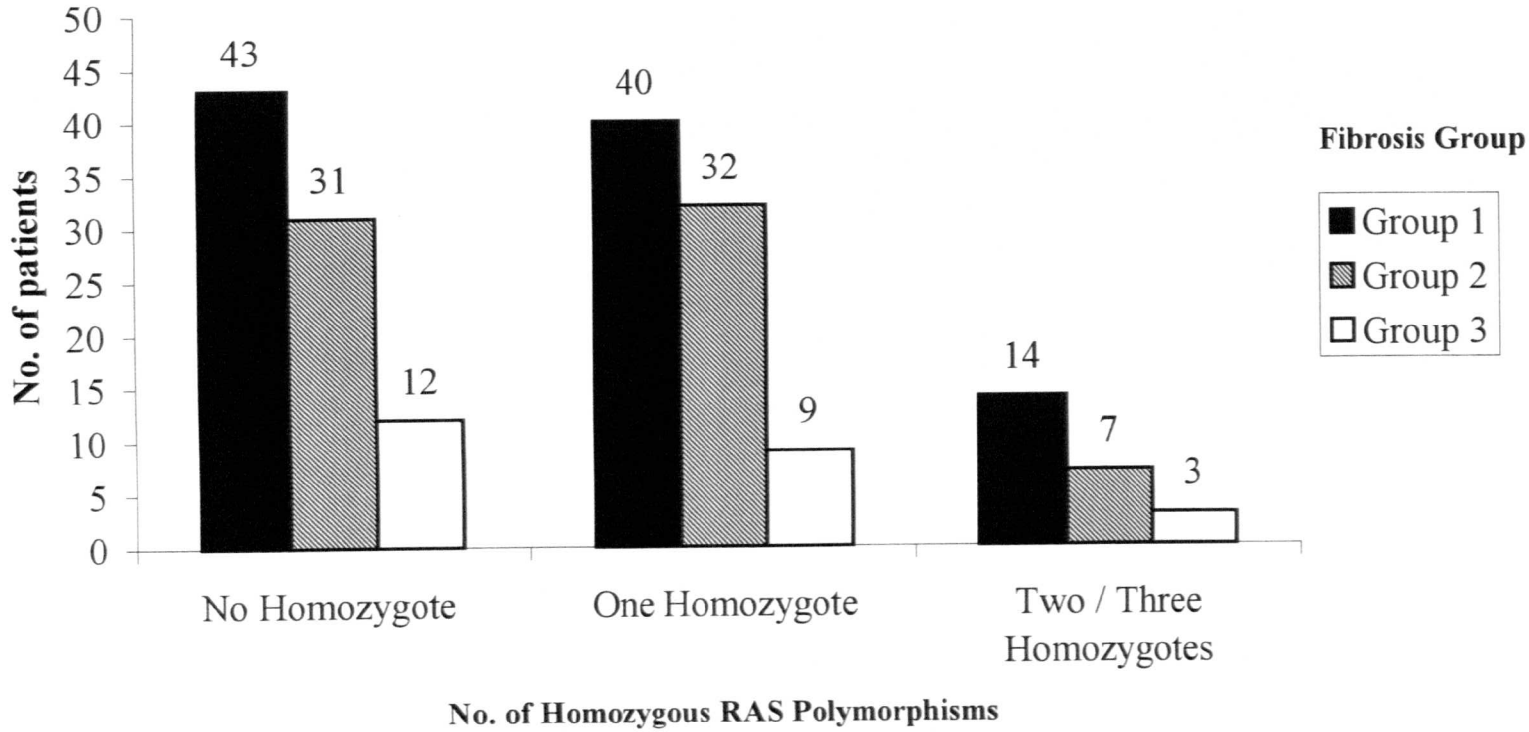


Figure 6.4 Distribution of liver biopsy Ishak fibrosis scores [0 or 1 (Group 1), 2 or 3 (Group 2) and 4 to 6 (Group 3)] in one-hundred and ninety one patients with chronic hepatitis C virus infection homozygous for none, one and two or more of the renin-angiotensin system (RAS) polymorphisms associated with increased activity of the renin-angiotensin system which were studied. There was no significant difference between the groups (p = 0.89).



CHAPTER 7

THE ROLE OF HEPATITIS G VIRUS CO-INFECTION ON THE SEVERITY OF HEPATITIS C RELATED LIVER INJURY

7.1 CONTENTS

7.2 SUMMARY

Following the discovery of the GB virus-C (GBV-C) or hepatitis G virus (HGV) in 1995 there has been much interest in whether it is the cause of significant liver disease alone or in combination with other known hepatitis viruses. The aim of this study was to assess the prevalence and risk factors for the acquisition of HGV in a group of asymptomatic blood donors found to have hepatitis C virus (HCV) infection at blood donation. Thereafter we compared the grade and stage of chronic hepatitis on liver biopsy in patients with and without HGV to assess the effect of HGV co-infection on the severity of liver disease in these HCV infected individuals. We studied 40 asymptomatic blood donors with serological evidence of HCV infection (ELISA-2 and RIBA-2 positive) and chronic hepatitis on liver biopsy. Full biochemical and haematological assessment was performed on the day of liver biopsy. Retrospective polymerase chain reaction testing of serum stored on the day of liver biopsy was performed for HCV and HGV RNA. The severity of chronic hepatitis on liver biopsy was graded and staged by a pathologist blinded to the HGV status of the patient.

HCV and HGV RNA were detectable in the serum of 37 (92%) and 7 (17%) respectively of the forty patients studied. Patients co-infected with HGV did not differ from those with serological evidence of HCV infection alone with regard to demographics and risk factors for acquisition of viral hepatitis. The severity of liver disease in both groups was comparable when assessed by standard liver function tests and by the frequency of abnormalities on standard liver function testing. There was no significant difference in the grade or stage of liver disease on

liver biopsy between patients with HCV infection alone and those with HGV co-infection.

Although HGV co-infection was observed frequently in these asymptomatic blood donors with HCV infection there was no histological or biochemical evidence that it influenced to the severity of HCV related liver injury.

7.3 INTRODUCTION

Since the discovery of the hepatitis C virus (HCV) there has been a continuing search for causes of non-A, non-B, non-C viral hepatitis agents. Hepatitis C virus infection accounts for up to one-third of community-acquired hepatitis and the majority of the post-transfusion associated non-A, non-B hepatitis however there remains a proportion of patients with these disease states who have negative serum markers for the known hepatitis viruses (A to E) (Rall, C. & Deinstag, J. 1995). In the early nineties two groups working independently identified novel flavivirus-like viral sequences from the serum of two patients with unexplained, putatively viral hepatitis. Using representational differential analysis, the full sequence of these viruses has been identified and named hepatitis G virus (HGV) and GB virus-C (GBV-C) virus respectively (Simons, J.N., et al. 1995, Linnen, J., et al. 1996). Characterisation of the full-length cloned genomic sequence of both viruses allowed them to be compared directly. Initial comparison of the NS3 helicase region of HGV and GBV-C sequences indicated that they were closely related (Linnen, J., et al. 1996). Direct comparison of the complete genomes revealed homology of 86% at the nucleotide level and greater than 95% amino acid sequence identity confirming them to be independent isolates of the same RNA virus from the Flaviviridiae family (Zuckerman, A.J. 1996). For clarity GBV-C and HGV are collectively referred to as HGV in this thesis.

In the initial report by Linnen et al. there was clear evidence that HGV virus was transmitted by transfusion to three patients who went on to develop post-transfusion fhepatitis (Linnen, J., et al. 1996). They proceeded to study patients with chronic liver disease and found that 19% (18 of 96 patients) of patients with

chronic HCV infection were co-infected with HGV. In that study they did not report what effect co-infection with HCV and HGV had on the severity and progression of HCV related liver injury.

The aim of this study was to assess the prevalence of HGV infection in asymptomatic blood donors already known to be infected with HCV. The role of HGV infection on the natural history of chronic HCV infection could then be explored to see whether co-infection with HGV could contribute to the variable rate of progression observed in patients with chronic HCV infection (Seeff, L.B. 1998a).

7.4 PATIENTS, MATERIALS AND METHODS

7.4.1 Patients

Between September 1991 and September 1993, 305,012 voluntary blood donations were processed by the Glasgow and West of Scotland Blood Transfusion Service and screened for anti-hepatitis C antibody. Seven hundred and twenty one units were reactive by second generation ELISA (Ortho Diagnostics, High Wycombe, UK). Two hundred and fifty one (35%) of these were also positive by supplemental recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryvilie, Ca, USA) with two or more antigens positive. These patients were counselled by the Blood Transfusion Service and referred for further investigation at a specialist Gastroenterology clinic at Gartnavel General Hospital. A full assessment of risk factors for acquiring hepatitis C infection was performed for each patient. Other causes of chronic liver disease were sought and excluded in the laboratory. All patients that attended the clinic were further investigated by liver biopsy regardless of the presence of liver function abnormalities.

Forty of these asymptomatic blood donors with serological evidence of hepatitis C virus infection and histological evidence of chronic hepatitis were screened retrospectively for hepatitis G virus infection by reverse transcriptase polymerase chain reaction.

7.4.2 Patient Assessment

Each patient underwent percutaneous liver biopsy. Formalin fixed, paraffin embedded liver sections were assessed by a single pathologist (R.N.M.

MacSween) using Ishak's modified histological activity index (Ishak, K., et al. 1995). The pathologist was blinded to the patient's HGV status.

Haematological and biochemical assessments were performed using routine laboratory methods on the day of liver biopsy. Additional sera which had been collected on the day of biopsy, was separated within four hours of collection and stored at -20°C to allow retrospective detection of HCV and HGV RNA by reverse transcription polymerase chain reaction (RT-PCR), which was not available locally at the start of this study.

7.4.3 HCV determination

All patients studied were reactive by second generation ELISA (Ortho Diagnostics, High Wycombe, UK) on initial screening and positive on supplemental testing by recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryville, Ca, USA) with two or more antigens positive. These tests were repeated and confirmed to be positive prior to inclusion in this study.

HCV RNA was detected using an in-house RT PCR method with primers for the 5' non-coding region of the HCV genome. RT PCR was performed as described (Dow, B.C., et al. 1993). This technique performed well in the EUROHEP HCV-RNA reference panel study (Damen, M., et al. 1996). HCV genotyping was performed on sera stored prior to treatment by restriction fragment polymorphism as previously described (McOmish, F., et al. 1993).

7.4.4 HGV determination

Screening for HGV RNA in serum was performed retrospectively on stored serum by RT-PCR with primers for the NS3 helicase region of the HGV genome (Heringlake, S., et al. 1996) (Figure 7.1). This method was developed and performed by me in the Institute of Virology at the University of Glasgow and is summarised here. The reaction mix for the reverse transcription (RT) step and the first and second round of polymerase chain reaction (PCR) are shown in Appendix 4.

RNA was extracted from 140 µl serum using a commercial spin column system for RNA isolation from liquid samples (QIAmp HCV-kit, Qiagen) according to the manufacturers recommendations. In a final step isolated RNA was eluted in 50 µl of diethylpyrocarbonate (DEPC) treated water (pre-heated to 80°C) from the spin columns (also pre-heated for 5 min. to 80°C). For reverse transcription 10 µl RNA and anti-sense primer HGV-G9 were heated at 70°C for 10 min, chilled quickly on ice, and converted to complementary DNA (cDNA) with reverse transcriptase (Superscript, GIBCO BRL) in a total volume of 20 µl. After one hour at 37°C the RT-reaction was stopped by heating to 95°C for 10 min. Five micro litres of cDNA was subjected to the first round of PCR using antisense primer HGV-G9, sense primer HGV-G7, and TAQ-DNA polymerase (GIBCO BRL) in a total volume of 25 µl. The final Mg-concentration for the reaction was 1mM. A touchdown PCR protocol was performed in the first 17 cycles [94°C – 2 min. (94°C – 30 sec, 60°C – 30 sec, 72°C – 45 sec)] reducing the annealing temperature 0.4°C per cycle, followed by 25 cycles with a constant annealing temperature of 53°C and a final extension step of 72°C for 7 minutes. One tenth (2.5 µl) of the product of the first

PCR reaction was subjected to a second semi-nested PCR with the same sense primer and an inner antisense primer HGV-G11 using a similar touchdown PCR protocol for 15 cycles [94°C – 2 min. (94°C – 30 sec, 61°C – 30 sec, 72°C – 45 sec)] reducing the annealing temperature 0.4°C per cycle followed by 25 cycles with a constant annealing temperature of 55°C and a final extension step at 72°C for 10 minutes. All RT and PCR reactions were performed using a Biometra Thermoblock.

A PCR product of 140 base pairs was detectable after electrophoresis on a 2% agarose gel against a 1 in 100 dilution of known HGV positive control serum (kindly provided by L. Jarvis and P. Simmonds, The Department of Medical Microbiology, The University of Edinburgh, Teviot Pl, Edinburgh) and molecular marker V (Boehringer-Mannheim). A sample of DEPC-treated water instead of RNA served as a negative control during sample preparation, cDNA synthesis and PCR amplification. Serum samples for each patient were analysed in two independent RT PCR runs. Primer sequences were derived from the published sequences of the NS3 region of the GBV-C prototype and the published isolates (GenBank accession numbers U23338 – U25545).

7.4.5 Statistical analysis and Ethics Approval

Statisitical analyis for the study was performed using SigmaStat (SPSS Science Software UK Ltd, Birmingham, UK). Comparison of those patients with and without HGV infection were made using Chi-square test or Fisher exact test when variables were nominal and using the t-test or Kruskal - Wallis test when continuous variables were evaluated. Consent to carry out this study was provided by the West Glasgow Hospitals University NHS Trust ethics committee.

7.5 RESULTS

7.5.1 Patient demographics

Thirty-seven (92%) of the forty patients studied had HCV RNA detectable on retrospective testing of serum obtained on the day of liver biopsy. Seven (17%) patients had HGV RNA detectable in serum. All three patients who had antibodies to HCV, chronic hepatitis on liver biopsy and had no detectable HCV RNA in serum were RT PCR negative for HGV RNA. Co-infected patients were comparable to those patients infected with HCV alone with regard to demographics, risk factors for parenteral transmission and prevalence of HCV genotypes (Table 7.1). Co-infected patients had a history of jaundice more frequently although this difference was not statistically significant (43% v 18%, p = 0.36).

7.5.2 Laboratory assessment

The biochemical and haematological assessment of samples obtained on the day of liver biopsy are summarised in Table 7.2. There was no significant difference in any of these parameters between the two groups. When the three patients who did not have HCV RNA detectable in serum (and had normal liver function tests) were excluded from the analysis there remained no significant difference in liver biochemistry and haematology between the two groups. The prevalence of abnormal liver function tests was comparable in the two groups when all patients and only those with detectable HCV RNA in serum were studied (Table 7.3).

7.5.3 Liver histology

The grade and stage of liver disease in patients infected with HCV alone and patients co-infected with HGV are shown in Figure 7.2. There was no significant difference in the grade or stage of liver disease on liver biopsy between the two groups. There remained no significant difference in necroinflammatory and fibrosis scores between the groups if patients who had no HCV RNA detectable in serum at the time of biopsy were excluded ($p = 0.53$ and $p = 0.23$ respectively).

7.6 DISCUSSION

In this study, we demonstrated that seven (17%) of 40 asymptomatic blood donors with serological evidence of HCV infection and chronic hepatitis on liver biopsy had stored serum samples positive for HGV RNA by RT PCR. This rate is comparable to the range of HGV RNA prevalence reported in HCV infected populations in other countries, such as England (15%), France (19%), Spain (28%), the United States of America (10% - 20%) and Australia (19%) (Petrik, J., et al. 1998, Bouchardeau, F., et al. 2000, Sauleda, S., et al. 1999, Slimane, S.B., et al. 2000, Brandhagen, D.J., et al. 1999, Lin, R., et al. 1998). In contrast the frequency of detectable HGV RNA in the plasma of healthy HCV negative non-remunerated blood donors in the East Coast of Scotland was 2.25% (23 of 1020 tested) (Blair, C.S., et al. 1998). Parenteral routes are the most important mode of HGV transmission (Nubling, C.M. & Lower, J. 1996) and there is evidence that intravenous drug users are at particular risk (Diamantis, I., et al. 1997). The increased prevalence of HGV observed in HCV infected individuals probably reflects the presence of risk factors for parenteral transmission of viruses in these individuals who would be excluded from blood donation. The identification of HGV RNA by RT PCR has limitations as a method of diagnosing HGV infection and only identifies patient who are viraemic at the time of testing. When serological methods for the detection of antibodies to the envelope protein E2 of HGV are employed to screen for past exposure to HGV in HCV infected patients rates of 40 - 50% are reported (Bouchardeau, F., et al. 2000, Sauleda, S., et al. 1999, Enomoto, M., et al. 1998). The prevalence of serological evidence of HGV infection in the Scottish blood donor population has not been established. Nevertheless the prevalence of HGV RNA in the blood donor population is

significantly higher than the prevalence of antibodies to HCV observed in blood donations in the West of Scotland (0.31% of donations) on the introduction of blood donor screening (Dow, B.C., et al. 1993). Given that HGV infection is common in the asymptomatic blood donor population and in HCV infected populations it is important to establish the clinical consequences of HGV infection.

Linnen at al showed that HGV was transmissible by transfusion and was associated with post-transfusion hepatitis in three patients. This does not establish HGV as the cause of post transfusion hepatitis in these patients. They proceeded to screen blood donors for HGV RNA and found comparable rates of detectable HGV RNA in the serum of blood donors with normal and those with elevated alanine aminotransferase levels (1.7% v 1.5%) (Linnen, J., et al. 1996). Further studies have failed to show an association between HGV infection alone and community acquired acute and chronic non-A-E hepatitis (Alter, H.J., et al. 1997, Alter, M.J., et al. 1997). In this study there was no evidence that HGV infection, assessed by the detection of HGV RNA in serum, was associated with biochemical or histological exacerbation of liver disease in asymptomatic blood donors with HCV infection. This is consistent with other reports (Alter, H.J., et al. 1997, Alter, M.J., et al. 1997, Slimane, S.B. et al. 2000, Lin, R. et al. 1998). There is therefore little evidence that HGV infection causes acute or chronic liver disease alone or in combination with other hepatitis viruses. Whether HGV is even a hepatotropic virus is now controversial. To allow replication in vivo it is essential for the positive stranded RNA of HGV to be converted to a negative stranded replicative template. Several groups have attempted unsuccessfully to show the presence of the negative strand of HGV RNA in human hepatocytes (Handa, A. & Brown, K.E. 2000, Shindo, M., et al. 1999, Kobayashi, M., et al. 1999, Mellor, J., et al. 1998,

Laskus, T., et al. 1997a, Laskus, T., et al 1997b) despite evidence of the positive strand of HGV RNA at low titres (Laskus, T., et al. 1997a, Shindo, M., et al. 1999, Mellor, J., et al. 1998). Seipp and colleagues first identified the replicative template of HGV in human hepatocytes using PCR based methods and in-situ hybridisation (Seipp, S., et al. 1999). This work has subsequently been reproduced by Halasz et al. using in-situ hybridisation in human hepatocytes from HGV infected patients without histological evidence of liver disease (Halasz, R., et al 2000). This suggests that even if HGV replication is occurring within hepatocytes it does not result in significant hepatitis and further questions the role of HGV as a hepatitis virus. It remains possible that HGV is more like cytomegalovirus, causing hepatitis only occasionally.

In summary although HGV co-infection was common in these HCV infected individuals due to shared risk factors, there was no evidence that HGV infection influenced HCV related liver disease. This is consistent with the findings of other investigators. In the absence of clinical and laboratory evidence that HGV is a hepatotropic virus or the cause of hepatitis in humans the significance of infection with HGV remains poorly understood. Certainly the variable rates of progression observed in HCV related liver disease cannot be explained by HGV co-infection.

Table 7.1 Demographics of patients infected with the hepatitis C virus (HCV) alone compared with those co-infected with HCV and hepatitis G virus (HGV). There was no significant difference between the patient groups ($p>0.05$)

Clinical Features	HCV alone (n = 33)		HGV and HCV co-infection (n = 7)	
Age, median years (range)	39	(26 – 53)	35	(29 – 44)
Sex, *N* males (%)	20	(61)	4	(57)
History of excess alcohol consumption, *N* (%)	2	(6)	1	(14)
Alcohol consumption at time of biopsy, median U / wk (range)	10	(0 – 100)	4	(0 – 175)
History of jaundice, *N* (%)	6	(18)	3	(43)
Source of hepatitis, *N* (%)				
Transfusion	13	(39)	2	(29)
Parenteral drug use	12	(36)	1	(14)
Occupational exposure	1	(3)	0	(0)
Tattoo / Body piercing	19	(58)	5	(71)
Unknown	5	(15)	1	(14)
HCV RNA RT-PCR negative, *N* (%)	3	(9)	0	(0)
HCV Genotyping *N*	30		7	
1 *N* (%)	14	(47)	2	(29)
2 *N* (%)	3	(10)	2	(29)
3 *N* (%)	13	(43)	3	(43)

Table 7.2 Laboratory assessment of disease severity in those patients infected with the hepatitis C virus (HCV) alone compared with those co-infected with HCV and hepatitis G virus (HGV). There was no significant difference for any parameter between the patient groups ($p>0.05$)

Median (Range)	HCV alone (n = 33)		HGV and HCV co-infection (n = 7)	
ALT (U/L)	115	(13 – 470)	91	(11 – 175)
AST (U/L)	56	(18 – 141)	63	(22 – 127)
Total bilirubin (μmol)	10	(1 – 27)	10	(3 – 17)
Albumin (g/L)	44	(40 – 51)	44	(40 – 51)
Gamma GT (U/L)	67	(7 – 378)	44	(7 – 378)
Prothrombin time (sec)	15	(12 – 18)	15	(14 – 18)
Leukocyte count ($x10^9$/L)	6.3	(3.5 – 9.7)	6	(3.5 – 11.1)
Haemoglobin (g/dL)	14.7	(12.2 – 17.4)	14.6	(11.8 – 16.2)
Platelet count ($x10^9$/L)	236	(125 – 424)	208	(142 – 297)

Normal ranges: Albumin 36-50 g/L, ALT 10-50 U/L, AST 10-35 U/L, Total bilirubin 3-18 μmol, Gamma GT 5-50 U/L, Haemoglobin 13-18 g/dL, Leukocyte count 4.5-6.5 $x10^9$/L, Platelet count 140-450 $x10^9$/L, Prothrombin time 15 sec.

Table 7.3 Prevalence of abnormal liver function tests in patients with hepatitis C virus (HCV) infection alone and with hepatitis G virus (HGV) co-infection. There was no significant difference in the prevalence of abnormal liver function tests between the two groups.

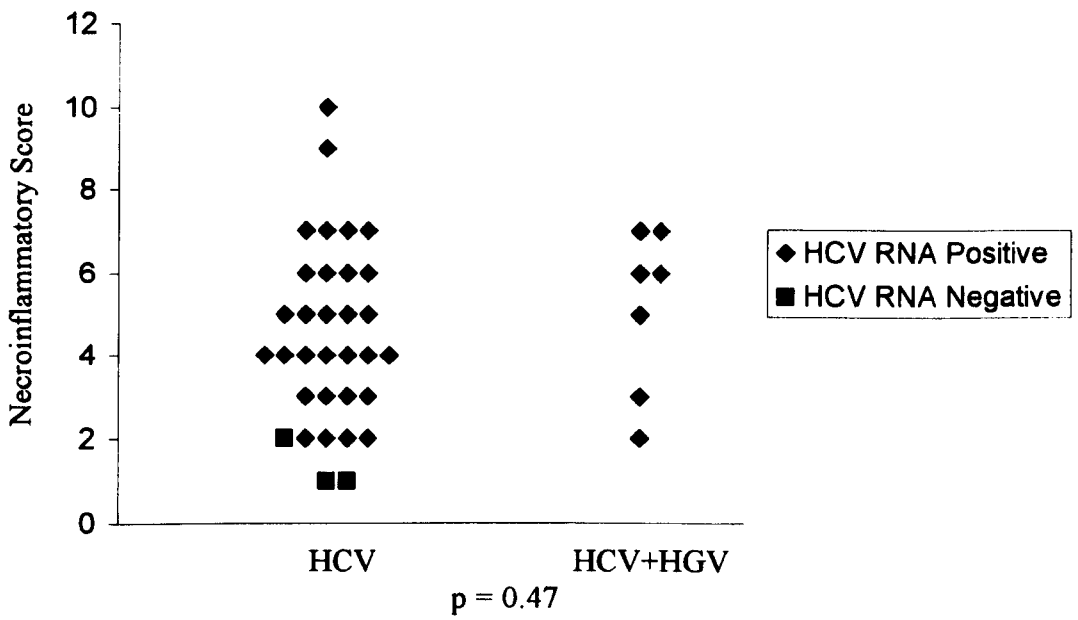
N (%)	HCV alone (n = 33)		HGV and HCV co-infection (n = 7)	
ALT >50 U/L	24	(73)	5	(71)
AST >35 U/L	25	(76)	5	(62)
Total bilirubin (>18 μmol)	3	(8)	0	(0)
Albumin <36 g/L	0	(0)	0	(0)
Gamma GT >50 U/L	17	(52)	2	(29)
Prothrombin time >15 sec	6	(18)	1	(14)

Figure 7.1 Primers for semi-nested RT-PCR protocol for the NS3 helicase region of the HGV genome utilised in this study.

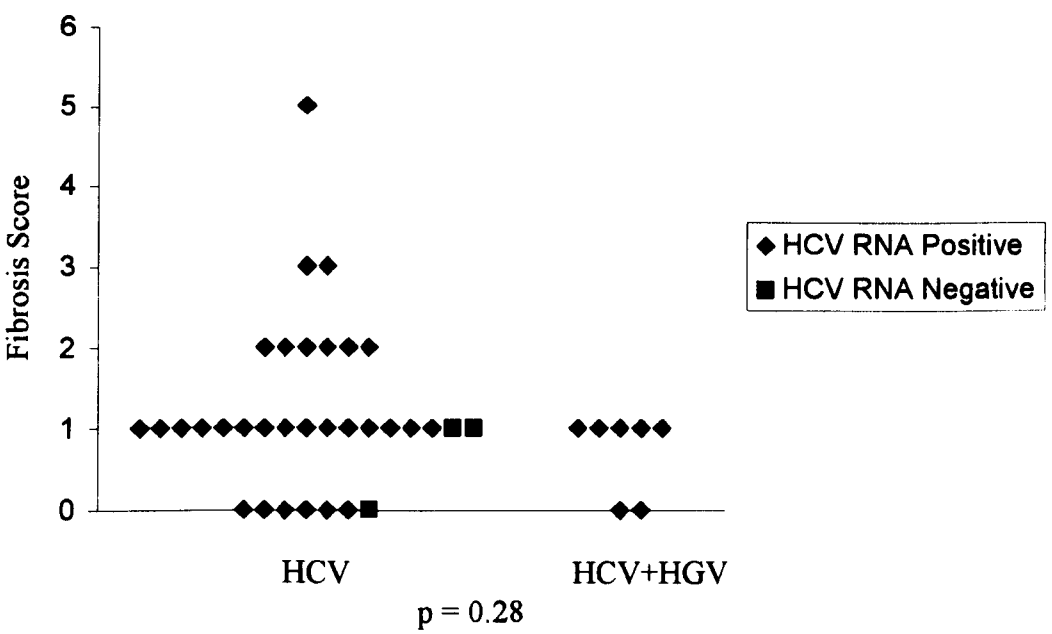
Primer		Sequence 5'→3'
HGV-G9	Reverse transcription / Outer antisense	TCYTTGATGATDGAACTGTC
HGV-G7	Sense (Inner and Outer)	GAGATYCCCTTYTATGGGC
HGV-G11	Inner antisense	TCYTTACCCCTRTAATAGGC

Figure 7.2 Comparison of Ishak grade and stage of liver histology in patients infected with hepatitis C virus (HCV) alone and those co-infected with the hepatitis G virus (HGV).

a. Necroinflammatory Scores



b. Fibrosis Scores



CHAPTER 8

A RANDOMISED CROSSOVER STUDY OF A NOVEL α - INTERFERON REGIME FOR THE TREATMENT OF ASYMPTOMATIC BLOOD DONORS WITH CHRONIC HEPATITIS C.

8.1 CONTENTS

8.2 SUMMARY

The management of asymptomatic blood donors with chronic hepatitis C virus (HCV) infection remains uncertain but the concern is that if left untreated they will develop progressive liver disease with the development of cirrhosis. There is little data on the efficacy and safety of treatment for these individuals. The aim of this study was to evaluate a novel regime of α - interferon in the management of these patients particularly to see if they would benefit from and tolerate this treatment. We studied 40 asymptomatic blood donors with serological evidence of HCV infection (ELISA-2 and RIBA-2 positive) and chronic hepatitis on liver biopsy in a prospective randomised crossover study. Each patient received a decreasing course of α - interferon (4.5MU-1.0MU) t.i.w. for 48 weeks preceded or followed by a year of observation. Patients were observed for a full year post-treatment. Twelve weekly serum ALT levels were monitored and serum was stored to allow retrospective testing for HCV-RNA. Liver histology was graded and staged at baseline, 48 and 96 weeks.

Six of the forty patients withdrew prior to starting treatment and a further four withdrew within twelve weeks of starting treatment. Response rates are reported for the thirty-four patients who commenced α - interferon therapy. Eight patients (24%) showed no ALT response to treatment and discontinued therapy at 12 weeks. Twenty-two (65%) patients had a normal ALT level at 12 weeks and went on to receive 48 weeks of therapy: Five (15%) had a breakthrough in ALT by 48 weeks despite continued treatment and 17 (50%) had a complete ALT response at 48 weeks. Fourteen (41%) of the complete responders had a sustained ALT response 1 year after completing treatment and 3 (9%) had a biochemical relapse

by 96 weeks. Eight (24%) of the patients with a complete sustained biochemical response to treatment cleared HCV RNA from serum and remained RT PCR negative one year after completing treatment.

Asymptomatic blood donors with chronic hepatitis C tolerate IFN therapy and have comparable response rates to other patient groups with chronic hepatitis C infection.

8.3 INTRODUCTION

Screening of blood donors for serological evidence of hepatitis C virus (HCV) was introduced in September of 1991. During the first 3 months of screening 0.31% of donations in the West of Scotland were reactive for anti-HCV antibody by second generation ELISA (Abott GmbH, Delkenheim, Germany) (Dow, B.C., et al. 1993). HCV carriers detected in this manner usually have mild symptoms and mild fibrosis on liver biopsy and are presumably at an early stage in the natural history of the infection (Bird, G.L.A., et al. 1994). The natural history of HCV infection in asymptomatic blood donors with HCV is not documented. The natural history of HCV infection is best documented in patients with post-transfusion hepatitis C where up to 85% of patients become chronic carriers and of these 20% develop progressive liver disease with the development of cirrhosis after 20-30 year (Seeff, L.B., et al. 1992). In contrast progression to cirrhosis was observed in only 0.4% of a cohort of 1,018 women infected with HCV from anti-D immunoglobulin after 20 years (Wiese, M. 2000). There are a number of reasons why treatment of these asymptomatic individuals may be indicated. There are obvious concerns that they may develop cirrhosis and hepatocellular carcinoma. In addition there is evidence that HCV infection can cause lymphoproliferative disorders of the B-cell lineage in the absence of significant liver disease and cases of hepatocellular carcinoma in these patients have also been described which supports the eradication of HCV infection even in those patients with mild disease (De Vita, S., et al. 1997, Zuckerman, E., et al. 1997, De Mitri, M.S., et al. 1995). Finally as asymptomatic HCV carriers are at risk of causing viral spread in the community or vertical spread to infants, their treatment may prevent new cases of infection.

A sustained biochemical and virological response to α - interferon monotherapy is seen in approximately 6% of interferon naïve patients after a 6 months and 16% after 12 months treatment, however most studies have specifically excluded patients with mild disease and normal serum transaminases (McHutchison, J.G. & Poynard, T. 1999). It is recognised that the best responses to antiviral therapy are seen in those with short duration of infection, low viral load and milder histological disease (Davis, G.L. & Lau, J.Y.N. 1997). Therefore asymptomatic blood donors with mild HCV infection diagnosed at screening might be expected to show improved response rates to α - interferon compared with other patient groups with chronic HCV. Few data exist to support this with only small pilot studies of treatment in patients with mild hepatitis as assessed by normal liver function tests and these include some patients with advanced histological disease (Marcellin, P., et al. 1997).

The aim of this study was assess the tolerability and response to treatment in asymptomatic patients with chronic hepatitis C infection detected at blood donor screening and to then study pre-treatment factors, such as HCV genotype and co-infection with hepatitis G virus, which might help predict the response to treatment.

8.4 PATIENTS, MATERIALS AND METHODS

This study was approved by the West Glasgow Hospitals University NHS Trust ethics committee and on entry each patient provided written informed consent.

8.4.1 Patients

As outlined in Chapter 7, forty asymptomatic blood donors with serological evidence of hepatitis C and histological evidence of chronic hepatitis were recruited into this randomised crossover study between August 1993 and September 1994. Patients were randomised into 2 groups, one which initially received 48 weeks of α - interferon (group A) and the other (group B), which underwent 48 weeks observation prior to receiving the same course of α - interferon.

8.4.2 Interferon treatment regime

Each patient received 4.5 MU of recombinant α - interferon (Roferon-A, Roche, Welwyn Garden City, Herts, UK) subcutaneously thrice weekly for 12 weeks. Any patient whose ALT was normal (<50 U / l) at 12 weeks went on to receive a decreasing dose of α - interferon thrice weekly for a further 36 weeks (3 MU, 2 MU, then 1 MU thrice weekly for 12 weeks respectively). The total dose of α – interferon given to each patient was 126 MU over 48 weeks. Non-responders with an elevated ALT at 12 weeks discontinued α - interferon therapy but remained under observation. Any patient who had a biochemical breakthrough (a rise in ALT out-with the normal range after twelve weeks while on treatment) had their

interferon dose increased to the previous higher dose and remained on that dose to 48 weeks.

8.4.3 Patient Assessment

Each patient underwent percutaneous liver biopsy at entry and again at 48 and 96 weeks. Formalin fixed, paraffin embedded liver sections were assessed by a single pathologist (R.N.M. MacSween) using Ishak's modified histological activity index (Ishak, K., et al. 1995). The pathologist was blinded to the patient's treatment status.

Haematological and biochemical assessments were performed using routine laboratory methods at baseline and while on treatment weekly until week four and again at six, eight and twelve weeks. Thereafter they were performed 12 weekly throughout the course of the study. Additional sera were collected, spun down within four hours of collection and stored at -20°C to allow retrospective detection of HCV-RNA by reverse transcription polymerase chain reaction (RT-PCR), which was not available locally at the start of this study.

8.4.4 HCV determination

All patients studied were reactive by second generation ELISA (Ortho Diagnostics, High Wycombe, UK) on initial screening and the presence of antibodies to HCV was subsequently confirmed by radio-immunoassay (RIBA-2, Chiron Corporation, Emeryville, Ca, USA) with two or more antigens positive. These tests were repeated and confirmed to be positive prior to inclusion in this study.

HCV RNA was detected using an in-house RT-PCR method with primers for the 5' non-coding region of the HCV genome (Figure 8.1). RT-PCR was performed as described (Dow, B.C., et al. 1993). This technique performed well in the EUROHEP HCV-RNA reference panel study and had a lower limit of detection of at most 2000 genomes / ml (Damen, M., et al. 1996). HCV genotyping was performed on sera stored prior to treatment by restriction fragment polymorphism as previously described (McOmish, F., et al. 1993).

RT PCR testing was performed retrospectively on all patients receiving treatment at entry and again after four, twelve and forty-eight weeks treatment.

8.4.5 HGV determination

Screening for HGV RNA in serum was performed retrospectively on stored serum by RT-PCR with primers for the NS3 region of the HGV genome. The method has been published previously and modified as discussed in Chapter 7 (Heringlake, S., et al. 1996).

8.4.6 Study end points and Statistics

Primary end points in assessing response to therapy were normalisation of serum alanine aminotransferase (ALT) and improvement in liver histology after completion of therapy. The secondary endpoints in assessing response to treatment were loss of detectable HCV RNA from serum and tolerability of interferon treatment.

Patients were classified according to their biochemical and virological responses to α - interferon treatment. Non response was defined as an elevated (>50 IU/L) ALT

after 12 weeks of treatment. An initial response was defined as a normal serum ALT after 12 weeks of interferon therapy and the response was considered complete if it remained normal until treatment was stopped at 48 weeks. Breakthrough was defined as an elevation in ALT on interferon therapy between 12 and 48 weeks. A sustained response was defined as a normal ALT for six months after completing interferon therapy. Relapse was defined as an elevation in ALT above normal in complete responders after interferon was discontinued. Responses were further defined as complete and sustained virological response if HCV RNA was not detectable in serum on completion and six months after interferon therapy respectively.

The two-sample Wilcoxon rank sum test and Chi-square tests were used to compare the two groups at entry into the study and to compare responders and non-responders to treatment. Changes in liver histology over the study were assessed using the two-sample Wilcoxon rank sum test.

8.5 RESULTS

Twenty patients were randomised into each study group. The patients in both groups were similar with respect to sex ratio, laboratory results, presence of HCV RNA in serum, and liver histology (Table 8.2). The patients from group A were significantly older and blood products were a more common source of infection in this group.

8.5.1 Patient follow-up

The progress of the study after randomisation is summarised in Figure 8.1. Eighteen patients from group A completed the treatment protocol. Two withdrew during the first 12 weeks of treatment; one could not tolerate self-injection while the other complied poorly with treatment. Four patients in group B were lost to follow up after completing 48 weeks of observation and a further 2 patients were withdrawn from group B prior to starting treatment as they were found to be persistently RT PCR negative for HCV RNA. Fourteen patients from group B went on to receive interferon therapy. Of these one stopped treatment after 1 month as he could not tolerate side effects and one defaulted after 12 weeks of treatment. Consequently 12 patients from group B completed treatment according to the protocol.

During the observation period of patients from group B, there was no significant change in the ALT levels and the mean ALT levels remained elevated as illustrated in Figure 8.2. None of the eighteen RT PCR positive patients from group B cleared HCV RNA from serum after 48 weeks observation. Patients from

group B showed no significant change in Ishak modified histological activity scores over the observation period (Figure 8.3).

8.5.2 Response to treatment

Response rates to α - interferon are reported for the 34 patients (20 from group A and 14 from group B) who commenced treatment, excluding the 6 patients who withdrew from group B before therapy was initiated. Response rates are reported on an intention to treat basis for the thirty-four patients who commenced α - interferon. The patients who defaulted from the study or withdrew did not differ significantly from those treated with regard to the histological severity of their liver disease ($p = 0.9$).

8.5.2.1 ALT response

Four (12%) patients withdrew within twelve weeks of starting therapy. The responses to α - interferon observed in the remaining thirty patients treated according to the protocol is summarised in Figure 8.4. Eight patients (24%) showed no biochemical response to α - interferon, with an elevated ALT level after 12 weeks of treatment, and discontinued treatment at that time. Twenty-two patients (65%) showed an initial response, with a normal ALT level after 12 weeks of treatment, and went on to complete 48 weeks of α - interferon. Five patients (15%) who had an initial response to α - interferon had a biochemical breakthrough, with a rise in ALT out-with the normal range despite continuing on α - interferon to 48 weeks. The remaining 17 patients (50%) were complete responders with normal ALT levels until they completed 48 weeks of treatment. Five of these patients had a normal ALT level on repeat testing prior to the

initiation of therapy and in each case the ALT remained normal until the completion of therapy. Within six months of completing α – interferon 3 patient (9%) showed a biochemical relapse with a persistent elevation of ALT level including one patient whose ALT had been persistently normal prior to therapy. The remaining fourteen patients (41%) had a complete sustained biochemical response, with normal ALT levels 1 year after stopping α - interferon.

8.5.2.2 Histological response

The changes in necroinflammatory activity on liver biopsy over the course of treatment and follow up periods are shown in Figure 8.5. There was no significant change in the scores for necroinflammatory activity and fibrosis in the group as a whole on treatment or during the follow up year. When each response group was considered individually a significant improvement in necroinflammatory activity by 48 weeks was confined to patients who had a complete sustained clearance of virus from serum ($p < 0.02$). There was no significant change in necroinflammatory scores in this group over the follow-up year ($p = 0.7$). There was no significant change in fibrosis scores in any of the individual response groups over the course of the study (Figure 8.6).

8.5.2.3 HCV RNA response

The RT PCR results in the 30 patients who received α - interferon treatment are shown in Table 8.3. One patient from group A had a complete sustained biochemical response to α - interferon but was found to be repeatedly RT PCR negative on testing serum from prior to entry into the study. The 29 remaining patients were PCR positive at entry into the study. Eight (24%) of the thirty four patients commencing α - interferon therapy experienced a sustained virological

response and remained RT PCR negative for HCV RNA in serum 1 year after completing therapy. Four patients with a sustained biochemical response were RT PCR positive for HCV RNA on completion of treatment. One patient having experienced a complete virological response had a virological relapse six months after completing therapy. Of the three patients who relapsed after completing therapy one was viraemic on completion of therapy and all were viraemic within six months of stopping therapy. Two patients experiencing a breakthrough on treatment remained RT PCR negative in serum to 48 weeks but all were viraemic within six months of completing α - interferon. Despite transient loss of HCV RNA from serum in three patients, all non-responders were viraemic by forty-eight weeks. Overall no patient viraemic after 4 weeks of treatment had a sustained clearance of HCV RNA from serum.

8.5.3 Predictors of a complete sustained virological response to interferon

Pre-treatment factors predictive of a sustained biochemical and virological response were analysed for those twenty-nine patients that completed the study treatment protocol and were HCV PCR positive prior to treatment. Patients that had a sustained to α - interferon therapy were comparable to those that did not with regard to baseline liver function tests and pre-treatment grade and stage of chronic hepatitis. Five patients had persistently normal ALT levels prior to treatment, one was PCR negative prior to treatment and one had a sustained virological response. Rates of sustained biochemical and virological response were comparable in patients with persistently normal and those with elevated ALT levels (one of 4, 25% v seven of 25, 28%, $p = 1.0$). Similarly patients with no fibrosis on pre-treatment liver biopsy had comparable sustained response rates to

those with fibrosis on pre-treatment biopsy (one of 8, 12% v seven of 21, 33%, p = 0.38)

The HCV genotypes of all 29 patients who were HCV PCR positive and commenced treatment were recorded. A complete sustained virological response was observed in four (27%) of the fifteen patients infected with genotype 1, two (40%) of the five with genotype 2 and two (22%) of the nine with genotype 3. No significant differences in response rates to α - interferon were detected between genotypes (p = 0.7).

Seven (18%) of the forty patients studied were found to be co-infected with HGV prior to the initiation of α - interferon therapy. No additional patients acquired HGV infection over the treatment period. Five of the seven patients co-infected with HGV received treatment according to the study protocol. The biochemical and HCV and HGV PCR responses to α - interferon are summarised in Table 8.4. Patients co-infected with HGV exhibited a comparable rate of sustained clearance of HCV RNA from serum with α - interferon compared with those patients not infected with HGV (40% v 25%, p = 1.0). HCV and HGV PCR responses were not directly associated. Three (60%) of the co-infected patients were HGV PCR negative at the end of treatment. The two patients HGV PCR positive at the end of treatment had a sustained clearance of HCV RNA from their serum and in both cases ALT levels were persistently normal after treatment.

8.5.4 Tolerability of α - interferon therapy

Twenty-eight (82%) of thirty-four patients who received α - interferon complained of adverse side effects on treatment and these were generally mild. The commonest complaints were of febrile reactions (17), myalgia or arthralgia (8), anorexia (6), headache (6), fatigue (6), pruritus (5), low mood (4), abdominal pain (4), exacerbation of psoriasis (2) and alopecia (2). Two (6%) patients could not tolerate treatment and discontinued therapy; one was not able to perform self-injection while the other experienced troublesome myalgia and fevers. No other alteration in α - interferon dose was required as a result of adverse side effects.

8.6 DISCUSSION

Infection with the hepatitis C virus becomes chronic in approximately 80% of those that acquire it, causing a chronic hepatitis in the majority of cases and over a number of years this can progress to cirrhosis. The natural history of the infection has largely been established in individuals who acquired HCV infection through blood transfusion where approximately 20% of individuals develop cirrhosis after 20 – 30 years (Seeff, L.B., et al. 1992). The natural history of the infection in asymptomatic individuals who are found to have chronic HCV infection at blood donor screening is not known. These individuals have mild symptoms and mild fibrosis on liver biopsy and are presumably at an early stage in the natural history of the disease (Bird, G.L.A., et al. 1995). Sustained response to treatment of chronic HCV infection has been shown to improve quality of life, halt progression of the liver disease and hopefully reduce the risk of subsequent hepatocellular carcinoma. Several organisations have published consensus conference recommendations concerning the indications for treatment of patients with chronic hepatitis C infection (Booth, J.C.L., et al. 2001, European Association for the Study of Liver Disease. 1999, National Institute of Health Consensus Development Panel Statement. 1997, Dhumeaux, D., et al. 1997). The consensus is consistent in recommending that treatment is restricted to patients with elevated transaminases for more than six months, active viral replication as indicated by positive polymerase chain reaction for serum HCV RNA, and moderate or severe inflammation on liver biopsy. Using these criteria asymptomatic blood donors with mild disease would not be considered suitable candidates for treatment and there are few data to guide their management. Most existing studies are small and have focused on patients assessed as having mild hepatitis on the basis of normal liver

function tests however this group of patients is ill defined and includes some patients with advanced liver disease (Marcellin, P., et al. 1997, Di Bisceglie, A.M. 1999). A single trial from Italy reported a 42% sustained virological response to interferon α2a monotherapy in thirty-two asymptomatic blood donors with mild disease on liver biopsy (Prati, D., et al. 2000). In the current study a complete sustained biochemical response to α - interferon monotherapy was observed in 41% of patients and a sustained virological response was observed in 24% of patients. The response rates in these two studies are at least as good as the response rate reported in HCV infected patients with a definite indication for antiviral therapy (10 – 25%) (Poynard, T., et al. 1996). The disparity in response rates between the current study and that of Prati et al. may be explained by the higher dose of α - interferon (4.5 MU thrice weekly for 6 or 12 months) used, the inclusion of patients only under 45 years of age in that study or the presence of a few patients with more severe liver fibrosis in the current study. Studies of patients with overt liver dysfunction have shown that sustained response rates to α - interferon are directly related to the total dose given and this may hold true for patients with mild liver disease also (Poynard, T., et al. 1996). In addition, in studies of patients with liver dysfunction who received treatment with α - interferon, mild chronic hepatitis on pre-treatment liver biopsy is an independent factor predictive of response to treatment in chronic hepatitis (Davis, G.L. & Lau, J.Y.N. 1997, Poynard, T. et al. 1998). In the current study, patients with no fibrosis on pre-treatment liver biopsy had lower response rates to α - interferon compared with patients with fibrosis although the difference was not statistically significant (12% v 33%). This may reflect an anergic immune response to HCV in these patients who will not respond to the immunostimulatory properties of α -

interferon (Prati, D., et al. 2000). The prevalence of HCV genotypes in these patients was similar to that detected in other local patient populations (see Chapter 3). Surprisingly, the response rates in patients infected with genotype 1 were similar, if not better, than that seen in patients infected with genotype 3 which contrasts with studies of patients with more severe chronic hepatitis and liver dysfunction (Davis, G.L. & Lau, J.Y.N. 1997). The sample size in this study was small but this raises the possibility that HCV genotype has less influence on treatment response in patients with mild disease.

The patients in this study were asymptomatic at the time of diagnosis of hepatitis C infection. Almost all patients who receive treatment doses (>3MU per injection) of α - interferon experience some adverse side effects. More severe side effects which may require dose modification are less common occurring in 10% to 15% of patients and severe or life threatening side effects occur in 0.1% to 1% of patients (Dusheiko, G. 1997). When this study was designed the optimal interferon dosage regime for chronic hepatitis C infection had not been established. Studies of initial induction regimens followed by dose reduction had been shown to be effective (Lee, W.M. 1997). In view of concerns about the tolerability of α - interferon therapy in this asymptomatic patient group, a novel regimen of progressively decreasing α - interferon dose was designed for those patients who responded to the initial twelve weeks of induction therapy. This regimen proved to be well tolerated by this previously asymptomatic population with only 6% of patients stopping treatment on the induction dose, a rate that is comparable to other studies (Dusheiko, G. 1997). No other dose modifications were required as a result of side effects which contrasts with the rates of dose reduction of between 9% and 22% observed in studies employing fixed dose regimens (Poynard, T., et al. 1996).

Given that no patient showed a sustained virological response to treatment if they remained PCR positive after four weeks of treatment, it may be possible to identify earlier those patients who are not showing a virological response to therapy, to avoid subjecting them to unnecessary therapy as is the case with patients with more severe liver disease and liver dysfunction (Tong, M.J., et al. 1997).

Combination therapy with α - interferon and ribavirin has been shown in randomised trials to produce improved response rates compared with α - interferon monotherapy in the management of treatment naïve patients with chronic HCV (McHutchison, J.G. & Poynard, T. 1999). α - Interferon monotherapy remains the treatment of choice for selected patient groups in whom therapy with ribavirin is contraindicated, in particular those with ischaemic heart disease, renal disease or in those receiving inadequate contraception (Glue, P. 1999). Ribavirin is teratogenic and is renally excreted. Conception in patients or their partners should be avoided during treatment and for six months thereafter. Haemolysis is the most significant adverse effect of ribavirin use and can be sudden and profound, particularly in patients with renal impairment. Thus combination therapy with α - interferon and ribavirin is not indicated for all treatment naïve patients and this novel regimen of α - interferon has been shown to be both a safe and effective alternative for these patients.

This study showed that asymptomatic blood donors with chronic hepatitis C infection detected at blood donor screening tolerated this novel α - interferon regimen and demonstrated similar if not superior response rates compared with other patient groups with chronic hepatitis C infection. The natural history of

chronic hepatitis C infection in asymptomatic blood donors is not established and it is unclear what the risks of disease progression are in this patient group. It may well be that untreated these patients have an excellent prognosis, however in waiting until there is evidence of significant disease the chances of a response to treatment may be reduced. Further data in this area are required however this study provides evidence that these patients tolerate and respond to α - interferon.

Table 8.1 RT-PCR Primers for the 5' non-coding region of the HCV genome utilised in this study.

Primer		Sequence 5'→3'	Nucleotide position*
HCV-22	Reverse transcription	TCGTCGTCATGGTGCACG	-8 to 9
HCV-21	Outer sense	CGACACTCCACCATGAATCAC	-332 to -302
HCV-24	Outer antisense	CATGGTGCACGGTCTACGAGACC	-20 to 3
HCV-23	Inner sense	TCACTCCCCTGTGAGGAACT	-305 to -286
NCR-4	Inner antisense	GCACCCTATCAGGCAGT	-54 to -38

* Numbering according to Choo Q.L., et al. 1991

Table 8.2 Entry characteristics of study groups.

Clinical Features	Group A (n=20)		Group B (n=20)	
Age, median years (range)	40	(28-53)	33*	(25-48)
Sex, *N* males (%)	12	60	12	60
History of jaundice, *N* (%)	4	(20)	5	(25)
Source of hepatitis, *N* (%)				
Transfusion	12	(60)	3**	(15)
Parenteral drug use	4	(20)	9	(45)
Occupational exposure	0	(0)	1	(5)
Tattoo / Body piercing	11	(55)	13	(65)
Unknown	3	(15)	3	(15)
Laboratory, mean[#] (range)				
ALT (U/L)	94	(11-220)	129	(13-470)
AST (U/L)	51	(18-126)	63	(18-141)
Total bilirubin (μmol)	10	(1-27)	10	(1-23)
Albumin (g/L)	44	(40-49)	44	(41-51)
Gamma GT (U/L)	62	(7-378)	63	(14-239)
Prothrombin time (sec)	14.6	(12-18)	14.9	(13-18)
Leukocyte count ($x10^9$/L)	6.5	(3.5-11.1)	6.2	(3.5-9.7)
Haemoglobin (g/dL)	14.6	(12.2-17)	14.9	(11.8-17.4)
Platelet count ($x10^9$/L)	239	(125-359)	224	(142-424)
HCV RNA RT-PCR negative, *N* (%)	1	(5)	2	(10)
HCV Genotyping *N*	19		18	
1 *N* (%)	10	(53)	6	(33)
2 *N* (%)	5	(26)	0	(0)
3 *N* (%)	4	(21)	12	(67)
Histology, median (range)				
Inflammation	4	(2-10)	6	(1-10)
Fibrosis	1	(0-5)	1	(0-3)

[#] Normal ranges: Albumin 36-50 g/L, ALT 10-50 U/L, AST 10-35 U/L, Total bilirubin 3-18 μmol, Gamma GT 5-50 U/L, Haemoglobin 13-18 g/dL, Leukocyte count 4.5-6.5 $x10^9$/L, Platelet count 140-450 $x10^9$/L, Prothrombin time 15 sec

* $p < 0.02$ ** $p < 0.003$

Table 8.3 Summary of RT PCR for HCV RNA results of the 30 patients completing the treatment protocol. The four patients who withdrew while receiving treatment were all RT PCR positive at entry, twelve weeks and thereafter.

No of patients HCV RNA negative at:	Biochemical response to treatment			
	Non Responders (n=8)	Breakthrough (n=5)	Complete Sustained Responders (n=14)	Relapsers (n=3)
entry	0	0	1	0
4 weeks	3	2	10	1
12 weeks	1	3	11	1
48 weeks	0	2	10	2
6 months post	0	0	9	0
1 year post	0	0	9	0

Table 8.4 Biochemical and virological responses to α - interferon observed in patients co-infected with hepatitis C and hepatitis G viruses.

No. (%)	Complete sustained virological response	Complete sustained biochemical response (HCV PCR positive)	Biochemical Relapse	Breakthrough	No response	Total
HGV PCR at pre-treatment						
HGV Negative	6	4	2	5	7	24
HGV Positive	2	1	1	0	1	5
End of treatment HGV PCR results in those HGV positive at entry						
HGV Positive	2	0	0	0	1	3
HGV Negative	0	1	1	0	0	2

Figure 8.1 Profile of study and flow of patients through the study.

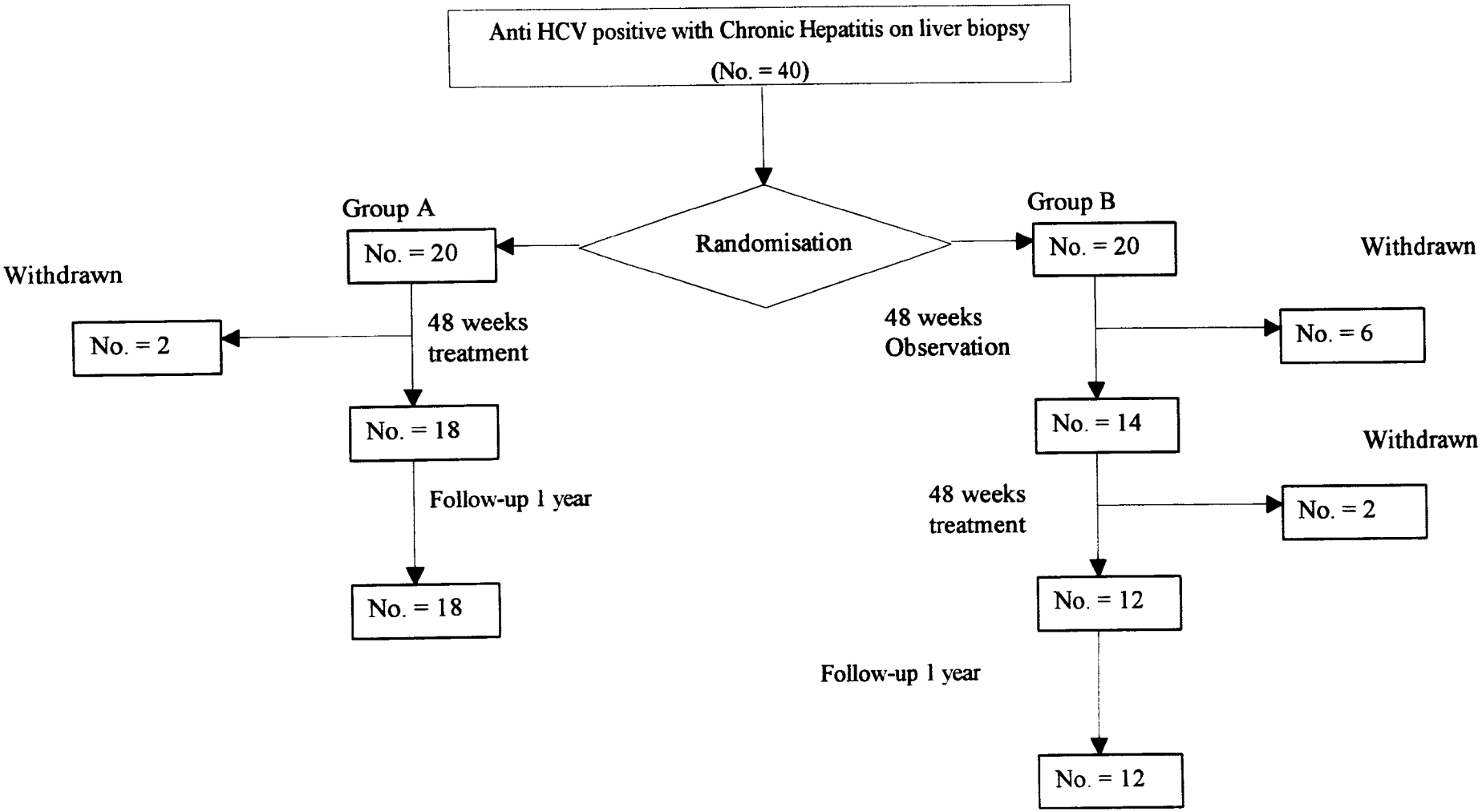


Figure 8.2 ALT levels in Group B during the observation period. Mean ALT levels with standard error of mean indicated by error bars.

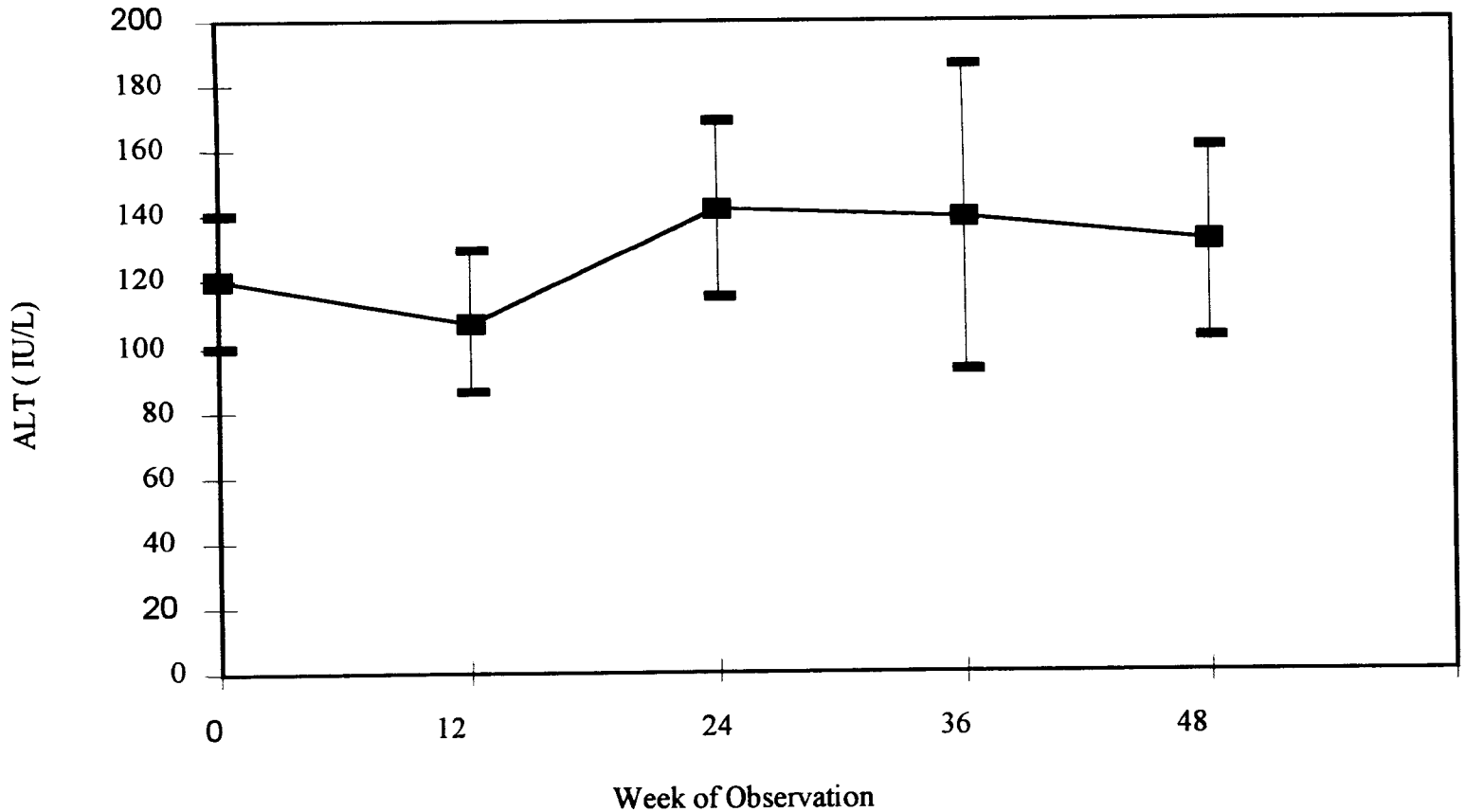
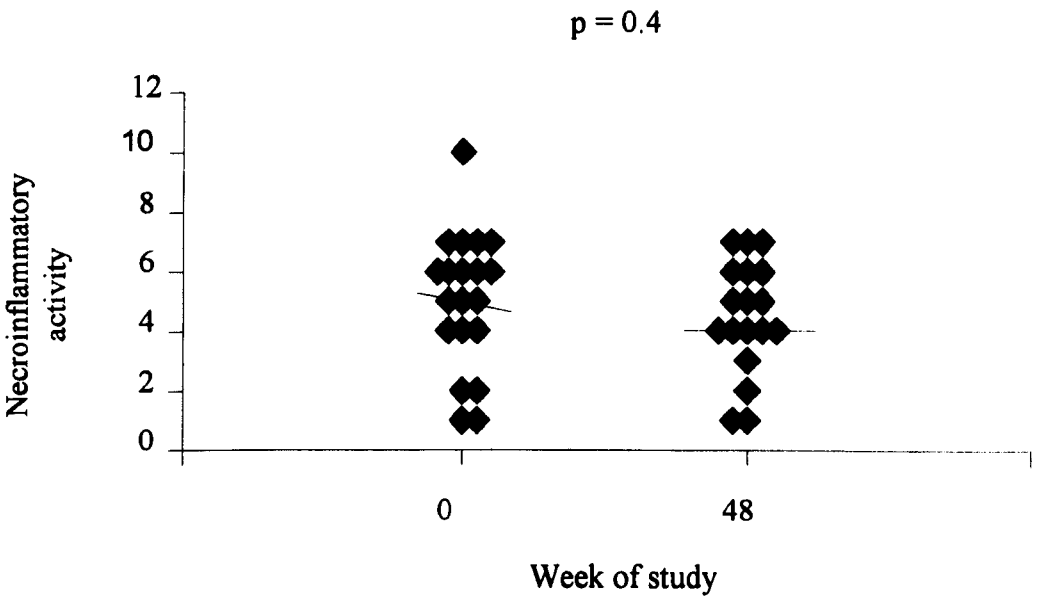


Figure 8.3 Group B liver biopsy scores (Ishak modified histological index) at entry and after the observation period. Medians marked with horizontal bar

(a) Necroinflammatory scores



(b) Fibrosis scores

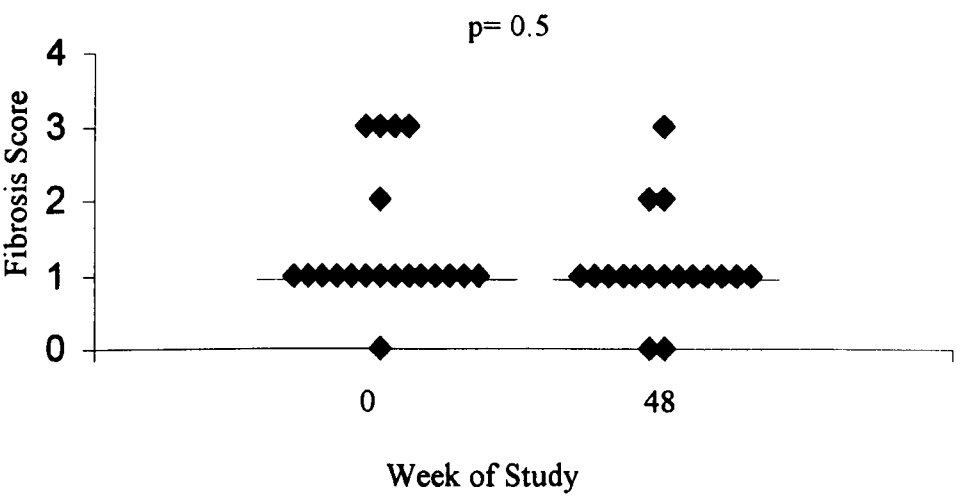


Figure 8.4 Biochemical responses to α - interferon among the 30 patients completing the treatment protocol. Mean ALT levels with standard error of mean indicated by error bars.

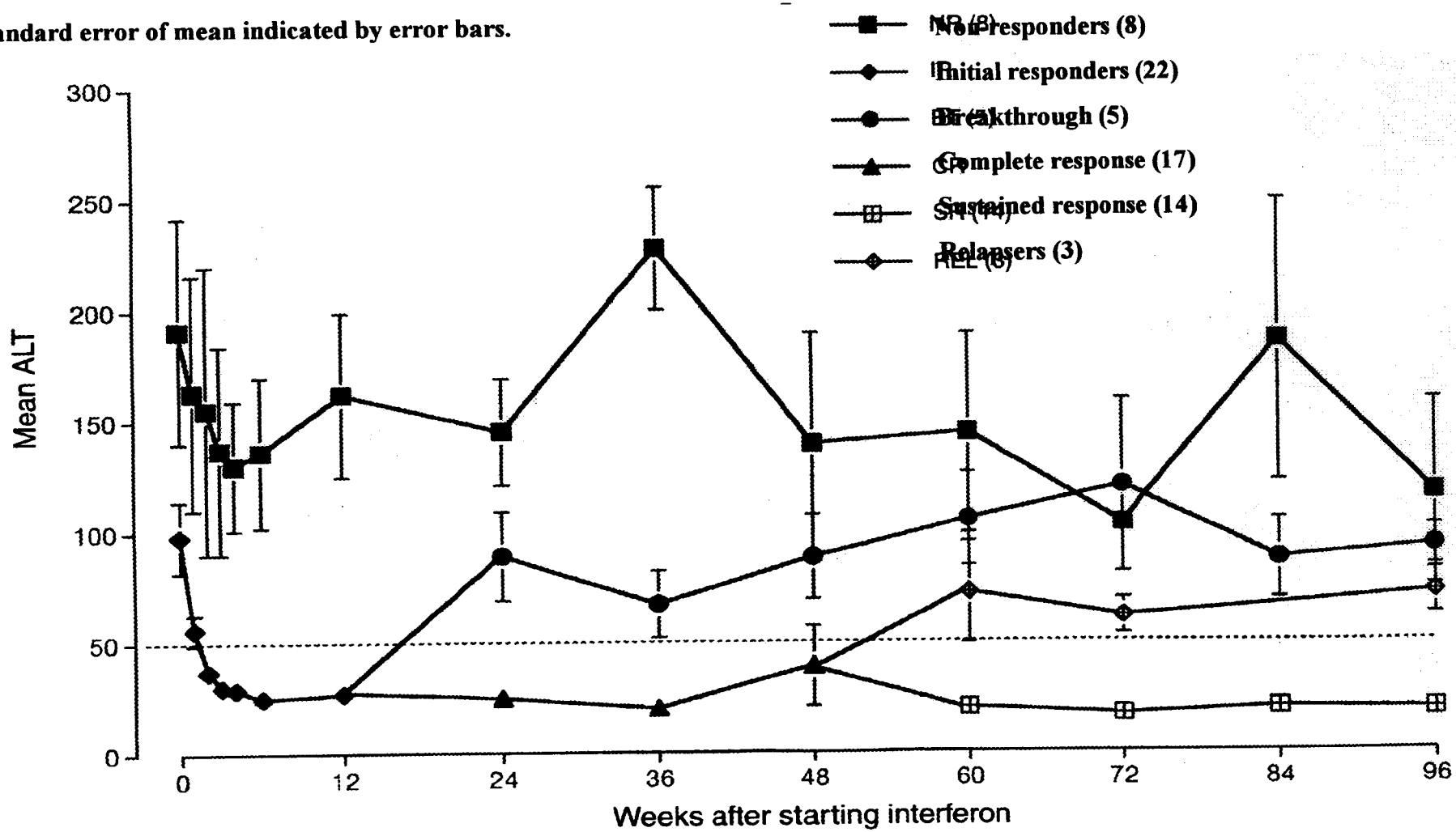


Figure 8.5 Changes in necroinflammatory activity on liver biopsy in the 30 patients treated according to the protocol. The scores for patients that had a complete sustained biochemical response to interferon and remained RT PCR negative (o) are joined. The scores for patients that had a complete sustained biochemical response but remained viraemic (△) and all other response groups (♦) are also shown. Significant improvement in necroinflammatory scores were seen only for patients who had a sustained clearance of HCV RNA from serum (p values shown refer only to patients that had a sustained clearance of HCV RNA from serum).

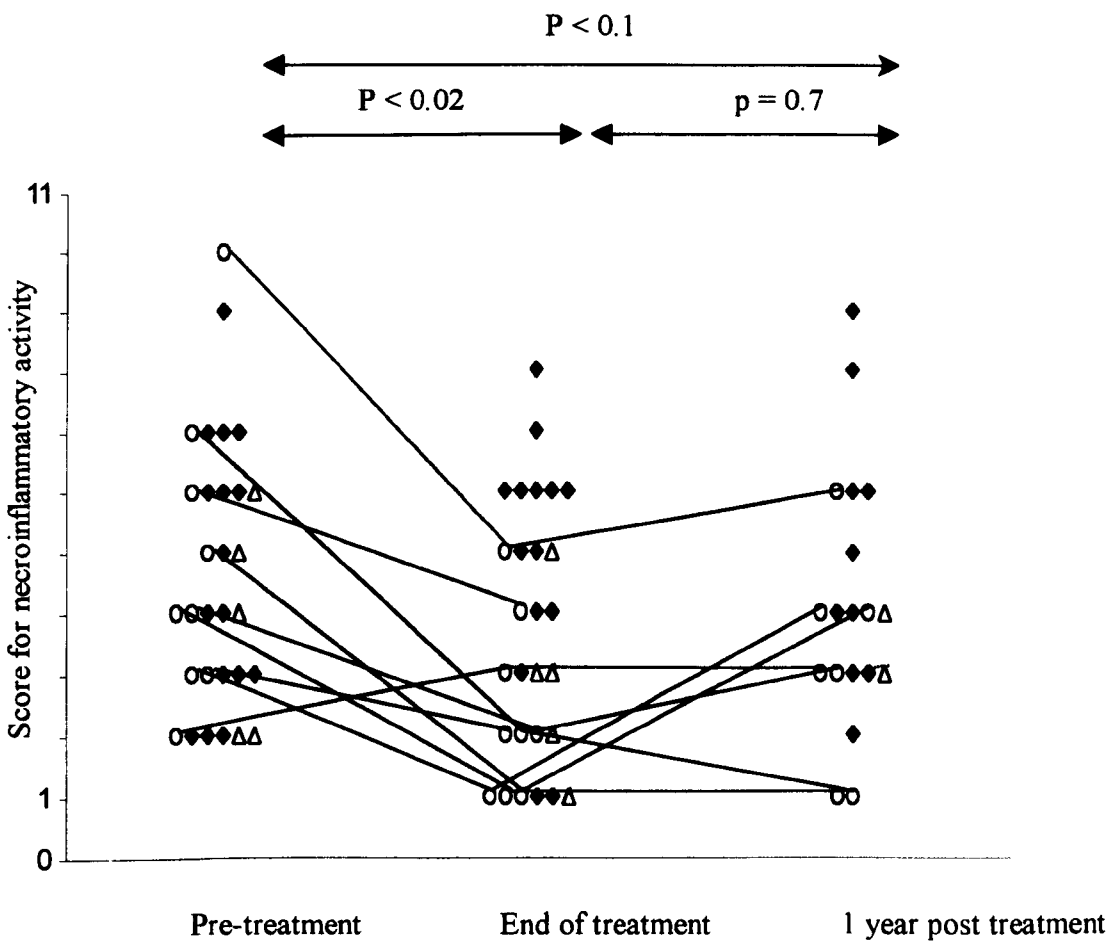
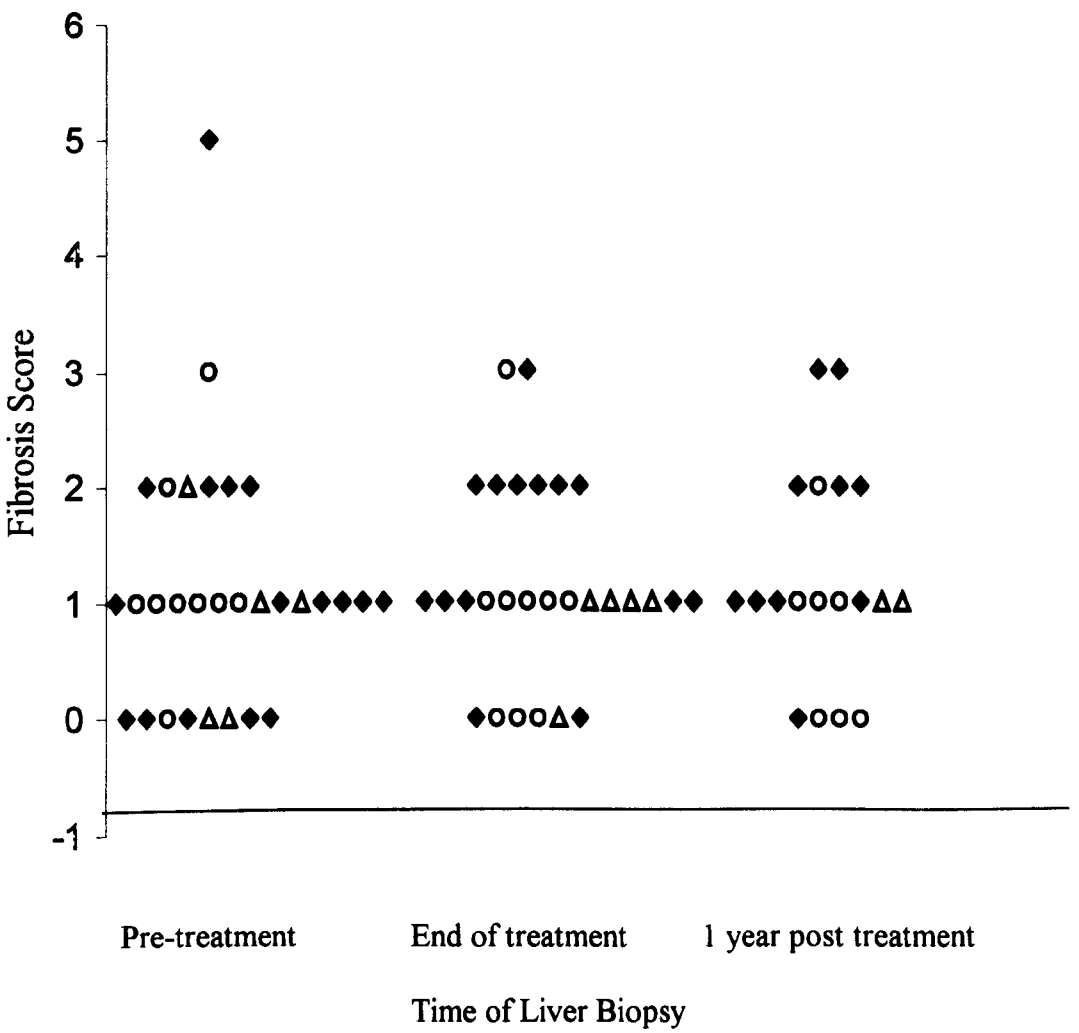


Figure 8.6 Change in fibrosis scores over the treatment period in the 30 patients receiving treatment as per protocol. The scores for patients that had a complete sustained biochemical response to interferon and remained RT PCR negative (o) are joined. The scores for patients that had a complete sustained biochemical response but remained viraemic (△) and all other response groups (♦) are also shown.



CHAPTER 9

CONCLUSIONS AND FURTHER RESEARCH

9.1 CONTENTS **Pages**

9.2 Summary of findings and further research from Chapter 3

This study indicates that the healthcare setting is unlikely to account for significant transmission of hepatitis C virus in an area with a large HCV infected injecting drug using population. Healthcare personnel and patients can both be reassured that the reservoir of healthcare personnel in the West of Scotland who are infected with HCV is extremely small (2.8 per 1000). It appears that working in the healthcare environment and performing exposure-prone procedures only rarely leads to the acquisition of hepatitis C virus by healthcare workers. In addition the risk posed to patients from hepatitis C virus infected healthcare workers is probably low. In the absence of guidelines for the management of healthcare workers infected with hepatitis C virus it is essential that needlestick injuries are reported and rigorously followed-up by Occupational Health Services to establish whether these conclusions are correct. In addition HCV infected healthcare workers should be advised to train in areas where exposure-prone procedures are not performed, as it is likely that in time their practises will be restricted.

9.3 Summary of findings and further research from Chapter 4

This study fails to show an advantage for α–GST in grading the severity of liver inflammation in chronic HCV infection but that α - GST may have a role in monitoring response to α – interferon therapy. An accurate non-invasive tool for the assessment of the severity of HCV related liver injury remains an important goal in the management of chronic HCV to avoid the necessity for repeat liver biopsy. A number of serum markers of liver fibrosis have been evaluated individually and have proved disappointing. This area is currently the target for a great deal of research and it is hoped that the combination of a number of these serum markers will provide a solution. Panels of these assays are being evaluated

in large prospective multicentre studies with longitudinal follow-up and results are awaited. In the mean time liver biopsy remains the gold standard for assessing and following up the severity of HCV related liver injury.

9.4 Summary of findings and further research from Chapter 5

In this chapter we found that although many patients with chronic hepatitis C virus infection have elevated serum iron studies, few have significant iron deposition within the liver. Carriage of the recognized mutations in the HFE gene, although frequently observed, do not account for the elevated serum iron studies and liver iron deposition that is present nor are they associated with any clinical, biochemical, virological or pathological feature. The concentration of liver iron did not have a significant role in the progression of HCV related liver injury. These findings indicate that iron depletion by venesection is unlikely to influence the natural history of chronic hepatitis C virus infection even in those patients with elevated liver iron concentrations. Further studies are required to establish whether the liver iron deposition in chronic hepatitis C virus infection is secondary to hepatocellular damage or whether it is a primary problem in the few patients in whom it is observed.

9.5 Summary of findings and further research from Chapter 6

This study reveals that genetic polymorphisms in the renin-angiotensin system, which are associated with increased activity in the systemic renin-angiotensin system, do not account for the variable rate of progression observed in chronic HCV infection. Whether a tissue renin-angiotensin system exists within the liver and whether angiotensin II stimulates human hepatic stellate cells to produce extracellular matrix has not been established. Further in-vitro studies are required

to establish whether exposure to angiotensin II stimulates the production of transforming growth factor - β1 by activated human hepatic stellate cells. Prospective controlled clinical studies of pharmacological blockade of the renin-angiotensin system are required to establish whether these therapies prevent the progression of hepatic fibrosis in chronic HCV and other chronic liver diseases.

9.6 Summary of findings and further research from Chapter 7

This study reveals that hepatitis G virus co-infection is common in HCV infected individuals due to shared risk factors. However there was no evidence that HGV infection influenced the biochemical and histological severity of HCV related liver disease. This is consistent with the findings of other investigators. In the absence of clinical and laboratory evidence that HGV is a hepatotropic virus or the cause of hepatitis in humans the significance of infection with this virus remains poorly understood and it should not be referred to as a hepatitis virus. Further research is required to establish whether the GB virus-C is a cause of disease in humans. Recently it was reported that patients who are co-infected with GB virus-C and the Human Immunodeficiency Virus (HIV) have a more favourable prognosis with delayed development of the acquired immunodeficiency syndrome (AIDS) compared with those infected with HIV alone(Stosor, V. & Wolinsky, S. 2001). Further study in this area is required. Certainly the variable rates of progression observed in HCV related liver disease cannot be explained by co-infection with HGV.

9.7 Summary of findings and further research from Chapter 8

This study revealed that asymptomatic blood donors with chronic hepatitis C infection detected at blood donor screening tolerated this novel α - interferon

regimen and demonstrated similar if not superior response rates compared with other patient groups with chronic HCV. The natural history of chronic hepatitis C infection in asymptomatic blood donors is not established and it is unclear what the risks of disease progression are in this patient group. Currently the consensus is that these patients should be left untreated however in waiting until there is evidence of significant disease the chances of a sustained virological response to treatment may be reduced. A large multi-centre NHS Healthcare Executive study is currently underway studying the combination of α - interferon and ribavirin in the management of patients with mild chronic hepatitis C virus infection and until the results of this study is available monitoring of these patients by serial liver biopsy remains the management of choice.

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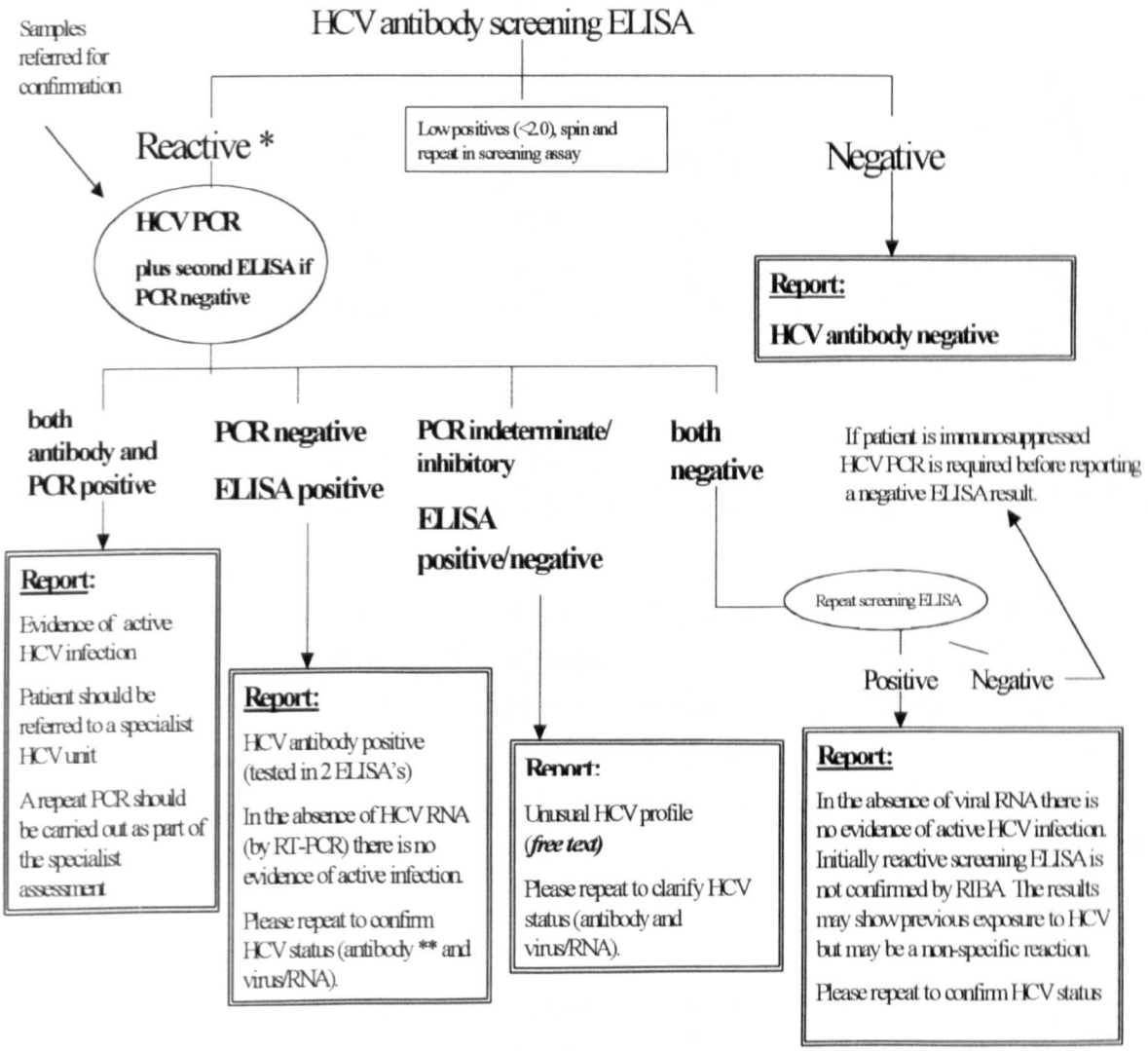
APPENDICES

Appendix 1. Algorithm for HCV testing employed by the Regional Virus Laboratory in Glasgow (Protocol updated May 2001). The assays employed for testing are an third generation ELISA (Ortho Diagnostics, Raritan, NJ), the Amplicor polymerase chain reaction (PCR) assay (Roche Diagnostics), and a third generation third generation RIBA (Ortho Diagnostics, Raritan, NJ).



* Consideration should be given to referring all ELISA reactive patients to a specialist centre.

** ELISA and RIBA

Appendix 2. Greater Glasgow Health Board Hepatitis C strategy review – inclusion and exclusion criteria for treatment of chronic hepatitis C.

All patients with HCV are potential candidates for therapy. However, until th natural history is better understood, those with the greatest risk of progressing t cirrhosis should receive priority. The following are suggested inclusion an exclusion criteria for selection of patients for combination therapy:

Inclusion criteria:

a. Age 70 years or less.
b. Either sex.
c. Serum PCR positive for HCV-RNA (within the last 6 months).
d. Elevated serum ALT for in excess of 6 months.
e. Liver biopsy evidence of moderate to severe chronic hepatitis C with or without cirrhosis (when feasible).

Exclusion criteria:

a. Marked obesity (body mass index > 30).
b. Symptomatic HIV positive patients (or asymptomatic with CD4 lymphocyte count < 350) or patients who are immunosuppressed.
c. Patients with cirrhosis and features of liver failure (consider for liver transplantation).
d. Evidence of primary hepatocellular carcinoma.
e. Patients with evidence of another cause for chronic hepatitis such as alcohol excess or auto-immune liver disease (smooth muscle antibody positive, high IgG).
f. Patients with significant co-morbidity due to neoplasia or cardiac, respiratory and renal disease.
g. Patients thought unlikely to be able to co-operate with subcutaneous injections or follow-up at the clinic.
h. Pregnancy, breast-feeding mother, or inability for either sex to use effective contraception during and for 6 months after therapy.
i. Patients with contraindications to use of interferon or ribavirin such as epilepsy or compromised CNS function, renal failure, congestive cardiac failure, psoriasis or known hypersensitivity to either product.

Appendix 3. Greater Glasgow Health Board Hepatitis C strategy review – pre-treatment assessment, treatment regimen and monitoring of therapy.

Pre-treatment assessment

Patients will have been selected according to criteria above. The following data should be available before treatment and one or more of these criteria indicate that the patient falls into a poor response category.

- Genotype 1 with high viral load
- Cirrhosis or bridging fibrosis on liver biopsy
- Males aged > 40 years

The following categories of patients will be considered:

a. Untreated patient (naive)

b. Relapse after previous interferon monotherapy

c. Non-responders to previous interferon monotherapy

Naive and relapse patients should be treated in the same manner as indicated below. There is no treatment yet recommended for non-responders, who should remain under observation.

Treatment regimen

All pre-menopausal women to have a negative pregnancy test before commencing therapy.

a. Standard

Alfa interferon 3 MU subcutaneously three times a week (TTW) plus oral Ribavirin 1-1.2 gm daily for 24 weeks.

b. Poor Response

Alfa interferon 3 MU subcutaneously TTW plus oral Ribavirin 1-1.2 gm daily for 24 weeks initially. If PCR negative at 24 weeks continue for a total of 48 weeks. If PCR positive at 24 weeks stop therapy.

Monitoring of therapy

It is recommended that patients are seen at 1, 2 and 4 weeks and thereafter every 4 weeks for 24 or 48 weeks during therapy. A clinical nurse specialist with experience in this area is ideally suited to this role, provided there is readily available medical support. Monitoring should include support and encouragement for the patient, reminder about the need for contraception, observation for any side-effects or complications, and blood tests including full blood count, renal function and liver function tests. Marrow suppression may require a reduction in dosage. Serum PCR for HCV-RNA should be checked at 24, 48 and 72 (poor response category) weeks and thyroid function tests at 24 and 48 weeks. Liver biopsy may be repeated (optional) six months after the end of treatment.

Appendix 4. HGV RT-PCR protocol. Reaction mixes per sample analysed.

RNA	10.5 µl
antisense primer HGV-G8 (20pmol/µl)	1 µl
Total RNA/anti-sense 1 mix	11.5 µl

Incubate for 10 min at 70°C, then quickly chill on ice

a. RT-Mix

5x RT-buffer	4 µl
DTT	2 µl
RNAsin (Promega)	1 µl
dNTP (10mM stock)	0.5 µl
Superscript RT (Gibco BRL)	1 µl
	8.5 µl
Plus RNA/anti-sense 1 mix	20 µl

Incubate for 1 hour at 37°C, 10 min at 95°C

cDNA	20 µl

Store at -20°C

Appendix 4 (Continued)

HGV RT-PCR protocol. Reaction mixes per sample analysed.

b. Mix for outer PCR

10x PCR buffer	2.5 µl
Mg 50mM (1mM final concentration)	0.5 µl
dNTP (10mM stock)	0.5 µl
Sense primer HGV-G7 (10 pmol / µl)	0.75 µl
Antisense primer HGV-G8 (10 pmol / µl)	0.75 µl
TAQ-polymerase (Gibco BRL)	0.25 µl
Water	14.75 µl
	20 µl
plus cDNA	5 µl

c. Mix for inner PCR

10x PCR buffer	2.5 µl
Mg 50mM (1mM final concentration)	0.5 µl
dNTP (10mM stock)	0.5 µl
Sense primer HGV-G7 (20 pmol / µl)	0.75 µl
Antisense primer HGV-G11 (20 pmol / µl)	0.75 µl
TAQ-polymerase (Gibco BRL)	0.25 µl
Water	17.75 µl
	23 µl
plus PCR 1 product	2.5 µl