Determinants of Hepatitis C Virus Clinical Outcomes

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Submitted for the degree of
Doctor of Philosophy (PhD)

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June 2003

ABSTRACT

Hepatitis C virus (HCV) infection is characterized by a broad spectrum of clinical outcomes. An estimated 14%-46% of individuals exposed to HCV are able to clear the virus, while the other portion develops chronic (persistent) infections. Among the individuals with chronic HCV who are treated with interferon-based therapies, only a portion are able experience sustained virological suppression. Similarly, a number of chronically infected individuals have autoimmune extrahepatic manifestations such as the presence of autoantibodies. The pathological mechanisms behind these phenomena are not known, but it is believed that host genetic factors may play a role. This thesis examines the hypothesis that host genetic factors may contribute to the diverse spectrum of HCV clinical outcomes. In addition, it examines the pathogenesis of antinuclear antibodies (ANA) in chronic HCV, and the effect of ANA positivity on the natural history of HCV. Correlations were observed between female gender and geographic location and ANA positivity. No relationships were observed for an effect of ANA positivity on response rates to interferon therapy. We observed a trend of ANA positivity with faster progression of HCV-related fibrosis, although this failed to achieve statistical significance. ANA-positive individuals tended to have more plasma cells in their liver than ANA-negative individuals. This study also observed a number of correlations between genotypes of the interferon induced genes encoding the myxovirus resistance 1 protein (MxA), 2'-5'oligo-adenylate synthase 1 (OAS-1), and protein kinase (PKR), as well as genes encoding cytotoxic T-lymphocyte antigen-4 (CTLA4), and inducible nitric oxide synthase (iNOS) (encoded by the *NOS2A* gene) with several outcomes including self-limiting versus chronic HCV infection, along with the response to interferon therapy. This study identified several factors to be correlated with ANA positivity in HCV. These factors may serve as future points for investigation by basic scientists understanding the mechanisms of HCV-mediated autoimmunity. Importantly, this study suggests that low titre ANA positivity should not be a contraindication to therapy. This study also highlighted the importance of several genetic pathways in HCV infection. These may serve as targets for future pharmacologic interventions or genetic tests designed to screen for those who will not benefit from interferon therapies.

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List of Abbreviations

HCV= hepatitic C virus

HBV= hepatitis B virus

HIV= human immunodeficiency virus

HCC= hepatocellular carcinoma

PCR= polymerase chain reaction

ANA= anti-nuclear antibodies

IIF= indirect immunofluorescence

MxA= myxovirus resistance 1

OAS-1= 2'- 5' oligoadenylate synthase

PKR= protein kinase

CTLA4= cytotoxic t-lymphocyte antigen 4

iNOS= inducible nitric oxide synthase

SR= sustained responder to interferon therapy

IR= initial responder to interferon therapy

RR= relapsed=responder to interferon therapy

NR= non-responder to interferon therapy

IFN= interferon monotherapy

IFN+R= interferon plus ribavirin combination therapy

Preface and Acknowledgements:

Statement of Originality and Contribution

This thesis was written by Leland J. Yee. Where the published work of others is used, or referred to, this is referenced. The work in this thesis was predominantly designed and conducted by Leland J. Yee. The contributions of others are detailed below:

For the study of anti-nuclear antibodies:

The study was designed by Leland Yee with Professor Andy Hall. All laboratory testing and scoring of anti-nuclear antibodies was conducted by Leland Yee. Double-reading of these tests was conducted by Dr. Peter Kelleher. All statistical analyses were conducted by Leland Yee. For the study of anti-nuclear antibodies and cognitive function in chronic hepatitis C, cognitive scores were provided courtesy of Dr. Dan Forton.

Genetic studies:

For the study of *cytotoxic T-lymphocyte antigen 4* polymorphisms and response to therapy, Leland Yee designed the study with Dr. Richard Kaslow. The genotyping system and primers were designed by Dr. James Tang. Genotyping was conducted by Leland Yee with Dr. Kevin Perez. All data analyses were performed by Leland Yee.

For the study of polymorphisms in interferon-stimulated genes and the outcome of hepatitis C, the study design was developed by Leland J. Yee with Dr. Mark Thursz. Genotyping was conducted by Leland J. Yee with Dr. Susanne Knapp and data analyses conducted by Leland Yee.

For the study of inducible nitric oxide synthase (*NOS2A*) gene polymorphisms and the outcome of hepatitis C infection, the study design was developed by Leland J. Yee with Dr. Mark Thursz and genotyping was conducted by Leland Yee and Dr. Knapp. Leland Yee analysed all the data.

Acknowledgements

I am grateful to a number of individuals who have assisted me greatly with this thesis. First, I would like to thank my supervisors, Professor Andy Hall and Dr. Mark Thursz for guiding me and assisting me with this thesis. Without their constant support and efforts, the studies included in this thesis, as well as the thesis itself, would have never come to fruition. Dr. Helen Weiss was an excellent statistical consultant, who was always willing to help me track down a solution to a problem and properly answer it. I am also indebted to numerous colleagues (too many to mention individually) who have given me advice and kept me relatively sane over the past few years.

I am also grateful to Drs. Peter Kelleher and Dirk van Leeuwen for spending extra time behind the microscope with me, teaching me the intricacies of reading immunodiagnostics slides and liver biopsies, respectively.

Chapter 1

Background: Hepatitis C Epidemiology and Natural History

1.1 The Hepatitis C Virus

Isolated in 1989, the Hepatitis C virus (HCV) accounts for the majority of cases of non-A, non B hepatitis.[1] HCV is a member of the flaviviridae family and is the sole member of the genus hepacivirus.[2,3] HCV is separated into 6 major genotypes, with several subtypes.[4,5]

The hepatitis C virus has a single-stranded RNA genome of approximately 9.6 kilobases that encodes a single, large polyprotein of about 3,000 amino acids.[6] Proteins encoded by the HCV genome are cleaved post-translationally into multiple structural and nonstructural polypeptides (Figure 1.1.1). Structural components include: the nucleocapsid core (C) and the envelope glycoproteins, E1 and E2. Nonstructural proteins (NS) include NS2 through NS5. While it is believed that NS3 has helicase and protease properties, and that NS5 has polymerase properties that aid in viral replication, the specific functions of the NS proteins have not been fully elucidated.[7] The 5' and 3' untranslated regions are highly conserved. The 5' untranslated region has an internal ribosomal entry site for the initiation

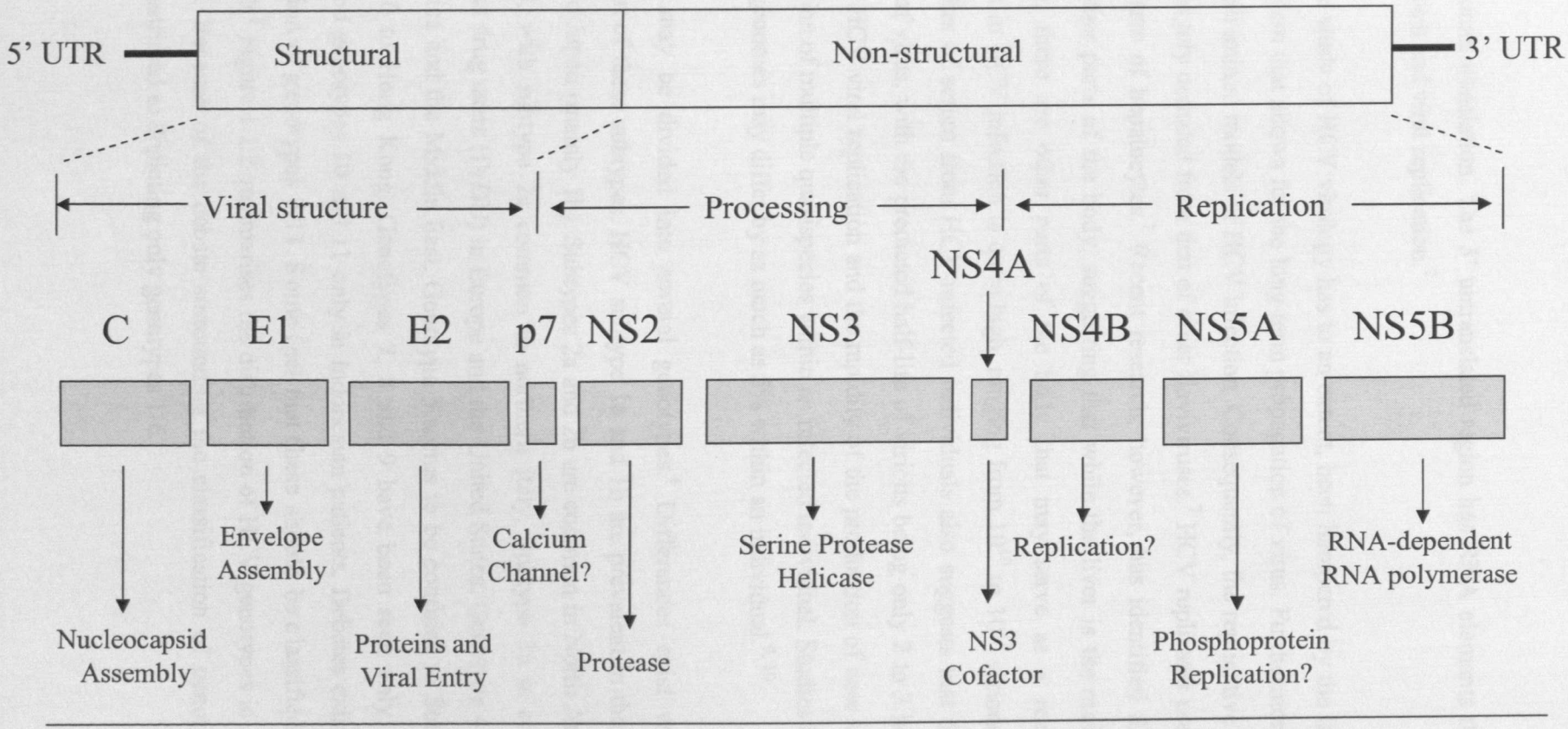


Figure 1.1.1. Cartoon diagram of the genomic structure of the hepatitis C virus. The 10 polypeptides cleaved from the polyprotein are shown in the bottom half, along with their suspected functions.

of viral protein translation. The 3' untranslated region has RNA elements that are crucial for translation and viral replication.[7]

The study of HCV virology has to an extent, been hampered by the lack of a viable culture system that allows for the long-term propagation of virus. Furthermore, there are no simple small animal models of HCV infection. Consequently, the replicative cycle of HCV has been largely deduced from that of other flaviviruses.[7] HCV replicates predominantly in the cytoplasm of hepatocytes.[7] Recent research, however, has identified the presence of HCV in other parts of the body, suggesting that while the liver is the main site of viral replication, there are other parts of the body that may serve as a reservoir. Virion production in HCV infection is quite high, ranging from 10^{10} to 10^{12} virions produced per day.[8] Studies of serum from HCV-infected individuals also suggests that there is a rapid turn-over of virus, with the predicted half-life of virions being only 2 to 3 hours.[8] The low fidelity of HCV viral replication and the rapidity of the production of new virions leads to the formation of multiple quasispecies within an infected individual. Studies have suggested that viral genomes may differ by as much as 5% within an individual.[9, 10]

HCV may be divided into several genotypes.[4] Differences exist with the global distribution of these subtypes. HCV subtype 1a and 1b are prevalent in the United States, Europe and Japan (mainly 1b). Subtypes 2a and 2b are common in North America, Europe and Japan, with subtype 2c common to northern Italy. Subtype 3a is common among intravenous drug users (IVDU) in Europe and the United States. Genotype 4 is prevalent in North Africa and the Middle East. Genotype 5 seems to be confined to South Africa, and genotype 6 to Hong Kong. Genotypes 7, 8 and 9 have been seen only in Vietnamese patients and genotypes 10 and 11 only in Indonesian patients. Debates exist regarding the classification of genotypes 7-11. Some feel that these should be classified as variants of genotype 6.[3] Figure 1.1.2 summarises the distribution of HCV genotypes in regions around the world. Because of the debate surrounding the classification of genotypes 7-11, the figure is restricted to depicting only genotypes 1-6.

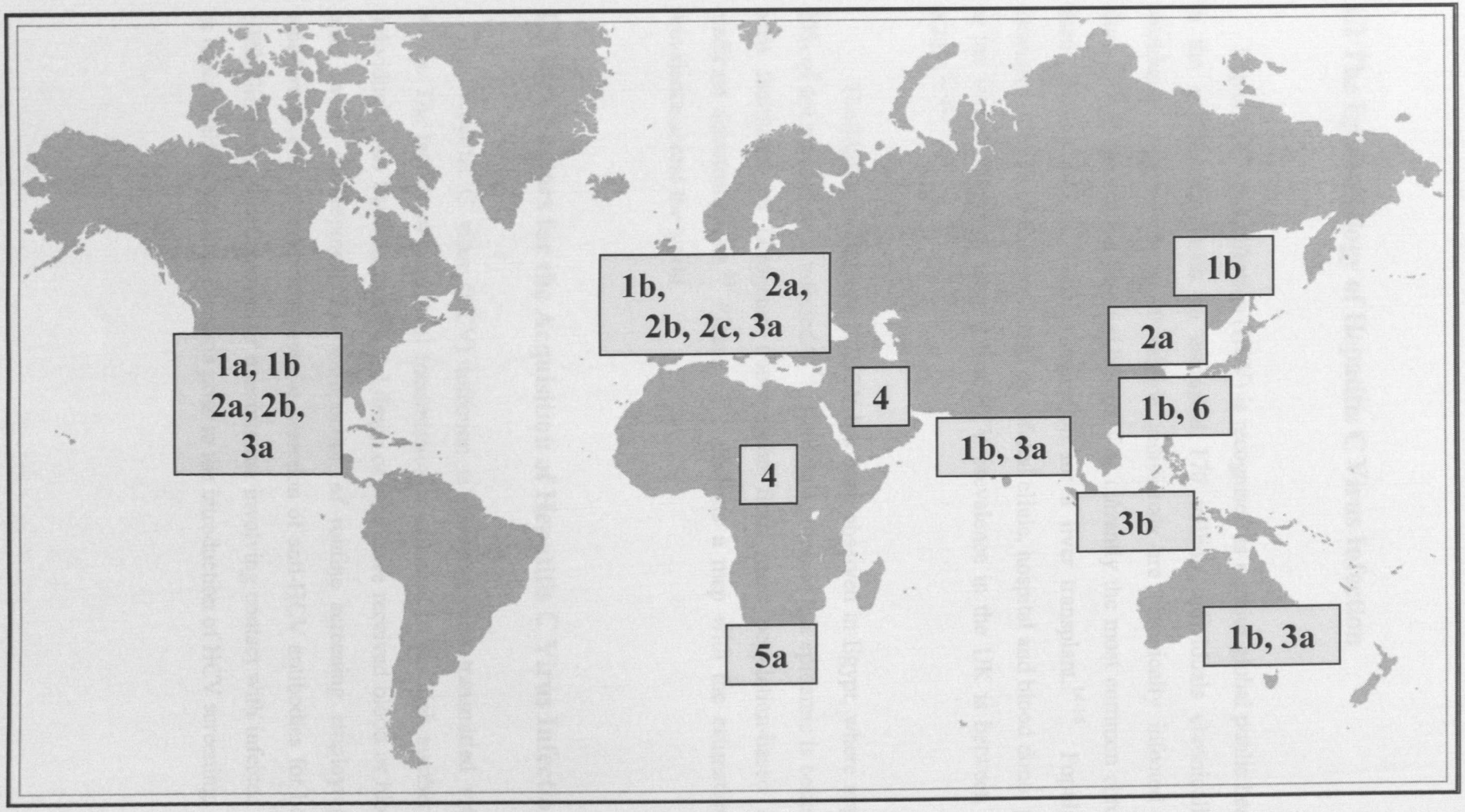


Figure 1.1.2. The distribution of hepatitis C virus genotypes in different regions of the world. Figure adapted from Fang J. *Clin Liver Dis*. 1997;1:503

1.2 The Epidemiology of Hepatitis C Virus Infection

Hepatitis C virus infection (HCV) is recognised as a major global public health burden in the world today, with an estimated 170 million individuals chronically infected worldwide. Approximately 10 million individuals are chronically infected in Europe alone.[11-13] In the United States (US), HCV is currently the most common chronic blood-borne disease and the leading indication for a liver transplant.[14-16] Population-based estimates of HCV prevalence based on referral clinic, hospital and blood donor populations in the United Kingdom suggest that HCV prevalence in the UK is between 0.08% and 0.72%.[17-20]

The highest prevalence of HCV has been observed in Egypt, where approximately 20% of the population is infected.[21-23] The propagation of this epidemic is believed to have been facilitated by iatrogenic transmission from mass population-based attempts to eradicate schistosomiasis.[23] Figure 1.2.1 presents a map with the estimations of HCV prevalence across the world.

1.3 Risk Factors for the Acquisition of Hepatitis C Virus Infection

Hepatitis C virus (HCV) infection is predominantly transmitted via parenteral routes. The facility of parenteral transmission is reflected in the high number of infected individuals who have either injected drugs or who have received blood or blood/products prior to the development and introduction of routine screening employing the more sensitive second generation assays for detection of anti-HCV antibodies for hepatitis C in 1992. Similarly, other sources of transmission involving contact with infected blood, such as haemodialysis was also common prior to the introduction of HCV screening.

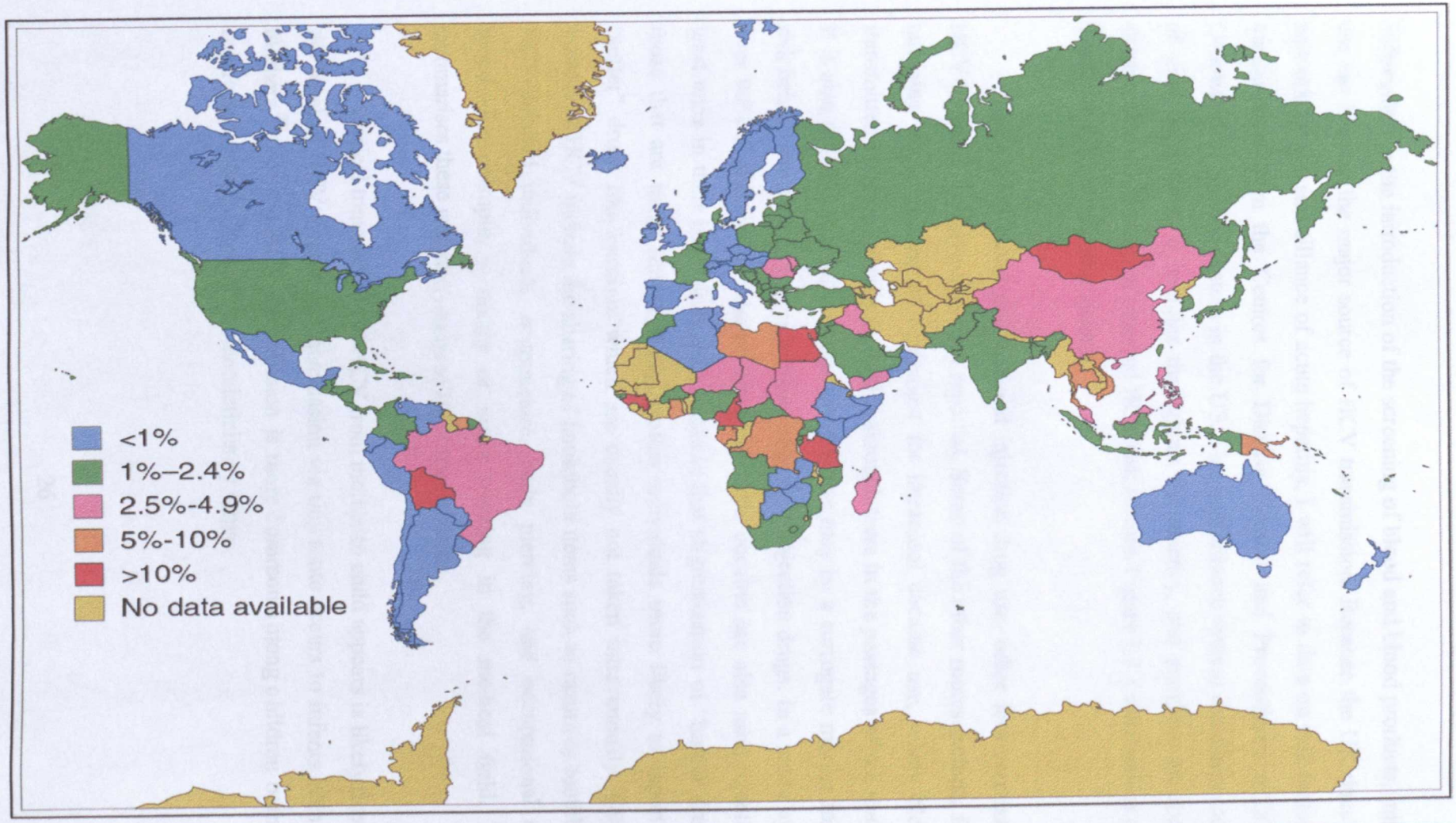


Figure 1.2.1. Map illustrating the prevalence of hepatitis C virus infection around the world. (Adapted from Cohen J. *Science* 1999 285; 26.

Subsequent to the introduction of the screening of blood and blood products, injection drug use has become the major source of HCV transmission. Because the UK does not have a network for the surveillance of acute hepatitis, I will refer to data on risk factors for HCV transmission from the Centres for Disease Control and Prevention's (CDC) Sentinel Counties Surveillance system in the US. This surveillance system examines incident cases of viral hepatitis in 6 counties throughout the country, and provides an opportunity to examine the distribution of reported HCV risk factors. Figure 1.3.1 summarises surveillance data from this surveillance system.

Aside from blood products and injection drug use, other less common routes of HCV transmission have also been reported. Some of the other routes include, for example, tattooing or the sharing of equipment for intranasal cocaine use, where blood may be transmitted from one person to another through tears in the passages of the nasal septum.[25] It is also possible that self-reported cocaine use may be a surrogate marker for other high risk behaviours such as past experimentation with injection drugs. In a study by Yee *et al.*, over 90% of individuals who reported intranasal cocaine use also used injection drugs at least once in their life.[26] It is quite possible that stigmatisation of "harder drugs" such as those that are taken intravenously, makes individuals more likely to report the use of "softer" drugs like cocaine, which are usually not taken intravenously. Other reported sources of HCV include the sharing of household items such as razors or toothbrushes with HCV-infected individuals, acupuncture, body piercing, and occupational exposure to blood—for example, a doctor or nurse working in the medical field. Table 1.3.1 summarises these reported routes of HCV transmission.

Vertical transmission of HCV from mother to child appears is likely dependent upon the level of HCV viraemia. Transmission via this route occurs to infants born to viraemic mothers.[27] Vertical HCV transmission is more common among children born to mothers coinfected with the human immunodeficiency virus

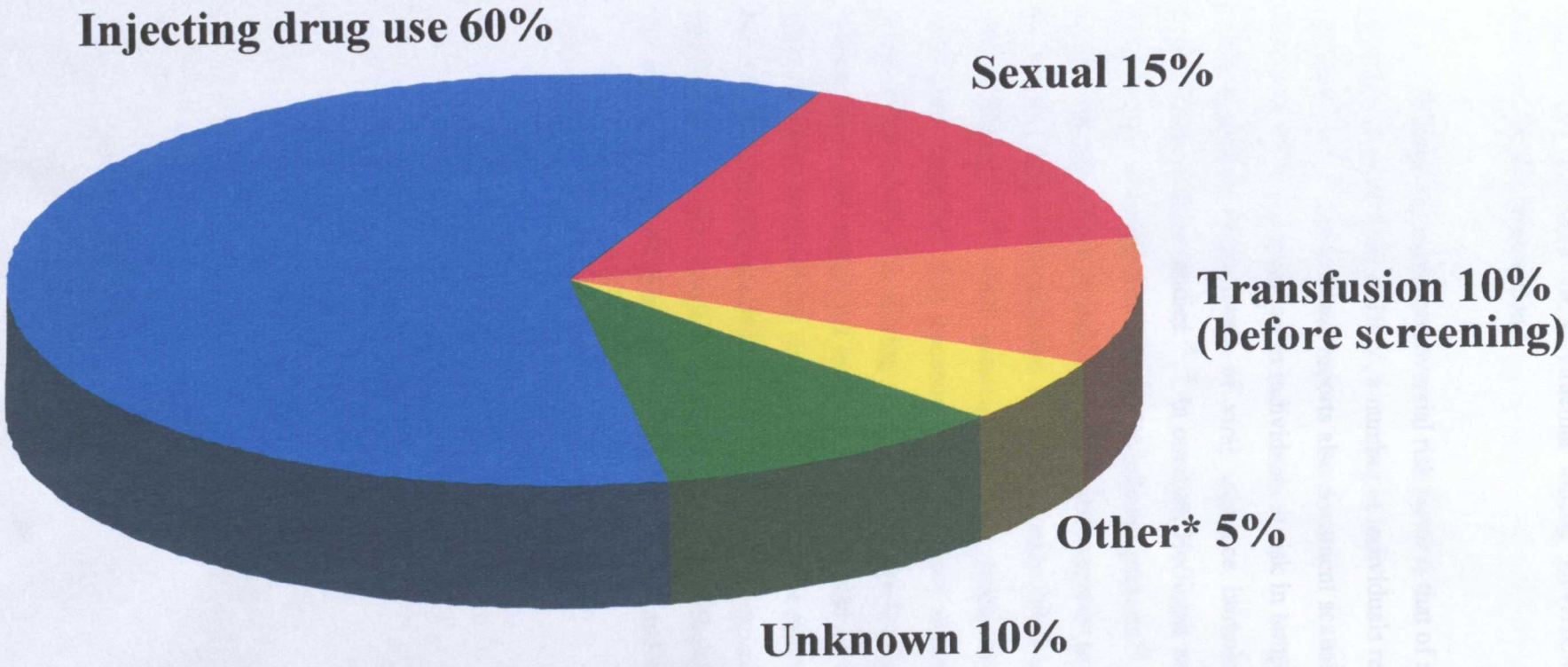


Figure 1.3.1. Reported major sources of hepatitis C acquisition, based on surveillance data from the United States. Source: Centres for Disease Control and Prevention (www.CDC.gov).

(HIV), and increased HCV viraemia among HIV/HCV coinfected individuals likely accounts for this observation.[27]

Perhaps the most controversial risk factor is that of sexual transmission.[28] In a recent population-based study of HCV, a number of individuals reported sex as the sole risk factor for hepatitis C.[16] Several case reports also document sexual transmission.[29, 30] These studies document HCV transmission in individuals at risk in temporal relationships with an HCV-infected partner. High levels of viral sequence homology also support the notion of transmission in these studies.[29, 30] In contrast, Hollinger and colleagues studied 18 couples (36 persons) recruited from 430 HCV-infected persons.[31] After screening for risk factors, all but only one of the 36 individuals reported exposure to parenteral risk factors. In half of the couples sexual transmission was immediately ruled-out, as the couples were infected with different HCV viral genotypes. In the remaining individuals, analysis of viral sequences suggested that transmission could have occurred in only 1 couple, and this couple later admitted to sharing equipment with each other for injection drug use.[31] Other studies, have also suggested low sexual transmission of HCV.[32-34] All sexual acts do not carry the same level of risk. Future studies must not only address these differences in risk, but also evaluate the role of comorbidities such as concomitant infection with HIV in HCV transmission via sex. Large prospective studies specifically designed to evaluate the risk of sexual exposure in HCV transmission must be conducted before this issue will be resolved.

Table 1.3.1. Summary of commonly recognised risk factors for HCV acquisition.

RISK FACTOR	REFERENCES
Receiving blood/blood products prior to 1992	Aach, 1991[35] Conroy-Cantilena, 1996[25] Alter, 1989[36]
Injection drug use	Conroy-Cantilena, 1996[25] Alter, 1999[16] Alter, 1990[37] JAMA
Intranasal cocaine use	Conroy-Cantilena, 1996[25]
Sharing of razors or toothbrushes	Mele, 1995 [38] Tumminelli, 1995[39]
Piercing	Conroy-Cantilena, 1996[25] Mele, 1995[38]
Sexual exposure	Alter, 1999[16] Alter, 1989[36]
Occupational exposure to blood	Alter, 1990[37]
Vertical transmission	Ohto, 1994[40]
Tattooing	Mele, 1995[38] Sun, 1996[41] Kaldor, 1992 [42]
Haemodialysis	Alter, 1990[37] Sandhu, 1999[43]
Acupuncture	Kioysawa, 1994 [44] Mansell, 1995 [11]

Individuals are often exposed to more than one risk factor. Surveillance of HCV risk factors often involves the use of hierarchical algorithms, where risk factors are placed in a hierarchical order based on probability of transmission. For example, sharing of equipment for injection drug use is placed higher on the algorithm than sexual exposure, because the probability of transmission via injection drugs is much higher. "Lower risk" routes of transmission may be masked by "higher risk" routes of transmission. While such a hierarchical system facilitates the surveillance of hepatitis C acquisition, it may not necessarily provide an accurate picture of HCV acquisition. Future studies of HCV transmission will have to address these factors as new studies are designed to assess the role of lower risk behaviours, such as sexual exposure, in the transmission of HCV. Figure 1.3.2 summarises the distribution of risk factors for HCV acquisition. A cohort effect is present, where older individuals tend to have exposure to blood and blood products, while younger individuals tend to report exposure to injection drug use. Some individuals report exposure to both blood products and drugs. Individuals in both age categories also report exposure to multiple other risk factors.

1.4 The Natural History of Hepatitis C Virus Infection: Progression and Therapy

The acute phase of HCV infection

HCV infection results in a diverse spectrum of possible outcomes. To begin with, an estimated 14% - 46% of individuals exposed to HCV are able to naturally clear the virus (self-limiting infections). While chronicity was traditionally defined as the persistence of elevated amino transferase levels for 6 months or more, it is now commonly defined as the persistence of HCV-RNA levels beyond 6 months or more. Unfortunately, the majority (54% - 86%) of individuals exposed to HCV develop persistent (chronic) HCV infections.

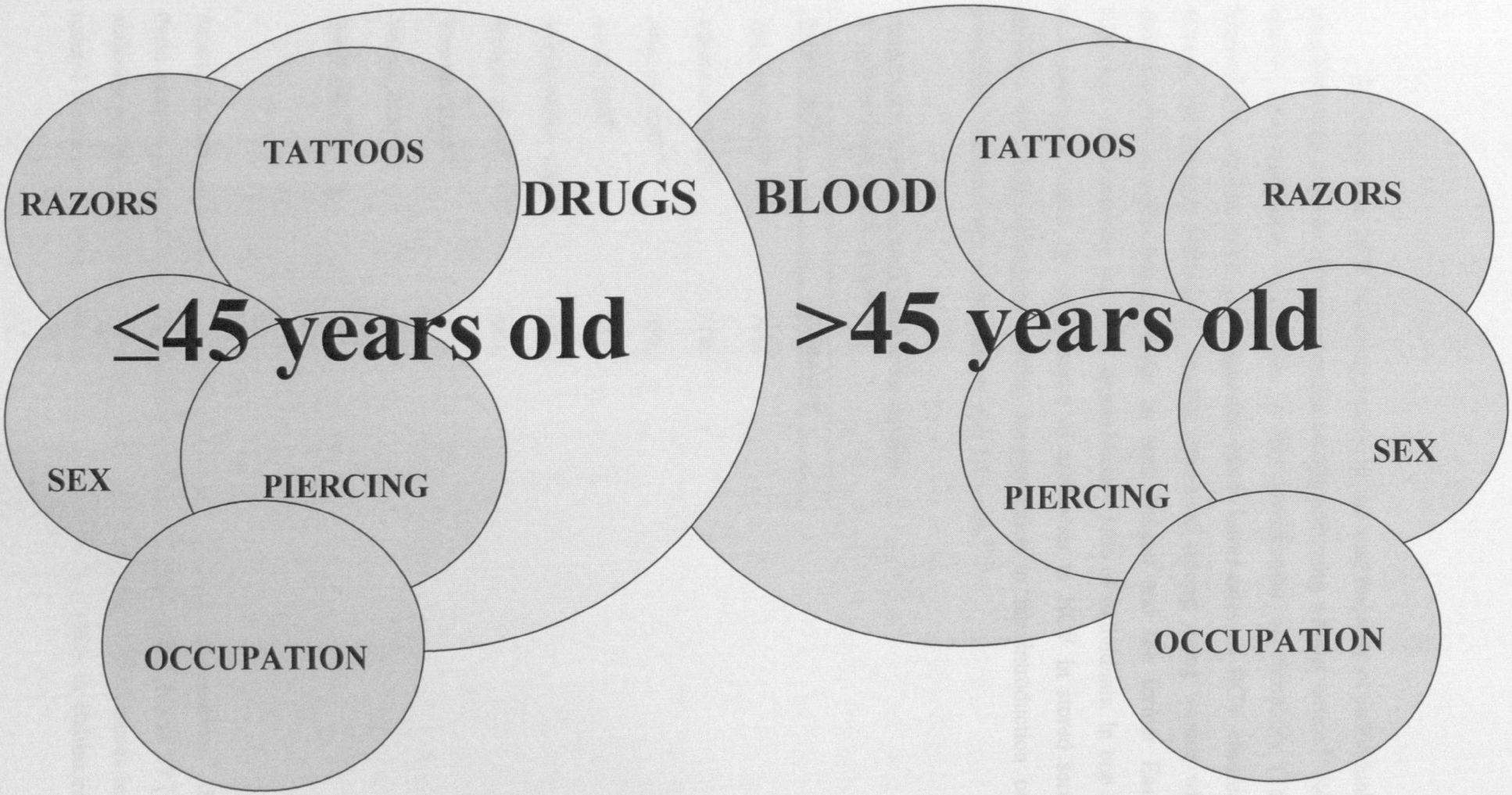


Figure 1.3.2. Schematic diagram illustrating the multiplicity of risk factors.

Infection with HCV is asymptomatic in the vast majority of individuals, with the transition from acute to chronic infection usually occurring without notice.[46] Retrospective studies have suggested varied rates in the development of chronicity (Table 1.4.1). Interestingly, studies have also suggested much lower rates of HCV chronicity among women and younger individuals. Studies conducted among young women who received contaminated anti-Rh-immunglobulin in both Ireland and the former East Germany suggested that chronicity occurs in approximately 55% of individuals. In one study, Vogt and colleagues tested for the presence of antibodies to HCV in stored sera from 458 children who had undergone cardiac surgery prior to the introduction of anti-HCV screening. The mean age of the patients was 2.8 years.[47]

Table 1.4.1. Table illustrating some of the variation in reported rates of HCV chronicity.

Author (year)	Chronicity
Alter, MJ 1992[48]	74%
Locasciulli, 1997[49]	71%
Alter, HJ 1997[50]	86%
Vogt, 1999[47]	55%
Kenny-Walsh, 1999[51]	55%
Rodger, 2000[52]	54%
Thomas, 2000[53]	86%
Wiese, 2000[54]	55%
Seeff 2001[55]	76%

Among the samples tested, 67 (14.6%) were found to be anti-HCV positive.[47] Follow-up of these samples 20 years later revealed that HCV-RNA was detectable in 37 (55%) of the antibody positive individuals. None had been treated for HCV. Differences in the rates of natural clearance of virus may occur by race as well. In a study in Baltimore, Maryland,

USA, Thomas and colleagues observed that individuals who were most likely to naturally clear HCV viraemia were non-black (Odds ratio (OR)=4.66; 95% Confidence Interval (95%C.I.): 2.44-8.90) and female (OR=1.58; 95%C.I.: 0.98 – 2.54).[56]

Progression of chronic HCV

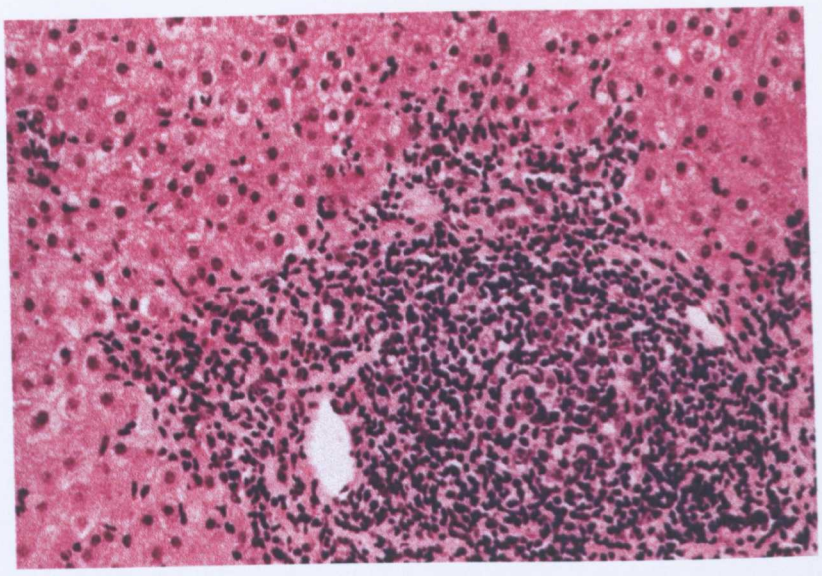
The clinical outcome of chronic HCV infection is varied as well. Among individuals with chronic infections, a portion progress to develop liver cirrhosis, while a portion of these cirrhotics continue to progress and develop the complications of end-stage liver disease, with some developing hepatocellular carcinoma. Just how many individuals progress to cirrhosis, develop end stage liver disease, or primary liver cancer is currently a matter of debate. Early studies of HCV natural history suggested that as many 20% of individuals progress to cirrhosis. However, recent studies involving individuals who were young and otherwise healthy at the time of infection, suggest that the occurrence of cirrhosis is less than 5%.[46]

It is also important to bear in mind several limitations regarding natural history studies. First, follow up of infected individuals has rarely exceeded two decades, so that outcome beyond this point is not yet known. Second, primary infection with HCV is usually asymptomatic. Aside from some unique incidents such as groups of women being infected with contaminated anti-Rh immunoglobulin in Ireland and Germany,[51, 54] estimates of durations of infection have been largely conducted on a retrospective basis by patient interview for risk factors for HCV acquisition. Consequently, problems such as recall bias come into play. In addition, HCV is a slowly-progressive disease. Many individuals experience other factors during the decades following infection, such as ingestion of large amounts of alcohol, or infection with other viruses such as the hepatitis B virus, so as to obscure "true HCV natural history."

The progression of fibrosis in chronic HCV infection is measured semi-quantitatively, commonly using either the METAVIR system, which has a scale of 0 to 4 for degrees of fibrosis (0 is no fibrosis and 4 is cirrhosis),[57] or the Knodell score, which has a scale of 0 to 6 for fibrosis (0 is no fibrosis and 6 is cirrhosis).[58] A characteristic feature of

chronic HCV infection is the presence of lymphoid aggregates. Figure 1.4.1. Part A. presents an example of lymphoid aggregates in a liver biopsy specimen stained with haematoxilin and eosin. Figure 1.4.1. Part B. presents a reticulin stain of a lymphoid follicle. Figure 1.4.2. illustrates some of the key clinical features of HCV-related end stage liver disease (ESLD). The computerised tomography (CT) scan presents a patient with a cirrhotic liver that is shrunken in size, covered with the nodules characteristic of cirrhosis, an enlarged spleen, and the presence of ascites. In addition, there is the presence of a notable hypervascular tumour in the liver. Figure 1.4.3. Part A. is a fine-needle biopsy of a cirrhotic liver. Figure 1.4.3. Part B. is a laparoscopic view of a cirrhotic liver. The nodular surface characteristic of cirrhosis may be clearly seen in this picture. Whether progression to cirrhosis is linear in these individuals is also a matter of debate.[59] Large cross-sectional studies have suggested that progression is linear, while follow-up studies have suggested varied progression.[60] More research is needed before this issue can be resolved.

A.



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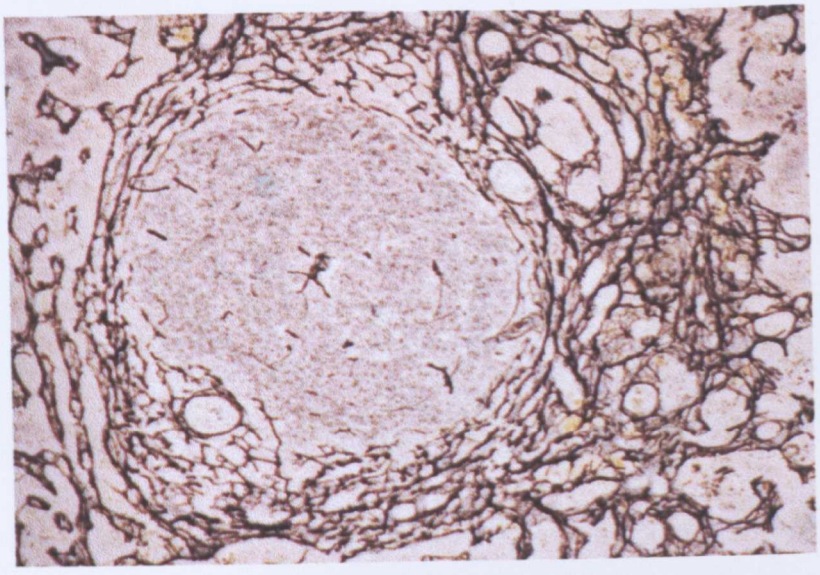


Figure 1.4.1. Lymphoid follicles in chronic hepatitis C virus infection. **Part A.** presents a haematoxylin and eosin stain of a liver biopsy specimen. The dark area is a lmyphoid follicle. **Part B.** is a reticulin stain of a lymphoid follicle.

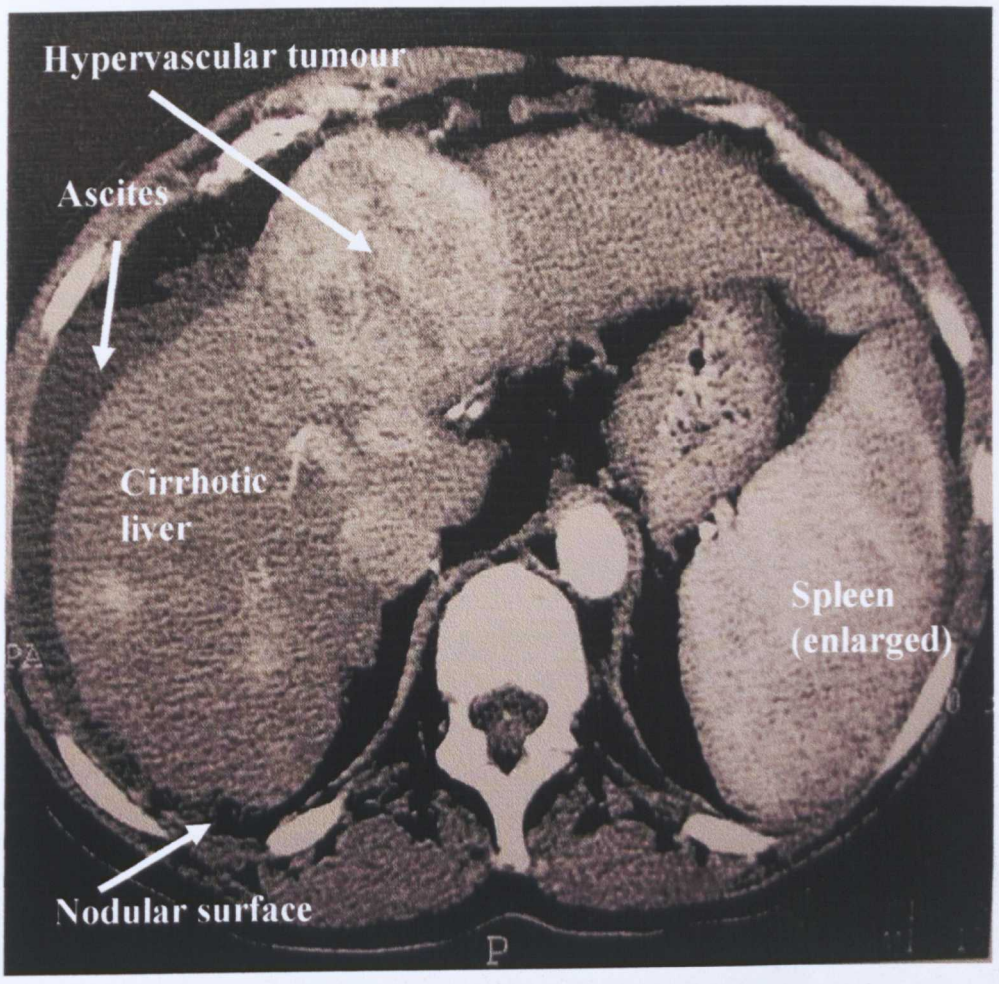
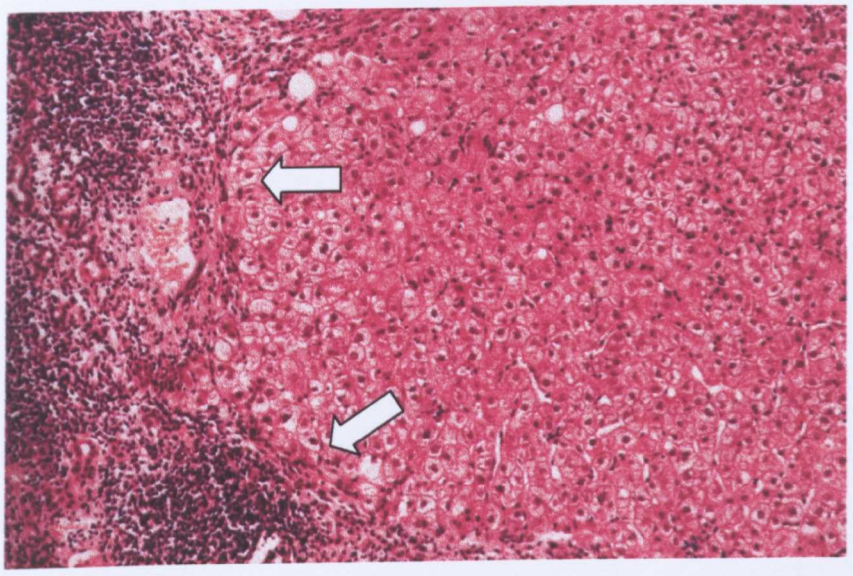


Figure 1.4.2. Computerised tomography (CT) scan of a patient with chronic hepatitis C virus (HCV) infection that depicts many of the characteristics of end-stage liver disease. The patient has an enlarged spleen and a cirrhotic liver with a nodular surface. There is also a sizeable tumour in the liver. Note the presence of ascites as well.

A.



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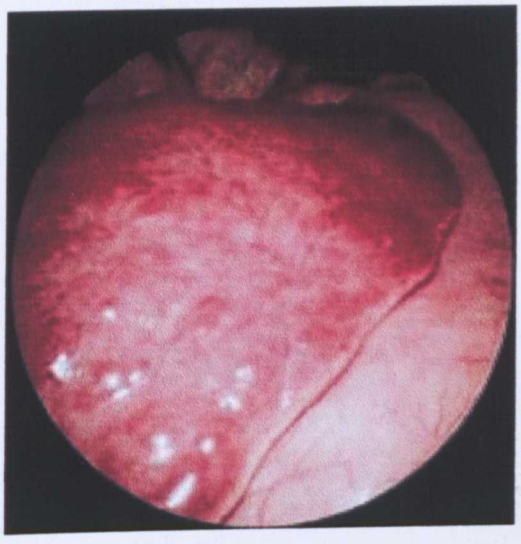

Figure 1.4.3. The cirrhotic liver. **Part A.** presents a haematoxylin and eosin stain of a liver biopsy specimen. The white arrow depicts the boundaries where a cirrhotic nodule has formed. **Part B.** is a cirrhotic liver as viewed by laparoscopy.

1.5 Therapy for hepatitis C virus infection

Interferon monotherapy

One of the first successful agents to be used against HCV infection was interferon-α. Interferons are cytokines with potent immunological effects that include anti-viral activity, and are divided into two main groups.[61, 62] Type I interferons include interferon-α (with at least 12 different subtypes), an interferon-β subtype, and an interferon-ω subtype. Type II interferons include interferon-γ. Type I interferons are released during a host response to an invading virus and bind to cell surface receptors and initiate the transcription of several proteins with potent anti-viral activities, such as protein kinase (PKR), 2'-5' oligo-adenylate synthase (OAS), and mxyovirus resistance-1 (MxA).[63] Type II interferons are produced by antigenically stimulated immunocytes and modulate the developing, specific immune reponse.[64] Interferons are released by the body within hours of any viral infection and inhibit viral replication in a non-specific manner.[63, 64] The importance of interferons in the host defense against viral infections are illustrated with transgenic animal models lacking type-1 interferon receptors. Following inoculation with small doses of viruses with low pathogenicity, these animals die rapidly.[65]

Trials of the use of recombinant interferon-α (3 million units administered by subcutaneous injection three times per week) for the treatment of chronic HCV showed promise. Treatment for 24 weeks resulted in a sustained response in 10-15% of individuals.[66,67,68] Subsequently, interferon-α became the first approved regimen for the treatment of chronic hepatitis C.

Interferon and Ribavirin combination therapy

In 1998, two landmark studies demonstrated higher response rates with the use of interferon-α in combination with ribavirin, than therapy with interferon-α alone.[69, 70] Ribavirin, a purine nucleoside analogue, is ineffective in lowering HCV-RNA levels when

used as monotherapy for HCV, although it did lower serum alanine transaminase levels in a significant number of patients.[71-73] In combination with interferon, however, addition of ribavirin results in increased end of treatment response rates. Combination interferon-α and ribavirin therapy results in sustained virologic response in approximately 25% of individuals with genotype-1 infections and about 40% of individuals with non-1 infections.

It is worth noting that the exact mechanisms of action of ribavirin are not known. There are 4 proposed mechanisms of action at present: 1) that ribavirin enhances T-cell responses by encouraging the expression of a T_H1 cell phenotype over that of a T_H2 phenotype;[74-76] 2) that it inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH), which produces GTP—a critical ingredient for successful RNA synthesis. By decreasing the net GTP pool, it is hypothesised that viral replication may be inhibited;[77, 78] 3) that it directly inhibits HCV via the NS5B region-encoded RNA dependent polymerase;[78] and 4) that it inhibits successful HCV replication as a mutagen that pushes the already error-prone RNA virus replication process to that of such immense proportions that viable virions can no longer be produced.[78] Further research into the pharmacology of ribavirin is needed to fully understand its mechanisms of action.

Another important feature resulting from these clinical trials was the demonstration that patients infected with genotype-1 viruses, which are more resilient to these interferon-based regimens, benefit from longer durations of therapy. Standard combination therapy consists of 3 million units of interferon-α- 2b administered by subcutaneous injection three times per week. In conjunction, 1200 mg of ribavirin is taken orally every day by individuals > 75 kg, or 1 000 mg of ribavirin is taken orally every day for individuals ≤ 75kg. Those with genotype-1 infections are now generally treated for a full year, while those with non-1 infections are treated for 6 months.

Pegylated interferons as monotherapy and combination therapy with ribavirin

Standard interferons have two important liabilities. First, the pharmacokinetics of standard interferons peak following administration and wane soon after, consequently limiting the amount of interferon available to fight virus during the troughs (Figure 1.5.1. Part A). Second, the need to administer three doses per week by subcutaneous injection

increases the possibility for non-compliance. Addition of a polyethylene glycol (PEG) moiety to the interferon molecule has greatly reduced its excretion, and increased the half-life of the protein (Figure 1.5.1 Part B).[79] Addition of the PEG molecule to interferon has resulted in higher sustained response rates in HCV patients

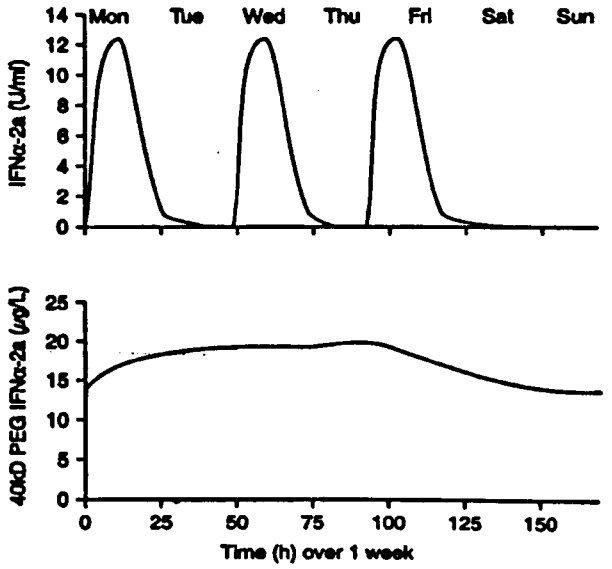

Figure 1.5.1 Pharmacokinetics of standard IFN and PEG-IFN. Levels of standard IFN peak and wane after each administration, while PEG-IFN maintains more steady levels over the entire week. (Adapted from Kozlowski 2001)[79]

compared to standard interferons (69% with PEG-interferon and 28% with standard interferon at 48 weeks of treatment; and 39% for PEG-interferon and 19% for standard interferon at 72 weeks of treatment).[80] Trails combining pegylated interferon with ribavirin observed sustained response in approximately 40 - 46%% of those with genotype-1 infections and 76% - 80% of those with non-1 infections.[81, 82]

Outcomes of anti-HCV therapy

Three main outcomes are observed in therapy for chronic HCV infection: (Lindsay 2002) 1) ***sustained response***—this is now commonly defined virologically as having undetectable serum HCV-RNA levels at 6 months after the discontinuation of treatment.

Older studies have defined response biochemically as a normalisation of ALT levels; 2) ***initial response***—this is a disappearance in serum levels of HCV-RNA at the end of the initial phase of treatment—commonly 12 weeks after the inception of the regimen. A portion of these individuals who have achieved initial response may also experience a virological relapse later on, so they are not true responders; and finally, 3) ***non-responders***—these are individuals who have detectable viraemia at week 12 of therapy. They are distinct from initial responders because they never achieved any sort of virological decline to undetectable levels, even during the initial course (first 12 weeks) of therapy. Figure 1.5.2 graphically illustrates these response phenotypes.

Side effects of therapy

Interferon therapy is associated with a number of side-effects, some of which may be debilitating and necessitate discontinuation of therapy. Common side effects include fatigue, influenza-like symptoms, haematologic abnormalities including haemolytic anaemia and neutropaenia, as well as neuropsychiatric symptoms including suicidal ideation.[83]

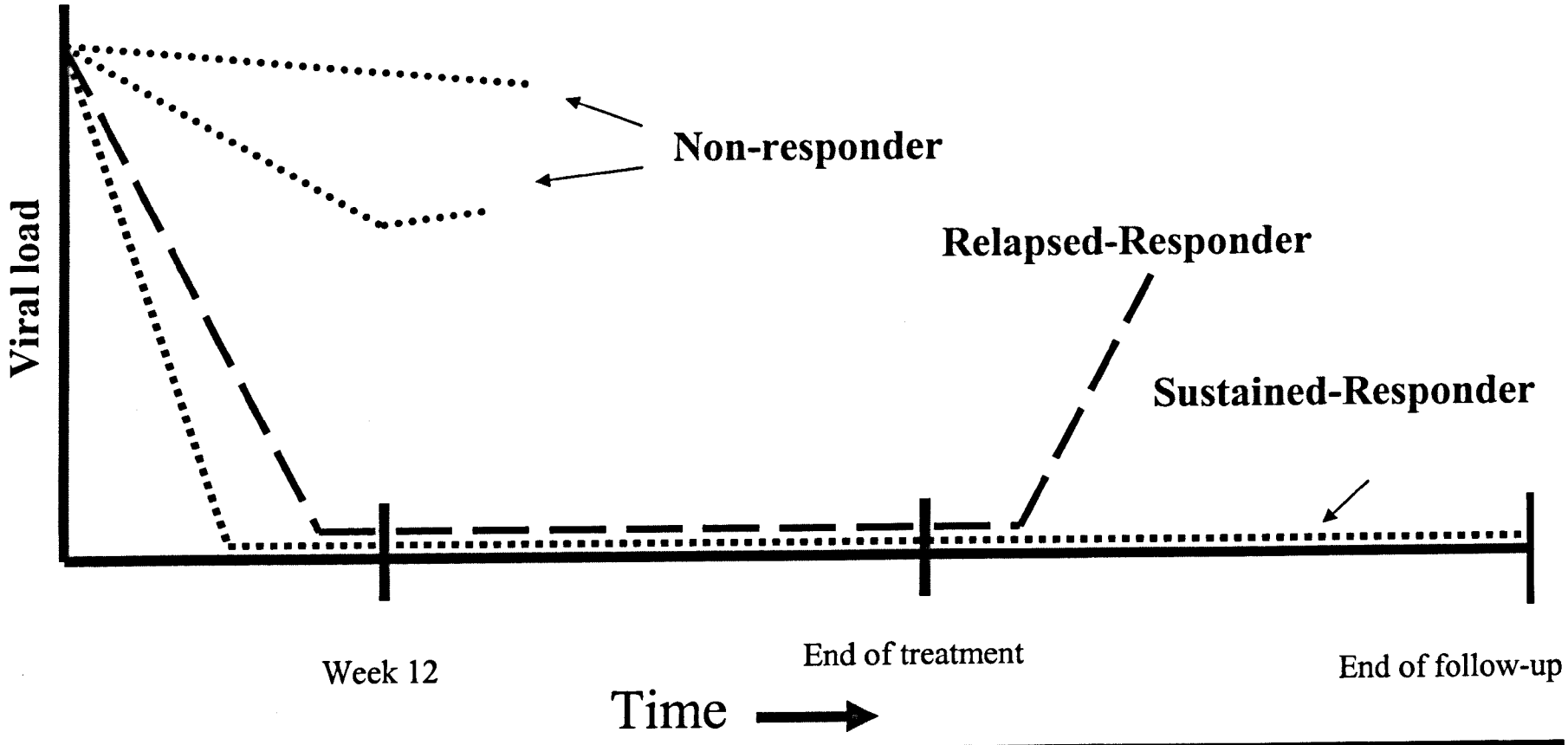

Figure 1.5.2. Cartoon diagram depicting the three main phenotypes observed following treatment of chronic hepatitis C virus infection with interferon. Non-responders may experience only a slight drop in viral levels or experience an initial moderate drop only to have viral levels resurge. These individuals never achieve undetectable levels of virus. Shortly after the inception of treatment, viral levels are not detectable amongst relapsed-responders. However, sometime following discontinuation of treatment, viraemia returns. Sustained responders generally achieve undetectable levels of HCV-RNA soon after initiation of treatment. Levels of viraemia remain undetectable until end of treatment. Six months after discontinuation of treatment (end of follow-up), levels are still undetectable.

Table 1.5.1 lists the frequency of the most commonly reported side effects for standard interferon-α plus ribavirin and pegylated interferon-α plus ribavirin.

Table 1.5.1. Frequency of some of the common side effects for interferon as mono-therapy, standard interferon with ribavirin (IFN+R) and pegylated interferon with ribavirin (PEG-IFN+R) during clinical trials.

Factor	IFN monotherapy (%)	IFN+R (%)	PEG-IFN+R (%)
Fatigue	78	55	54
Headache	53	52	47
Pyrexia	48	56	43
Myalgia	55	50	42
Rigors	NR	35	24
Insomnia	NR	39	37
Nausea	36	33	29
Alopecia	24	34	28
Irritability	28	28	24
Arthralgia	16	25	27
Anorexia	NR	22	21
Dermatitis	NR	18	21
Diarrhoea	34	NR	NR
Depression	14	30	22

Abbreviations: NR=Not Reported
Adapted from Davis 1989, Fried 2002 and Fried 2002.[66, 82, 83]

Predictors of response to therapy

The multiple, often debilitating side effects accompanying interferon-based therapies has prompted a search for predictors of who will most likely respond to these interferon-based therapies. Currently, commonly employed predictors of response involve viral factors. Non-1 viral genotype and low pre-treatment viral levels are all favorable prognosticators.[69, 70] More recently, there has been an interest in the role of viral dynamics during the period immediately following the inception of treatment in predicting therapeutic response. Early rapid decline in viral levels is a favorable indicator. The drop in viral levels following the start of treatment is biphasic. The initial drop is believed to reflect the clearance of free virions. The slope of this drop is dependent upon the dose of interferon.

The second slope reflects the clearance of virally infected cells.[8] Figures 1.5.3. and 1.5.4. illustrate these concepts. Figure 1.5.3 presents data from the Consensus interferon trials. Among those individuals who achieve sustained virologic response, the majority experienced viral disappearance within the first 4 weeks following initiation of therapy.[84] Figure 1.5.4. is a schematic diagram that illustrates the biphasic nature of viral dynamics in the period immediately following treatment of HCV infection with interferon.

The search for host factors that predict therapeutic response has also examined host demographic factors as well as host genetics. Women have been observed to respond better to treatment with interferon.[69, 70, 81, 82, 85] Race may also affect response to therapy.[86] In particular, blacks in the United States have also been observed to have a poor response to treatment.[87, 88] Other factors such as body weight and obesity have also been explored.[89] There has also been a search for genetic factors that predict outcome of therapy. This is reviewed in Chapter 2.

1.6 Comorbid Conditions in Hepatitis C Virus Infection

Chronic HCV infection is associated with a number of clinical/biological extrahepatic manifestations. Table 1.6.1 lists some of these comorbid conditions associated with HCV infection. Extrahepatic comorbidities include the presence of autoimmunity (non organ-specific autoantibodies) to more autoimmune disease-like manifestations, such as the Sicca syndrome, presence of cryoglobulins, or Raynaud's phenomena.[90, 91] Other conditions include peripheral blood count abnormalities,[92] or the presence of Type II diabetes mellitus.[93-98] More recently, reports have suggested an association between chronic HCV infection and cognitive dysfunction including memory impairment ("brain fog").[99]

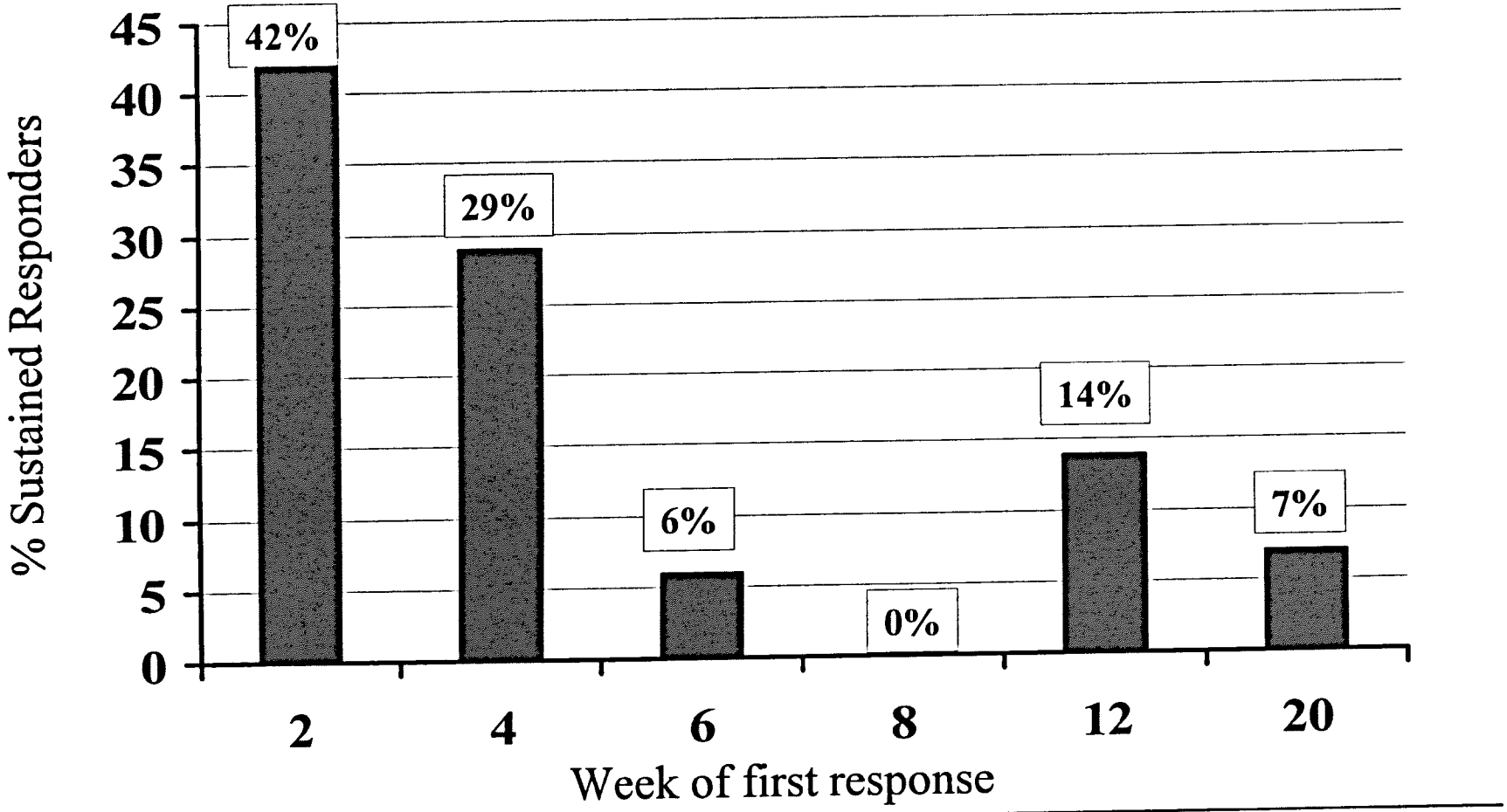

Figure 1.5.3. Data from the Consensus interferon trials illustrating the correlation between early virologic response (defined as undetectable levels of serum HCV-RNA) and sustained response to therapy. The majority of sustained responders achieved their first response by week 4.
Data from Lee et al. *Hepatology* 1998.[84]

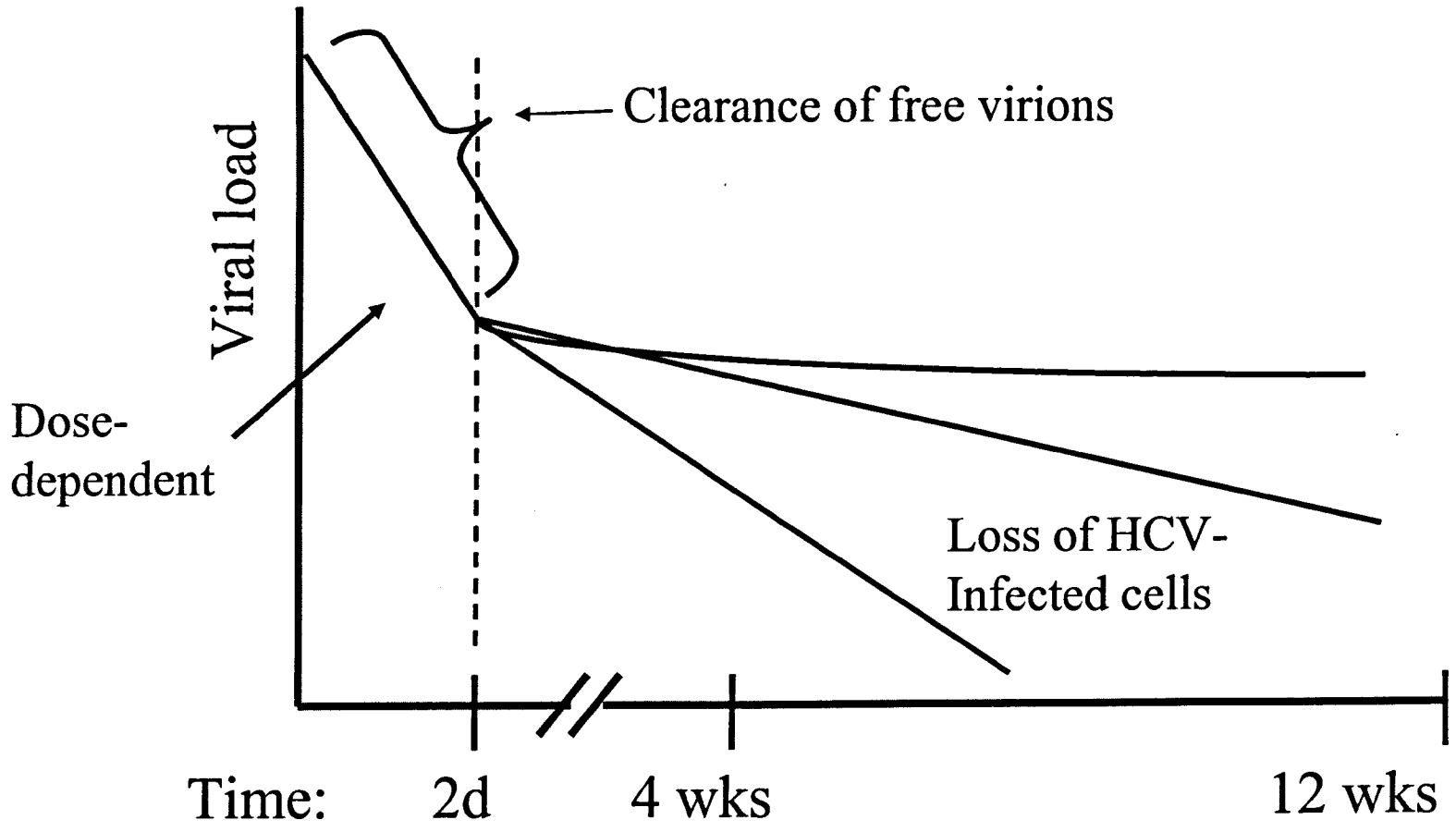

Figure 1.5.4. Schematic diagram illustrating the biphasic decline of serum HCV levels during the initial period following the start of interferon treatment. The first phase is believed to reflect the clearance of free virions, The slope is dose-dependent. The second phase is believed to reflect the clearance of HCV-infected cells. Adapted from Neumann et al. *Science* 1998.[8]

HCV is also associated with a number of non-clinical/biological comorbidities. Perhaps the most important non-clinical/biological co-morbidity is that of substance abuse (injection drugs and alcohol).[100-103] The prevalence of antibodies to HCV has been reported to be higher in alcoholic patients with clinically apparent liver disease than those without clinically apparent liver disease in France, Germany, the United States, Italy, Japan, and Sweden.[104-110] Strong correlations between behavioural patterns such as alcohol use and past or current drug abuse are likely to account for this strong connection. Given the observed synergistic effect of concurrent alcohol abuse and infection with chronic HCV in accelerating disease progression, this comorbidity is a factor with important clinical and public health ramifications.

1.7 Autoimmunity in hepatitis C virus infection

Many of the comorbidities associated with HCV infection are autoimmune in nature.[111-114] In this section I will highlight a few of the most common and important autoimmune comorbidities. Perhaps the most common autoimmune manifestation is the presence of non organ-specific autoantibodies (NOSA).[114-116] Table 1.7.1 highlights some of the autoantibodies associated with chronic HCV infection. The prevalence of these autoantibodies varies greatly, depending upon the population studied.[114] Differences in methodologies used to assess the presence of autoantibodies as well as choice of cut-off titres may also account for some differences. HCV-associated autoantibodies may differ from those seen in patients with primary rheumatic disorders. For example, HCV-associated anti-cardiolipin antibodies (ACA) generally exist in low titres and are not associated with lupus anticoagulants, anti-beta-2 glycoprotein 1, antithrombin antibodies, or clinical manifestations such as thrombocytopaenia.[117] While the prevalence of some of these autoantibodies in HCV-infected individuals is high, there are little data examining correlates of autoantibody positivity and the effect of positivity on HCV clinical outcome.

Table 1.7.1. Autoantibodies associated with chronic HCV infection.

Autoantibody	Reported prevalence
Rheumatoid factor	60%
Cryoglobulins	40%
Antinuclear antibody	4% - 41%
Antithyroid antibodies	10%
Anticardiolipin antibodies	20%
Anti-smooth muscle antibodies	7% - 20%
Anti-liver kidney microsomal-1 antibodies	1% - 3%
Anti-neutrophil cytoplasmic antibodies	10%

Adapted from Lövy 2000.[114]

There have been reports associating chronic HCV infection with other clinical manifestations including cryoglobulinaemia, thyroid dysfunction and Sjögren's syndrome. The presence of cryoglobulins was the initial and most widely recognised extrahepatic manifestation of HCV infection.[118, 119] Cryglobulins are immunoglobulins directed against immunoglobulins that reversibly precipitate at cold temperatures and consist of two types. Type I cryoglobulins are monoclonal, while type II cryoglobulins are polyclonal and typically contain a mixture of IgG and IgM molecules.[90] Immune complex formation and cryoprecipitation may occur and result in glomerulonephritis or vasculitis.[90] Arthralgias, peripheral neuropathy, hepatosplenomegaly, and lymphadenopathy may occur, and cutaneous lesions are common and may include palpable purpura and urticaria.[90] Treatment with interferon-α has generally been reported to mitigate HCV-induced cryoglobulinaemic symptoms.[118, 119]

Thyroid dysfunction has also been reported in HCV infection. Although the association of HCV with autoimmune thyroid disease is controversial,[120-122] the presence of thyroid autoantibodies is relatively common.[123-127] Interferon therapy for HCV has also been shown to induce the formation of anti-thyroid autoantibodies *de novo* in some patients.[128-131] Exacerbation of thyroid autoimmunity may occur in some individuals with preexisting diathesis, such as the presence of antimicrosomal antibodies.[129-131]

Associations of Sjögren's syndrome and HCV has been reported to be as high as 10%-20%.[132-135] Other studies, however, have refuted these findings.[136-140] If primary Sjögren's syndrome is defined as sicca syndrome in the presence of anti-Ro (SSA) and anti-La (SSB) antibodies, then the prevalence is quite low.[90] HCV infection, however, is more commonly associated with sialoadenitis (in the absence of anti-Ro (SSA) and anti-La (SSB) antibodies) and occasionally Sicca symptoms.[113, 141-144] The cause of this association is not clear, although in one study transgenic mice expressing HCV-envelope proteins developed an sialoadenitis-like exocrinopathy.[145]

Table 1.7.2 outlines some of the extrahepatic manifestations reported among individuals with chronic HCV infection. There are many other autoimmune associations reported with HCV infection, including autoimmune haemolysis and thrombocytopaenia,[146-148] Beçhet's syndrome,[149] and myasthenia gravis.[150] Many of these reports await further evidence to confirm a true association with HCV.

Table 1.7.2. Some of the extrahepatic manifestations reported in individuals with chronic HCV infection.

Extrahepatic manifestations
Fatigue
Arthalgia
Paresthesia
Myalgia
Pruritis
Diabetes
Raynaud's phenomena
Thyroid abnormalitites
Psoriasis
Non organ-specific autoantibodies
Peripheral blood count abnormalities
Cognitive dysfunction

Adapted from: Poynard and Gumber SC.[91, 151]

1.8 Viral Factors and the Outcome of Hepatitis C Virus Infection

The association of the various HCV genotypes with differences in pathogenicity has not been clearly established.[9, 152, 153] Some reports have suggested that infection with genotype 1b is associated with more severe disease and a more aggressive course than other genotypes.[16, 154-161] and is more prone to chronicity.[162] In contrast, there are a number of studies that have found no such associations.[153, 163-166] An explanation for this discrepancy centres on a possible cohort effect. Zein *et al* found that individuals with genotype 1b tended to be older than those infected with other genotypes and that genotype 1b may have been present before the other genotypes, and thus patients with genotype 1b infection may have just been infected for longer durations of time.[161] Similar observations have been made in France and Spain.[167, 168] In short, this explanation suggests that genotype 1b is a surrogate for more severe disease because people with this strain of virus are most likely to have been infected for longer durations of time and that the genotype 1b strain, *per se*, is not more pathogenic than others.

Infection with any HCV genotype may lead to cirrhosis, end-stage liver disease and hepatocellular carcinoma, and the frequency of these outcomes appear to be similar with each of the different viral genotypes.[7] However, there are two major differences with respect to viral genotypes. First, it is clear that genotype 1 viruses are more resistant to therapeutic intervention with interferon-based regimens.[69, 70] Unfortunately, in developed countries such as those of Western Europe, genotype 1 virus is the predominant viral subtype.[16, 164, 169] Second, infection with genotype 3 has been associated with a greater likelihood of developing steatosis on liver biopsy.[170] Interestingly, clearance of genotype 3 has been associated with a resolution of this steatosis.[170]

1.9 The Immune Response to Hepatitis C Virus Infection

The Immune Response in Self-limiting Infections

In addition to the generation of anti-HCV antibodies, individuals who are able to experience self-limiting HCV infections appear to mount strong CD4+ and CD8+ T-cell responses against multiple epitopes on HCV structural as well as non-structural proteins.[171, 172] T cells are likely to play a major role in the clearance of virally-infected cells. Lack of such a strong T-cell response during the initial phase following HCV infection is likely to result in persistent infection.[172, 173] A chimpanzee model of HCV infection has also demonstrated a strong intrahepatic T-cell response among animals with self-limiting infection than in those with chronically evolving infections.[174]

A strong T_H1 cytokine response is likely important in self-limiting infections as well.[175-178] In a prospective study of the immune response in acute HCV infection, Kamal and colleagues examined CD4+ T-cell responses in individuals with and without co-infection with *Schistosoma mansoni*,[179] and organism that is known to elicit a strong T_H2-type cytokine response.[180-182] This group observed one-third (5 of 15) patients with acute HCV mono-infection to experience self-limiting infections, while all individuals with HCV and *S. mansoni* co-infection progressed to chronic hepatitis.[179] Using interferon-γ production as a measure of the T_H1 cytokine profile and interleukin-10 as a measure of the T_H2 cytokine profile, co-infected patients were observed to exhibit a low T_H1 cytokine profile.[179] In addition, proliferation assays were used to assess CD4+ T-cell responses to HCV (Core, NS3, NS4 and NS5 antigens).[179] Patients with self-limited HCV mono-infection exhibited CD4+ T-cell responses that were significantly greater in vigour, frequency, and breadth than in those with chronic HCV mono-infection or in those with chronic HCV and *S. mansoni* co-infection, thereby providing further evidence supporting the notion that a strong T_H1 profile is important in self-limiting HCV infection.[179] There is a similar correlation with strong CD8+ T-cell responses in self-limiting infections. Gruner and colleagues examined CD8+ T-cell responses among patients with self-limiting and

chronic HCV infection.[183] They observed that individuals with self-limiting HCV infection had higher numbers of interferon-γ-producing HCV-specific CD8+ T-cells.[183]

The importance of a strong cell-mediated immune response has also been demonstrated in several studies that have documented cellular immune responses to HCV-antigens in the peripheral blood of individuals who were both anti-HCV and HCV-RNA negative.[184, 185] In a study following a cohort of women who were exposed to the same strain of HCV via a contaminated batch of human Rhesus immunoglobulin approximately 2 decades earlier, circulating HCV-specific T-cells persisted in peripheral blood approximately 20 years after resolving HCV infection, while anti-HCV antibodies were not detectable in many of the individuals.[178] This study not only underscores the importance of strong cellular immune responses to HCV, but suggests that the occurrence of self-limiting infections in the general population may be underestimated, because documentation of initial infection is often not available and surveys of HCV only measure HCV exposure via humoral parameters rather than cellular ones.[178] Another study by Wedemeyer and colleagues suggested that the presence of HCV-specific T cell responses in the absence of HCV-infection may be the result of the induction of cross-reactive T-cells by heterologous viruses.[186] In a database search, a high degree of sequence homology was observed between the HCV-NS3 peptide epitope and a sequence of the influenza A virus neuraminidase protein. The researchers observed a high degree of cross-reactivity between the two viruses in both *in vitro* assays and *in vivo* animal models, suggesting that host responses to an infectious agent may be influenced by cross-reactive memory cells that are induced by past exposure to heterologous viruses.[186]

These observations elicit the question as to whether individuals who have cleared HCV infection are protected from viral recrudescence upon reinfection with HCV. Bassett and colleagues rechallenged chimpanzees with homologous and heterologous HCV and did not observe sterilising immunity.[187] However, the clinical course of HCV reinfection was less severe in these animals.[187] Similar observations for the lack of protective immunity were observed by Farci and colleagues.[188]

Immune-Mediated Liver Injury in Chronic HCV Infections

HCV is not directly cytopathic. Consequently, it is believed that liver injury in chronic HCV infection is likely to result from immune-mediated clearance of infected hepatocytes.[189] Murine models have provided evidence of the role of antigen-specific T-cells in hepatocyte damage. For example, hepatitis C virus transgenic mice develop acute hepatitis upon transfer of syngeneic hepatitis C virus-specific CD8+ T-cell clones.[190] Similarly, a hepatotropic isolate of lymphocytic choriomeningitis virus induced liver cell injury mediated by class I-restricted CD8+ T cells.[191]

On the human level, several observations also support the role of immune-mediated liver damage: 1) liver cell damage coincides with the development of the host immune response and not the initiation of viral replication during primary HCV infection,[192] 2) viral replication in chronic HCV occurs in some individuals with the absence of liver damage,[193] 3) liver-infiltrating HCV immune effector cells have been recovered in several studies.[194-196], and 4) immunosuppression of HCV patients is generally associated with a transient surge in viraemia and a temporary normalisation of transaminases, while removal of immunosuppression may lead to exacerbation of hepatitis,[197, 198]

Immune Response in the Context of Interferon-Based Therapies

A strong and robust T-cell response is also associated with sustained response to interferon-based regimens for HCV. Cramp and colleagues prospectively studied the role of virus-specific T-helper lymphocyte responses along with interferon-γ and interleukin-10 (IL10) production before, during and after treatment with interferon alone or in combination with ribavirin. They observed that T-cell reactivity was uncommon prior to therapy, but increased with therapy, with peak activity occurring between weeks 4-8. Those who cleared virus were patients who developed HCV-specific T-cell proliferation with increased interferon-γ production. In addition, patients treated with combination interferon plus ribavirin had markedly lower levels of IL10 production, with lymphocyte proliferation being similar in both treatment groups. These observations suggest that those who are able to mount strong CD4+ T-cell responses with strong type I cytokine production (i.e.,

interferon-γ) and lower type II cytokine production (i.e., IL10) are more likely to achieve sustained virologic response.

1.10 Host Genetic Factors and the Natural History of Hepatitis C Virus Infection

Studies of twins suggest that genetic factors may play a significant role in modulating the immune response to infectious agents. Twin heritability studies have found a high genetic contribution for the immune response to several infectious diseases.[199-203] This is in contrast to many autoimmune diseases, where concordance among monozygotic twins is almost always under 50%, suggesting a more significant role for environmental factors in autoimmune disease pathogenesis.[204, 205] For this reason, much interest has been focused in recent years on the possible contributions of host genetic factors to the often diverse clinical manifestations seen in many infectious diseases.

With the discovery of the major histocompatability complex and its role in immunity, there has been historically, a great interest in the potential role of HLA antigens in the natural history of infectious diseases. More recently, however, there has been an interest in the role single base-pair exchanges, termed single nucleotide polymorphisms (SNP), in the regulatory regions of genes of key immunomodulatory proteins, or proteins involved in drug or metabolic pathways. The human genome project has facilitated the exploration of the role of these polymorphisms in disease outcomes. A systematic review of the scientific literature regarding the potential role of host genetic diversity on the outcome of HCV infection is provided in Chapter 2.

1.11 External Factors and the Natural History of Hepatitis C Virus Infection

One of the most important external modulators of HCV natural history is that of alcohol consumption. Several studies have shown an association between increased alcohol consumption and more advanced liver disease among individuals with chronic HCV infection.[53, 60, 206-210]

In a retrospective study conducted by the HCV National Steering Group in the United Kingdom, 923 individuals were identified as having become infected with HCV following a blood transfusion, along with 475 transfusion-recipient controls. Study participants were asked to complete a validated questionnaire on alcohol consumption.[211] Individuals who drank more were at increased risk for cirrhosis (RR=2.84). The Dionysus Study, a population-based study of 6,917 unselected residents of two cities in Italy, found that among the 2.3% of individuals positive for the HCV virus, those who drank more than 30g of alcohol per day for more than 10 years had a 3-fold higher risk of cirrhosis (95%C.I.: 1.2- 7.4; p=0.01).[210]

Several other studies have also suggested that increased alcohol consumption may adversely affect the outcome of interferon therapy as well. In a study by Loguercio, response of patients to interferon was inversely proportional to the amount of alcohol ingested.[212] A study by Tabone an colleagues observed lowered rates of sustained virologic response among individuals who drank alcohol: 33% of non-drinkers responded, 20% of those who drank 25g – 50g of alcohol per day, and 9% in those who drank more than 75g per day.[213] No differences were detected with respect to non-response suggesting that those who consumed more alcohol and failed to respond were relapse-responders.[213] The biological mechanisms behind these epidemiologic observations have not yet been fully elucidated.

Co-infection

Another important external factor affecting the natural history of HCV infection is co-infection with other infectious agents. The most important of these is co-infection with the human immunodeficiency virus (HIV). Because HCV and HIV are both parenterally transmitted infections, concomitant infection with the two agents may be common among specific populations such as injection drug users. It is estimated that in the United States alone, 25% of HIV-infected persons are co-infected with HCV.[56] With rapid improvements in HIV therapies, liver disease has emerged as an important medical problem in co-infected individuals.

HIV-infection adversely impacts the outcome of HCV infection at all stages.[56] While self-limiting infection occurs in approximately 14% - 46% of HCV mono-infected patients, only 5% - 10% of individuals co-infected with HIV experience self-limiting infections.[48, 53, 214, 215] Among those with chronic HCV infections, co-infection with HIV has been associated with higher HCV-RNA levels, an accelerated progression of liver fibrosis, and an increased likelihood of developing liver failure and hepatocellular carcinoma.[53, 216-223]

1.12 Hepatitis C Virus Infection: The Burden of Disease

Sequelae of persistent HCV infection remain a major public health burden worldwide. HCV, along with hepatitis B, are the main causes of primary liver cancer worldwide. Liver cancer is currently the fifth most common cancer in the world, with 437,000 incident cases annually.[224] Developing countries are affected more than developed countries by liver cancer (Table 1.12.1.)

Table 1.12.1. Cancer cases attributable to viral hepatitis C and hepatitis B in 1990 in developed and developing countries.

	Developed Countries		Developing Countries	
	Males	**Females**	**Males**	**Females**
Hepatitis C	11,300	5,700	68,200	24,500
Hepatitis B	12,400	6,200	154,800	55,500

	World Total		
	Male	**Female**	**Both**
Hepatitis C	79,500	30,200	109,700
Hepatitis B	167,200	61,700	228,900

(Adapted from Parkin DM *et al.*, *Cancer Surveys*, Vol. 33, 1999)[225]

Random estimates of HCV prevalence have not been conducted in the United Kingdom. While populations like blood donors groups, clinic populations, analyses of stored sera or patient databases have been employed, probability cluster sample analyses have not been conducted on the general population. Table 1.12.2. presents data on HCV prevalence in England and Wales. These data were based on an epidemiologic survey of residual sera collected from specimens submitted to the Public Health Laboratory Service Laboratories in England and Wales for routine examination.[226]

Table 1.12.3. presents estimates of HCV incidence and prevalence from a study conducted in Tayside, Scotland. This study studied the liver disease virology dataset from Ninewells Hospital.[227]

Table 1.12.2. Number of individuals who tested positive out of the total number of sera tested and the percent positive for antibodies to hepatitis C from England and Wales.

	A. Males	B. Females
Birth cohort	**Total**	**Total**
1976-1980	5/424 (1.18%)	0/457 (0%)
1971-1975	4/748 (0.53%)	1/853 (0.12%)
1966-1970	11/1045 (1.05%)	7/1152 (0.61%)
1961-1965	21/1043 (2.01%)	7/1190 (0.59%)
1956-1960	10/854 (1.17%)	8/1063 (0.75%)
1951-1955	11/617 (1.78%)	2/789 (0.25%)
1946-1950	7/426 (1.64%)	3/474 (0.63%)
1936-1945	3/646 (0.46%)	1/705 (0.14%)
1926-1935	3/609 (0.49%)	4/518 (0.77%)
1880-1925	2/973 (0.21%)	5/1096 (0.46%)
Total	77/7385 (1.04%)	38/8297 (0.46%)

(Adapted from Balogun M.A. *J Infect.* 2002.)[226]

Table 1.12.3. Incidence and prevalence of HCV in Tayside, Scotland (per 100,000 population).

Year	Incidence	Prevalence
1991	1.02	---
1992	6.60	7.11
1993	9.36	14.93
1994	9.87	22.50
1995	20.98	41.96
1996	27.44	65.80
1997	23.96	86.65
1998	20.01	103.64

(Adapted from: Steinke DT, *et al. Gut*, 2002)[227]

Random surveys of HCV prevalence in the general population have been conducted in the United States (US).[16] Models estimating disease burden and economic costs have been subsequently calculated based on these data. While it is difficult to extrapolate estimates of costs from studies conducted in the US to the UK because of the different healthcare systems, the estimates of HCV disease burden provide a glimpse of the impact of HCV on the healthcare system in a developed country. Accordingly, I will summarise some important findings below.

While the introduction of screening the blood supply for hepatitis C has greatly reduced transmission via that route, transmission via injection drug use remains an important route of transmission. Although the number of incident cases has greatly declined, the prevalent pool continues to rise. This is mainly a result of the fact that there is generally a large lag between the acquisition of infection and the onset of symptoms (sometimes a decade or two). Using United States-based estimates, the Centres for Disease Control project a 4-fold increase in the numbers of individuals with long-standing infection from 1990 to 2015.[228] A recent study by Davis and colleagues projected the burden of liver disease based on existing incident and prevalent HCV cases. They found that if the incidence of new infections remains stable, the prevalent pool will rise 4% annually and plateau at 2008. The maturation of existing cases will cause increases in cirrhosis by 61%, decompensated liver disease by 279%, hepatocellular carcinoma by 68%, the need for a

liver transplant by 528% and the occurrence of liver-related deaths by 223%.[229] The majority (77%) of liver-related deaths in the United States during 1999 were a result of HCV infection.[16, 230]

A recent study from the United States by Wong and colleagues estimated that from 2010-2019, HCV may lead to the loss of 1.83 million years of life among those under 65, at a societal cost of billions of dollars.[231] The impact of HCV on liver transplantation is highlighted in Table 1.12.4. These data from the United States United Network for Organ Sharing (UNOS) show that both the number of patients registered on the liver transplant waiting list and the number of transplants conducted as a result of HCV-related liver disease have risen dramatically over the past few years.[230]

Table 1.12.4. Impact of HCV on liver transplantation over time.

A. Number of patients on the liver transplant waiting list in the United States as a result of HCV infection and those on the list as a result of other causes.

	1995	1996	1997	1998	1999	2000
HCV	2086	2354	2798	3225	3670	3886
Other causes	5251	5704	5832	6318	6848	7007
% from HCV	28%	29%	32%	34%	35%	36%

B. Number of liver transplants as a result of HCV in the United States and those transplanted as a result of other causes.

	1991	1992	1993	1994	1995	1996
HCV	343	565	796	930	1129	1190
Other causes	2588	2464	2608	2661	2750	2825
% from HCV	12%	19%	23%	26%	29%	30%

	1997	1998	1999	2000
HCV	1268	1517	1625	1679
Other causes	2832	2899	2856	2900
% from HCV	31%	34%	36%	37%

Data from the United Network for Organ Sharing (UNOS) and excerpted from Kim R. Hepatology 36 (5, Suppl. 1), 2002.[230]

The burden of disease resulting from hepatitis C infection is severe in both developing countries, as evidenced by the statistics concerning liver cancer, and developed countries, as reflected in studies estimating HCV disease burden.

1.13 Chapter 1 References

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Chapter 2

Host Genetic Diversity and the Outcome of Hepatitis C Virus Infection: A Systematic Literature Review

2.1 Genetic Heterogeneity and the Outcome of Hepatitis C

As mentioned in Chapter 1, the natural history of hepatitis C virus (HCV) infection is characterised by diverse clinical outcomes. Some individuals infected with HCV are able to clear the virus, while others develop persistent (chronic) infections. There is some evidence to suggest that if people are infected at a younger age or if they are female then the risk of persistent infection is reduced, but other factors which determine whether the infection is self limiting or persistent have not been identified. It is unlikely that viral sequence variation is a major determinant of the outcome at this stage as any viral strain with a reduced capacity to establish persistent infection would rapidly disappear.

Treatment of persistent HCV infection has enjoyed a remarkable increase in success rates over a relatively short period, but even with pegylated interferons in combination with Ribavirin, about 50% of individuals with HCV genotype 1 and 30% with non-1 HCV genotypes will fail to achieve sustained virological responses.[1, 2] Viral genotype is clearly a major determinant of the outcome of treatment along with gender and ethnic background. However, these variables do not account for all of the variability in the outcome of HCV infection or of treatment.

It is clear that the outcomes of infectious diseases are determined by a number of factors which can be broadly classified as host, organism and environment. Under the category of host some of the most important factors are genetic. Studies amongst monozygotic twins suggest that host genetic factors account for 50% or more of the variability in the major outcomes in infectious disease.[3] Infection of siblings is rare with HCV so it is unlikely that confirmatory twin studies will be reported, as has been done with hepatitis B virus infection.[3, 4] To date, genetic studies have not been conducted employing siblings with vertically-acquired HCV. However, the emergence of reproducible genetic associations with the outcomes of HCV, along with the parallels from other infectious diseases such as malaria and hepatitis B,[4, 5] provide strong circumstantial if not direct evidence of the importance of genetics in the natural history of infectious diseases.

Identification of disease susceptibility genes is usually achieved using three approaches: 1) linkage analysis in affected sibling pairs, 2) linkage analysis in rodent models of the infection, and 3) disease association studies.[6] There are examples suggesting that some outcomes of HCV infection run in families.[7] However, this situation is sufficiently rare to preclude the use of linkage analysis to identify susceptibility genes. As suitable small animal models of HCV infection are not available, disease association studies are the principal approach used to identify disease susceptibility genes in HCV. This approach has the advantage of statistical power but is limited by the need to select candidate genes. Candidate genes are chosen by the investigator based on their knowledge of HCV or infectious disease pathophysiology.

To examine the extent of the literature examining correlations between host genetic markers and the outcome of hepatitis C virus infection, I conducted a systematic review of the literature. In addition, I have attempted to highlight any consistent observations and trends in the literature. Meta-analyses are also presented to further explore observed trends.

2.2 Systematic Review Methods

Search strategy

A series of subject and keyword searches were conducted first using the 1966-15 April 2003 Medline and then repeated using the 1980-15 April 2003 EMBASE database. First, "hepatitis C" was used as a subject heading, with the subgroup "genetics." Next, a series of keyword searches were used to first identify articles related to "hepatitis C" and then combined with the following keywords using the "and" statement: "polymorphism, HLA, MHC, and genetics." In addition, the citations of the bibliographies of these articles were reviewed for additional references. Finally, the search was repeated in the 1980-15 April 2003 PubMed database. Articles were restricted to those in the English language. All articles were evaluated for study design, technical aspects such as genotyping methods (serological versus molecular) and statistical rigour.

Meta-analysis

All meta-analyses were conducted using the STATA® software. Forest plots, tests of heterogeneity and pooled estimates with weighted adjustments for sample size were calculated using this software.[8]

2.3 Systematic Review Results

Among papers identified in the literature search, 88 were disease association studies that directly pertained to the topic of genetics and the susceptibility to HCV infection, the progression of HCV infection, the response to therapy, or extra-hepatic manifestations in HCV. Table 2.3.1 lists the papers and the major findings for studies concerning genetics and the susceptibility to HCV infection. Table 2.3.2 lists the studies and their findings on the association of genetics with the response to anti-HCV therapies. Table 2.3.3 concerns those studies of genetics and the progression of HCV. Major findings are listed as well.

Table 2.3.4 lists studies concerning genetics and comorbid conditions in HCV. The relevant findings of these studies are shown. Tables 2.3.1 through 2.3.4 are located at the end of the chapter.

2.4 Systematic Review of Genetics and HCV Outcome: Data Synthesis

Susceptibility to persistent HCV infection

Early candidate gene studies focused on the genes of the major histocompatibility complex (MHC) and the outcome of HCV. Given that MHC gene products encode key components of the antigen presentation and processing pathways, it is not surprising that these early studies focused on this region of the human genome. More recently, however, the body of literature on genetic factors in HCV infection has grown to include many non-MHC candidate genes as well. There are no estimates of the relative contribution of MHC versus non-MHC genes to clinical outcome in HCV, but in other infections, such as hepatitis B, the contributions appear to be well balanced or supportive of a greater effect from non MHC genes.[4]

A total of 34 studies address susceptibility to HCV infection (Table 2.3.1).[9-42] Several consistent associations have been observed with specific alleles and the susceptibility to HCV, and are highlighted in Table 2.4.1. A number of studies in several populations have reported an association between DQB1*0301 with viral clearance.[9, 10, 17, 24, 27, 30, 32, 42, 43] One study, however, reported an opposite effect for DQB1*0301.[38] Several of these studies on susceptibility to HCV compared individuals with chronic HCV infection to healthy controls. The use of healthy individuals poses a study design problem as an estimated 54% - 86% of these controls, if exposed to the hepatitis C virus, would develop chronic infections, while the others experience viral clearance.

Another allele that has been correlated with self-limiting HCV is DRB1*1101.[9, 10, 27, 30, 40] It is also worth noting that in one Italian study, a protective role was found for the

DR5 serogroup against HCV infection.[41] The molecularly-defined DRB1*1100 and DRB1*1200 group of alleles are part of the serologically-defined DR5 group. In one study, the DRB1*1101 allele was correlated with susceptibility to vertically transmitted HCV infection.[25]

These two DR and DQ alleles are in linkage disequilibrium with each other and this makes it difficult to establish which allele is truly responsible for influencing the outcome of HCV infection. However, the strength and patterns of linkage disequilibrium vary in different ethnic groups and the replication of the association of DQB1*0301 in multiple and diverse populations suggests that this allele may exert a stronger effect on viral clearance than DRB1*11.[44]

Table 2.4.1. Summary of replicated correlations of particular alleles and self-limiting HCV infection.

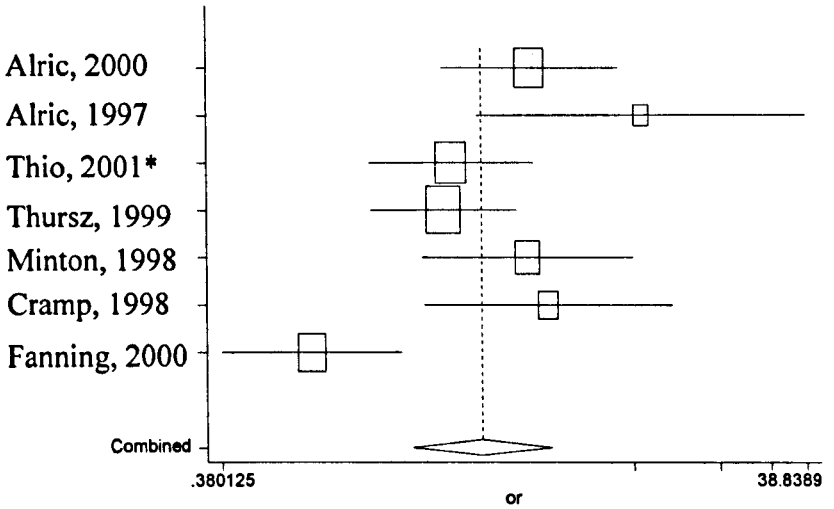
Allele	Studies
DQB1*0301	Alric, 1997 Alric, 2000 Cramp, 1998 Mangia, 1999 Minton, 1998 Thursz, 1999 Thio, 2001 Zavaglia, 1998
DRB1*1101	Alric, 1997 Alric, 2000 Minton, 1998 Thursz, 1999 Yenigun, 2002

To examine the associations between DRB1*0301 and the DRB1*1101 alleles and self-limiting HCV more closely, I conducted a meta-analysis employing data from the published literature. To ensure that similar studies were pooled, only studies that employed molecular genotyping techniques and compared those with self-limiting infections (HCV

antibody positive, but PCR negative) to those with chronic infections (HCV antibody positive and PCR positive) were employed. Only data involving Caucasian individuals were utilised in the analysis of DQB1*0301. Because many studies involving the DR locus only reported alleles to their 2-digit specificities, studies reporting 4-digit allele specificities were collapsed into their respective 2-digit categories and all meta-analyses were conducted with these data. In other words, all alleles such as DRB1*1101, DRB1*1103, etc. were grouped together as DRB1*11. Similar to the analysis of DQB1*0301 above, only those studies of Caucasian individuals employing molecular genotyping techniques that compared those with self-limiting infections to those with chronic infection were included. Figure 2.4.1. presents the results of these meta-analyses. Part A. presents the data for DQB1*0301, while Part B. presents the data for DRB1*11. In addition, the identification of individuals with self-limiting infections has largely been retrospective in nature. Quantification of possible confounding factors such as viral genotypes, dose of virus, etc. has been impossible in most cases.

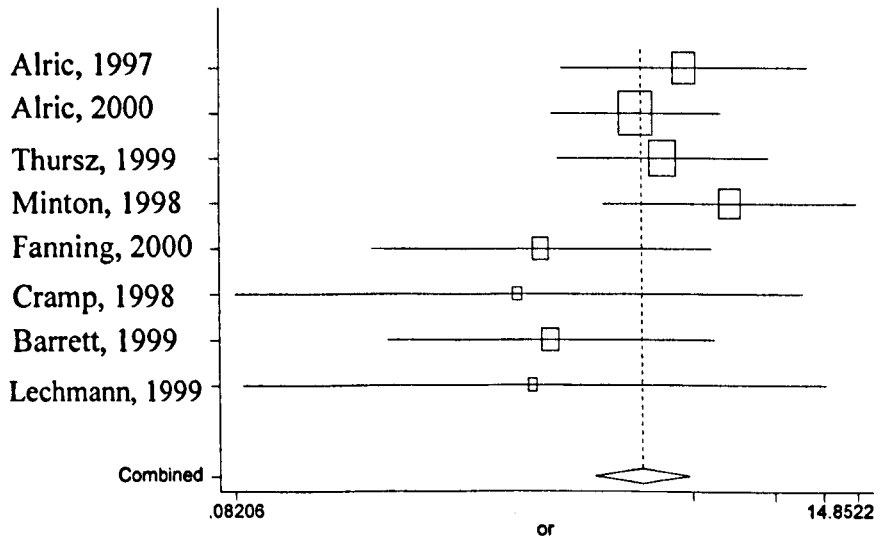
The analysis of DQB1*0301 and self-limiting infection shows a pooled estimate of 2.4 (95%C.I.: 1.8-4.8) (Figure 2.4.1. Part A.), Considerable heterogeneity exists with respect to the studies of DQB1*0301 and viral clearance (Q=21.1; p=0.004). Latent ethnic differences may explain some of this variability. While this analysis was restricted to Caucasian individuals, a wide range of sub-ethnic groups are included, from northern to southern Europeans. In contrast, studies of DRB1*11 and self-limiting infection are less heterogeneous (Q=6.9; p=0.503). The pooled estimate for the effect of DRB1*11 on self-limiting infection is 2.5 (95%C.I.: 1.7 – 3.7). The studies involving DRB1*11 are also much smaller in size and have less precision (Figure 2.4.1. Part B.). These meta analyses must be interpreted with caution. Considerable publication as well as and investigator-submission biases exist. Many studies showing no association between class II alleles and self-limiting infection may not be in the published literature both because journals tend to dislike publication of negative studies or investigators do not bother writing-up negative results. Alternatively, negative results may eventually be published in non-English journals.

A. DQB1*0301



Pooled estimate: 3.0 (95%C.I.: 1.8 – 4.8) Test for heterogeneity: Q=21.1; p=0.002

B. DRB1*11



Pooled estimate: 2.5 (95%C.I.: 1.7 – 3.7) Test for heterogeneity: Q=6.9; p=0.503

Figure 2.4.1. Meta analyses. Summary estimates of studies showing an association between DQB1*0301 and self limiting HCV infection (Part A.). Summary of estimates of studies showing an association between DRB1*11 and self-limiting HCV infection (Part B). Only data pertaining to Caucasian individuals in comparisons between individuals with self-limiting versus persistent HCV infections are employed in this analysis.

*This paper presented data on Caucasians only for DQB1*0301 and not DRB*11

There is an unusual situation in Ireland where a large number of women were infected with HCV by contaminated RhD immunoglobulin.[45] The viral strain of the infecting inoculum and the dose were consistent in this outbreak and associations has been reported for HLA-DRB1*0101 with self limiting infection.[12, 18] Outside this unique population, however, HLA-DRB1*0101 has not been shown to influence the outcome of HCV infection. This population provides an interesting opportunity for the study of immunogenetic determinants of HCV susceptibility. To begin with, this cohort is quite homogenous. It is comprised of younger child-bearing age women of a similar ethnic background. Second, all individuals were infected with the same strain of virus with similar doses. Variations in viral strain, size of viral inoculum and host racial heterogeneity that may potentially mask genetic effects are eliminated in this situation. At the same time, these attributes pose problems with respect to generalisability. It is unclear whether findings in this cohort may be extrapolated to both genders or to other ethnic groups. This population is a very homogenous group of Irish women, and correlations may not be readily extracted to other ethnic groups. It is also possible that the dose and strain of HCV that infected these women makes this cohort unique. Different immunodominant epitopes may exist on different strains of HCV. It is possible that the strain of HCV found in the Irish outbreak elicits immune responses from specific alleles such as DRB1*0101. This may explain why the association of DRB1*0101 with self-limiting infection has only been reported in this population.

It is difficult to dismiss the possibility that MHC class I genes influence the outcome of HCV infection. There have been very few reports of disease association studies using these loci as candidate genes. Thio et al. reported associations of MHC class I alleles with viral clearance: HLA-B*57 (Odds ratio (OR)=0.62; 95% CI, 0.39 to 1.00), and HLA-Cw*0102 (OR= 0.43; 95% CI, 0.21 to 0.89) as well as persistence: HLA-A*2301 (OR= 1.78; 95% CI, 1.01 to 3.11) and HLA-Cw*04 (OR= 1.78; 95% CI, 1.21 to 2.59).[33]

Although most association studies of genetic susceptibility to HCV infection have involved the MHC, several studies have also addressed the potential role of non-MHC loci.

One of the more interesting stories concerns the potential role of the chemokine receptor (CCR5) in resistance to HCV infection.[39] CCR5 has gained considerable attention in the past few years because of its role as a co-receptor for the human immunodeficiency virus (HIV). Homozygotes for a deletion variant (CCR5*Δ32) are conferred a relatively high resistance to HIV infection. Although, CCR5's role as a co-receptor for HIV is clearly not directly relevant to HCV infection, CCR5 is the principal ligand for the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES. Because chemokines and chemokine receptors are important in lymphocyte recruitment and it is possible that the CCR5*Δ32 variant may influence the outcome of other infections, such as HCV, via this pathway. In a study by Woitas an colleagues, a higher than expected frequency of CCR5*Δ32 homozygotes was observed in patients with HCV infection. It is not surprising in many respects that this study observed a differential distribution of the frequency of CCR5*Δ32 in those with HIV compared to other populations, such as those with HCV mono-infection, as by definition, possession of CCR5*Δ32 makes it more difficult for one to be infected by HIV via this chemokine co-receptor.[29, 46, 47] A recent large study by Promrat and colleagues observed a similar distribution of CCR5*Δ32 homozygotes between patients with chronic HCV and healthy controls.[29] In a study by Mangia and colleagues, and another study by Zhang and colleagues, it is suggested that the differential distribution of CCR5*Δ32 observed by Woitas and colleagues is primarily the result of reduced frequency of CCR5*Δ32 individuals in the HIV cohorts.[46, 47]

Studies have addressed genetic variation in the regulatory regions of immunomodulatory cytokine genes as well. Several studies have focused on genes such as Interleukin-10 (IL10) and tumour necrosis factor (TNF), and have observed no association with respect to tumour necrosis factor alpha HCV susceptibility,[36, 38] but one study observed an association with the IL10 G/G genotype and chronic infection when compared to healthy controls (OR=2.75 (adjusted for age and sex); 95%C.I.: 1.01-7.50; p=0.048).[36] Associations were observed between the Haptoglobin1-1 allele and risk for HCV infection ($p < 0.001$).

Response to anti-HCV therapies

Twenty-five studies addressed the potential role of genetics in the response to interferon therapy or variations in viral load (Table 2.3.2).[9, 19, 29, 30, 36-39, 48-65] Unlike studies of susceptibility to HCV, the majority of studies have concerned regions outside the MHC, with an emphasis on single nucleotide polymorphisms. Three main outcomes are commonly examined in studies of the response to anti-HCV therapies: 1) ***sustained response***—now commonly defined virologically as having undetectable serum HCV-RNA levels at 6 months after the discontinuation of treatment. Older studies have defined response biochemically as a normalisation of ALT levels; 2) ***initial response***—this is a disappearance in serum levels of HCV-RNA at the end of the initial phase of treatment—commonly 12 weeks after the inception of the regimen. A portion of these individuals who have achieved initial response may also experience a virological relapse later on, so they are not true responders; and finally, 3) ***non-responders***—these are individuals who have detectable viraemia at week 12 of therapy. They are distinct from initial responders because they never achieved any sort of major virological decline to undetectable levels, even for a short period of time during the initial course (first 12 weeks) of therapy.

Relapse responders represent an intermediate phenotype between sustained responders and non-responders. As a result, they may be classified independently as their own phenotype in some studies, included with non-responders in some studies, or included with sustained responders in other studies to create an "initial reponse" phenotype that includes all individuals who a decline in HCV-RNA levels during the initial phase (first 12 weeks) of therapy. Also, some early studies define response to therapy solely based on normalisation of ALT levels. This is not surprising as shortly after the discovery of hepatitis C, therapy was largely based around ALT-normalisation. Additionally, it is worth noting that some studies deal with interferon monotherapy, while others deal with interferon and ribavirin combination therapy. Studies have not yet emerged involving the newer pegylated interferons, both as monotherapy or in combination with ribavirin.

Unlike the replicated associations of DQB1*0301 and DRB1*11 with self-limiting infection, clear and replicated associations have not been observed with alleles of the MHC and therapeutic outcome. In fact, there have been some contradicting observations. Kikuchi and colleagues observed that the HLA-DR4 group was associated with non response.[58] In contrast, Sim and colleagues observed the DRB1*0404 allele (a subset of the DR4 serogroup) to be correlated with response.[63] One major explanation for the observed differenced between these two studies is that the populations were different with respect to race, and gene frequencies may vary greatly from race to race. While the Kikuchi study examined Japanese patients, the Sim paper studied Caucasians from Canada.[58, 63] Both studies defined response based on biochemical parameters rather than virological ones. Geographic variations in viral genotype also exist, with genotype-1 predominating in North American countries such as Canada, and genotypes 2 and 6, in addition to genotype-1, also existing in parts of eastern Asia, and it is possible that these viral genotypic differences may mask host genetic associations, as Kikuchi examined differences in HCV genotype, while Sim did not.[58, 63] The human MHC region is one of the most genetically diverse regions in the human genome, and studies must be sufficiently powered to detect meaningful differences between groups.

Contradictory associations have also been observed with respect to association studies of cytokine gene polymorphisms and the response to interferon-based therapies for HCV. Because of the important roles of IL10 in the immune response, several studies have examined the role of functional polymorphisms in this gene with initial response and sustained response. In a small-scale study, an Australian group looked at single nucleotide polymorphisms and the resulting haplotypes that these alleles form and correlated them with initial response to interferon monotherapy.[52] They observed the *IL10* -592A and -819T alleles to be correlated with initial response (OR=5.0, 95%C.I.: 1.4-19.0; p=0.01). In another study employing patients treated with interferon + ribavirin combination therapy, Yee et al found that carriage of the -592A or -819T alleles, and the corresponding extended haplotype as defined by the IL10R allele: -591A+ -819T+ -1082A+ -2763C+ -3575T+ 108bp IL10R, conferred an advantage for sustained response to therapy (OR=2.2, p=0.016 for the individual SNPs and OR=2.65, p=0.01 for the haplotype, respectively).[65] Homozygosity for the -592A or -819T alleles, or the homozygosity for the corresponding

extended haplotype showed a very strong association with sustained response (OR=16.6, p=0.013 for the homozygous genotypes and OR=13.7, p=0.025 for the haplotype, respectively). A recent study failed to confirm such an association between IL10 polymorphisms and the response to therapy.[38] A couple of studies have examined the role of tumour necrosis factor variants and the response to therapy. Both studies did not detect a relationship between polymorphisms in this gene and response to therapy.[62, 65]

One study examined the role of polymorphisms in genes involved in several genes involved with the interferon response pathways. In particular, a Japanese group has examined the role of polymorphisms in the *MxA* gene and response to interferon monotherapy in a Japanese population and observed that the *-88MxA-(G/G)* genotype to be correlated with non response and the *-123MxA-(C/C)* genotype to be correlated with non-response.[56, 57]

Studies of HCV progression

A total of 28 studies were found to address the topic of host genetics and HCV progression, and have largely been inconsistent (Table 2.3.3).[9, 11, 19, 21, 24, 26, 29, 30, 37, 53-55, 66-83] There is a trend with DRB1*11 alleles and less severe liver disease. Asti observed DRB1*1104 (OR=4.82) to be associated with normal ALT.[11] Kuzushita reported DRB1*1101 to be correlated with normal ALT (OR=0.3).[21] Renou reported that the DRB1*11 allele was correlated with normal ALT (OR=2.36).[78] Haruna observed the DRB1*1101 allele to be associated with less piecemeal necrosis (OR not reported), and Hüe observed DRB1*11 to be associated with lower Knodell scores (OR=0.35).[53, 70] Tillman also observed DRB*11 to be lower among cirrhotics (RR=0.29).[81] Haruna reported an association between DRB1*1201 with less severe liver disease (OR not reported) as well.[53] It is interesting to note that the DRB1*1100 and DRB1*1200 alleles form the DR5 serogroup. Whether these observed associations represent some sort of underlying biological mechanisms with these alleles is not currently known. Future studies are needed to address this question. Almost all of the studies reviewed did not adjust for potential

environmental confounders, such as alcohol use. Future studies should examine genetic associations in conjunction with other potential environmental as well viral factors.

Nine studies directly examined the role of *HFE* gene variants and the progression of liver disease.[61, 69, 84-89] Two major polymorphisms are of importance in the literature: *Cys282Tyr* and *His63Asp* (often abbreviated C282Y and H63D, respectively in single letter amino acid notation). Of these two polymorphisms, heterozygotes for *Cys282Tyr* have been shown to have normal to slightly elevated hepatic iron content.[90] Given the observation that a number of individuals with chronic HCV infection have excess hepatic iron,[91] some investigators have examined whether heterozygosity for this variant is associated with advanced liver disease. The results of these studies have been discrepant, with 4 studies suggesting an association between possession of *HFE* variants and increased fibrosis or cirrhosis,[61, 69, 88, 92] and the other studies suggesting that there are no associations. Methodological differences may account for these differing observations. For example, studies employed different scoring systems for histological fibrosis. Some studies conducted categorical analyses, while others compared averages of these semi-quantitative categories. All of the studies were cross-sectional in design and correlations were conducted with respect to the stage of fibrosis, irrespective of the duration of HCV infection.

Studies of extrahepatic manifestations

Fifteen studies addressed correlations between host genetics and the occurrence of extrahepatic manifestations in HCV (Table 2.3.4).[37, 69, 84, 86, 88, 93-103] Two studies observed an association between DRB1*11 and the presence of cryoglobulins (OR not reported in Amaroso study; OR=3.4 in Cacoub study).[93, 94]. Both studies examined Italian populations. Two other studies conducted in ethnically dissimilar populations found an association between the class II DR3 allele with the presence of cryoglobulins (RR6.14 and RR=3.7, respectively).[98, 103] Hwang and colleagues studied Chinese patients, while Lenzi and coworkers studied Italians.[98, 103]. Lenzi also examined class I loci as well and found an association with B8 and the presence of cryoglobulins. Linkage between B8 and DR3 is

quite strong, and this haplotype has been linked with several autoimmune diseases including autoimmune hepatitis type I, primary biliary cirrhosis and rheumatoid arthritis.[104-108]

A few studies have also addressed the potential role of the *HFE* gene variants (*Cys282Tyr* or *His63Asp* polymorphisms) and iron levels. No consistent observations with respect to this locus have been observed, with several studies reporting no association between this variant and increased iron,[85, 86, 102] and another study reporting an association between this locus and higher mean ferritin levels.[88] Differences in study design— for example the definition of iron (i.e., serum ferritin levels versus iron stains of liver biopsies) may play a major role in these differences. Some studies employed use of the 5-point METVIR scoring system for liver fibrosis while others used the 7-point Knodell histological activity index. In addition, use of these semi-quantitative scoring systems is accompanied by a degree of inter-observer variation. These differences preclude the use of meta-analyses to pool data from these studies.

2.5 Concluding Remarks

A few genes have been associated with the outcomes of HCV infection. The most replicated association is with the class II DQB1*0301 allele and self-limiting HCV infection. With respect to the response to therapy, two studies have shown an association between the *-88MxA-(G/G)* genotype and non response to interferon monotherapy. There appears to be a correlation between the alleles in the DR5 serogroup with milder liver disease. Two studies have shown a correlation between the DRB1*11 allele and the presence of cryoglobulins, while another two studies have shown correlations between the DR3 allele and the formation of cryoglobulins.

Reproducibility is essential in this field. Unfortunately, there tends to be a bias in the reported literature towards the publication of positive results irrespective of the quality of the study. Another concern is that demonstration of an association with an allele, even

after confirmation, does not necessarily imply that the allele is functionally responsible for changes in the disease susceptibility or resistance. Allelic variants are carried on long segments of DNA which are likely to contain other polymorphisms and are inherited as haplotypes. The patterns of linkage disequilibrium between polymorphic loci are complex and unpredictable. Therefore comprehensive investigation is required to identify causative variants from those which are associated through linkage disequilibrium. The resolution of both of these issues will require much larger study populations.

There has been a tendency in recent years to dismiss the potential contribution of genetic association studies to the scientific literature due to the lack of data on the functional effects of many genotypic variants that have been studied. It is important to bear in mind that the understanding of genetics in health and disease consists of several different parts: 1) the identification of new polymorphisms, 2) the association of these polymorphisms with disease outcomes, and 3) the understanding of the functional effects of these variants. These three factors may not necessarily be completed at the same time, and the importance of studies addressing only one or two of these aspects should not be dismissed. With the human genome project it is likely that the identification of new polymorphisms and the association of new polymorphisms with disease outcomes will far exceed the speed at which functional studies are conducted. Similarly, the generation of many negative studies will occur. Given the importance of replication in this field it is important that all of the evidence be considered, regardless of whether the associations are positive or negative.

The primary aim of investigating the genetic determinants for the outcomes of HCV infection is to understand why some individuals develop self limiting infection, why others are resistant to therapy, and why some individuals with chronic HCV progress more rapidly with respect to their HCV-related liver disease than others. However, there may be, in the future, clinical benefits arising from these studies. Host genes which influence the resistance to persistent HCV infection may provide novel therapeutic targets and knowledge of MHC associations may be used to identify epitopes derived from HCV antigens to be used as vaccine candidates. For example, the identification of the importance of CCR in HIV infection has led to the development of CCR5 antagonists aimed at the

competitive inhibition of HIV attachment.[109-112] Furthermore, it could be anticipated that genetic factors which influence the response to therapy will be used in the future as prognostic indicators or to modify therapeutic regimens in the way that we currently use viral genotypes. Similarly, drugs may be specifically tailored to individuals with a certain genetic makeup.[113]

While there are some consistent observations (i.e., DQB1*0301 and self-limiting infection), there are more inconsistent observations. Several factors may account for these inconsistencies. First, there are differences with respect to the populations studied. Gene frequencies vary across races, and it is quite plausible that one ethnic group may have the preferential use of different allele(s) in viral eradication than those from other ethnic groups. Further studies of the functional aspects of HLA molecules will better elucidate the picture of immunogenetic determinants of disease outcome in light of biological plausibility. Confounding from other environmental as well as viral factors (such as alcohol consumption as well as viral genotypes, respectively) as well as other as yet unidentified factors may also contribute to differences. Variations in study design may also play a role in the differences observed in the present review. Many of the studies were conducted on relatively small samples. Smaller samples decrease the power of the study (likelihood of detecting a difference between groups, assuming that a true difference exists) as well as the precision of the measurements of association.[114] The issue of sample size is even more critical in studies concerning the MHC, which is known to be one of the most genetically diverse regions of the human genome. Lack of an association may not mean that associations do not exist; rather, they may be a reflection of the lack of an under-powered study's ability to detect meaningful relationships. Finally, differences in study design may account for some of these differences as well. As with any case control study, the case definition as well as control group selection will affect study outcome. For example, in the study of self-limiting versus persistent infection, some groups compare individuals with self-limiting infection to those with persistent infection, while others compare chronically infected individuals to a group of healthy HCV-negative individuals.[44]

Interestingly, most of the studies reviewed did not examine gene environment interactions or control for behavioural confounders such as alcohol use. Future studies of

HCV natural history will have to be sufficiently powered to accommodate adjustment for not only potential confounding factors but the possibility of genetic interactions with environmental or viral factors. Importantly, quantitative evaluation of drinking behaviours will need to be made and will require the use of validated survey instruments.

There is an urgent need for the establishment of large cohorts for the systematic study of host genetics and the outcome of hepatitis C. These cohorts must be defined using rigorous epidemiologic methods. In addition, these cohorts should be sufficiently large to detect meaningful relationships between genetic markers and disease outcomes. Replication of observed relationships is an important aspect of disease association studies. It would be optimal for future studies to involve two unrelated cohorts, where hypotheses may be generated in one cohort and replicated in another.[30] The identification of genetic polymorphisms in the human genome has been greatly facilitated by the human genome project. However the correlation of these genetic markers with disease outcomes has just begun. The success of this field will rely on the careful evaluation of these markers in properly designed epidemiologic studies along with sufficient replication of putative predictive markers in different populations.

Table 2.3.1. Summary of studies of host genetics and susceptibility to hepatitis C virus infection.

Study	Population	No. HCV Patients	No. of Controls	Allele or Haplotype	OR	Significance
Alric L, 1997	French	103	25	DQB1*0301 with clearance DRB1*1101 with clearance	10.64 5.96	p <0.01 p <0.02
Alric L, 2000	French	63 with clearance	282 with persistence	DRB1*1101 with clearance DQB1*0301 with clearance DQB1*02 with persistence	NG NG NG	pc=0.012 pc=0.003 pc=0.04
Asti M, 1999	Italian	41 HCV with normal ALT for 1 year 99 with chronic HCV 179 uninfected controls		Tap1C higher in normal controls vs. all HCV+ individuals	0.16	pc=0.0002 (95%C.I.: 0.06-0.42)
Barret S, 1999	Irish	73 with clearance	84 with viral persistence	DRB1*01 with clearance	4.9	pc=0.01
Bosi I, 2002	Italian	17 infants with clearance	18 infants with persistance	DR13 in children protective against HCV vertical transmission	NG	p < 0.01
Caocci G, 1998	Sardinian	53 with HCV	220 healthy individuals	HLA A and B DRB1*1302 associated with HCV+ DRB1*0405 associated with HCV+ among patients with genotype 1b vs controls	— RR=7.5 RR=4.7	No assoc pc < 0.005 pc < 0.02

Chen D, 1996	German	186 HCV+ with renal disease	328 HCV+ with renal disease	No correlations observed between HLA class I and class II (DRB1, DQA1, and DQB1) with susceptibility to HCV	—	No assoc
Congia M, 1996	Italian	116	30	DRB1*1601-DQB1*0502 (DR2) with protection from HCV acquisition	NG	pc=0.0092
Cramp M, 1998	British	49	55	DRB1*04 allele with clearance DQA1*03 allele with clearance DQB1*0301 with clearance	4.52 4.69 5.09	p=0.0022 p=0.0012 p=0.0078
		104	134 healthy	DQB1*0302 less frequent among HCV antibody positive individuals compared to those anti-HCV negative	NG	p=0.0063
Fanning L, 2000	Irish	84 with clearance	72 with HCV persistence	DRB1*01 with clearance	0.37	p <0.05 (95%C.I.: 0.13-1.01)
				DQB1*0501 with DRB1*0701 reduced among those with persistence	NG	p < 0.05
Hennig B, 2002	European	147	426	LDL receptor exon 10 (G to A) with chronicity	0.46	p=0.005 (95%C.I.: 0.27-0.78)
Hohler T, 1997	German	75	101	DRB1*0301 with susceptibility to HCV	RR=3.9	p <0.001
				DRB1*1301 with with protection from HCV	RR=0.2	p < 0.008
				DQA1*0103 with protection from HCV	RR=0.31	p < 0.003
				DRB1*1301-DQA1*0103 with protection from HCV	RR=0.08	p < 0.005

Kuzushita N, 1998	Japanese	130 HCV+	916 healthy	DRB1*0405 associated with HCV DQB1*0401 associated with HCV	1.4 1.5	p < 0.05; pc=0.7 p < 0.03; pc=0.4
Lechman M, 1999	German	21	49	DR15 with clearance DRB1*1501 with clearance	RR=6.5 RR=13.9	pc=0.02 pc=0.03
Louagie H, 1996	Dutch	239 HCV+	220 healthy controls	Hepatoglobin Hp1-1 higher among HCV+ Hepatoglobin Hp2-2 lower among HCV+	NG NG	p < 0.001 p <0.05
Mangia A, 1999	Italian	35 acute	149 chronic	DRB1*1104 associated with clearance DQB1*0301 associated with clearance DRB1*1104-DQB1*0301 with clearance	4.51 4.52 7.38	pc=0.054 (95%C.I.: 2.01-10.1) pc=0.0039 (95%c.I.: 2.15-9.51) pc=0.009 (95%C.I.: 2.58-21.59)
Martinetti M, 1997	Italian	24 children born to HCV+ mothers who are HCV+	17 children born to HCV+ mothers who are HCV-	DRB1*1101 with susceptibility DRB1*1104 with susceptibility DRB1*Gly86 with susceptibility DRB1*Val86 with clearance	9.0 NG 5.33 0.19	p=0.035 NG p=0.033 p=0.033
McKiernan S, 2000	Irish	95	148	DRB1*0101 with clearance DQB1*0501 with clearance	RR=0.27 RR=0.38	pc < 0.0005 pc=0.002 (95%C.I.: 0.24-0.62)

				DRB1*03011 with persistence	RR=2.61	pc=0.001 (95%C.I.: 1.58-4.32)
				DQB1*0201 with persistence	RR=2.69	pc=0.0004 (95%C.I.: 1.63-4.44)
				DQB1*0603 with persistence	NG	p=0.005 (pc=NS)
				DQB1*0202 with persistence	NG	p=0.005 (pc=NS)
				DRB1*0401 with clearance	RR=0.52	p=0.009 (pc=0.4) (95%C.I.: 0.32-0.86)
Minton E, 1998	British	35	135	DRB1*11 with clearance	RR=0.19	p < 0.001 (95%C.I.: 0.39-0.91)
				DQB1*0301 with clearance	RR=0.75	p <0.002 (95%C.I.: 0.61-0.94)
Okada F, 2001	Japan	75	232 healthy	p53Pro homozygotes greater among HCV-infected males with genotype 1b	7.17	p=0.039 (95%C.I.: 1.32-38.93)
				p53Pro allele frequency greater among males infected with genotype 1b HCV	2.21	p=0.01 (95%C.I.: 1.04-4.67)
Promrat K, 2003	American	339	2380 blood donors	CCR5*D32 similar between HCV infected and controls		No assoc
Thursz M, 1999	European	Cohort 1: 85	170	DRB1*1101 with clearance	2.14	p=0.013 (95%C.I.: 1.11-4.12)
		Cohort 2: 57	152	DQB1*0301 with clearance	2.22	p=0.004 (95C.I.: 1.24-3.96)
				DRB1*0701 with chronicity	2.04	p=0.027 (95%C.I.: 1.03-4.17)
				DRB4*0101 with chronicity	2.38	p=0.002 (95%C.I.: 1.29-4.35)

Tibbs C, 1996	British	104	177	DQA1*03 with protection from chronicity	RR=0.37	pc=0.046
				DRB1*0403 with protection from chronicity	RR=0.35	pc=0.03
				DQB1*0302 with protection from chronicity	RR=0.37	pc=0.038
				DR4 with protection from chronicity	RR=0.42	NG
				DPB1*0402 with chronicity	RR=2.7	pc=0.057
Thio C, 2001	American	200	374	DQB1*0301 with viral clearance among all subjects studied	0.72	p=0.031 (95%C.I.: 0.53-0.97)
				DQB1*0501 with clearance among all subjects studied	0.65	p=0.014 (95%C.I.:0.46-0.92)
				DRB1*0101 with clearance among all subjects studied	0.45	p=0.001 (95%C.I.: 0.28-0.73)
				DRB1*0301 with persistence among all subjects studied	1.78	p=0.01 (95%C.I.: 1.13-2.79)
				DRB1*0101-DQB1*0501 with clearance among all subjects studied	0.48	p=0.003 (95%C.I.: 0.30-0.78)
				DRB1*0301-DQB1*0201 with persistence among all subjects studied	1.80	p=0.012 (95%C.I.: 1.14-2.85)
				DRB1*0301-DQB1*0201-DQA1*0501 with persistence among all subjects	1.79	p=0.013 (95%C.I.: 1.13-2.83)
				DQB1*0501 with clearance among whites	0.47	p=0.01 (95%C.I.: 0.27-0.84)
				DRB1*0101 with clearance among whites	0.32	p=0.0004 (95%C.I.: 0.17-0.60)
				DRB1*0101-DQB1*0501 with clearance among whites	0.34	p=0.001 (95%C.I.: 0.18-0.65)
				DRB1*0301 with persistence among whites	2.36	p=0.010 (95%C.I.: 1.23-4.52)
				DRB1*0301-DQB1*0201 with persistence among whites	2.51	p=0.007 (95%C.I.: 1.28-4.91)

				DRB1*0301-DQB1*0201-DQA1*0501 with persistence among whites	2.48	p=0.008 (95%C.I.: 1.27-4.86)
				DQB1*0301 with viral clearance among blacks	0.65	p=0.054 (95%C.I.: 0.42-1.00)
				DRB1*0101-DQB1*0501 with clearance in whites	0.32	(95%C.I.: 0.17-0.60)
				DRB1*0301-DQB1*0201 with persistence in whites	2.36	(95%C.I.: 1.23-4.52)
Thio C, 2002	American	231	444	A*1101 with clearance	0.49	(95%C.I.: 0.27-0.89)
				B*57 with clearance	0.62	(95%C.I.: 0.39-1.0)
				Cw*0102 with clearance	0.43	(95%C.I.: 0.21-0.89)
				Cw*04 with persistence	1.78	(95%C.I.: 1.21-2.59)
				A*2301 with persistence	1.78	(95%C.I.: 1.01-3.11)
van Vlierberghe H, 2001	Belgian	142	196 healthy controls	Higher hepatoglobin Hp1-1 phenotype higher among chronic HCV	NG	p < 0.01
				Cys282Tyr allele higher among HCV patients	NG	p < 0.05
Vejbaesya A, 2000	Thai	43 with clearance	57 with persistence	DRB1*0301 with persistence	NG	pc=0.03
				DQB1*0201 with persistence	NG	pc=0.04
			140 healthy Thais	DRB1*0701 decreased in HCV-infected compared to normal controls	NG	pc=0.003
				DQA1*0201 decreased in HCV-infected compared to normal controls	NG	pc=0.001
Vidigal P, 2002	Various	80	37	IL10 -1082 G/G	—	p=0.048
				TNF	—	No assoc

				TGF-B	—	No assoc
Vitte R, 1995	French	108	181	HLA A, B, C, DR, and DQ	—	No assoc
Wawrzynowicz-Syczewska M, 2000	Polish	129	103	DQB1*0602 with clearance	2.2	p=0.03
				DQB1*02 with clearance	2.3	p=0.002
				DQB1*0301 with chronicity	0.5	p=0.015
				DRB1*1501 with clearance	2.38	p=0.014
				DRB1*0701 with clearance	2.3	p=0.0064
				DPB1*0101 with clearance	2.46	p=0.02
				DRB1*1501-DQB1*0602 with clearance	2.2	p=0.027
				DRB1*0701-DQB1*02 with clearance	3.23	p=0.0006
Wawrzynowicz-Syczewska M, 2000	British	57 acutes	93 chronics	IL-1 A and clearance	—	No assoc
				IL-1B and clearance	—	No assoc
				IL-1 RN and clearance	—	No assoc
				TNF -308 and clearance	—	No assoc
				TNF-238 and clearance	—	No assoc
				IL 10 (-591+ -819 + -1082 haplotypes) and clearance	—	No assoc
Woitas R, 2002	German	153	102[a] 102[b] 130[c]	CCR5*Δ32 homozygosity more common in chronic HCV than all three control groups	—	p <0.02
Yenigun A, 2002	Turkish	49	43 healthy	DRB1*11 reduced among patients	0.3	p < 0.01 (95%C.I.: 0.1-0.7)

Zavaglia C, 1996	Italian	117	489 healthy controls	DR5 is protective in susceptibility to HCV	RR=0.4	pc=0.005
Zavaglia C, 1998	Italian	99	179	DQA1*0201-DQB1*0201 with susceptibility to HCV	1.8	p=0.04 (95%C.I.: 0.96-3.5)
				DRB1*1104-DQA1*0501-DQB1*0301 with protection from HCV	0.31	p=0.0025 (95%C.I.: 0.12-0.7)

Abbreviations: NG=Not given in publication; OR=Odd's ratio; RR=relative risk; 95%C.I.=95% Confidence Interval; p_c=p-value corrected for multiple comparisons; No assoc=No association observed.

a=healthy control population b=patients with HIV mono infection c=patients with HCV/HIV coinfection

Table 2.3.2. Summary of studies of host genetics and the response to therapy hepatitis C virus infection or variations in HCV viral load.

Study	Population	Therapy	No. Treated/ Studied	Correlation of Allele, Genotype or Haplotype	OR	Significance
Almarri A, 1998	Egyptian	Mono	55	DR2 with response (Response defined biochemically)	NG	$p < 0.005$
Alric L, 1997	French	—	103	DQB1*0201 with lower HCV levels	NG	$p < 0.05$
Alric L, 1999	French	Mono	170	DQB1*06 with sustained response	NG	$p < 0.02$
Coelho-Borges, 2002	Brazilian	IFN+R	34	HFE wild type with SR	NG	p=0.03
Dincer D, 2001	Turkish	Mono	22	DRB1*07 with Non-response DRB1*13 with non-response	NG	$p < 0.05$
Dorak M, 2002	American	IFN+R	250	CCR5-D32 and promoter genotypes and sustained response to therapy	—	No assoc
				CCR E/E genotype with greater decline in viral loads during treatment	???	p=0.016
				CCR C/G*2 genotype with lower median baseline HCV viral load	???	p=0.05
Edwards-Smith C, 1999	Australian	Mono	53	IL10 -1082 G/G or G/A with IR	3.5	p=0.055 (95%C.I.: 1.0-13.2)

				IL10 -819 C/T or T/T with IR	4.7	p=0.018 (95%C.I.: 1.3-17.8)
				IL10 - 592 A/A or A/C with IR	5.1	p=0.12 (95%C.I.: 1.4-19.1)
				-592A+-819T+-1082A with IR	4.5	p=0.024 (95%C.I.: 1.2-16.8)
				-592C+-819C+-1082G with NR	0.22	p=0.026 (95%C.I.: 0.06-0.87)
Haruna Y, 2000	Japanese	—	155	DRB1*1301 with higher HCV RNA levels	NG	p=0.0007
Fanning L, 2001	Irish	Natural variance of HCV viral load without treatment	57	DRB1*15 and/or DQB1*0602 with greater viral load fluctuation	—	p <0.05
				DRB1*0701 and/or DQB1*0201 with relatively stable viral loads	—	p <0.05
Haruna Y, 2000	Japanese	—	155	DRB1*1301 with higher HCV RNA levels	NG	p=0.0007
Hennig B, 2002	European	Mono & IRN+R	221	LDL receptor 3' UTR (G to A) with IR	3.58	p=0.023
Hijikata M, 2000	Japanese	Mono	52	-88*MxA*-(G/G) rarer in SR	—	p=0.0321
				-88*MxA*-(G/T) with SR	—	p=0.0170
Hijikata M, 2001	Japanese	Mono	52	-123*MxA*-(C/C) with NR	—	p=0.0028
Kikuchi I, 1998	Japanese	Mono	67	B54 and non-response	7.46	p = 0.0258

				A24-B54-DR4 and non-response (Response defined biochemically)	24.12	p=0.0378
Miyaguchi S, 1997	Japanese	Mono	172	B55 with SR	—	p<0.01
				B62 with SR	—	p<0.01
				Cw3 with SR	—	p<0.01
				Cw4 with SR	—	p<0.05
Par A, 1998	Hungarian	Mono	33	DR3 more frequent in non-responders	NG	NG
				B8-DR3-DQ2 more frequent in non-responders	NG	NG
Pirisi M, 2000	Italian	Mono	53	C282Y or H63D not correlated with response	NG	p=0.546
Promrat K, 2003	American	Mono and IFN+R	121	CCR5 promoter 59029 -A allele marginally associated with SR	3.07	p=0.048
Rosen H, 2002	Caucasian	Mono and IFN+R	155	TNF alpha	—	ns
Sim H, 1998	Canadian	Mono	70	DRB1*0404 with SR (Response defined biochemically)		p=0.019
Sugimoto Y, 2002	Japanese	Mono	193	LMP7-K with SR	2.3	p=0.053 (95%C.I.: 1.1-4.6)
				Tap1, Tap2 and LMP2	—	No assoc

Thursz M, 1999	European	Mono	288	DR5 with SR	1.79	p=0.036 (95%C.I.: 1.0-3.19)
Vidigal P, 2002	American	IFN+R	75	TGFb +29 C/C with non-response	4.64	p=0.029 95%C.I.: 1.17-18.43
Vitte R, 1995	French	Mono	73	HLA A, B, C, DR, and DQ	—	No assoc
Wawrzynowicz-Syczewska M, 2000	Caucasian	Mono	55	DRB1*0701-DQA1*0201-DQB1*02 and SR	4.89	p=0.00048
Wawrzynowicz-Syczewska M, 2000	Polish	29 IR	26 NR	IL-1 A and response IL-1B and response IL-1 RN and response TNF -308 and response TNF-238 and response IL 10 (-591+ -819 + -1082 haplotypes) and response	— — — — — —	No assoc No assoc No assoc No assoc No assoc No assoc
Woitas R, 2002	German	Viral Load Study	153	CCR5-Δ32 homozygosity with increased HCV-viral load	—	p=0.045
Yee L, 2001	American	IFN+R	104	IL10 -819 T allele with SR IL10 -592 A allele with SR IL10 -819 T/T with SR IL10 -592 A/A with SR IL10R108+-3575T+-2763C+-1082A+	2.2 2.2 16.6 16.6 2.65	p=0.016 p=0.016 p=0.013 p=0.013 p=0.10

-819T+-592A with SR IL10R108+-3575T+-2763C+-1082A+ -819C+-592C with NR	0.39	p=0.16
IL10R108+-3575T+-2763C+-1082A+ -819T+-592A homozygosity with SR	13.7	p=0.25

Abbreviations: NG=Not given in publication; OR=Odd's ratio; RR=relative risk; 95%C.I.=95% Confidence Interval; Mono= Interferon Monotherapy; IFN+R=combination interferon + ribavirin; SR=sustained responder; NR=non-responder; pc=p-value corrected for multiple comparisons; No assoc=No association observed.

Table 2.3.3. Summary of studies of host genetics and the progression of liver disease in hepatitis C virus infection.

Study	Population	No. of Cases	Controls	Correlation of Allele, Genotype or Haplotype	OR	Significance
Akuta N, 2001	Japanese	27	323	Tap2*0201 with cirrhosis	NG	p <0.05
Alric L, 1997	French	96	subgroup analyses of the 96	DQB1*0301 with lower mean Knodell scores	NG	p=0.045
Asti M, 1999	Italian	41 HCV with normal ALT 99 with chronic HCV	179 uninfected	Tap1C with protection from chronic liver disease compared to healthy controls	0.23	pc=0.005
				DRB1*03 with protection from chronic liver disease compared to healthy controls	0.09	pc=0.008
				TNFB1 with normal ALT when compared to those with progressive liver disease	2.95	p=0.021 (95%C.I.:1.17-7.40)
				TAP2C with normal ALT when compared to those with progressive	3.0	p=0.040 (95%C.I.: 1.04-15.34)
				DRB3*03 with normal ALT when compared to those with progressive liver disease	16.47	p=0.042 (95%C.I.: 1.0-244.68)
				DRB1*1104 with normal ALT when compared to those with progressive liver disease	4.82	p=0.058 (95%C.I.:0.94-24.52)
Brandhagen D, 2000	American	42 liver transplanted	87 without liver transplants	DRB1 not correlated with progression of chronic HCV	NG	No assoc

Coelho-Borges S, 2002	Brazilian	34	subgroup analyses of the 34	HFE mutations with iron status	NG	No asoc
Czaja A, 1996	American	64	subgroup analyses of the 64	No correlations between DR antigens	—	No assoc
Erhardt A, 2003	German	401	295 healthy	In multivariable analyses, C282Y hetero-zygotes associated with liver cirrhosis compared to wild type	5.9	p < 0.009 (95%C.I.: 1.6 – 22.6)
				In multivariable analysis, H63 hetero-Zygotes associated with liver cirrhosis Compared to wild type	2.9	p < 0.05 (95C.I.: 1.0 – 8.4)
Fanning L, 2001	Irish	57	subgroup analyses of the 57	DRB1*0701 with lower HAI score	—	p=0.031
Haruna Y, 2000	Japanese	155	subgroup analyses of the 155	DRB1*1302 in those without bile duct damage	NG	pc=0.002
				DRB1*1302 with those with less portal lymphocyte infiltration	NG	p=0.03
				DRB1*1101 with those with less piecemeal necrosis	NG	p=0.004

				DRB1*1502 with those with piecemeal necrosis	NG	p=0.015
				DRB1*1502 with those with more portal lymphocyte infiltration		p=0.03
				DRB1*1201 and DRB1*0802 with those with no bile damage	NG	p=0.02
				DRB1*1201 and DRB1*0802 with those wihtout piecemeal necrosis	NG	p=0.03
Hennig B, 2002	European	143	58	LDL receptor Exon 8 (A to G) with mild fibrosis	2.68	p=0.006 (95C.I.: 1.38-5.20)
Hézode C, 1999	French	209	subgroup of 209 studied	Cirrhosis more frequent in patients with than without liver iron accumulation But C292Y and H63D are not major determinants	NG	p=0.004
Hüe S, 2002	French	197 with Knodell F0-F3 (CAH)	36 with Knodell of F4	DRB1*11 with less severe liver disease	0.35	p=0.04 (95%C.I.: 0.1-0.9)
				DRB1*03 assoc with cirrhosis	2.1	p=0.04 (95C.I.: 1.1-4.1)
				DQB1*0201 associated with cirrhosis	1.97	p=0.02 (95C.I.: 1.2-3.3)
Higashi Y, 1996	Japanese	60	293 healthy	B*61 with cirrhosis	RR=2.06	p < 0.02
				Cw3 and cirrhosis	RR=2.07	p < 0.02
				DR4 and cirrhosis	RR=1.99	p < 0.02
				DR9 and cirrhosis	RR=0.35	p < 0.01
				DQB1*0301 and cirrhosis	RR=0.44	p < 0.05
				DQB1*0303 and cirrhosis	RR=0.37	p< 0.01
				DQB1*0401 and cirrhosis	RR=1.9	p < 0.05

				DQB1*0402 and cirrhosis	RR=2.5	p < 0.05
				Cw3-DR4-DQB1*0401 or *0402	5.05	p < 0.0002
				B*61-DR4-DQB1*0401 or *0402	6.21	p <0.0005
Höhler T, 1998	German	82	99 healthy	TNF 238.2 (A allele) with HCV patients	NG	pc <0.017
Knöll A, 1998	German	101	Subgroups of the 101 were analysed	C282Y heterozygotes had similar fibrosis stage scores than those who were wild-type	NG	NG
Kazemi-Shirazi L, 1999	Austrian	184	487 healthy	HFE variants were not correlated with Increased hepatic fibrosis	NG	NG
Kuzushita N, 1996	Japanese	19 with normal ALT	49 with abnormal ALT and CAH	DR13 associated with normal ALT compared to those with abnormal ALT	NG	pc < 0.003
Kuzushita N, 1998	Japanese	194 with chronic liver disease	66 with persistently normal ALT	B54 assoc with chronic liver disease compared to HCV+ with normal ALT	10.1	p <0.003; pc=0.049
				DRB1*0405 with chronic liver disease compared to HCV+ with normal ALT	2.7	p < 0.02; pc=0.3
				DRB1*1101 with normal ALT compared to chronic liver disease	0.3	p < 0.05; pc=1.2
				DRB1*1302 with normal ALT compared to chronic liver disease	0.2	p < 0.01; pc=0.2
				DQB1*0401 with chronic liver disease compared to HCV+ with normal ALT	2.7	p < 0.02; pc=0.2

				DQB1*0604 with normal ALT compared to chronic liver disease	0.3	p < 0.03; pc=0.3
				B54-DRB1*0405-DQB1*0401 higher risk if chronic liver disease compared to those with DRB1*0405-DQB1*0401 without B54	13.2	p=0.0015
Kuzushita N, 1999	Japanese	36 with normal ALT	109 with chronic liver disease	Tap2*0101, *0102, *0201 similar between groups	NG	No assoc
				Tap2*0103 with normal ALT	0.31	pc=0.01 (95%C.I.: 0.15-0.66)
				Tap2*0103 along (without DRB1*1302-DQB1*0604) with normal ALT	0.24	pc= <0.05 (95%C.I.: 0.087-0.67)
Martinelli A, 1999	Brasilian	135 men	subgroup analyses	Liver fibrosis among HFE carriers more intense than among non-carriers	NG	p=0.01
Mangia A, 1999	Italian	44 cirrhotics with <25 yrs HCV infection	76 cirrhotics with > 25 years HCV infection	DQB1*0502 with more rapidly progressive liver disease with genotype 1b infections	8.15	pc=0.098 (95%C.I.: 1.49-44.4)
McKiernan S, 2000	Irish	145	subgroup analyses of the 145	DRB1*03011 with low inflammatory score	NG	p=0.008
				DQB1*0201 with low inflammatory score	NG	p=0.008
Negro F, 2000	Swiss	120	Subgroup analysis of 120	Occurrence of C282Y or H63D not correlated with fibrosis	NG	p=0.632

Peano G, 1994	Italian	51	36 symptom free	DR5 with protection from severe liver disease	RR=0.14	pc=0.005
Pellegris G, 2002	Italian	161 with HCC	749 healthy blood donors	Cw7 in HCC patients with liver cirrhosis compared to healthy controls	NG	p=0.0000074
				DQ1 in HCC patients with liver cirrhosis compared to healthy controls	NG	p=0.000025
				Cw7 in HCC patients without liver cirrhosis compared to healthy controls	NG	p=0.0025
				B8 in HCC patients without liver cirrhosis compared to healthy controls	NG	p=0.00027
	NOTE: In this study, patients with HCC had HBV or HCV.					
Pirisi M, 2000	Italian	58	138 Healthy	Carriage of either C282Y or H63D higher Among patients than controls	NG	p=0.01
				Increase of either mutation in stages above 1	NG	p=0.034 for trend
Powell E, 2000	Australian	128	Analyses based on subgroups of the 128 with HCV	TGFß codon 25 Arg/Arg homozygotes with increased hepatic fibrosis	0.25	padj=0.018 (95C.I.: 0.08-0.79)
				Angiotensinogen (AT)-6 A/A homozygotes with increased hepatic fibrosis	0.34	padj=0.030 (95%C.I.: 0.12-0.90)
				IL10 and fibrosis	—	No assoc
				ACE (angiotensin converting enzyme) intron 16 and fibrosis	—	No assoc
Promrat K, 2003	American		Analyses based on subgroups of	RANTES -403A with mile inflammation (HAI score)	0.34	p=0.03 (95%C.I.: 0.13-0.90)

			the 339 with HCV			
Renou C, 2002	French	83 with normal ALT	233 with elevated ALT	DRB1*11 with normal ALT	2.36	pc=0.03 (95%C.I.: 1.39-4.00)
				DQB1*0401/0402 with normal ALT	NG	p=0.01
				DRB1*08 with normal ALT	2.76	p=0.02 (95%C.I.:1.12-6.77)
				DRB1*01 with elevated ALT	0.26	p=0.004 (95C.I.:0.10-0.70)
Reynolds W, 2002	American	166	All analyses based on subgroups of the 166 with HCV	Myeloperoxidase -493 GG with lower fibrosis scores	—	p < 0.05
Smith B, 1998	British	137	All analyses based on subgroups of the 137 with HCV	C282Y heterozygosity with more fibrosis	7.6	p=0.01 (95%C.I.: 1.9-31.2)
Sonzogni L, 2002	Italian	394	All analyses based on subgroups of the 394 with HCV	microsomal epoxide hydrolase gene mEH 113His/His homozygosity associated with HCV-related HCC	2.2	p=0.03 95%C.I.: 1.0-4.6
				slow/very slow mEH phnotypes with HCC	2.8	pc=0.006 (95C.I.: 1.5-5.3)
				Very slow mEH phenoptype with HCC	1.5	pc=0.038 (95%C.I.: 0.6-3.4)
Thorburn D, 2001	British	164	Subgroup analyses of the 164	No association of increased fibrosis score and HFE variants	NG	p=0.16
				No association of cirrhosis with HFE variants	NG	p=0.37

Thursz M, 1999	European	321	321	DRB1*0701 with moderate or severe fibrosis	0.65	p=0.019 (95%C.I.: 0.45-0.95)
				DRB1*1501 with moderate or severe fibrosis	0.58	p=0.01 (95%C.I.: 0.38-0.90)
				DRB4*0101 with with moderate or severe fibrosis	0.72	p=0.044 (95%C.I.: 0.52-1.0)
				DRB3*0101 with mild fibrosis	1.46	p=0.03 (95%C.I.:0.92-2.34)
				DQB1*0501 with mild fibrosis	1.50	p=0.016 (95%C.I.: 1.06-2.11)
				DQB1*0604 with mild fibrosis	1.98	p=0.026 (95%C.I.: 1.04-3.81)
Tillmann H, 2001	German	69 in group 1	331 blood and liver donors	In group 1: A19 with cirrhosis	RR=1.98	p=0.032
				In group 1: B35 with cirrhosis	RR=1.98	p=0.049
				In group 1: B37 with cirrhosis	RR=4.32	p=0.035
		39 in group 2	170 blood donors	In group 1: Cw4 with cirrhosis	RR=1.98	p=0.035
				In group 1: DR3 with cirrhosis	RR=1.96	p=0.038
				In group 1: DR5 with protection from cirrhosis	RR=0.3	p=0.001
				In group 1: DQ3 with protection from cirrhosis	RR=0.44	p=0.029
				In group 2: B8 with cirrhosis	RR=2.64	p=0.027
				In group 2: DRB1*11 lower in cirrhotics	RR=0.29	p=0.04
				In group 2: DQB1*03 lower among cirrhotic patients	RR=0.53	p=0.033
Vitte R, 1995	French	108	181	HLA A,B, C, DR, DQ	—	No assoc

Wozniak M, 2002	British	111	subgroup analyses of the 111	ApoE-epsilon4 allele with mild liver disease	varies by subgroup	$p < 0.006$
Yee L, 2000	American	30	114	TNF2(-238A) TNF3 (-308A)	3.2 5.1	p=0.03 p=0.03

Abbreviations: NG=Not given in publication; OR=Odd's ratio; RR=relative risk; 95%C.I.=95% Confidence Interval; pc=p-value corrected for multiple comparisons; No assoc=No association observed.

Table 2.3.4. Summary of studies of host genetics and comorbid manifestations of hepatitis C virus infection.

Study	Study Size	Population	Extra-hepatic manifestation	Correlation of Allele, Genotype or Haplotype	OR	Significance
Amoroso A, 1998	193	Italian	Mixed Cryoglubulinaemia	DRB*11 with cryoglobulins	NG	p=0.0035
				Immunoglobulin heavy chain gamma1 switch region RFLP–5.4 kb allele with cryoglobulins	NG	pc=0.002
				Immunoglobulin heavy chain gamma1 switch region RFLP–5.5 kb allele with protection from cryoglobulins	NG	pc=0.001
Cacoub P, 2001	158	French	Mixed Cryoglubulinaemia	DRB1*11 (DR11) with cryoglobulins	3.4	pc=0.017
				DR7 with protection from cryo	0.34	p=0.012
Czaja A, 1996	64	Mixed	Autoantibodies or concurrent immunologic disease	DR1 was lower among individuals with autoimmune manifestations compared to those without	NG	p=0.002
				DR1 was lower among individuals with autoimmune manifestations compared to normal subjects	NG	p=0.003
Erhardt A, 2003	696	German	Iron in HCV	Heterozygotes for *HFE* had higher ferritin	NG	p < 0.0005

				Heterozygotes for *HFE* had higher serum iron	NG	p < 0.0005
				Heterozygotes for *HFE* had higher iron stains	NG	p< 0.006
Hezode C, 1998	211	French	Iron in HCV	Cys282Tyr mutation	NG	No assoc (p=0.41)
Hijikata M, 2000	46	Japanese	Anti-LKM-1 antibodies	CYP2D6 allele with LKM+	NG	0.0035
				CYP2D6*10 allele with LKM-	NG	0.0069
				CYP2D6*1 homozygosity with LKM+	NG	0.0021
Hwang S, 2002	122	Chinese	Mixed Cryoglobulinaemia autoantibodies	DR3 with cryoglobulins	RR=6.14	pc=0.009 (95%C.I.: 2.03-15.84)
				DR4 with autoantibodies	RR=3.97	pc=0.036
Jurado A, 1997	88	Spanish	LKM positivity	DR7 associated with HCV+ LKM	RR=6	pc=0.0086
			Note: This study was essentially a comparison of HLA among 49 with AIH-type 2 with 30 HCV patients who were LKM-.			
Kakizaki S, 1999	439	Japanese	autoimmune thyroid disorders during IFN therapy	A2 with thyroid disorders	10.6	0.00011
				B46 with thyroid disorders	4.8	0.0099
				Cw7 with thyroid disorders	3.0	0.025
Kakizaki S, 2000	566	Japanese	autoimmune thyroid disorders during IFN therapy	A*0206 with autoimmune thyroid disorders during IFN treatment	4.7	0.0097

Kazemi-Shirazi L, 1999	184	Austrian	Iron in HCV	H63D mutation higher among patients with higher iron	NG	45% in patients vs. 21.8% in controls
				C282Y	NG	No assoc
Knoll A, 1998	101	German	Iron in HCV	Cys282Tyr mutation	---	No assoc
Lal P, 2000	26	US	Iron in HCV	C282Y NOT corr with increased iron in HCV cirrhosis	NG	No assoc
Lenzi M, 1998	55	Italian	Mixed Cryoglobulinaemia	DR3 with cryoglobulins	RR=3.7	pc=0.03
				B8 with croglobulins	RR=5.9	pc=0.0005
				B8 and DR3 with cryoglobulins	RR=7.2	pc=0.0003
Smith B, 1998	137	British	Iron in HCV	Cys282Tyr (HFE) heterozygotes with higher mean ferritin	---	p=0.0005
				Cys282Tyr (HFE) heterozygotes with higher iron stauration	---	p=0.0001
				Cys282Tyr (HFE) heterozygotes with higher positive iron stain	---	p=0.02
				Cys282Tyr (HFE) heterozygotes with more fibrosis	---	p=0.01
				Cys282Tyr (HFE) mutation carriers more likely to be cirrhotic	7.6	p=0.01
				Cys282Tyr (HFE) heterozygotes and inflammation scores	---	No assoc

Vitte R, 1995	285	French	anti-tissue antibodies or lymphocytic sialadenitis	HLA A, B, C, DR, and DQ	—	No assoc

Abbreviations: NG=Not given in publication; OR=Odd's ratio; RR=relative risk; 95%C.I.=95% Confidence Interval; pc=p-value corrected for multiple comparisons; No assoc=No association observed.

2.6 Chapter 2 References

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Chapter 3

General Methods: Cohorts and Data Collection Management

3.1 The Cohorts

The present study employed the use of 3 cohorts: The St. Mary's Hospital Hepatitis C Cohort (London, UK), and the Hepatitis C European Network for Cooperative Research (HENCORE), a multi-centre European study of HCV that includes individuals from the St. Mary's Hospital Hepatitis C Cohort, and the Alabama Hepatitis C Cohort (Alabama, USA). As outlined below, each cohort was constructed for slightly different research questions and accordingly, the data collected differs.

The HENCORE Cohort

The Hepatitis C European Network for Cooperative Research (HENCORE) study is a multi-centre study of hepatitis C involving eight tertiary care referral centres in seven countries, at eight centres: Lund, Sweden; London, United Kingdom; Hannover, Germany; Barcelona, Spain; Athens, Greece; Torino, Italy; Padova, Italy and Lyon, France. The study enrolled approximately 1600 Caucasian individuals with the aim of establishing guidelines about HCV screening, control and management. Figure 3.1.1 presents a map of Europe with the locations of each of the participating centres indicated. Enrolment was between October 1995 and October 1997.[1]

Liver biopsies and histology

Most study participants received a liver biopsy at enrolment, which was scored by a single pathologist using the Ishak scoring system with the modified Knodell activity index.[2, 3] Pre-treatment sera were collected on these patients at the time of enrolment, as was a sample of their DNA.

Clinical and demographic data

Standard data including baseline ALT values, along with demographic variables such as gender, age at treatment were collected on participants and entered into a database. Appendix 1 lists these demographic variables. Estimates of the year of HCV infection were obtained on a number of patients via interview by the attending physician.

Anti-HCV Therapy

Approximately 30% of patients in this cohort were given interferon monotherapy for their hepatitis C. Briefly, 3 million units of interferon monotherapy was administered by subcutaneous injection three times weekly for 24 weeks irrespective of the patient's HCV viral genotype, which was the standard practise at the time the study was carried-out. Patients were given a qualitative test for presence of HCV-RNA to assess response to therapy. Per standard definitions, those with positive tests at the end of treatment were termed "non-responders," while those with negative tests at the end of treatment and positive tests at 6 months after discontinuation of therapy were termed "relapsed responders," and those who were negative at the end of treatment and negative at 6 months after discontinuation of treatment were termed "sustained responders."

Figure 3.1.1. Map illustrating the locations of the medical centres participating in the Hepatitis C European Network for Cooperative Research (HENCORE) Study. Centres are located in: London, UK; Lyon, France; Barcelona, Spain; Athens, Greece; Hannover, Germany; Padova, Italy; Torino, Italy; and Lund, Sweden. (Map source: U.S. Central Intelligence Agency)

The St. Mary's Hospital Cohort

The St. Mary's Hospital Cohort consists of approximately 1,800 subjects who have been seen at the Hospital's Liver Centre for routine care of their HCV infection. The cohort is approximately 80% Caucasian, with others from parts of Africa, the Middle East and the Indian subcontinent. A portion of this cohort forms part of the HENCORE cohort. When medically indicated, a liver biopsy was performed on each patient and staging of fibrosis and necro-inflammation was conducted by a single pathologist using the Ishak score with modified Knodell index.[2, 3] Approximately, 30% of individuals were treated for their HCV using interferon monotherapy, as per the HENCORE study. Pre-treatment serum and DNA samples have been collected and stored on almost all subjects. Clinical and demographic data such as possible risk factors and current/ past alcohol were also collected and stored.

The Alabama Hepatitis C Cohort

The Alabama Hepatitis C Cohort is a multi-centre drug therapy cohort consisting of patients from the University of Alabama at Birmingham (UAB) in Birmingham, Alabama, USA and the University of South Alabama in Mobile, Alabama, USA. The study aimed to enrol a total of 400 individuals between the two centres with chronic hepatitis C for treatment with standard interferon-α + ribavirin (Rebetron®; Schering-Plough Corporation, USA), with approximately 100 patients recruited at the University of South Alabama and approximately 300 from the University of Alabama at Birmingham. Both sites are major tertiary care referral medical centres. In particular, UAB University Hospital is a major organ transplant centre in the USA, and as a result patients are from a diverse geographic area covering most of the state of Alabama, with a few patients coming from neighbouring states as well. Figure 3.1.2 presents a map of the Southern states of the USA, with the locations of Birmingham and Mobile highlighted. By the end of 2001, new recruitment was ended in light of newer protocols employing the more efficacious pegylated interferons, either alone, or in combination with ribavirin.[4-6] The cohort is 90% Caucasian and 10% black.

A.

B.

Figure 3.1.2 Locations of study centres for the Alabama Hepatitis C Cohort. **Part A**. shows the Southern states of the United States of America, with the state of Alabama circled. **Part B**. The state of Alabama, with the cities of Birmingham and Mobile depicted. Outlines within the map indicate counties. (Map source: Cartographic Research Lab, Department of Geography, University of Alabama at Tuscaloosa).

The treatment protocol

Treatment using interferon-α and ribavirin (IFN+R) is based on viral genotype. Because early virologic response is highly predictive of therapeutic outcome, treatment was initially for 12 weeks. If patients responded to therapy at the end of these 12 weeks (defined as non-detectable viral levels by PCR), treatment was continued. For those with the more resilient genotype-1 virus, treatment was continued for an additional 48 weeks (1 year of total treatment). For those with non-1 genotypes, treatment was continued for an additional 12 weeks (6 months of total treatment). Figure 3.1.3 summarises the protocol. All patients received 3 million units of interferon-α three times per week by subcutaneous injection. In addition, patients > 75 kg received 1200 mg of orally administered ribavirin twice daily, while those < 75 kg received 1000 mg or ribavirin twice daily. A sample of DNA was collected on patients enrolled in this protocol.

Patient inclusion/exclusion criteria

This cohort aimed to enrol individuals with compensated chronic HCV infection who were otherwise "healthy."[7, 8] All patients with other forms of chronic liver disease such as chronic HBV co-infection and concomitant forms of autoimmune liver disease were excluded. Alcohol consumption was a contraindication for inclusion in this study and all patients must have been off alcohol for at least 6 months prior to the start of enrolment screening to be eligible. In addition, individuals co-infected with the human immunodeficiency virus (HIV) were excluded. All patients were adults over the age of 18 and under the age of 70. Severe cirrhosis (Child's B/C) and decompensated liver disease were also a contraindication for enrolment in the study. Individuals who had mild cirrhosis (Child's A) and compensated liver disease were not excluded in this protocol.

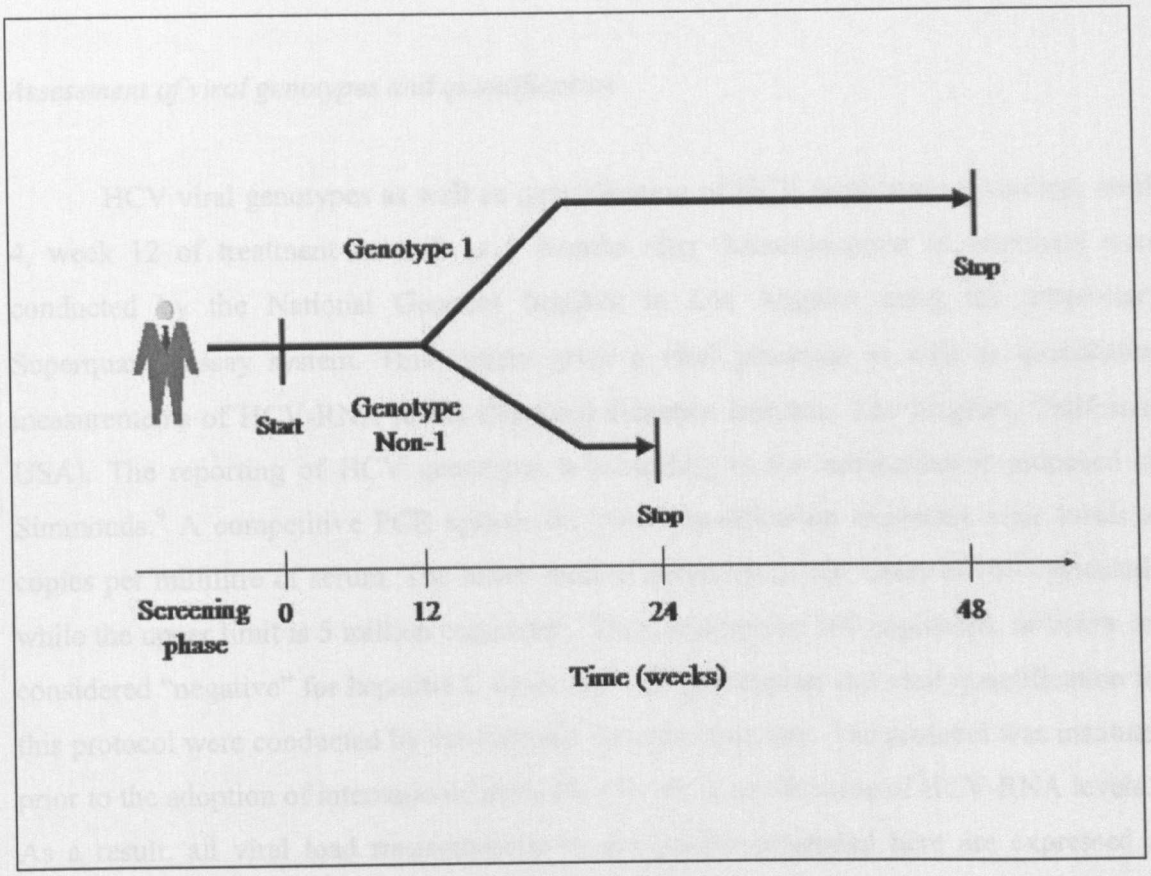

Figure 3.1.3. The UAB HCV treatment protocol. After undergoing screening, eligible patients are given 12 weeks of interferon and ribavirin therapy (3 million units of interferon-α three times per week, along with 1200 mg ribavirin daily if >75 kg, or 1000 mg ribavirin daily if <75 kg). If a patient is positive for HCV-RNA at week 12, treatment is terminated and the patient is considered a non-responder. If the patient is PCR negative for HCV-RNA at week 12 and they have genotype-1 infection, they are given 48 weeks of therapy. If they have a non-1 genotype infection, they are given 24 weeks of therapy. Six months after the cessation of therapy patients are tested for the presence of HCV-RNA again. If they are negative they are considered "sustained responders." If they are positive, they are considered "relapsed responders."

Assessment of viral genotypes and quantification

HCV viral genotypes as well as quantification of HCV viral load at baseline, week 4, week 12 of treatment as well as 6 months after discontinuation of treatment were conducted by the National Genetics Institute in Los Angeles using the proprietary Superquant® assay system. This system gives a viral genotype as well as quantitative measurements of HCV-RNA levels (National Genetics Institute, Los Angeles, California, USA). The reporting of HCV genotypes is according to the nomenclature proposed by Simmonds.[9] A competitive PCR system for viral quantification expresses viral levels as copies per millilitre of serum. The lower limit of detection of this assay is 100 copies/mL, while the upper limit is 5 million copies/mL. Thus, readings at 100 copies/mL or below are considered "negative" for hepatitis C virus. All viral genotyping and viral quantification for this protocol were conducted by the National Genetics Institute. The protocol was instituted prior to the adoption of international units (IU) for the quantification of HCV-RNA levels.[10] As a result, all viral load measurements in the studies presented here are expressed as copies per millilitre of serum, rather than international units. Because the HENCORE study was conducted largely before the widespread use of viral quantitation and treatment according to viral genotypes (with genotype-1 HCV receiving a full year of treatment and non-1 genotypes receiving half a year), such data are not available in the HENCORE cohort.

Clinical and demographic data

Standard clinical data such as ALT values, routine biochemistries (platelet and neutrophil counts, etc), were collected on all patients and stored in a database, along with demographic variables such as gender. The process for collection of demographic variables has been previously described, and consists of a detailed patient interview instrument that I developed previously.[11] Estimates of the year of HCV infection were obtained on approximately half of the individuals in this cohort by this patient interview. Standard biochemical and laboratory data were collected on each patient at each clinic visit and were entered by the biostatistics unit of the UAB Comprehensive Cancer Centre Data handling in

this cohort will be discussed in greater detail below. A list of key variables in this database is presented in Appendix 2.

Liver Biopsies and Histology

All patients in this cohort received a liver biopsy prior to enrolment in the study. However, systematic scoring of hepatic biopsies for extent of fibrosis using either the METAVIR or ISHAK systems was not routinely conducted on all patients.[2, 12] Instead, consultant liver pathologists described the biopsy sample and whether the patient was eligible for enrolment. Table 3.1.1 summarises the characteristics of each of the cohorts employed in the present study.

Table 3.1.1. Comparison of cohort characteristics. The HENCORE cohort and the Alabama Hepatitis C Cohort.

	Alabama Cohort	**HENCORE cohort**
Therapy		
Type of regimen	interferon+ribavirin	interferon monotherapy
Viral genotyping	all patients	some
Viral load quantification	all patients	none
Fibrosis Progression		
Systematic scoring using Ishak	none	all patients
Systematic scoring using METAVIR	some	none

3.2 Ethical approval

Ethical approval for the conduct of the present studies were obtained from the St. Mary's Hospital Local Research Ethics Committee and the University of Alabama at Birmingham Institutional Review Board. Studies of autoimmunity in hepatitis C were granted by the St. Mary's Hospital Local Research Ethics Committee and the Ethics Committee of the London School of Hygiene and Tropical Medicine. Ethical approval for the use of data on treatment outcomes for patients participating in the HENCORE study was obtained from the ethics committees of each of the participating centres.[1]

3.3 Data Collection and management

The HENCORE and St. Mary's Hospital Database:

Clinical and demographic information, including estimates of the time of HCV acquisition were obtained on patients by staff of each of the individual centres. Copies of these data forms were sent to both London and Lyon in paper form. Entry of the information on these forms into Access® was conducted independently in both centres and cross-validated by comparing the databases from the two centres and discrepancies were corrected by checking the original forms.

With respect to genotyping, the data are downloaded from the genotyping computer and cross-checked by two observers. These are then uploaded into the Access® database. For the present study, I subset data from this database, which contains information on both HENCORE and St. Mary's Hospital patients, along with relevant genotyping information. Appendix 1 lists some of the key demographic variables in this database. Subset data were then converted these data to SAS version 8® for data analyses.

Data management for the study of antinuclear antibodies:

Standard clinical and demographic data were obtained from the Access® database described above. Data were subset and stored in a database I created using EpiData® (Copenhagen, Denmark: www.epidata.dk). Data generated from the studies of anti-nuclear antibodies (ANA) were double entered, cross-validated and entered into this EpiData® database. Data were then converted to SAS® for analyses.

The Alabama Hepatitis C Cohort

Clinical and standard demographic data were collected as each patient presented to the clinic at the time of their visits. These data were recorded, double-entered and cross-validated as part of standard operating procedure for this protocol. Information on risk factors from a detailed patient interview was also collected on standardised forms, double-entered and cross-validated. Appendix 2 lists the key demographic and clinical variables available in this database. In addition to standard double-entry and cross-validation of data, frequency distributions of each of the major variables were conducted after each 50 updates to the database to search for obvious outliers and major shift in the distribution of data. All data were kept in a FoxPro® database at the Biostatistics Centre of the UAB Comprehensive Cancer Centre.

For genetic data, all genotypings are double-read and the two readings are cross-validated. Mismatches are discussed between the two observers and where irresolvable, the genotype assays are repeated. These cross-validated data are then double entered along with the demographic and clinical data and subjected to the same data consistency techniques as above.

3.4 Genotyping techniques

Genotyping of single nucleotide polymorphisms was conducted using three methods in the present study.

HENCORE cohort:

Real-time PCR (kinetic thermal cycling)

Genotyping of single nucleotide variants in this cohort was largely by kinetic thermal cycling using real-time polymerase chain reaction (PCR).[13] Real-time PCR detects specific amplification products in real time, and data on PCR products are gathered during the amplification process. This technique employs the use of a fluorescently labeled oligonucleotide probe and thus eliminates the need for post-PCR processing, such as the need to run amplification products out on agarose gels and stain them with ethidium bromide. With the elimination of post-amplification processing, the process is faster, and allows for a much higher throughput.

Allele-specific PCR

In this technique, primers are designed for specific alleles at each locus. Two reactions are needed for each locus. In the first reaction, a primer specific for allele X is used with a common reverse primer. In the second reaction, a primer specific for allele Y is used along with the same common reverse primer. Amplicons are typically small, with on average, only 50 base pairs of length.

These reactions are carried-out on 96 well plates (Figure 3.4.1.). In the odd columns primers specific for allele X are placed, along with the common reverse primer; in the even columns, primers for allele Y are placed (Figure 3.4.1). So for each sample, two wells are required. For example, Sample 1 will be placed in well A-1 and well A-2, with A-1 containing the primer specific for allele X and well A-2 containing the primers specific for allele Y. This is repeated throughout the tray. Each tray also contains a negative control of

water along with the primers to check for contamination, along with three positive controls—an example of an individual homozygous for allele X and another for allele Y and a heterozygous individual. The two amplifications are compared by the computer. A cut-off threshold is manually set by the operator to prevent the computer from reading non-specific amplification products or gene products with poor amplification. Presence of a homozygote for allele X or Y will result in product in only 1 of the wells. Figure 3.4.2 presents an example of this scenario. Presence of a heterozygous individual will result in products in both wells (Figure 3.4.2).

Genotyping by restriction digest

A portion of individuals in the HENCORE cohort were genotyped by restriction fragment length polymorphism (RFLP). In this technique, restriction enzymes are employed. Restriction enzymes are enzymes obtained from bacteria that cut specific pallendromic patterns of DNA. For example, the enzyme, *Eco*RI cuts both strands of DNA at the same site between the G and the A:

5'- G ↓ AATTC-3'
3'- CTTAA ↑ G-5'

Consequently, these enzymes may be employed for genotyping of allelic variants. A simplified scenario based on the aforementioned *Eco*RI example is present below:

Initially, a segment of DNA is amplified containing the locus that is to be genotyped. This product is then incubated with a restriction enzyme. If an individual possesses a specific allele that will allow an enzyme to cut, the original longer segment of DNA will be cut into two segments when the product is run out on a gel. If an individual does not have this particular sequence of DNA, then only one longer segment will be present.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UNKN X1514	UNKN X1514	UNKN X646	UNKN X646	UNKN X1321	UNKN X1321	UNKN L160	UNKN L160	UNKN X497	UNKN X497	UNKN X1212	UNKN X1212
B	UNKN X487	UNKN X487	UNKN I171	UNKN I171	UNKN L106	UNKN L106	UNKN X795	UNKN X795	UNKN X164	UNKN X164	UNKN X793	UNKN X793
C	UNKN X180	UNKN X180	UNKN X451	UNKN X451	UNKN X920	UNKN X920	UNKN L093	UNKN L093	UNKN L128	UNKN L128	UNKN X1029	UNKN X1029
D	UNKN X1119	UNKN X1119	UNKN X598	UNKN X598	UNKN L104	UNKN L104	UNKN X583	UNKN X583	UNKN X1022	UNKN X1022	UNKN X1394	UNKN X1394
E	UNKN X1104	UNKN X1104	UNKN L	UNKN L	UNKN X853	UNKN X853	UNKN X79	UNKN X79	UNKN X881	UNKN X881	UNKN Control XY	UNKN Control XY
F	UNKN L093	UNKN L093	UNKN X1044	UNKN X1044	UNKN L157	UNKN L157	UNKN L153	UNKN L153	UNKN X1477	UNKN X1477	UNKN Control Y	UNKN Control Y
G	UNKN X1463	UNKN X1463	UNKN A011	UNKN A011	UNKN X1286	UNKN X1286	UNKN X1282	UNKN X1282	UNKN L183	UNKN L183	UNKN Control X	UNKN Control X
H	UNKN X59	UNKN X59	UNKN X272	UNKN X272	UNKN X1127	UNKN X1127	UNKN X6052	UNKN X6052	UNKN L174	UNKN L174	UNKN water	UNKN water

Controls

Figure 3.4.1. Sample of the layout used for PCR trays used in automated genotyping of single nucleotide polymorphisms (SNPs). Trays contain 96 wells, capable of genotyping 48 samples. Samples are loaded into pairs of wells. For example the same sample is loaded into well A1 and A2, and another sample in B3 and B4, etc. Wells in the odd columns contain the primers specific for the first allele and those in the even columns contain primers specific for the alternate allele. Water, which serves as a negative control, is loaded into wells H11 and H12.

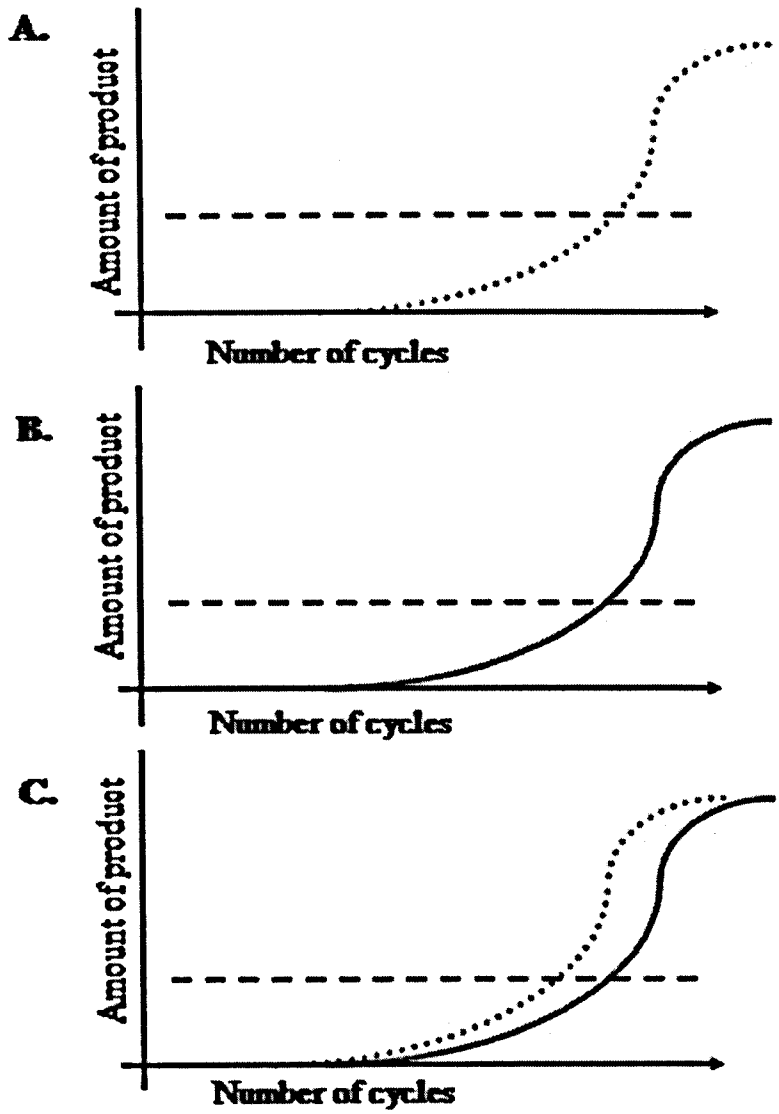

Figure 3.4.2. Schematic diagrams illustrating allele-specific amplification using kinetic thermal cycling. **A.** Example of an individual homozygous for allele X. Only product for this allele is amplified (dotted line). **B.** Example of an individual homozygous for allele Y. Only product for this allele is amplified (solid line). **C.** Example of an individual heterozygous for both alleles (XY). Both products are present (dotted line and solid line). Horizontal hashed lines represent the cut-off value set by the observer so that only products above that line are counted by the computer. This prevents the counting of non-specific or poorly amplified products.

Genotyping by PCR-SSP

Genotyping of single nucleotide allelic variants in the Alabama hepatitis C cohort was by polymerase chain reaction (PCR) with sequence-specific primers (SSP). In this method, primers are designed for specific alleles at each locus. Two reactions are needed for each locus. In the first reaction, a primer specific for allele A is used with a common reverse primer. In the second reaction, a primer specific for allele B is used along with the same common reverse primer. In both reactions, a pair of primers is used to amplify a "housekeeping gene," which is another gene that is not related to the gene under study. This serves as a positive control in the assay. Amplicons of each of the specific alleles is around 400-500 base pairs in length in this assay. The resulting PCR products are run on agarose gels and stained with ethidium bromide for analysis. Amplicons of the house keeping gene is typically larger (around 600-800 base pairs in length) to allow for adequate discrimination of allele-specific amplicons from the housekeeping gene amplicon.

This assay is carried-out in a 96-well tray, with each tray containing 48 samples (2 wells for each sample, with each well containing a reaction specific for each of the alleles). After PCR amplification, the resulting PCR-products are run-out on a gel and stained with ethidium bromide and then photographed. Figure 3.4.3 provides an example of this type of genotyping method. This figure presents an ethidium bromide-stained gel of PCR-SSP typing of variants for the Tapasin gene.

3.5 Statistical Methods

Statistical software

Power calculations were conducted using the EpiInfo® 2000 software (Centers for Disease Control and Prevention, Atlanta, USA). Statistical analyses were conducted using the SAS® statistical package (Cary, North Carolina, USA). Conversions to SAS®

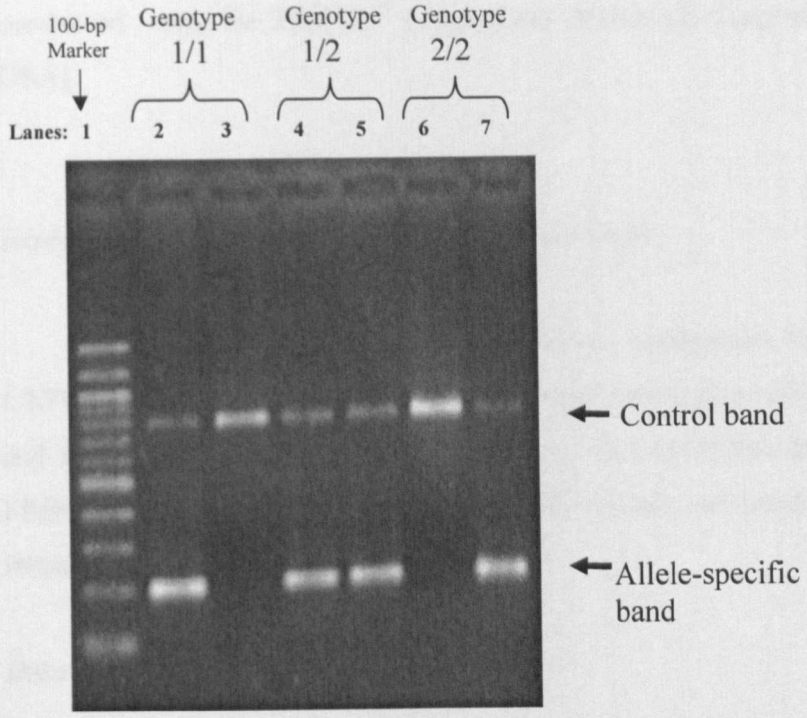

Figure 3.4.3. Example of genotyping using PCR-SSP (polymerase chain reaction with sequence specific primers) for dimorphic allelic variants. Lane 1 contains the molecular marker (100 base pair ladder). Lane 2 and 3 show an example of an individual who is homozygous for allele 1-- presence of band in only the lane with the reaction mixture specific for allele 1 (lane 2), while lanes 4 and 5 show an example of an individual who is heterozygous for allele 1 and alleles 2 --presence of a band in each of the allele 1and allele 2-specific reaction mixes (lanes 4 and 5, respectively). Lanes 6 and 7 show an example of an individual who is homozygous for allele 2 -- presence of band in only the lane with the reaction mixture specific for allele 2 (lane 7). All reaction mixtures contain primers specific for a "housekeeping gene" that serves as a positive control band.

from the Access® (Microsoft Corporation; Seattle, Washington, USA) and EpiData® (Copenhagen, Denmark) databases was achieved using the StatTransfer® programme (Circle Systems, Inc., Seattle, Washington, USA). Additional analyses and graphics were conducted using the EXCEL® programme (Microsoft Corporation, Seattle, Washington, USA).

Exploration of continuous and categorical variables

For examination of the distribution of continuous variables, the SAS® PROC UNIVARIATE (procedure used to obtain basic descriptive statistics such as median, mode and range) and PROC MEANS (procedure used to obtain the mean), while the PROC FREQ (procedure to give frequency distributions) command was used to examine the frequency distributions of categorical data.

Data cleaning and missing data

All datasets were cleaned by initially examining frequency distributions of variables (both categorical and continuous) to detect obvious outliers. The dataset was then visually scanned to pick up any unusual observations. Such observations were cross-checked against original data entry forms to identify and rectify errors.

Information was missing on gender and ethnicity was missing on approximately 29% and 30% of individuals, respectively, in the HENCORE database. Individuals with missing data were dropped from any analyses involving those variables.

Univariable associations of categorical data

For univariable associations of categorical data, standard Chi-square (χ^2) statistics for contingency tables were used. For calculations involving a cell with a count ≤5, Fisher's exact *p*-values and confidence limits were calculated using the SAS® software. Otherwise, standard maximum likelihood *p*-values and confidence limits were obtained. The PROC FREQ command (to obtain frequency distributions for categorical data) was used in this procedure along with the CHISQ CMH (chi-square and Cochrane Mantel-Hanzel statistics).

Univariable comparisons of non-parametric data

For comparisons of non-parametric data, the SAS® PROC NPAR1WAY (for non parametric 1-way tests) command with the WILCOXON stipulation (to specify the use of the Wilcoxon tests) was used to employ the Wilcoxon-rank sum test. This test was employed in Chapter 6 for the study of *CTLA4* gene variants viral dynamics to examine differences in viral load at each time point.

Multivariable analyses

For multivariable analyses of categorical data, logistic regression was employed using the PROC LOGISTIC procedure in SAS®. For the analyses of matched-pairs, conditional logistic regression was used using the PROC PHREG procedure in SAS®.

Longitudinal Analyses

For evaluation of viral dynamics over the course of anti-HCV treatment, longitudinal analyses by mixed modelling was used.[14] The PROC MIXED procedure in SAS® was employed.[15] Briefly, this procedure is a mixed model procedure that incorporates

both fixed and random effects. It is more robust than standard repeated measures by analysis of variance and allows for unequal spacing of measurement points.

Spatial analyses

For analysis of geographic distributions of anti-nuclear antibodies (ANA) in patients chronically-infected with HCV two methods were employed. First, multi-level modelling was employed with the PROC MIXED procedure (procedure for random and fixed effects mixed modelling) using SAS® software.[15, 16] Second, logistic regression was employed with study centres listed in hierarchical order by geographic latitude.

Genetic Studies: associations of allele frequencies with clinical outcomes

In genetic association studies, the frequency of individual alleles was assessed by direct counting. Variants on both chromosomes in each individual are assessed in the calculation of allele frequencies. Thus, there are twice as many alleles as individuals (2N) in a study.

Genetic Studies: associations of genotype frequencies with clinical outcomes

Frequencies of genotypes were assessed by direct counting. Briefly, the combination of alleles on both chromosomes at a particular genetic locus are assessed as a single unit in each individual. Thus, for a dimorphic single nucleotide polymorphism, three possible variants may exist for each individual:

Table 3.5.1. Possible combinations of genotypes at each dimorphic locus for single nucleotide polymorphisms.

Combination	Chromosome 1	Chromosome 2
Possibility 1	Variant 1	Variant 1
Possibility 2	Variant 1	Variant 2
Possibility 3	Variant 2	Variant 2

Unlike allele frequencies, the proportion of individuals with each genotype should add up to 100%. For univariable calculations of genotypes with clinical outcomes, the standard Chi-square test. Maximum likelihood or Fisher's exact confidence-limits and p-values were calculated as appropriate (see aforementioned section on univariable associations of categorical data).

Genetic Studies: associations of allele carrier frequencies with clinical outcomes

Allele carrier frequencies, also sometimes referred to as "phenotype" frequencies, assess whether an individual carries a particular allele. Allele carrier frequencies were also assessed in the present study by direct counting. Univariable associations of allele carriage with clinical outcomes, the standard Chi-square test were calculated. Maximum likelihood or Fisher's exact confidence-limits and p-values were calculated as appropriate (see aforementioned section on univariable associations of categorical data). Possible interactions between genetic variants and other factors as gender and alcohol use were also explored.

Correction for multiple comparisons

To control for the possibility of chance associations, many investigators employ the use of the Bonferroni correction for multiple comparisons. This technique multiplies the p-value by the number of comparisons made. This correction reduces type I error at the expense of type II error.[17] Due to the hypothesis-generating nature of this study, I have deliberately opted not to employ the use of the Bonferroni correction. Rather than risk the possibility of dismissing an association solely based on the p-value, which varies according to both study size and the strength of association,[18] I have not employed this correction. However, the number of comparisons made will be clearly presented to allow the reader to judge the quality of data for his/herself.

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Chapter 4

Studies of Data Consistency and Reproducibility

4.1 Study aims and methods: agreement and reproducibility in genetic and autoantibody data collection

To systematically examine the consistency and reproducibility of the genetic and autoantibody data generated in the present study, I conducted a series of evaluations to statistically ascertain these factors.

4.2 Evaluation of automated genotyping results

As mentioned previously, every automated genotyping run is validated by a human reader. By checking the amplification curves themselves, the reader is able to validate the automated scoring system and look for samples that may have had poor amplification not picked-up by the automated system; for example, those samples that do actually amplify, but amplify only moderately late, or those with poor amplification curves in general. To evaluate this process, assessment was made of the consistency and reproducibility of this process by conducting a series of tests to evaluate the level of observer with machine agreement, observer with observer agreement, and intra-observer agreement using the *kappa* (κ) correlation coefficient.[1] Calculations were made using the SAS® software (Cary, North Carolina, USA). Briefly, the kappa correlation coefficient is used to assess the degree of intra-observer agreement. The kappa score ranges between 0 and 1. Data collected as part of an observer agreement study are arranged in a contingency table, where column levels represent the ratings of one observer and the row levels, the rating of the other observer. Each cell represents one possible observation of each observer, with the diagonals

of the table represent the agreements between the two observers. [2] Kappas less than 0.4 represent "slight agreement." Those greater than or equal to 0.4 and less than 0.8 are represent "moderate agreement." Kappas greater than or equal to 0.8 are represent "excellent agreement."[3]

For this evaluation we selected, at random, one of the loci we genotyped for this project: an A to G single nucleotide polymorphism for 48 individuals at the –277 position of the *NOS2A* gene, which encodes the protein inducible nitric oxide synthase. Genotyping at this locus was conducted using kinetic thermal cycling as described earlier. These results were first validated by the first observer (LJY) and then independently by the second observer (Dr. Susanne Knapp, Research Associate, Imperial College Faculty of Medicine at St. Mary's Hospital, London, UK). Next, both observers independently repeated their evaluations of the automated genotyping results approximately 4 months after the initial scoring to limit memory of the first reading. Discrepancies in genotyping were reviewed jointly and a consensus was reached between the two observers after reviewing the raw data (amplification curves). If discrepancies could not be resolved, or one of the two observers felt that the curves were spurious (i.e., insufficient amplification of one of the alleles) the discrepant sample was re-genotyped.

Several comparisons were made to assess the genotyping process:

1) The first study evaluated the agreement between the first observer (LJY) and the automated genotyping system.

2) The second evaluation compared the level of agreement between the second observer (Dr. Knapp) and the automated system.

3) The third comparison examined the consistency within the first observer (LJY)

4) The fourth comparison examined the consistency within the second observer (Dr. Knapp).

5) The fifth assessment evaluated the level of agreement between the first observer (LJY) (Second reading from analysis 3 above) and a second reader (Dr. Knapp) (First reading from analysis 4 above). The use of the first or second readings of LJY and Dr. Knapp for the genotype results used in this comparison were selected at random.

4.3 Anti-nuclear antibodies in hepatitis C –positive patients

To evaluate the determination of antinuclear antibody (ANA) positivity and negativity, we compared scorings by a first reader (LJY) and a second reader (Dr. Peter Kelleher, consultant clinical immunologist, St. Mary's Hospital, London, UK) of all samples we evaluated for ANA from the St. Mary's Hospital cohort (N=207). To evaluate agreement regarding reading of ANA autoantibody patterns and titres, all positive samples from the St. Mary's Hospital cohort (N=18) and the Padova, Italy cohort (N=22) (Total: N=40) were double read by the first reader (LJY) and the second reader (Dr. Peter Kelleher) for both ANA pattern and titre. Agreement between the two observers was assessed by the *kappa* (κ) correlation coefficient.[1] Additionally, inter-observer agreement was assessed for the first reader. For this study, all samples positive on the initial screen at a 1:40 dilution were titred. Titre readings were assessed at: 1:40, 1:80, 1:160, 1:320 and 1:640 dilutions. The 1:40 dilution from this titre process was used as a second independent reading to assess the consistency of pattern scoring by the first observer (LJY). Due to limited resources, titres are assessed for positive individuals only once. For this reason a study assessing consistency of titre readings within the first observer (LJY) was not conducted.

In summary, four comparisons were made to assess the consistency and reproducibility of ANA data:

1) The level of agreement between the first observer (LJY) and the second observer (Dr. Kelleher) with respect to ANA positivity and negativity

2) The level of agreement between the first observer (LJY) and the second observer (Dr. Kelleher) with respect to the pattern of autoantibody staining.

3) The level of agreement between the first observer (LJY) and the second observer (Dr. Kelleher) with respect to the patient's ANA titre.

4) The level of consistency within the first observer (LJY) between a first and second scoring of ANA patterns on the same samples on two separate occasions.

4.4 Results: agreement and reproducibility in genetic and autoantibody data collection

Evaluation of automated genotyping results

Table 4.4.1 summarises the agreement between the first observer (LJY) and the automated genotyping system on genotyping of 48 samples. There was a high level of agreement between the observer and the automated genotyping system (κ=0.93; 95%C.I.: 0.84-1.0). Disagreements generally concerned heterozygous samples, with the automated system calling one sample a heterozygote and the first reader calling it an AA homozygote. In another sample, the automated system called this person a heterozygote, while the first observer called this individual a GG homozygote.

Table 4.4.2. presents the agreement between the second observer (Dr. Knapp) and the automated genotyping system of 48 samples (κ=0.93; 95%C.I.: 0.81-1.0). Disagreements concerned heterozygote samples. In one sample, the automated system called one individual an AA homozygote, while the second observer called it an AG heterozygote. In another sample, the automated genotyping system called an individual an AG heterozygote, while the second observer called that person an AA homozygote.

Table 4.4.1. Summary of observer agreement. Agreement between the first observer (LJY) and automated genotyping.

		Automated genotyping				
		AA	AG	GG	Failed	Total
First Reader (LJY)	AA	18	1	0	0	19
	AG	0	21	0	0	21
	GG	0	1	7	0	8
	Failed	0	0	0	0	0
	Total	18	23	7	0	48

κ =0.93 95%C.I.: 0.84 - 1.0

Table 4.4.3 and 4.4.4 present the results of intra-observer agreement for the first reader and second reader, respectively (κ=0.90; 95%C.I.: 0.79-1.0 and κ=0.93; 95%C.I.: 0.90- 1.0, respectively). Discrepancies between the first and second readings for the first reader generally concerned the classification of heterozygotes. In the first reading, one individual was classified as an AG heterozygote and as an AA homozygote in the second reading, while another individual was classified as an AG heterozygote in the first reading and a GG homozygote in the second reading. A third individual was classified as an AA homozygote in the first reading, and as an AG heterozygote in the second reading.

Table 4.4.2. Summary of observer agreement. Agreement between the second observer (Dr. Knapp) and automated genotyping.

		Automated genotyping				
		AA	AG	GG	Failed	Total
Second Reader (Dr. Knapp)	AA	17	1	0	0	18
	AG	1	22	0	0	23
	GG	0	0	7	0	7
	Failed	0	0	0	0	0
	Total	18	23	7	0	48

κ =0.93 95%C.I.: 0.84 - 1.0

The only disagreement between the first and second reading by the second observer concerned the classification of an individual as an AG heterozygote in the first reading and as an AA homozygote in the second.

Table 4.4.5. evaluates the agreement between the first and second observers. Discrepancies generally concerned the classification of heterozygotes. Three individuals were classified by the second reader as heterozygotes and as AA homozygotes by the first reader. Another individual was classified as an AA homozygote by the second reader and as an AG heterozygote by the first. A fifth individual was classified as an AG heterozygote by the second reader and as a GG homozygote by the first reader.

Table 4.4.3. Summary of intra-observer agreement. Agreement between a first and second reading on two separate occasions of the same automated genotyping results by the first reader (LJY).

First Read

Second Read	AA	AG	GG	Failed	Total
AA	18	1	0	0	19
AG	1	20	0	0	21
GG	0	1	7	0	8
Failed	0	0	0	0	0
Total	19	22	7	0	48

κ =0.90 95%C.I.: 0.79 - 1.0

Table 4.4.4. Summary of intra-observer agreement. Agreement between a first and second reading on two separate occasions of the same automated genotyping results by the second reader (Dr. Knapp).

First Read

Second Read	AA	AG	GG	Failed	Total
AA	16	1	0	0	17
AG	0	24	0	0	24
GG	0	0	7	0	7
Failed	0	0	0	0	0
Total	16	25	7	0	48

κ =0.97 95%C.I.: 0.90 - 1.0

Table 4.4.5. Summary of observer agreement. Agreement between the first observer (LJY) and second reader (Dr. Knapp) of automated genotyping results.

		Second reader (Dr. Knapp)				
		AA	AG	GG	Failed	Total
First Reader (LJY)	AA	16	3	0	0	19
	AG	1	20	0	0	21
	GG	0	1	7	0	8
	Failed	0	0	0	0	0
	Total	17	24	7	0	48

κ =0.83 95%C.I.: 0.69 - 0.98

4.5 Results: Anti-nuclear antibodies in hepatitis C –positive patients

Table 4.5.1 highlights the agreement between the first observer (LJY) and the second reader (Dr. Peter Kelleher) with respect to the determination of ANA positivity or negativity on Hep-2 cells as detected by indirect immunofluorscence (IIF) of 40 samples. The main discrepancies concerned LJY calling a sample positive, while Dr. Kelleher calling it a negative. Although one sample was called negative by LJY and positive by Dr. Kelleher. For all discrepancies, Dr. Kelleher's determinations of patterns were used in the final dataset.

Table 4.5.2 presents the agreement between the first and second observers with respect to the classification on ANA patterns. There were only two discrepancies. The

Table 4.5.1. Summary of intra-observer agreement. Agreement for scoring of ANA positivity Between the first observer (LJY) and the second observer (Dr. Kelleher).

Dr. Peter Kelleher

		Positive	Negative	Total
LJY	**Positive**	18	8	26
	Negative	1	180	181
	Total	19	188	207

κ =0.78 95%C.I.: 0.64 - 0.92

Table 4.5.2. Summary of intra-observer agreement. Agreement for scoring of ANA pattern for a first observer (LJY) compared to a second observer, Dr. Peter Kelleher, consultant clinical immunologist, St. Mary's Hospital, London UK).

Second Observer

(Dr. Kelleher)

		Homogenous	Speckled	Nucleolar	Other	Total
First	**Homogenous**	3	0	0	1	4
	Speckled	0	30	0	0	30
Observer	**Nucleolar**	0	0	3	0	3
(LJY)	**Other**	1	0	0	2	3
	Total	4	30	3	3	40

κ =0.88 95%C.I.: 0.73 - 1.03

primary discrepancy was with the classification of homogenous and "other" patterns. One sample was labelled "other" by the first reader and homogenous by the second, while the other was labelled homogenous pattern by the first reader and "other" by the second.

Table 4.5.3 presents the agreement between the first and second observers with respect to scoring of ANA titre. All four discrepancies concerned a tendency for observer one to score at a lower titre than observer two. One individual was scored as having a

Table 4.5.3. Summary of intra-observer agreement. Agreement for scoring of ANA titre for a first observer (LJY) compared to a second observer, Dr. Peter Kelleher, consultant clinical immunologist, St. Mary's Hospital, London UK).

		Second Observer (Dr. Kelleher)				
		1:40	1:80	1:160	1:320	Total
First Observer (LJY)	1:40	17	1	0	0	18
	1:80	0	14	2	0	16
	1:160	0	0	5	0	5
	1:320	0	0	1	0	1
	Total	17	15	8	0	40

κ =0.84 95%C.I.: 0.69 - 0.99

titre of 1:40 by the first observer, while the second observer scored this individual at 1:80. Two individuals were scored by observer one at the 1:80 level, while observer two scored them at the 1:160 level. The final discrepancy occurred when the first observer scored a sample at 1:320 while the second scored that sample at 1:160.

Table 4.5.4 summarises the agreement within observer one with respect to the scoring of ANA pattern. The only discrepancy between the two readings was the scoring of one individual as a homogenous in the first reading session and as an "other" in the second.

Table 4.5.4. Summary of intra-observer agreement. Agreement for scoring of ANA pattern within the first observer (LJY) of two separate scorings of the same patient samples.

		First Session				
		Homogenous	Speckled	Nucleolar	Other	Total
Second Session	Homogenous	4	0	0	0	4
	Speckled	0	30	0	0	30
	Nucleolar	0	0	3	0	3
	Other	1	0	0	2	3
	Total	5	30	3	2	40

κ =0.94 95%C.I.: 0.83 - 1.05

4.6 Discussion: automated genotyping

These studies suggest that there is a high level of both intra- and inter-observer agreement with respect to data generated in genotyping assessment. In the method of the kappa statistic, as developed by Cohen, kappa values greater than 0.80 are considered to have "excellent levels of agreement," kappa values greater than or equal to 0.4 and less than 0.80 are considered to have "moderate" agreement. Kappa values less than 0.4 have "slight" agreement.[1,2]

Both the first and second observers had a very high level of agreement with the automated genotyping system (κ=0.93 for both). Moreover, both observers showed a high

level of internal consistency when comparisons were made of scorings conducted on two separate occasions, with the second observer showing a slightly higher level of agreement between her first and second readings than the first (κ=0.97 for the second and κ=0.90 for the first). Both individuals also had a moderately strong level of agreement with respect to each other (κ=0.83).

Discrepancies between either observer and the automated system generally concerned the classification of heterozygous individuals. As mentioned previously in Chapter 3, a common problem with heterozygous individuals is the preferential amplification of one of the alleles over the other. It is possible that poor amplification of template DNA that may have produced sufficient amplicons to be detected by the automated system, but the over numbers of amplicons and quality of the resulting aggregate product may not have been adequate for the individual who is checking the data to label as a heterozygote. Such an example can be seen in Table 4.4.1 where the two disagreements concerned individuals labeled as an AG heterozygote by the automated system and AA and GG homozygotes by the first reader. In fact, the second reader scored one of the AG heterozygotes from the automated genotyping system as an AA homozygote. The other disagreement between the second and the automated system also concerned a heterozygote individual, where a sample was labeled AG by Dr. Knapp and AA homozygote by the automated system.

The results of this study suggest that the automated genotyping system employed in the present study is consistent and reproducible, with a high level of both intra- and inter-observer consistency. The relatively small sample sizes precluded the achievement of nominal statistical significance for some of the comparisons made in the present study, although all comparisons had a strong degree of correlation ($\kappa \geq 0.83$).

4.7 Discussion: anti-nuclear autoantibody screening

This study suggests that there is moderate to strong agreement between LJY and Dr. Kelleher regarding the scoring of ANA positivity (κ= 0.78), ANA pattern (κ= 0.81), as well as ANA titre (κ= 0.84)

Discrepancies between LJY and Dr. Kelleher regarding autoantibody pattern generally concerned the more uncommon patterns placed in the "other" category in the present study (Table 4.5.2). In addition, LJY had a tendency to score lower than Dr. Keller with respect to autoantibody titre (Table 4.5.3). Furthermore, LJY exhibits a high degree of intra-observer consistency with respect to his scoring of ANA patterns (κ= 0.94) (Table 4.5.4).

In summary, moderate to strong correlations were demonstrated with respect to both inter- and intra-personal consistency and reproducibility for scoring of ANA positivity, ANA pattern and ANA titre. The results of this study suggest that the ANA data generated in the present study by LJY are consistent and reproducible.

4.8 Chapter 4 References

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Chapter 5

Autoimmunity in Hepatitis C: Correlates of Antinuclear Antibody (ANA) Positivity and Its Clinical Relevance

5.1 Background: Autoimmunity in hepatitis C virus infection

Antinuclear antibodies (ANA) are a variety of antibodies directed against constituents of cell nuclei including DNA, RNA and various nuclear proteins.[1] Patients with various autoimmune abnormalities including connective tissue or rheumatic diseases such as systemic lupus erythmatosus (SLE) frequently present with high levels of ANA.[1] For the diagnosis of some diseases such as SLE, positive titres of ANA form an integral part of the diagnosis, and a negative ANA rules out the disease.[1, 2] ANA positivity is seen with several other disorders such as rheumatoid arthritis and scleroderma. Positive ANA results have also been reported in healthy, older people.[3] These individuals, unlike those with autoimmune diseases such as SLE, tend to have very low titres.[3]

The prevalence of extrahepatic manifestations including autoimmune conditions in chronic hepatitis C virus (HCV) infection have been reported to be high.[4, 5] Autoimmune manifestations range from the presence of non-organ specific autoantibodies to the Sicca syndrome or cryoglobulinaemia. A myriad of autoantibodies have been described in chronic HCV including rheumatoid factor (RF), smooth muscle antibodies (SMA), and antinuclear antibodies (ANA).[4-6] The prevalence of some antibodies such as antinuclear antibody (ANA) has been reported to be as high as 41% in individuals with chronic HCV infection.[4-7] Interestingly, serological surveys also suggest that there is a gradient in the prevalence of autoimmune manifestations, with a higher prevalence in southern Europe than Northern Europe.[4]

In addition to autoimmune manifestations, chronic HCV infection has also recently also been associated with cognitive dysfunction. In a recent study, Forton and colleagues examined mono-infected HCV and HBV patients with mild histology to rule out any encephalopathy from advanced liver disease, and found individuals with HCV viraemia to have significantly lower cognitive scores than individuals who had cleared HCV viraemia.[8] They also observed a number of these individuals with cognitive impairment to have elevated levels of choline/creatine ratios by magnetic resonance spectroscopy compared to healthy volunteers and individuals with chronic hepatitis B virus infection.[9, 10]

The systemic autoimmune disease systemic lupus erythematosus (SLE) is characterised by diverse and varied manifestations, ranging from skin rash to cerebral infestations that may be as mild as subtle cognitive dysfunction or as severe as life-threatening coma. The mechanisms accounting for such cognitive and psychological impairments is unknown. Some studies suggest that anti-DNA antibodies may be involved.[11] Recently, DiGiorgio and colleagues identified that a subset of anti-DNA antibodies cross-reacts with the N-methyl-D-aspartate (NMDA) receptors, NR2a and NR2b, in neuropsychiatric lupus.[12] The NR2 receptors are the ligand for the neurotransmitter glutamate and are expressed on neurons throughout the forebrain.[13-16] Studies suggest that the NR receptor is involved with learning and memory.[17] In major psychosis, glutamate receptors can display altered expression, while over-stimulation of NR2 can cause neuron death from over excitation through an excessive influx of calcium into cells.[18-21] Stimulation of NR2 by phenylcyclohexylpiperidine, a glutamate receptor antagonist, cases hallucinations and paranoia.[22-24]

In the present study I identified correlates of ANA positivity in a large multi-centre cohort of individuals with chronic hepatitis C. I also examined the effect of ANA positivity on HCV clinical outcome and looked for possible geographic variations in ANA prevalence. Given the relatively high reported prevalence of ANA in individuals with chronic hepatitis C, and reports of cognitive dysfunction in individuals with hepatitis C, we also hypothesised that the presence of ANA is correlated with cognitive dysfunction in HCV.

5.2 Methods

Indirect Immunofluorescence: the principle

ANA pattern and titre was assessed by indirect immunofluorescence on Hep-2 cells using standard techniques.[2, 25, 26] Hep-2 ANA substrate slides were purchased from The Binding Site Limited (Birmingham, UK). A single slide contains 10 wells coated with Hep-2 cell ANA substrate.[27] Hep-2 cells are an epithelial cell line derived from a human carcinoma of the larynx, and have replaced frozen rat liver sections as the main substrate for demonstration of ANA.[2, 25, 27] Hep-2 cells are characterised by several advantages over frozen rat livers. First, Hep-2 cells have extremely large nuclei compared to rat liver cells, allowing for better visualization of nuclear patterns and consequently allow for an increase in assay sensitivity. Cells derived from the same Hep-2 line are characterised by less batch to batch variability when compared to frozen rodent tissue. Hep-2 cells are an actively dividing cell line which also allows for the exposure of greater numbers of antigens not normally expressed in resting cells of rat liver sections.[27]

The principle of this process relies on the indirect visualisation of ANA bound to substrate fixed on slides (ANA substrate slides from The Binding Site Limited, Birmingham, UK), that is then viewed indirectly via immunofluorescence from a secondary antibody that is bound to antinuclear antibodies. Figure 5.2.1 presents a diagram of this process. As illustrated in part A of Figure 5.2.1, patient sera is placed in

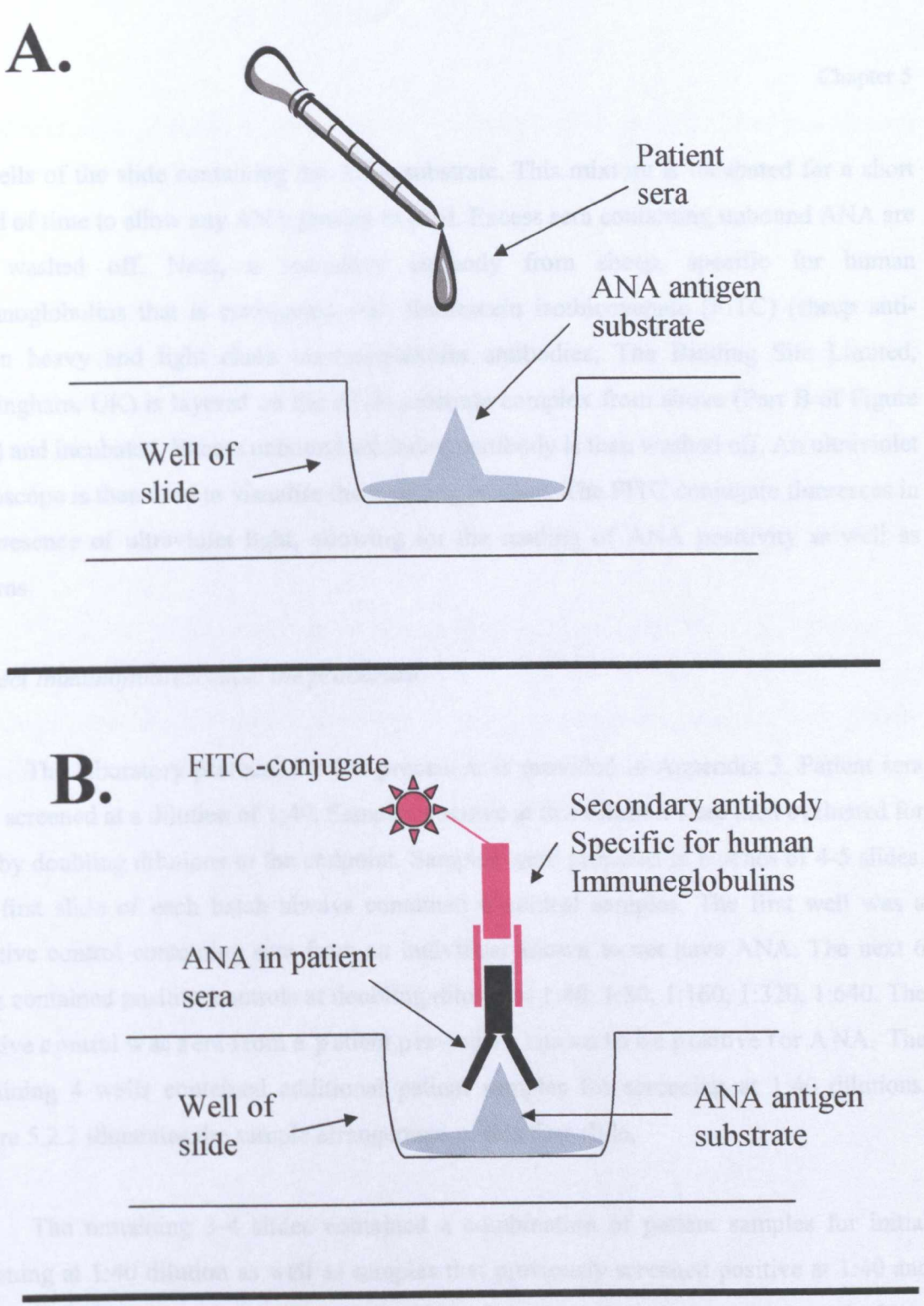


Figure 5.2.1. Schematic diagram of the indirect immunofluorescence (IIF) process. **Part A.** Patient sera is placed in the well of the slide containing the ANA antigen substrate. Unbound ANA are then washed off. **Part B.** A secondary antibody specific for human immunoglobulins that is conjugated with FITC is then laid on top of the ANA-substrate complex from part A. Unbound antibodies are washed off. The slides are then visualised using an ultraviolet light microscope.

the wells of the slide containing the ANA substrate. This mixture is incubated for a short period of time to allow any ANA present to bind. Excess sera containing unbound ANA are then washed off. Next, a secondary antibody from sheep, specific for human immunoglobulins that is conjugated with fluorescein isothiocyanate (FITC) (sheep anti-human heavy and light chain immunoglobulin antibodies; The Binding Site Limited, Birmingham, UK) is layered on the ANA-substrate complex from above (Part B of Figure 5.2.1) and incubated. Excess unbound secondary antibody is then washed off. An ultraviolet microscope is then used to visualise the resulting product. The FITC conjugate fluoresces in the presence of ultraviolet light, allowing for the reading of ANA positivity as well as patterns.

Indirect Immunofluorescence: the procedure

The laboratory protocol for this procedure is provided in Appendix 3. Patient sera were screened at a dilution of 1:40. Samples positive at this dilution were then evaluated for titre by doubling dilutions to the endpoint. Samples were prepared in batches of 4-5 slides. The first slide of each batch always contained 6 control samples. The first well was a negative control containing sera from an individual known to not have ANA. The next 6 wells contained positive controls at doubling dilutions: 1:40, 1:80, 1:160, 1:320, 1:640. The positive control was sera from a patient previously known to be positive for ANA. The remaining 4 wells contained additional patient samples for screening at 1:40 dilutions. Figure 5.2.2 illustrates the sample arrangement of this first slide.

The remaining 3-4 slides contained a combination of patient samples for initial screening at 1:40 dilution as well as samples that previously screened positive at 1:40 and subsequently needed tittering. To assess ANA titre, these samples were run at doubling dilutions to the endpoint. Figure 5.2.3 gives an example of a possible arrangement for these slides.

To facilitate categorical analysis, positive samples were grouped into 3 possible categories for ANA pattern: speckled, homogenous or nucleolar. Figure 5.2.4 presents

examples of these patterns. A fourth category of "other" was created for other less frequent patterns that do not fit into the above categories. Figure 5.2.5 presents examples of other possible patterns that would be included in this category.

645 patients from three European liver centres were screened for ANA using this method. Participating centres included Lund, Sweden (225 patients); London, United Kingdom (207 patients); and Padova, Italy (213 patients). Patients from Lund and Padova were individuals from the HENCORE study and were subject to the enrollment criteria of that study (discussed in Chapter 2). For the patients from London, the 207 patients were selected at random from the entire pool of individuals in the St. Mary's Hepatitis C Cohort.

Caucasians in this study are defined as Northern European, Southern European, or Eastern European. Because of the frequencies of all of the other categories of non-Caucasian races are too small to provide meaningful categorical analyses individually, analyses was conducted in the cohort as a whole (all races) and then with Caucasians only.

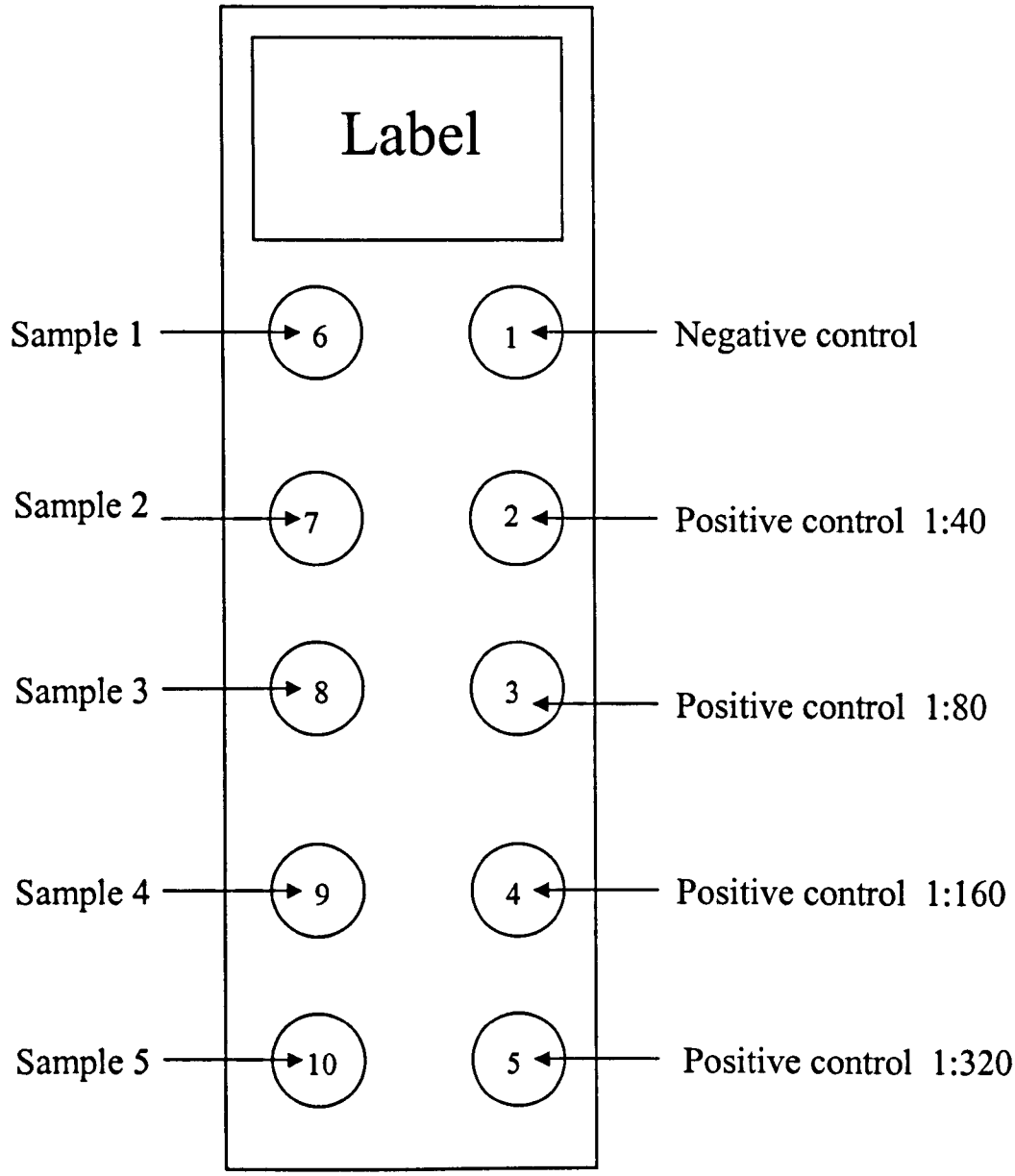


Figure 5.2.2. Example of control slide set up. The first slide in every batch of ANA slides is set up in this manner. The slide contains one negative control as well as a series of positive controls at different dilutions. The remaining wells on the slide are used to screen samples.

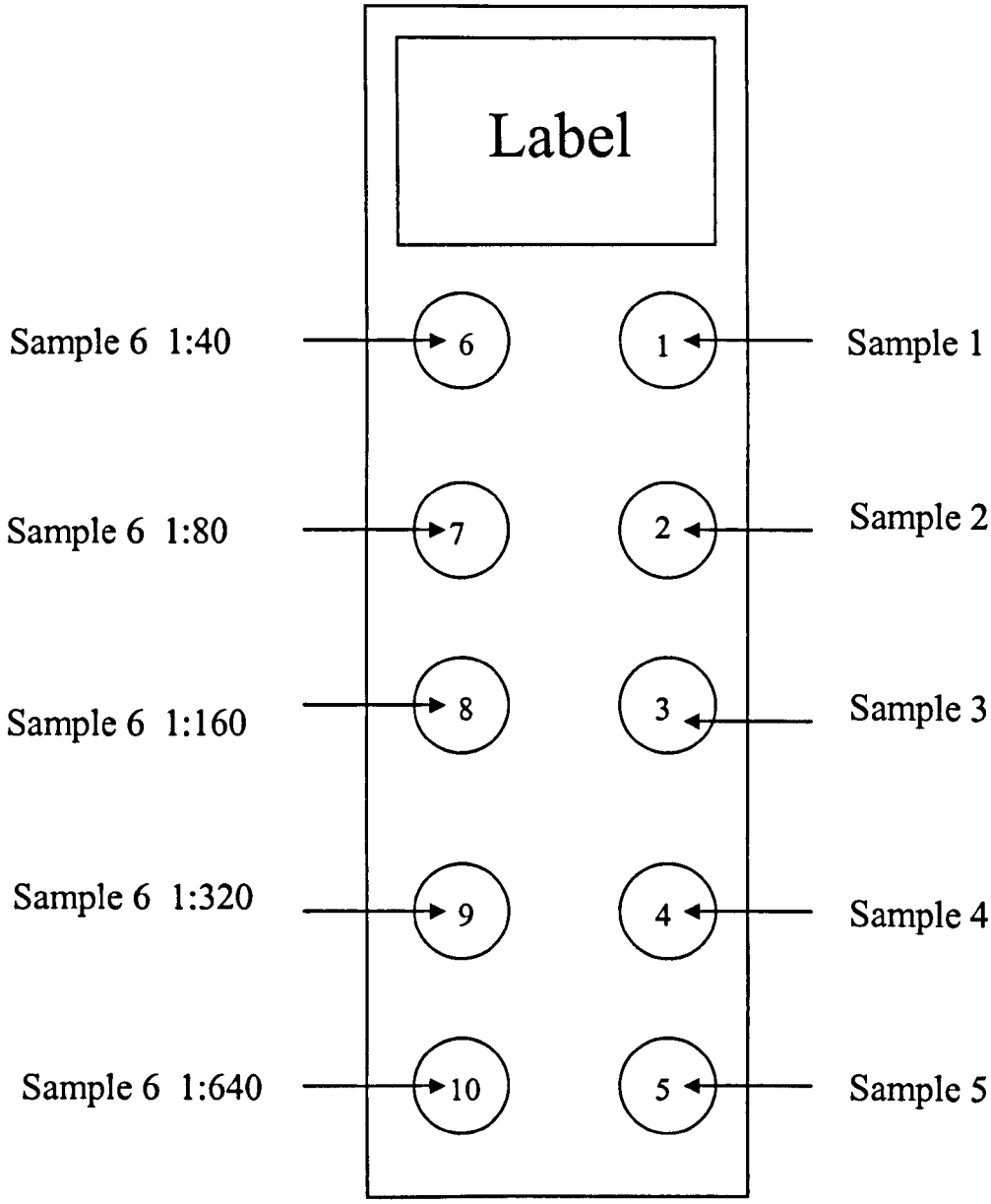


Figure 5.2.3. Example of sample slide set up. Sample slides contain patient samples for preliminary ANA screening as well as dilutions of previously positive ones for auto-antibody titre determination. Here, samples 1-5 and 7 are samples for screening at 1:40 dilution, while sample 6 (wells 6-9) is a sample that screened positive on a previous run and is currently being assayed for titre.

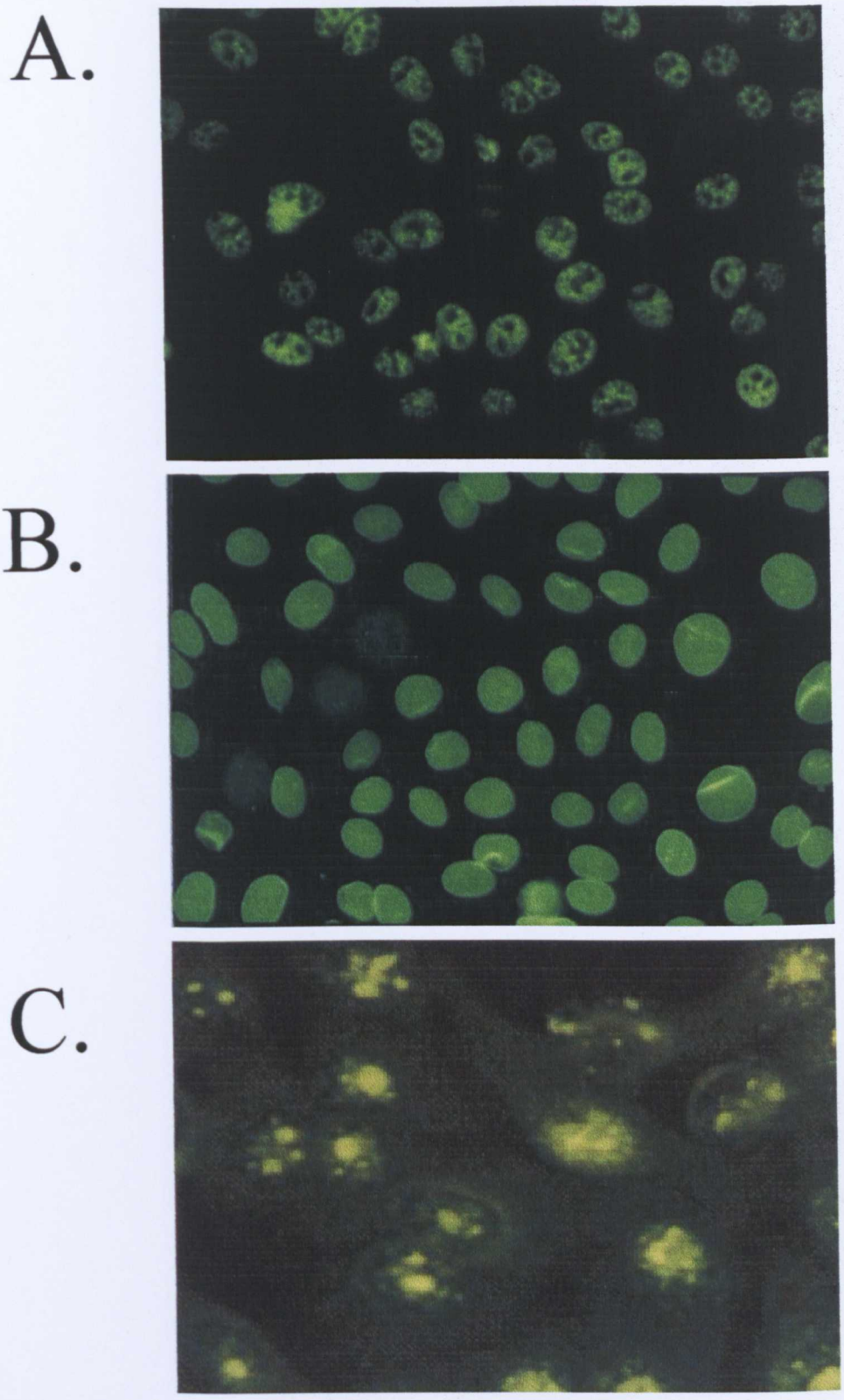


Figure 5.2.4. The major ANA patterns considered in the present study. Patterns are detected by indirect immunofluorescence (IIF) on Hep-G2 cells. The patterns shown are: **A.** Speckled. **B.** Homogenous **C.** Nucleolar.

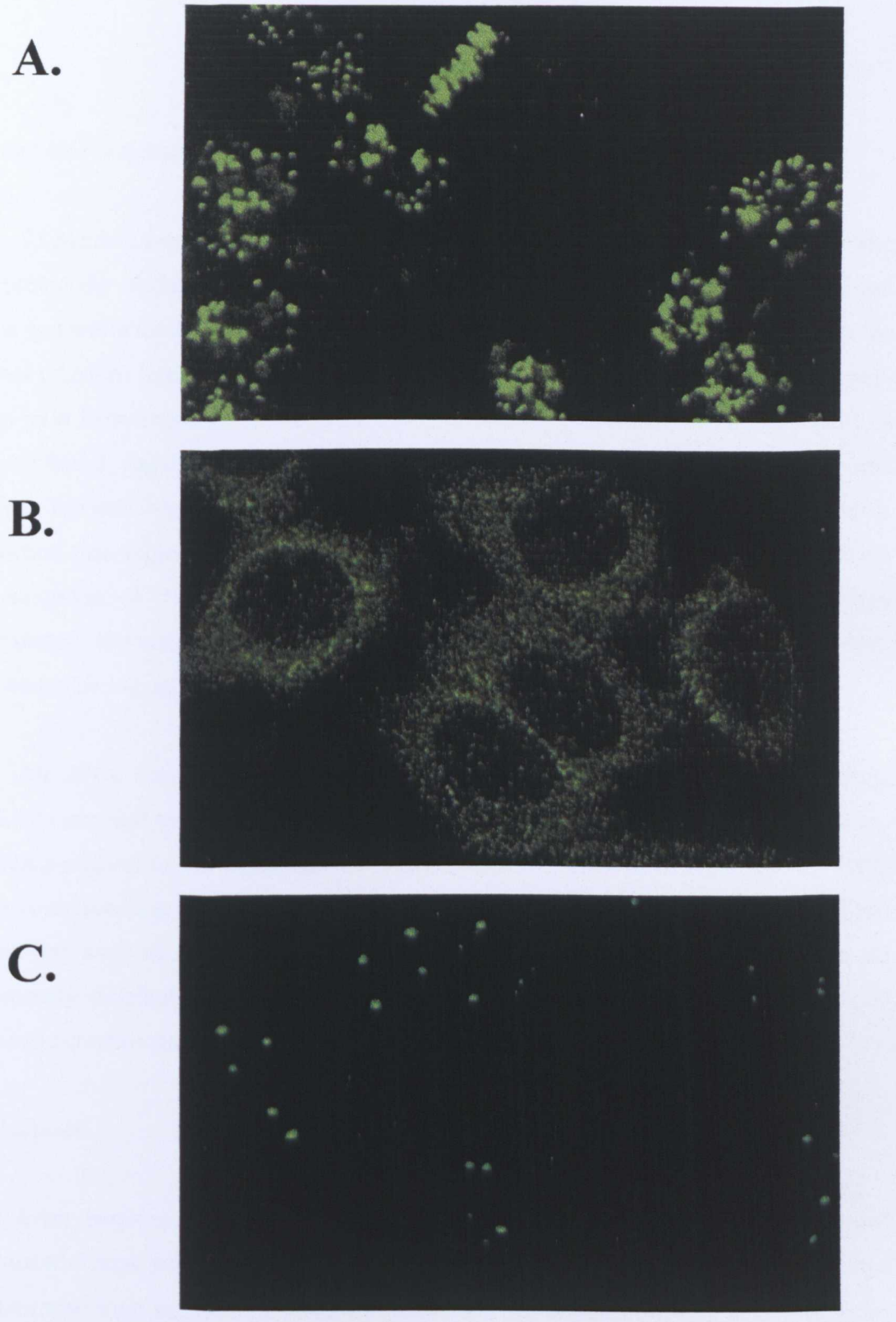


Figure 5.2.5. Example of other possible less frequent ANA patterns. **A.** Centromere pattern. **B.** Cytoplasmic speckling. **C.** Nuclear dots.

(Photograph: The Binding Site Limited, Birmingham, UK)

ANA and HCV-related cognitive impairment

23 patients were obtained at random from a group with chronic HCV infection who were previously evaluated by Forton *et al.* for levels of choline and creatine in the basal ganglia and white matter by magnetic resonance spectroscopy.[9] They were screened for the presence of ANA. Individuals in this group were a collection of HCV patients with biopsy-proven mild liver histology due to HCV mono-infection.[8] Individuals were subjected to a computer-based cognitive assessment test and also completed depression, fatigue and quality of life tests.[8] Individuals with HCV viraemia exhibited significantly more cognitive dysfunction than a group of individuals who had cleared HCV viraemia.[8] Further analysis of a subgroup of 17 of the 23 individuals with magnetic resonance spectroscopy demonstrated elevated choline/creatine ratios in the basal ganglia and white matter, suggesting a biological cause to this abnormality.[8-10]

All ANA assays were conducted without knowledge of the patient's cognitive scores. We intended to analyse this study by comparing the mean choline/creatine scores of the ANA positives to the mean choline/creatine scores of the ANA negatives, the HCV positive individuals as a whole, and to a group of healthy volunteers using Student's t-test and one-way analysis of variance (ANOVA) for normally distributed data. If the data are not normally distributed, log-transformed data were to be used in the calculations. All choline and creatine data were kindly provided courtesy of Dr. Daniel M. Forton.

Liver biopsies

Liver biopsies stained with haematoxilin and eosin for individuals from United Kingdom who were positive for ANA were matched 1 to 1 to an individual from the United Kingdom who were negative for ANA by gender and age at treatment (± 2 years). Biopsies were evaluated by a single pathologist (Dr. Robert D. Goldin, consultant histopathologist, St. Mary's Hospital, London, UK), who was blinded to the ANA status of the individual biopsies and all other clinical information. In addition to scoring of inflammation (grade) and fibrosis (stage) using the modified Histological Activity Index (Ishak) semiquantitative scoring system, the biopsies were scored for the amount of fat (less than 10% or more),

portal lymphoid aggregates (presence or absence) , bile duct damage (presence or absence), esosinophils (conspicuous or not) and plasma cells (conspicuous or not) Analyses were as matched pairs using conditional logistic regression.

Data collection, management and statistical analyses

As already described in Chapter 2, all ANA data, along with clinical information, were kept in a database I created using the EpiData® programme.(Copenhagen, Denmark) Relevant demographic and clinical data were directly downloaded from the HENCORE database, while results of ANA tests were recorded on data entry sheets (Appendix 4), double-entered and cross-validated. The dataset was converted to SAS® (Cary, North Carolina, UK) by the StatTransfer ® programme (Seattle, Washington, USA) for statistical analyses. Appendix 5 provides the data dictionary for this database.

Standard statistical techniques including Chi-square tests for contingency tables for univariable data and logistic regression for multivariable analyses were used to assess correlates of ANA positivity. Both maximum likelihood and Fisher's exact p-values and confidence limits were calculated as appropriate. To facilitate categorical analyses, continuous variables including duration of HCV infection, current age, and age at HCV acquisition were dichotomised at the median (20, 48 and 27, respectively). HCV viral genotypes were dichotomised into type 1 and non-1. Amount of alcohol consumption was divided into those who were "high drinkers" (> 40 units per week) and "low drinkers" ($\leq$ 40 units per week). To increase the likelihood of detecting an effect with fibrosis score and necro-inflammatory score with ANA positivity, the extremes were analysed. Individuals with a fibrosis score $\geq$5 were compared to those with scores $\leq$2. For analyses of necro-inflammatory score those with a score of $\geq$7 were compared to those $\leq$2. Because 98% of the patients with a reportable HCV risk factor had a history of either injection drug use or the receipt of blood or blood products, we restricted our analyses of source of HCV to these two factors. For analyses of the effect of geographic location, centres were compared in a hierarchical order by geographic latitude. Factors with a p-value $\leq$ 0.20 in univariable analysis were entered into a multivariable logistic regression model.[28] For analyses of geographic distribution of ANA, data were analysed by both logistic regression and by multi-level modeling using the SAS® system.[29, 30]

For analyses of the effects of ANA positivity on progression of fibrosis, ordered logistic regression was used with the stages of fibrosis as the outcome variable. ANA positivity and estimated duration of HCV infection were included as the explanatory variables. The patient was assumed to have a fibrosis score of 0 at the time of infection, and a rate calculated by taking the difference between the patient's current biopsy score and the assumed zero fibrosis at the time of infection and adjusting for the patient's estimated duration of infection in a multiple logistic regression model.

5.3 Results

ANA cohort demographics

Table 5.3.1 present the distribution of demographic characteristics of the ANA study cohort. Data are presented for the cohort as a whole as well as by individual centre. Patients from Italy tended to be older, while the estimated durations of HCV infection did not vary significantly between each study centre. The similar durations of HCV infection and older patient age in Italy suggest that HCV patients in Italy were infected at an older age. This is confirmed in the distributions of age at infection, where individuals in Italy tended to be infected in their mid-thirties, while individuals in the other centres were infected in their mid to later twenties. Males predominated in the cohort as a whole and in each study centre. The male to female ratio was similar between the three centres. Viral genotype-1 predominated in all of the study centres. Most patients had mild to moderate fibrosis (≤ fibrosis score of 3) in all of the centres.

Table 5.3.1. Demographics of the ANA cohort as a whole and by individual centre.

FACTOR	CENTRES COMBINED (N=645)	LUND (N=225)	LONDON (N=207)	PADOVA (N=213)
Patient age (mean ± SD)	50.9 ± 12.6	48.0 ± 10.2	48.4 ± 11.1	56.1 ± 14.5
Duration of HCV infection	21.1 ± 9.3	21.0 ± 7.0	23.1 ± 9.8	19.9 ± 9.9
Age at infection (mean ± SD)	30.6 ± 14.2	27.3 ± 12.2	25.0 ± 11.3	36.1 ± 15.0
Males : Females	58.7% : 41.3%	58.2% : 41.8%	61.1% : 38.9%	56.8% : 43.2%
Viral genotype (genotype 1: non-1)*	55.2% : 44.8%	53.9% : 46.1%	64.3% : 35.7%	52.3% : 47.7%
Fibrosis score distribution				
0	5.0%	0%	11.8%	0%
1	17.8%	16.5%	22.5%	12.5%
2	26.7%	27.8%	21.9%	32.5%
3	18.1%	19.1%	17.8%	17.5%
4	12.9%	18.3%	3.6%	20.8%
5	8.4%	9.6%	8.3%	7.5%
6	11.1%	8.7%	14.1%	9.2%
Race				
Northern European	51.0%	90.2%	60.7%	0.9%
Southern European	37.2%	1.8%	11.0%	98.2%
Indian/Indian Subcontinent	1.9%	0.5%	5.8%	0%
Black African	1.6%	0.9%	4.2%	0%
North African	3.4%	0.4%	10.5%	0%
Eastern European	2.1%	4.0%	2.1%	0%
Afro-Caribbean	1.0%	0%	3.1%	0%
Oriental	0.6%	0%	2.1%	0%
South American	0.6%	0.4%	0.5%	0.9%
Middle Eastern	0.6%	1.8%	0%	0%

*These samples were collected during the era of interferon monotherapy, prior to the treatment of HCV according to viral genotype. As a result not every individual was genotypes for virus. These frequencies are based on retrospective genotyping of collected patient serum samples.

ANA positivity: prevalence and geographic differences

Among the 645 patients studied, 50 (7.75%) were positive for ANA at 1:40 or greater, with 4.4% positive in Lund, 8.7% in London, and 10.33% in Padova. Table 5.3.2 summarises the observed prevalence data from each of the individual centres as well as the cohort as a whole. Amongst the cohort as a whole, there was an increase in ANA prevalence in a southerly direction (OR=0.66; 95%C.I.: 0.46-0.94; p=0.023). Similar observations were observed by multi-level modelling, comparing Northern and Southern Europeans (F=2.96; p=0.0860).

Table 5.3.2. Summary of ANA positivity by study centre.

LOCATION	NO. POSITIVES	PREVALENCE
Lund, Sweden (N=225)	10	4.4%
London, UK (N=207)	18	8.7%
Padova, Italy (N=213)	22	10.3%
Centres combined (N=645)	50	7.8%

Figure 5.3.1 summarises the distributions of ANA pattern as well as titre among the total cohort as well as the individual centres. 84% of positive individuals had low titres of ANA (1:80 or lower) in the entire cohort and 100% had titre of 1:160 or lower (Figure 5.3.1). Speckled pattern was the most commonly observed ANA pattern. Of the 50 ANA positive samples in the combined cohort, 34 (68%) were speckled, 4 (8%) homogenous, and 3 (6%) were nucleolar. Nine (18%) had other patterns, which included 3 with cytoplasmic speckling and 6 with nuclear dots. No significant differences with respect to the distribution of ANA pattern were observed between the distribution of these patterns among the three centres (Figure 5.3.1).

Correlates of ANA positivity

Table 5.3.3 Part A presents univariable correlates of ANA positivity at ≥ 1:40. Duration of HCV infection, age at HCV infection, current age, self-reported alcohol consumption, fibrosis stage, source of infection, and necro-inflammatory score were not correlated with ANA positivity. Female gender (OR=3.2; 95%C.I.: 1.72 – 5.95; p=0.0001) and geographic location (OR=0.66; 95%C.I.: 0.46 – 0.94; p=0.023) were associated with ANA positivity. The association with female gender persisted when the analyses were restricted to Caucasians only (OR=2.7; 95%C.I.: 1.3 – 5.57; p=0.0078). In a multivariable model of the effect of gender on ANA positivity, controlling for geographic location, the current age of the patient and fibrosis, only gender retained its significance (Table 5.3.3 Part B.). The association of female gender with ANA positivity (OR=3.3; 95%C.I.: 1.46 – 7.37; p=0.0025) was observed when positivity was defined as having a titre ≥1:80 (Table 5.3.4). Geographic location was no longer statistically significant (OR=0.76; 95%C.I.: 0.48 – 1.21; p=0.2510) at this titre. Gender retained its significance in a multivariable model at a titre of ≥ 1:80 (OR=3.4; 95%C.I.: 1.36 – 8.54; p=0.0087).

Similar results for the effect of geographic location were observed when analysed by multilevel modelling at a titre of ≥ 1:40 (F=2.9; p=0.0585) and ≥ 1:80 (F=2.6; p=0.0722). The small numbers of ANA-positive individuals precluded achievement of statistical significance when this statistical method was applied.

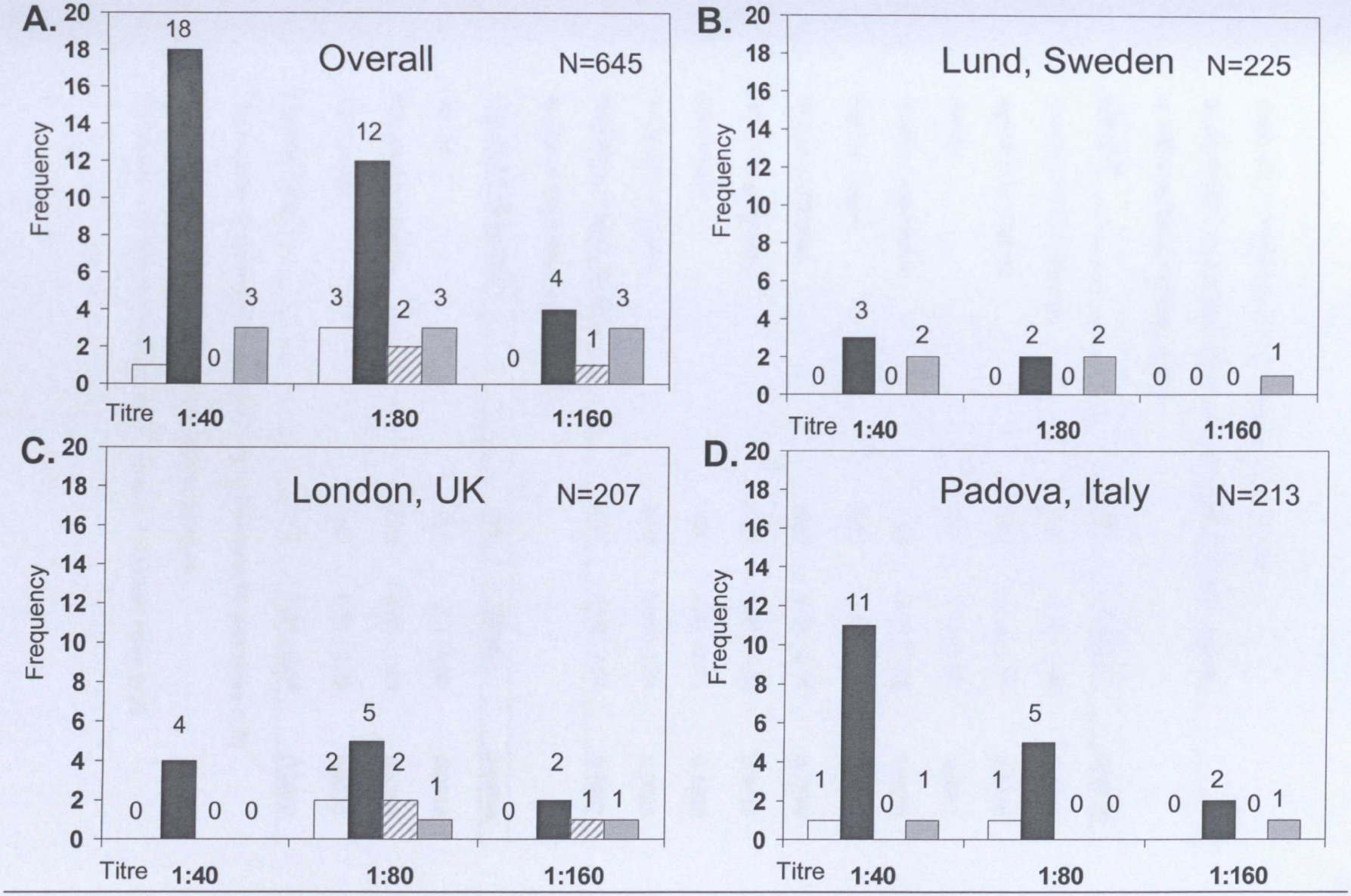


Figure 5.3.1. Observed distributions of ANA pattern and titre. ANA titre and pattern distributions overall and among each of the study centres. The frequencies of each of the patterns is indicated above each of the bars. □= homogenous pattern, ■ = speckled, ▨ = nucleolar, and ▩ = other.

Table 5.3.3. Correlates of ANA positivity at ≥ 1:40 titre.

A. Univariable correlates of ANA positivity. **B.** Multivariable analysis.

A. UNIVARIABLE CORRELATES

FACTOR	OR	95%C.I.	p-value
Duration of HCV Infection	0.72	0.38 - 1.44	0.3219
Age at HCV Infection	0.92	0.29 -1.70	0.7796
Gender	3.2	1.72 - 5.95	0.0001
Alcohol consumption	0.63	0.19 - 2.08	0.4416
Fibrosis stage[a]	0.59	0.29 - 1.17	0.1277
Source of infection[b]	0.88	0.38 - 2.03	0.7569
HCV viral genotype	0.84	0.45 – 1.55	0.5742
Current age	1.51	0.84 - 2.71	0.1632
Geographic location	0.66	0.46 - 0.94	0.0225
Necroinflammatory score[c]	1.17	0.46 - 2.94	0.7457

B. MULTIVARIABLE ANALYSES

FACTORS IN MODEL	OR	95%C.I.	p-value
Gender	3.0	1.54 - 5.93	0.0013
Geographic location	0.74	0.49 - 1.10	0.1299
Current Age	1.42	0.72 - 2.79	0.3150
Fibrosis stage[a]	0.77	0.28 - 2.09	0.6070

[a]Comparison of severe fibrosis score (≥ 5) versus lower fibrosis score (≤ 2).

[b]Injection drug use or exposure to blood/blood products

[c]Comparison of high necroinflammatory score (≥ 7) to lower score (≤ 2)

Table 5.3.4. Correlates of ANA positivity at ≥ 1:80 titre.

A. Univariable correlates of ANA positivity. **B.** Multivariable analysis.

A. UNIVARIABLE CORRELATES

FACTOR	OR	95%C.I.	p-value
Duration of HCV Infection	0.62	0.26 - 1.48	0.2791
Age at HCV Infection	0.53	0.21 - 1.33	0.1701
Gender	3.3	1.46 - 7.37	0.0025
Alcohol consumption	0.36	0.05 - 2.73	0.3055
Fibrosis stage[a]	0.81	0.24 - 2.81	0.7458
Source of infection[b]	1.72	0.54 - 5.51	0.3564
HCV viral genotype	0.99	0.45 – 2.18	0.9783
Current age	1.46	0.68 - 3.14	0.3304
Geographic location	1.0	0.44 - 2.45	0.9963
Necroinflammatory score[c]	1.25	0.35 - 4.50	0.734

B. MULTIVARIABLE ANALYSES

FACTORS IN MODEL	OR	95%C.I.	p-value
Gender	3.4	1.36 - 8.54	0.0087
Geographic location	0.89	0.53 – 1.50	0.6628
Current age	1.52	0.62 – 3.76	0.3643
Fibrosis stage[a]	0.88	0.25 - 3.18	0.8489

[a]Comparison of severe fibrosis score (≥ 5) versus lower fibrosis score (≤ 2).

[b]Injection drug use or exposure to blood/blood products

[c]Comparison of high necroinflammatory score (≥ 7) to lower score (≤ 2)

Impact of ANA positivity on HCV clinical outcome

ANA positivity had no significant effect on HCV clinical outcome. There was a trend with ANA positivity and faster progression of HCV fibrosis (OR=1.8; 95%C.I.: 0.81 – 4.2; p=0.1452), but this did not achieve statistical significance. Alterations in response rates were not observed when stratified by ANA positivity. Among all of those who were treated, 26.7% achieved sustained response; among ANA negative individuals who were treated, 26.3% achieved sustained response, while among ANA positive individuals 33.3% achieved sustained response; 95%C.I.: 0.51 – 3.9; p=0.5126). ANA titre did not play a significant difference on these results, as similar observations were obtained for the effects of ANA positivity on fibrosis progression and response to therapy if ANA positivity is defined at ≥ 1:80. Among ANA negative individuals who were treated, 26.3% achieved sustained response, while among ANA positive individuals 20% achieved sustained response; 95%C.I.: 0.14 – 3.4; p=0.6595) when positivity was defined at this titre.

ANA and cognitive dysfunction

Only 3 of the 23 individuals studied in this group were positive for ANA. The extremely low frequencies of ANA positivity prohibited any further meaningful statistical analyses. I, nevertheless, wanted to see where these three individuals stand on the spectrum of cognitive scores. If they all had scores indicative of severe cognitive impairment, then it is possible that further studies might be warranted. Analyses of these three patients also showed that there were no consistent trends with respect to choline/creatine ratios in both the basal ganglia and white matter when compared to the mean score of HCV infected individuals as well as a healthy control population (Table 5.3.4). The scores of patient two are relatively similar to the HCV population as a whole and the healthy controls. The other two patients exhibit only slightly higher scores.

Table 5.3.5. Summary of ANA positive patients' choline/creatine scores as assessed by magnetic resonance spectroscopy (MRS).*

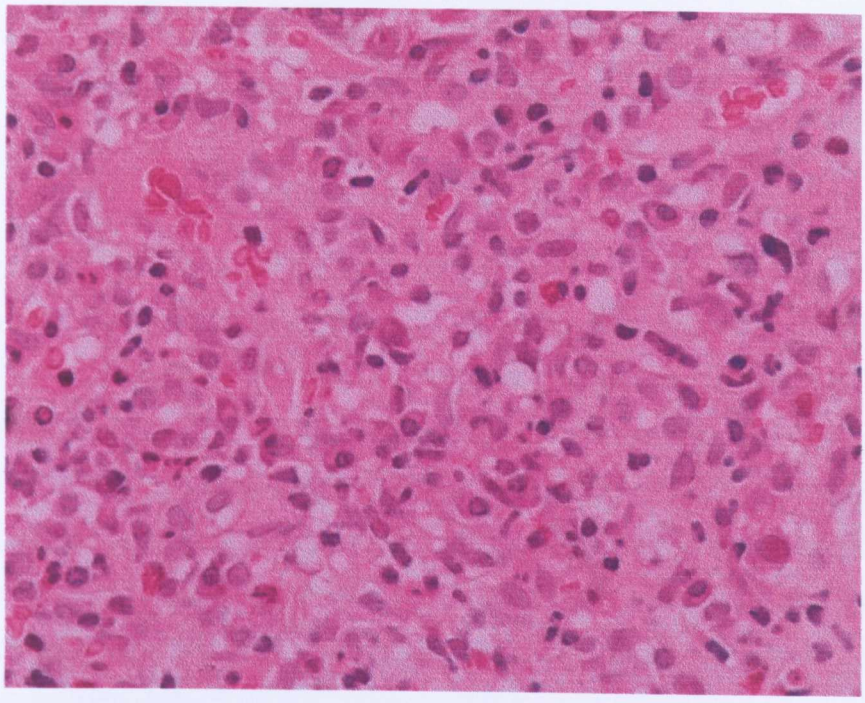
Patients	Basal ganglia Choline/creatine ratio	White matter Choline/creatine ratio
Patient 1	1.16	1.44
Patient 2	1.08	1.14
Patient 3	1.14	1.22
All HCV Patients (N=36) Mean ± SD	1.16 ± 0.19	1.31 ± 0.22
Healthy controls (N=29) Mean ± SD	1.06 ± 0.12	1.16 ± 0.12

*All MRS data were provided courtesy of Dr. Daniel M. Forton.

Liver biopsies

Among the 18 individuals from the United Kingdom who were positive for ANA, 17 had biopsies available for examination. Among the 17 pairs of biopsies examined (N=34 samples total), there were no differences observed with respect to the distribution of fat, eosinophils, and bile duct damage between ANA positive liver biopsies and ANA negative biopsies ($p > 0.320$ for all). In contrast, plasma cells were more frequent in those who were ANA positives (Hazard Ratio=9.0; p=0.037) (Figure 5.3.2). There were fewer lymphoid aggregates among ANA positive samples, although the difference was not statistically significant (Hazard Ratio=0.291 p=0.118) (Figure 5.3.2)

A.



B.

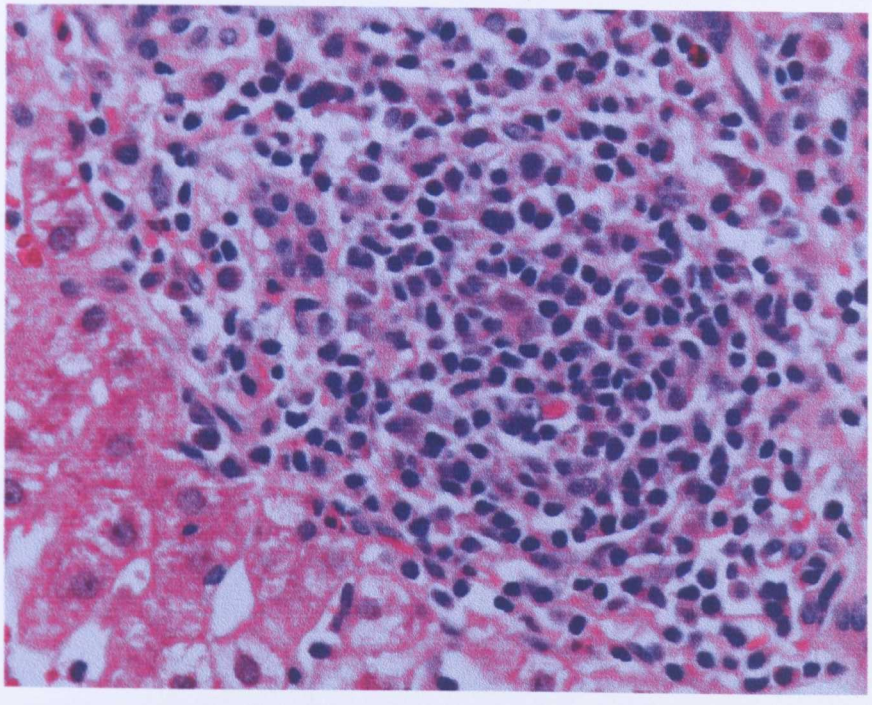


Figure 5.3.2. Haematoxylin and eosin stained liver biopsies (Original magnification x 400). **Part A.** presents a biopsy from an ANA positive individual. **Part B.** presents the biopsy from an ANA negative individual. Conspicuous plasma cells are present in the ANA positive individual. In the ANA negative individual, there is a lymphoid aggregate but only scanty of plasma cells.

5.4 Conclusions

This study observed a prevalence of low levels of ANA among 7.8% of individuals in three centres across Europe. This study confirms previous studies showing an increased prevalence of ANA in individuals with chronic hepatitis C.[6, 7, 31] We observed an 8.7% ANA prevalence in London. This is over two-fold higher than the normal background prevalence of ANA in west London, which is approximately 2%-3% (Dr. Peter Charles, Charing Cross Hospital, London, UK: personal communication). An ANA prevalence of 10.3% was also observed in chronic HCV patients in Italy. This is comparable to a previous large, random, population-based study of ANA prevalence in Italy, which reported prevalence to be about 10%-14%.[31, 32]

The present study observed ANA prevalences that are smaller than some figures reported by other groups. Several factors may account for this difference. First, there may be a different mixture in the cases seen by the other study centres, where individuals with autoimmune co-morbidities or a predisposition for autoimmunity could be preferentially selected in some way. Second, there may be differences in viral strains causing these differences, although viral genotype is not associated with ANA positivity either in this study or in others.[31, 32] Third, there may be technical differences. Differences may exist in the techniques employed to assay ANA. Our study employed the use of Hep-2 cells, while some older studies employed the use of rat liver ANA substrate. Fourth, there may be true differences in the populations where host-related factors such as genetics may play a pathogenic role.

This study observed a significant geographic gradient in ANA prevalence, where the prevalence of ANA increases in a southerly direction. This observation supports other studies of autoimmunity in hepatitis C where the prevalence of autoimmune manifestations appears to be greater in Southern Europe compared to Northern Europe.[4] For example, HCV-related cryoglobulinaemia has been reported extensively in southern Europe, but not in northern Europe.[4]

The present study observed that women are more likely than men to develop ANA. This is consistent with the association of female gender with autoimmunity and a previous study of patients with HCV that showed women tended to develop autoantibodies.[33, 34] While the mechanism behind the association of gender with autoimmunity has not yet been elucidated, it is believed that hormones such as oestrogen may play an important role.[33] Furthermore, it is possible that certain individuals with a genetic predisposition are more likely to develop autoantibodies.[35] This study also supports previous observations that HCV genotype and route of HCV acquisition are not correlated with autoantibody formation.[31, 32]

The observation that ANA positive patients have more plasma cells in their liver biopsies raises the possibility that increased plasma cells may be a marker for B cell polyclonal activity with a secondary clinical manifestation of increased serum immunoglobulins with or without autoantibody production. A study by Watanabe et al. examined liver biopsies from patients with primary biliary cirrhosis who were anti-mitrochondrial antibody (AMA) positive and patients who were AMA-negative and ANA-positive and found patients with ANA to have significantly greater numbers of plasma cells.[36] Future studies may examine the pathological bases for this correlation more extensively.

Importantly, our study suggests that positivity for ANA at low titres does not alter response to interferon therapy and positivity for ANA at low levels should not be a contraindication for treatment. There was an association of ANA positivity with an almost two-fold higher chance of having faster fibrosis, although this did not reach statistical significance. Our observation with respect to the response to interferon therapy confirms observations from a previous study showing that the presence of serum autoantibodies does not alter response rates to interferon therapy in HCV.[34] Studies have suggested that levels of alanine amino transferase (ALT) and gamma-glutamyl transferase (GGT) are associated with the formation on non-organ-specific autoantibodies in HCV.[31, 34] These factors were not assessed in the present study. It would be interesting for future studies to explore in greater detail whether these and other biochemical markers of liver function such as albumin levels or prothrombin time are correlated with ANA positivity. Future studies are also needed to address the possible role of host and environmental factors in autoantibody formation in HCV.

Studies of healthy individuals suggest that a number have low levels of ANA.[3, 26] Tan and colleagues reported the prevalence of ANA in healthy individuals to be 31.7% at a dilution of 1:40, 13.3% at 1:80, 5.0% at 1:160, and 3.3% at 1:320.[3, 26] They also did not observe any significant differences in the occurrence of ANA positivity across different age groups (20-60 years).[3, 26] The extraordinarily high prevalence reported in this study is likely to reflect the increased sensitivity of the Hep-2 cell substrate over the rat liver substrate, and illustrates how assay methodology and reader subjectivity are present in this widely-employed standard clinical technique.

The mechanisms for autoantibody positivity in individuals with are not known. A couple of hypotheses have received much attention in recent years. One hypothesis is that viral infection may induce the release of cytokines that activate auto-reactive T-cells and induce autoimmune pathogenesis.[37] Another hypothesis is that molecular mimicry is involved.[38] Infection with HCV or hepatitis delta can induce antibodies to the same antigens recognised in autoimmune hepatitis.[37, 39] Antibodies to liver, kidney microsome antigens (LKM) found in some individuals with chronic HCV have been found to react to linear and conformational epitopes of the cytochrome mono-oxygenase, P450 2D6 (CYP 2D6).[39-42] Such studies of the epitopes of ANA found in individuals with chronic HCV infection have not been conducted. Future studies into the aetiology of ANA formation in HCV may focus on characterisation of ANA epitopes.

It would be interesting for future studies to explore whether the mechanisms affecting ANA positivity in patients with HCV infection are the same as those in other autoimmune conditions. It is possible that the presence of HCV in some individuals induces low titres of natural IgG antibodies directed to nuclear epitopes, and future work on epitope mapping may increase the understanding of ANA formation in chronic HCV. Future studies may also address possible host genetic as well as environmental pathogenic mechanisms of ANA formation in HCV. This will, however, necessitate large study populations to provide enough cases to enable studies with sufficient power to detect meaningful associations.

5.5 Chapter 5 References

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Chapter 6

Host genetics and Hepatitis C Outcome

6.1 Introduction

A number of studies have suggested an important role for host genetic diversity in influencing the outcome of hepatitis C virus (HCV) infection (see Chapter 2 for systematic review). In this study I examined the possible correlations between genetic variants in several genes and the outcome of HCV infection. I used the candidate gene approach to see whether specific genes in key pathways of the host immune response to the hepatitis C virus affect the susceptibility to persistent HCV infection or the response to interferon therapy.

Interferon-Stimulated Genes

The interferon system is a crucial component of the immune response to infectious agents. Type I interferons induce different proteins with anti-viral action including 2'-5'oligo-adenylate synthetase (OAS), double stranded RNA-dependent protein kinase (PKR) and myxovirus resistance 1 (MxA).[1] Upon binding to double stranded RNA (dsRNA), OAS catalyses the formation of 2'-5'-linked oligoadenylate and activates RNAseL, which breaks down viral and cellular RNA.[2, 3] PKR is also activated by dsRNA which leads to the phosphorylation of its substrate, eIF2, which inhibits the guanosine nucleotide exchange factor, eIF2B, and halts viral replication.[4, 5] PKR may function to shut-down protein synthesis following infection of a cell and limit the proliferation of uninfected cells.[6] Interactions between the Hepatitis C virus (HCV) and PKR are believed to be an important mechanism behind the resistance of HCV to interferon therapies.[7] MxA protein has selective activity against several viruses.[8-10] However, the precise mechanism of action has not been elucidated. Studies of *in vivo* MxA levels in responders to interferon treatment of HCV have shown greater levels in virological responders than non-responders.[11] Similarly, MxA mRNA levels have been

reported to significantly increase after initiation of interferon therapy only in those who respond.[12] Given the importance of the PKR, OAS and MxA in the immune response, polymorphisms in the genes encoding these three proteins were selected as candidate genes in this project.

Inducible Nitric Oxide Synthase (NOS2A) Haplotypes and the Outcome of Hepatitis C

Nitric oxide (NO) is an important signaling molecule involved with combating microbial infections. It is formed by the oxidative deamination of the amino acid L-arginine to L-citrilline by nitric oxide synthases (NOS).[13] NO possesses potent anti-microbial effects, including the ability to inhibit the growth of many infectious organisms *in vitro*.[14] Three isoforms of NOS enzymes are currently known to exist: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase ((iNOS) also referred to as NOS2). Of these three isoforms, iNOS is absent in resting cells, but is capable of being rapidly expressed in response to pro-inflammatory stimulti such as cytokines, including interferon. When present, iNOS is capable of synthesising 100-1000 times more NO than the other forms over a prolonged period of time.[13] These properties make iNOS an important part of the host response to infectious agents. Experiments employing iNOS inhibitors and iNOS knockouts have demonstrated, *in vivo*, the important role of iNOS in the host defense to infectious agents.[15-18]

iNOS is encoded by the *NOS2A* gene, and several polymorphisms have been described in the *NOS2A* gene at positions -277, -1026, and -1659, numbered relative to the transcription initiation site. Strong linkage disequilibrium exists between these loci and several conserved, well-defined haplotypes have been observed in Caucasian populations.[19] With the importance of iNOS in the host defense against infectious agents, I selected the *NOS2A* gene as another candidate gene. Given the strong linkage disequilibrum between variants in the *NOS2A* gene and the well-defined and conserved haplotypes that these variants form,[19] I will examine in this study, the correlations of *NOS2A* gene haplotypes with self-limiting versus persistent HCV infection as well as the response to interferon therapy. The use of haplotypes have also proven useful for the identification of susceptibility regions in previous studies as well.[20]

T-Lymphocyte Response and Response to Therapy

As summarised in chapter 1, interferon α- (the standard short-acting forms and the increasingly used newer long-acting pegylated interferons) alone or in combination with ribavirin (IFN+R) are currently the standard regimen for the treatment of chronic hepatitis C virus (HCV) infection,[21-23] but is frequently accompanied by significant side effects that may often be debilitating.[24] Moreover, only 40-80% of suitable candidates treated with this regimen achieve sustained response (SRs)—defined as having achieved an undetectable HCV-RNA level at 6 months after discontinuation of therapy.[25, 26] The shortcomings of this regimen have prompted a search for factors that could predict those most likely to benefit from it.

Clearance of HCV viraemia naturally and in the context of IFN+R therapy has been correlated with a strong immune response. Both CD4+ T-helper and CD8+ cytotoxic T-lymphocyte (CTL) responses are important in the response to HCV infection.[27-34,35-37] Host genetic factors that govern these responses may also modify the course of HCV infection. Polymorphisms in HLA molecules as well as cytokine genes appear a ssociated with natural clearance and histologic progression of HCV infection (Chapter 2)

The cytotoxic T-lymphocyte antigen-4 (CTLA4), encoded by a gene on chromosome 2q33, is expressed on activated CD4+ and CD8+ T-cells.[38] It binds to the ligands B7-1 (CD80) and B7-2 (CD86),[39-41] and down-regulates T-cell function.[40, 42-45] CTLA4 is expressed 24- 48 hours after T-cell activation and the expression is inversely correlated with CD28 expression, al alternative B7-1 ligand which induces T cell activation. Mice deficient in CTLA4 exhibit polyclonal T-cell activation and proliferation.[46-49] A single nucleotide polymorphism (SNP) resulting in a C-to-T transition at position –318 of the *CTLA4* promoter has been described.[50] In addition, a G-to-A transition at position 49 in exon 1 of the *CTLA4* gene encodes an alanine (Ala)-to-threonine (Thr) substitution in codon 17 of the leader peptide.[51, 52]

Recent functional studies have produced evidence that polymorphisms at position –318 of the promoter, position 49 in exon 1, and the resulting haplotypes

formed by the variants of these two loci exert a differential functional effect on CTLA4-driven down-regulation of T-cell activation.[53-57]

Considering the possible importance of CTL responses in HCV clearance, the involvement of CTLA4 regulating those responses, I selected *CTLA4* as another candidate gene. I specifically tested the hypothesis that CTLA4 variants are associated with sustained response to IFN+R therapy.

Table 6.1.1. summarises the genes included the in the present study. Information on the chromosomal location of the gene along with the aforementioned biological functions are also listed.

Table 6.1.1. Summary of genes studied, their chromosomal location, and the biological function of the proteins encoded by the genes.

Gene	Chromosomal Location	Biological Function
MxA	21q22.3	MxA protein has anti-viral properties
OAS-1	12q24.2	Breaks down viral and cellular RNA
PKR	2p22.3	Inhibits viral replication
NOS2A	17q11.2	iNOS protein has anti-viral effects
CTLA4	2q33	Downregulator of T-lymphocyte response

6.2 Methods

Patients

Interferon-stimulated genes

For this study, I examined 638 Caucasian subjects enrolled in the HENCORE (Hepatitis C European Network for Collaborative Research) study; the details of this study have been published before and are described in chapter 3.[58] Briefly, this is a multicentre European study of HCV that enrolled individuals from nine centres between October 1995 and June 2001 and the cohort includes individuals with both self-limiting and persistent HCV infections. A portion of these individuals have also been treated with interferon monotherapy. Patients were recruited randomly or sequentially in each centre in order to minimise selection bias. Each patient gave informed consent and ethical approval was obtained from the local research ethics committee at each centre.

Patients were classified into the following groups as follows: 1) self limiting HCV infection: individuals with antibodies to HCV who have no evidence of viraemia on at least two occasions and who have persistently normal liver transaminase levels; 2) persistent HCV infection: individuals who have evidence of viraemia for at least six months; 3) sustained treatment response: patients with persistent HCV infection treated with interferon α alone who had normal liver transaminases and no evidence of viraemia six months after the end of treatment; 4) response-relapse: patients with persistent HCV infection treated with interferon α who had normal liver transaminases and no evidence of viraemia at the end of treatment, but in whom viraemia returned during the follow-up period of 6 months; 5) non-response to treatment: patients with persistent HCV infection treated with interferon α who never lost viraemia during treatment. Individuals considered "initial responders" to interferon were those who experienced a loss of HCV viraemia during the first 12 weeks of treatment, regardless of whether they ultimately achieved sustained response (sustained responders + relapse responders).

NOS2A promoter haplotypes and hepatitis C outcome

For this study, we studied 619 Caucasian patients enrolled in the HENCORE (Hepatitis C European Network for Collaborative Research) study. The characteristics of this cohort as well as the definition of patient groups are the same as those stated above for the study of interferon-stimulated genes.

HCV antibodies and viraemia

For patients in the aforementioned studies, antibodies to HCV antigens were detected with either an enzyme linked immunoassay (EIA) or a recombinant immunoblot assay (RIBA) containing 4 HCV antigens on a cellulose acetate strip used according to the manufacturer's instructions (Abbott Laboratories, North Chicago, IL). The presence or absence of viral particles in serum was determined by reverse transcription polymerase chain reaction using a commercially available assay (Amplicor, Roche) with a sensitivity of approximately 200 genomes/ml.

CTLA4

Among individuals with chronic hepatitis C referred to the UAB Liver Center, 312 received INF+R therapy under a clinical trial protocol. Suitability for therapy was determined by inclusion/exclusion criteria and guidelines established internationally.[25, 26] Briefly, all had compensated liver disease due to HCV, and none had other forms of chronic liver disease or other major co-morbid conditions. All were seronegative for the human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV).[25, 26]

By December of 2000, approximately 170 individuals had completed therapy. In a nested case-control study, the first 79 consecutive Caucasian responders and the first 79 consecutive Caucasian non-responders who had completed therapy and consented to DNA genotyping were included in the present study. Individuals with non-1 genotype infections who responded (reached undetectable viral RNA levels) by week 12 received treatment for a total of 6 months, while those with genotype-1 infections who responded by week 12 continued to receive therapy for 1 year.

HCV genotypes and HCV-RNA levels were assessed before, at weeks 4 and 12 during, and at 6 months after discontinuing treatment (National Genetics Institute, Los

Angeles, CA). A sustained responder (SR) was defined as having had an undetectable plasma HCV RNA level at 6 months after discontinuation of therapy. This included individuals who were weakly positive for HCV-RNA at week 12 (under 1,000 copies/mL) but had no detectable HCV-RNA at 6 months after discontinuation of therapy (delayed responder). A non-responder (NR) was defined as having either detectable virus at week 12 of therapy, at which time treatment was discontinued, or detectable virus at 6 months after discontinuation of therapy despite a virologic response during the initial course (flare-up). From trial patients who met these criteria, 79 SRs and 79 NRs were included.

ALT was assessed by standard clinical laboratory techniques (Kirklin Clinic Laboratory, Birmingham, AL). For categorical analyses, a threshold value of 1.5 times above the upper limit of normal ($\geq$ 72 or $<$ 72) was used, a commonly accepted definition of "high" ALT level. HCV isolates were dichotomised as either 1 or non-1 genotype because infections with genotype 1 viruses are less responsive and require a longer course of treatment.[25, 26] Baseline viral load was divided into "high" for individuals with $\geq$ 2,000,000 copies/mL and "low" for individuals $<$ 2,000,000 copies/mL, the value adopted by most treatment algorithms for assessing probable response.[21, 25, 26] The threshold value for "older" and "younger" age was 40 years as used by previous studies.[25, 59]

Laboratory procedures

Screening for novel polymorphisms

This study also involved the identification of several novel polymorphisms in the promoter region and the functional domains of the *PKR* and *OAS-1* genes, along with the promoter region of *MxA* by direct sequencing of these regions, Sequencing was performed using Big-Dye Terminator Chemistry (Applied Biosystems, Warrington, UK) and an ABI377 automated DNA sequencer. Sequences were aligned and analysed using Sequence Navigator Software (Applied Biosystems, Warrington, UK). Screening for these polymophisms was conducted by Dr. Susanne Knapp (Imperial College, UK). I later used these polymorphisms to examine possible correlations with HCV outcome.

Genotyping of allelic variants

OAS-1

An A to G SNP at position 84 bp in the untranslated 3'end of exon 8 of OAS-1 (position 347 of HUMOAS08/M18101) was genotyped using allele specific real time PCR. Each reaction comprised 0.2 μM of each of the primers specific for exon 8 (Table 6.2.1); 2.5 units of Stoffel Gold Polymerase (David Birch, RMS); 1x Stoffel Gold buffer (10 mM Tris-HCl, 10 mM KCL at pH 8.0); an additional 30 mM KCl for a final concentration of 40 mM ; 2 mM $MgCl_2$; 50 μM each dATP, dCTP, and dGTP; 25 mM TTP; 75 mM dUTP: 2 units of UNG (PE), 0.2 x SybrGreen I (Roche Molecular Probes); 2 μM ROX (Roche Molecular Probes); 5 % DMSO; and 2.5 % Glycerol. Kinetic PCR reactions were performed on a GeneAmp 5700 Sequence detection System (PE Applied Biosystems). An initial incubation step of 2 min at 50°C (to allow UNG mediated elimination of carry-over PCR product contamination),

Table 6.2.1. Sequences of primers used to genotype polymorphisms in the *OAS* gene.

Gene	Site	Oligo Name	Oligo sequence (5' to 3')	Annealing positions and reference sequence*
OAS	exon 8	reverse A-allele	CTCACTGAGGAGCTTTGTCt	323-343 of M11810
	exon 8	reverse G-allele	CTCACTGAGGAGCTTTGTCc	323-343 of M11810
	exon 8	forward common primer	CAGGTGGGACTCTTGATCCAG	366-348 of M11810

*Sequence identification numbers are Genbank Accession Numbers.

and an enzyme heat activation step of 12 min at 95 °C were followed by 40 two-step amplification cycles of 20 sec at 95 °C for denaturation and 20 sec for 58 °C for annealing and extension, and a final 5 minutes extension at 72 °C. All PCR reactions were performed on 5-50 ng genomic DNA in a total volume of 20 μl.

MxA

The biallelic G/T polymorphism in the promoter region of MxA at position –88 from the transcription start site,[60] was genotyped by restriction fragment length

polymorphism (RFLP) using the enzyme HhaI (New England Biolabs). The primer sequences for the MxA-88 position can be found in Table 6.2.2 and were used to amplify a fragment of 351 bp. Amplification was carried out in a volume of 20 µl, containing 10-100 ng DNA, 2.5 mM $MgCl_2$, 500 nM of each primer, 500 µM dNTP's, 1x PCR buffer (Qiagen), 1 unit Taq DNA polymerase (Qiagen), 0.16 ul TaqStart Antibody (BD Clontech). The cycling conditions in an Applied Biosystems 2400 or 9700 machine were: denaturation at 94 °C for 5 minutes, subsequently 35 cycles of denaturation at 94 °C for 30 seconds; annealing at 58 °C for 30 seconds; and extension at 72 °C for 1 minute. This was followed by a final extension step at 72 °C for 7 minutes. For the HhaI restriction digest 8 µl of the PCR product were digested for at least 4 hours or over night in a volume of 20 µl and 5 units of HhaI according to manufacturers specifications. 10 µl of the digested PCR product were run out on 2% agarose gels and analyzed. In the presence of the G allele the 351 bp long product is

Table 6.2.2. Sequences of primers used to genotype polymorphisms in the *MxA* gene.

Gene	Site	Oligo Name	Oligo sequence (5' to 3')	Annealing positions and reference sequence*
MxA	promoter -88	forward	TGAAGACCCCCAATTACCAA	269-287 of X55639
	promoter -88	reverse	CTCTCGTTCGCCTCTTTCAC	619-600 of X55639
	promoter -545	forward	GGCCTGGCCTGACAACTAT	29666-29684 of AL773577
	promoter -545	reverse	CATCCAAGCCTGCACGTAT	30068-30050 of AL773577

*Sequence identification numbers are Genbank Accession Numbers.

cut into 260, 51, 23 and 16 bp. A SNP (G/A) at position –545 from the transcription start site was genotyped using BsaI (New England Biolabs) to differentiate between the alleles. A 402 bp fragment was amplified at the conditions previously described, using primers of Table 6.2.2. Restriction digestion was carried out using 8 µl of the PCR product in 20ul volume in the presence of 2 units BsaI. Presence of the A-allele was indicated by the absence of the BsaI cutting site, whereas the G allele results in the creation of a 287 bp and a 115 bp fragment.

PKR

Point mutations in the promoter region of PKR were genotyped by sequence analysis. In addition restriction fragment length polymorphism (RFLP) with the enzyme

SgrAI was used to genotype the SNP at position -168 (168 bp from the transcription start) of the PKR gene. A 282 bp fragment was amplified using the forward primer PPKR-168F and the reverse primer PPKR-168R (see Table 6.2.3). In the presence of the T allele a second SgrAI site was introduced which leads to the separation of 19, 97 and 166 bp fragments after digestion and gel separation, whereas in presence of the T allele the SgrAI digest only gives rise to a 116 and 166 bp fragment.

A short tandem repeat (CGG) in exon 1 of the 5' untranslated region of PKR was genotyped on the automated ABI377 sequencer (Perkin Elmer Applied Biosystems). PCR products were obtained using a FAM labelled sense primer (primer PPKR3S, see Table 6.2.1) and allowed size discrimination of the fluorescently labelled products. The products were diluted between 1:5 and 1:20 in water and 1 µl of the diluted product was added to 4 µl of formamide, TAMRA 350 size marker and Bluedextran/EDTA buffer (ratio 3:1:1). The samples were heated to 94°C for 5 minutes and then cooled on ice immediately. 2 µl of this solution was loaded onto a denaturing 4.25% polyacrylamide gel and run on an ABI 377 genotyping system. Allele discrimination was performed using Genescan and Genotyper software.

Table 6.2.3. Primers used to genotype polymorphisms in the *PKR* gene.

Site	Oligo Name	Oligo sequence (5' to 3')	Annealing positions and reference sequence*
Promoter region including exon-1	PPKR3S	AGGGTTCCTGGCCGTGCAGG	442-461 of HSU51035; 441-460 from Kuhen *et al.*, 1997
Promoter region including exon-1	PPKR4A	CCGCGCTCCCTCGGCTGC	751-734 from Kuhen *et al.*, 1997.
Promoter region	PPKR-168F	GACTAGGCCAGCGGAGAAC	
Promoter region	PPKR-168R	GCTTCGGGAGAGCTGGTT	
exon-4	PKR4S	ATATTCTCTTTGTAATCAGG	2-21 U5635
exon-4	PKR4A	AAAAATGGCAATCACTCACC	160-141 U5635
exon-6	PKR6S	CCTTCTATGATTTCTCCTAG	1-21 U5637
exon-6	PKR6A	ATCCAAAGGCAATACGTACC	167-147 U5637
exon-11	PKR11S	ACAGTGTTTTATCTTTAAGG	2-21 of U50642
exon-11	PKR11A	GTAAACATTTACTACTTACTCG	162-141 of U50642
exon-12	PKR12S	CCCTGTTCCTTTTAACTAGG	2-21 of U50643
exon-12	PKR12A	CTCAGGATCATAATCACTGC	159-140 of U50643
exon-13	PKR13S	CTGTGAATTTTATACCCAGG	1-21 of U50644
exon-13	PKR13A	GTATTACTTTTTCCACTTACC	221-201 of U50644
exon-17	PKR17S	GACTCTCACTGTCATTGCAG	1-20 of U50648
exon-17	PKR17A	GTGTCATTGCACTCCAGCCT	401-420 of U50648

*Reference sequences are Genbank accession numbers.

NOS2A promoter

Three single nucleotide polymorphisms were targeted in the present study: An A to G substitution at position -277, a G to T substitution at position -1026, and a C to G substitution at position -1659 of the *NOS2A* gene were genotyped by allele specific real time PCR.[61] The primers used for this procedure are provided in Table 4. Each reaction was comprised of 0.2 μM of each of the primers specific for exon 8 (Table 6.2.4.); 2.5 units of Stoffel Gold Polymerase (David Birch, RMS); 1x Stoffel Gold buffer (10 mM Tris-HCl, 10 mM KCL at pH 8.0); an additional 30 mM KCl for a final concentration of 40 mM ; 2 mM $MgCl_2$; 50 μM each dATP, dCTP, and dGTP; 25 mM TTP; 75 mM

dUTP: 2 units of UNG (PE), 0.2 x SybrGreen I (Roche Molecular Probes); 2 μM ROX (Roche Molecular Probes); 5 % DMSO; and 2.5 % Glycerol. Kinetic PCR reactions were performed on a GeneAmp 5700 Sequence detection System (PE Applied Biosystems). An initial incubation step of 2 min at 50°C (to allow UNG mediated elimination of carry-over PCR product contamination), and an enzyme heat activation step of 12 min at 95 °C were followed by 40 two-step amplification cycles of 20 sec at 95 °C for denaturation and 20 sec for 58 °C for annealing and extension, and a final 5 minutes extension at 72 °C. All PCR reactions were performed using 5-50 ng genomic DNA in a total volume of 20 μl.

NOS2A Haplotype Assignment

Several conserved haplotypes have been reported in Caucasian populations (Table 6.2.5).[19] Haplotypes in the present study were assigned per these previously published descriptions.[19]

Table 6.2.4. Primers used for genotyping the three single nucleotide polymorphism in the promoter region of the *NOS2A* gene.

SNP	Primer specificity	Primer sequence
NOS2A -277 (A/G)	forward common primer	5'-CTGGCTCCGTGGTGCC-3'
	A-specific reverse primer	5'-CAGGGTGGCTGCTAAGAT-3'
	G-specific reverse primer	5'-CAGGGTGGCTGCTAAGAC-3'
NOS2A -1026 (G/T)	forward common primer	5'-GGCATTATAAGGAATGAAATTATAGGCC-3'
	G-specific reverse primer	5'-GATTACAAGGGTTAGCCACC-3'
	T-specific reverse primer	5'-GATTACAAGGGTTAGCCACA-3'
NOS2A -1659 (C/T)	reverse common primer	5'-GGGATGGTATGGTGCTGATG-3'
	T-specific forward primer	5'-CCTTGAACAAGGCAGAACT-3'
	C-specific forward primer	5'-CCTTGAACAAGGCAGAACC-3'

Table 6.2.5. Major Caucasian *NOS2A* haplotypes and their designations.

Designation	-277 (A/G)	-1026 (G/T)	-1659 (C/T)
Haplotype 1	A	G	C
Haplotype 2	G	T	C
Haplotype 3	G	G	C
Haplotype 4	G	T	T
Haplotype 5	A	T	C

Burgner D. *et al.* [19]

Genotyping of *CTLA4* allelic variants

Polymerase chain reaction with sequence specific primers (PCR-SSP) was used to define the SNP in position 49 of exon 1 in the *CTLA4* gene. Four PCR-SSP reactions were used to type two *CTLA4* SNPs at nucleotide positions 49 in exon 1 and –318 in the promoter, with each reaction including a pair of SNP-specific primers (Table 6.2.6) and a pair of control primers (C5, 5'-TgC CAA gTg gAg CAC CCA A-3') and (C3, 5'-gCA TCT TgC TCT gTg CAg AT-3') specific for human DRB1 intron 3, as described by Olerup *et al.* The first two SSP reactions defined the 49A/G alleles when each of the 49A and 49G-specific primers was paired with the general primer SSPg. The second two SSP reactions combined one of the –318C- and –318T-specific primers with another general primer SSPf. The PCR mix (10 µl each) consisted of 1x buffer C (60 mM Tris-HCl, pH 8.5, 15 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$), 50-70 ng of genomic DNA, 0.3 units of AmpliTaq polymerase, 120 nM of each control primer, 250 nM each of *TAP1*-specific primer (positive control), 0.4 mM each of dGTP, dCTP, dTTP and dATP, 10% (v/v) glycerol, and 0.02% cresol red. PCR cycling began with 10 higher-stringency cycles of denaturing at 95°C for 25 sec, annealing at 62°C for 45 sec, and extension at 72°C for 45 sec, followed by 22 additional lower-stringency cycles of denaturing at 95°C for 25 sec, annealing at 58°C for 40 sec, and extension at 72°C for 40 sec. Half of each PCR reaction product was loaded directly onto 1.5% agarose gels for

electrophoresis, and the SSP-banding patterns were recorded on photographs of ethidium bromide-stained gels. PCR-SSP-based typing of the –318C/T variants were validated by 100% match with results from restriction fragment length polymorphisms (RFLP) as –318T contains an MseI site (TTAA). The strategy for typing –318C/T and 49A/G was similar to procedures developed independently by another group of investigators,[54] except that the orientation of the 49A/G-specific primers differed and our PCR-SSP was done at a higher stringency as verified by close resemblance between primer melting temperatures and PCR annealing temperatures. PCR-SSP-based genotyping of *IL10* variants was performed according to procedures reported previously.[62]

Table 6.2.6. Oligonucleotides used to define *CTLA4* polymorphisms by PCR-SSP

Oligo Specificities	Oligo name	Oligo sequence (5' → 3') [a]	Annealing positions [b]
CTLA4 49A (17Thr)	SSP1	gCT CAg CTg AAC CTg gCT A	1223 → 1241
CTLA4 49G (17Ala)	SSP2	CTC AgC TgA ACC Tgg CTg	1224 → 1241
CTLA4 general	SSPg	ACA gAg CCA gCC Aag CCA	1424 → 1441
CTLA4 -318C [c]	SSP3	CCA CTT AgT TAT CCA gAT CCT C	854 → 875
CTLA4 -318T [c]	SSP4	CCA CTT AgT TAT CCA gAT CCT T	854 → 875
CTLA4 general [c]	SSPf	gCT TTg ATC CAg ATA TgT ATT ACA C	1056 → 1080

Statistical analyses:

Standard univariable analyses were conducted using contingency tables. Odds Ratios (OR) and 95% confidence intervals (95%C.I.) were calculated along with maximum likelihood and Fisher's exact p-values, as appropriate using the EpiStat module of EpiInfo 2000 (Centres for Disease Control and Prevention, Atlanta, Georgia, USA), or the STATA® and SAS® statistical packages (see Chapter 3 for a detailed discussion of the analytical methods).

To analyse proportions of individuals carrying a variant, the number of patients with that variant was divided by the total number of patients (N); SRs and NRs were compared with Chi-square statistics for contingency tables. Odds ratios (ORs), 95% confidence intervals and p-values (both maximum likelihood and Fisher's exact) were calculated as appropriate.

Alleles at adjacent loci in linkage disequilibrium form haplotypes. To measure the strength of linkage disequilibrium between variants at the promoter and exon 1 loci, delta and χ^2 values were calculated according to standard methods.[63] Haplotypes were inferred from the relative values of these statistics for each allele combination. For the study of CTLA4 variants, the putative promoter and exon-1 haplotypes were verified by comparison with previous studies.[42, 64]

Comparisons of the effects of carriage of variants on baseline viral load levels were tested using the Wilcoxon rank-sum method (2-tailed p-value) including all 158 Caucasian patients with all viral genotypes. Multiple logistic regression was used to adjust for covariates thought to modify therapeutic response using the entire cohort of 158 Caucasian patients and then in those with genotype-1 infections only. SRs to IFN+R were compared with NRs as the referent group.

To assess the effects of genetic variants on viral dynamics during the course of treatment, longitudinal analysis using mixed models was employed.[65] This time series analysis method is analogous to the repeated measures procedure based on the analysis of variance (ANOVA). This form of longitudinal analysis is more robust than the conventional repeated measures procedure and allows for unequal measurement intervals. The SAS® PROC MIXED procedure in SAS® software (Cary, NC) version 8.1 was used for longitudinal analysis, ensuring a parametric distribution appropriate for this statistical procedure by taking natural log-transformed viral load measurements (baseline, week 4 and week 12). For corroboration, the differences in viral load measurements at three time points: baseline, week 4 and week 12 of therapy, were compared using univariable techniques (Wilcoxon rank sum test).

6.3 Results

Interferon-Stimulated Genes and Hepatitis C

Cohort characteristics

The present cohort was 52.8% male and 47.2% female. The average age at infection among those with self-limiting infection was 26.8 years ± 14.6 years st. dev., and 32.6 years ± 14.7 years st. dev. among those with persistent infection. Viral genotyping was available on 111 patients in the present study. 61.3% of those viral genotyped had genotype-1 infections, while 38.7% had non-1 genotype infections. Among the 638 Caucasian individuals included in the study, 94 had self-limiting HCV infection and chronic (persistent) HCV infection. 166 individuals were treated with interferon monotherapy and 93 achieved sustained response and 12 were relapsed-responders (105 initial responders), while 61 were non-responders.

Polymorphisms in the OAS, PKR and MxA genes

The following polymorphisms were identified: an A/G substitution at position –545 of the *MxA* gene, a C/T substitution at position –168 and a G/T substitution at position –180 of the *PKR* gene, an A/G substitution in the 3' untranslated region (3'-UTR) of *OAS-1* at position 347 of M11810 and a CGG trinucleotide repeat in the promoter region of the *PKR* gene. Subsequent comparison of the –545*MxA*(A/G) SNP and the 3'-UTR *OAS-1*-(A/G) SNP with known polymorphisms in the GenBank database suggests that these SNPs correspond to the previously described polymorphisms rs462903 and rs2660, respectively. The *-88MxA* polymorphism has been taken form the literature,[60] and the CCG repeat in the 5'-UTR of the PKR gene has subsequently been published.[66, 67]

MxA

The single nucleotide polymorphism (SNP) at position –88 with respect to the transcription initiation site appeared to influence the natural outcome of HCV infection

in addition to the response to interferon therapy (Table 6.3.1). The TT genotype was found more frequently in patients who had self limiting infection (OR = 3.30, 95% CI 0.80 – 14.15; p = 0.05) together with the GT genotype (OR = 1.59, 95% CI 0.98- 2.59; p = 0.05) indicating a gene dosage effect. The GT genotype was also found more frequently in patients with an initial response and those with sustained response to interferon compared to those with non-response (Initial Response: OR = 2.1, 95% CI 1.05 – 4.07; p = 0.02. Sustained Response: OR = 1.8, 95% CI 0.85 – 3.82; p = 0.1). The TT genotype was not detected in these groups. The SNP at position –545 was not found to be associated with the outcome of HCV infection or treatment response.

OAS-1

The SNP in the 3'UTR of the *OAS-1* gene shows evidence of association with self limiting HCV infection but not with the response to interferon therapy (Table 6.3.2). The GG genotype at this locus was found in 9% of patients with self limiting infection compared to 18.8% of patients with persistent infection (OR=0.43; 95%C.I.: 0.21 – 0.86; p=0.01).

PKR

Polymorphisms in the PKR gene appear to influence both the natural outcome and the response to interferon therapy in HCV infection (Table 6.3.3 for self-limiting versus persistent HCV infection; Table 6.3.4 for initial response to interferon monotherapy versus non-response; and Table 6.3.5 for sustained response compared to non-response). Heterozygotes for the SNP at position –168 relative to the

Table 6.3.1. Summary of univariable correlations between *MxA* polymorphisms and the outcome of hepatitis C virus infection among Caucasian patients. **Part A.** presents the distributions for self-limiting versus persistent infection. **Part B.** presents distributions for initial response (IR) versus non-response (NR) to interferon mono therapy. **Part C.** presents the distributions for sustained responders (SR) versus non-responders (NR) to interferon mono therapy.

PART A.						
Locus	**Variant**	**Self-limiting N (%)**	**Persistent N (%)**	**OR**	**95%C.I.**	**p-value**
MxA	***-88***					
	GG	88 (65.2)	222 (77.1)	0.56	0.35 - 0.89	0.010
	GT	41 (30.4)	62 (21.5)	1.59	0.98 - 2.59	0.048
	TT	6 (4.4)	4 (1.4)	3.30	0.80 - 14.19	0.050*
	-545					
	AA	14 (7.9)	42 (27.3)	0.58	0.54 - 3.27	0.120
	AG	48 (61.5)	87 (56.5)	1.23	0.68 - 2.23	0.462
	GG	16 (20.5)	25 (16.2)	1.33	0.63 - 2.82	0.420
PART B.						
Locus	**Variant**	**IR N (%)**	**NR N (%)**	**OR**	**95%C.I.**	**p-value**
MxA	***-88***					
	GG	108 (73.5)	97 (85.1)	0.49	0.25 - 0.95	0.020
	GT	39 (26.5)	17 (14.9)	2.06	1.05 - 4.07	0.023
	TT	0 (0.0)	0 (0.0)	-----	-----	-----
	-545					
	AA	23 (30.3)	16 (21.6)	1.57	0.71 - 3.52	0.230
	AG	40 (52.6)	46 (62.2)	0.68	0.33 - 1.36	0.238
	GG	13 (17.1)	12 (16.2)	1.07	0.42 - 2.73	0.880
PART C.						
Locus	**Variant**	**SR N (%)**	**NR N (%)**	**OR**	**95%C.I.**	**p-value**
MxA	***-88***					
	GG	73 (76.0)	97 (85.1)	0.56	0.26 - 1.18	0.100
	GT	23 (24.0)	17 (14.9)	1.80	0.85 - 3.82	0.096
	TT	0 (0.0)	0 (0.0)	-----	-----	-----
	-545					
	AA	22 (30.1)	16 (21.6)	1.56	0.7 - 3.53	0.238
	AG	39 (53.4)	46 (62.2)	0.70	0.34 - 1.42	0.238
	GG	12 (16.4)	12 (16.2)	0.46	0.17 - 1.26	0.092

*Fisher's exact p-value. OR=Odds ratio; 95%C.I.= 95% Confidence Interval.

Table 6.3.2. Summary of univariable correlations between *OAS-1 3'-UTR* polymorphism and the outcome of hepatitis C virus infection among Caucasian patients. **Part A.** presents the distributions for self-limiting versus persistent infection. **Part B.** presents distributions for initial response (IR) versus non-response (NR) to interferon mono therapy. **Part C.** presents the distributions for sustained responders (SR) versus non-responders (NR) to interferon mono therapy.

PART A.

Locus	Variant	Self-limiting N (%)	Persistent N (%)	OR	95%C.I.	p-value
OAS-1	*3'-UTR*					
	AA	45 (36.9)	185 (35.9)	1.05	0.68 - 1.61	0.830
	AG	66 (54.1)	234 (45.3)	1.42	0.94 - 2.15	0.082
	GG	11 (9.0)	97 (18.8)	0.43	0.21 - 0.86	0.010

PART B.

Locus	Variant	IR N (%)	NR N (%)	OR	95%C.I.	p-value
OAS-1	*3'-UTR*					
	AA	97 (36.1)	51 (34.7)	1.06	0.68 - 1.65	0.780
	AG	117 (43.5)	72 (49.0)	0.80	0.52 - 1.22	0.283
	GG	55 (20.4)	24 (16.3)	1.32	0.75 - 2.31	0.310

PART C.

Locus	Variant	SR N (%)	NR N (%)	OR	95%C.I.	p-value
OAS-1	*3'-UTR*					
	AA	51 (35.7)	51 (34.7)	1.04	0.63 - 1.74	0.860
	AG	60 (41.9)	72 (49.0)	0.75	0.46 - 1.23	0.223
	GG	32 (22.4)	24 (16.3)	1.48	0.79 - 2.77	0.190

Abbreviations: OR=Odds ratio; 95%C.I.= 95% Confidence Interval.

Table 6.3.3. Summary of univariable correlations between *PKR* polymorphisms and self-limiting versus persistent infections.

Locus	Variant	Self-limiting N (%)	Persistent N (%)	OR	95%C.I.	p-value
PKR	***-180***					
	GG	2 (4.7)	8 (10.5)	0.41	0.06 - 2.27	0.226
	GT	17 (39.5)	20 (26.3)	1.83	0.77 - 4.38	0.134
	TT	24 (55.8)	48 (63.2)	0.74	0.32 - 1.69	0.430
PKR	***-168***					
	CC	6 (9.8)	39 (19.2)	0.46	0.16 - 1.21	0.090
	CT	37 (60.7)	77 (37.9)	2.75	1.45 - 5.24	0.002
	TT	18 (29.5)	87 (42.9)	0.56	0.29 - 1.08	0.060
PKR	***CGG repeat***					
	Long/Long	43 (70.5)	180 (78.9)	0.64	0.32 - 1.26	0.162
	Short/Long	18 (29.5)	39 (17.1)	2.03	1.01 - 4.07	0.030
	Short/Short	0 (0.0)	9 (3.9)	5.3	0.30 - 92.75	0.201

*Fisher's exact p-value. OR=Odds ratio; 95%C.I.= 95% Confidence Interval.

Table 6.3.4. Summary of univariable correlations between *PKR* polymorphisms and initial response (IR) to interferon mono therapy compared to those with non response (NR) to therapy.

Locus	Variant	IR N (%)	NR N (%)	OR	95%C.I.	p-value
PKR	***-180***					
	GG	2 (6.9)	6 (14.6)	0.43	0.06 - 2.68	0.320
	GT	9 (31.0)	10 (24.4)	1.40	0.43 - 4.58	0.538
	TT	18 (62.1)	25 (61.0)	1.05	0.35 - 3.12	0.930
PKR	***-168***					
	CC	19 (17.0)	18 (22.2)	0.72	0.33 - 1.56	0.360
	CT	43 (38.4)	27 (33.3)	1.25	0.66 - 2.37	0.471
	TT	50 (44.6)	36 (44.4)	1.01	0.54 - 1.87	0.978
PKR	***CGG repeat***					
	Long/Long	76 (85.4)	74 (71.8)	2.29	1.05 - 5.07	0.023
	Short/Long	12 (13.5)	22 (21.4)	0.57	0.25 - 1.32	0.154
	Short/Short	1 (1.1)	7 (6.8)	0.16	0.01 - 1.30	0.050

Abbreviations: IR=initial response; NR=non-response; OR=Odds ratio;
95%C.I.= 95% Confidence Interval.

Table 6.3.5. Summary of univariable correlations between *PKR* polymorphisms and sustained response (SR) to interferon mono therapy compared to those with non response (NR) to therapy.

Locus	Variant	IR N (%)	NR N (%)	OR	95%C.I.	p-value
PKR	***-180***					
	GG	2 (7.4)	6 (14.6)	0.47	0.06 - 2.91	0.370
	GT	9 (33.3)	10 (24.4)	1.55	0.47 - 5.16	0.421
	TT	16 (59.3)	25 (61.0)	0.93	0.31 - 2.82	0.887
PKR	***-168***					
	CC	12 (15.2)	18 (22.2)	0.63	0.26 - 1.51	0.250
	CT	31 (39.2)	27 (33.3)	1.29	0.64 - 2.59	0.437
	TT	36 (45.6)	36 (44.4)	1.05	0.54 - 2.05	0.890
PKR	***CGG repeat***					
	Long/Long	42 (89.4)	74 (71.8)	3.29	1.10 - 10.52	0.017
	Short/Long	5 (10.6)	22 (21.4)	0.44	0.13 - 1.34	0.113
	Short/Short	0 (0.0)	7 (6.8)	0.14	0.01 - 2.42	0.115

Abbreviations: IR=initial response; NR=non-response; OR=Odds ratio;
95%C.I.= 95% Confidence Interval.

transcription initiation site were more likely to have self limiting HCV infection (OR=2.75; 95%C.I.; p=0.002) but this genotype had no significant influence on the outcome of treatment. The SNP at position –180 had no significant influence on the outcome of HCV infection or treatment.

Associations were sought for individual alleles of the CGG repeat polymorphism in the PKR promoter region. As trinucleotide repeats may influence gene transcription, alleles were categorised as short (< 9 repeats) or long (≥ 9 repeats). Individuals with two short alleles were found more frequently amongst those who had self limiting infection (OR 18.8, p = 0.11) as were individuals with one short allele (OR = 2.03, 95% CI 1.01 – 4.07; p = 0.03). Conversely, patients with two long alleles were found more frequently in the initial responder group (OR= 2.29, 95% CI 1.05 – 5.07; p = 0.02) and sustained responder group (OR = 3.29, 95% CI 1.10 – 10.52; p= 0.02). In a global test, all polymorphic sites approximated to Hardy Weinberg Equilibrium.

NOS2A haplotypes and hepatitis C

Cohort characteristics

The present cohort was 55.7% male and 44.3% female. The average age at infection was 43.5 years ± 15.8 years st. dev. Viral genotyping was available on 177 patients in the present study. 66.7% of those viral genotyped had genotype-1 infections, while 33.3% had non-1 genotype infections. Among the 619 individuals included in the study, 76 had self-limiting HCV infection and 543 chronic (persistent) HCV infection. 314 individuals were treated with interferon monotherapy and 112 achieved sustained response and 102 were relapsed-responders (214 initial responders), while 100 were non-responders.

NOS2A haplotypes and HCV outcome

Five major *NOS2A* haplotypes were observed with the following frequencies: haplotype 1: 60.9%; haplotype 2: 16.2%; haplotype 3: 8.7%; haplotype 4: 12.8%; and haplotype 5: 1.4%, and is comparable with frequencies reported in other Caucasian cohorts.[20] Homozygosity for haplotype 2 (Table 6.3.6) was associated with self-limiting infection (OR=3.02; 95%C.I.: 1.10-8.0; p=0.0206), while possession of the combination of haplotypes 2 and 4 was also associated with self-limiting infection (OR=4.57; 95%C.I.: 1.32-14.32; p=0.0018).

The combination of haplotypes 1 and 4 were associated with the initial response to interferon therapy (OR=2.32; 95%C.I.: 1.05-5.68; p=0.0275) (Table 6.3.7). Marginally significant associations with non-response were observed with possession of haplotypes 1 and 5 (OR=0.09; 95%C.I.: 0.01 – 1.93; p=0.0556) and homozygosity for haplotype 3 (OR=0.27; 95%C.I.: 0.04 – 1.43; p=0.0594). No significant associations were observed between *NOS2A* haplotypes and sustained response to interferon therapy (Table 6.3.8).

The relative infrequency of these haplotypes precluded multivariable analysis. As an alternative, I stratified the observed haplotypic effects by patient gender and viral genotype. Stratification of the association between haplotype 2 homozygous and self-limiting infection yielded a stronger effect among men than women (OR=3.72; 95%C.I.:

0.95 – 14.60; p=0.0790 for men, and OR=1.30; 95%C.I.: 0.26 – 6.39; p=0.6691 for women). Stratification of the haplotype 2/4 combination by gender yielded similar strength's of association for both males and females (OR=3.2; 95%C.I.: 0.32 – 31.34; p=0.3335 for men, and OR=3.4; 95%C.I.: 1.05 – 11.06; p=0.0317 for women). Stratification of the association between the combination of haplotype 1/4 and initial response to therapy yielded a stronger effect among women than men (OR=0.99; 95%C.I.: 0.39 – 2.54; p=0.9854 for men, and OR=7.6; 95%C.I.: 1.74 – 33.27; p=0.0015 for women). Stratification of the association of the combination of haplotypes 1/4 and initial response by viral genotype yielded a stronger effect among those with genotype-1 virus infections (OR=6.79; 95%C.I.: 1.51 – 30.52; p=0.0048 for genotype-1 infections and OR=1.8; 95%C.I.: 0.21 – 15.45; p=1.0 for non-1 genotypes).

Table 6.3.6. Association of *NOS2A* haplotypes among 619 Caucasian individuals in the HENCORE Study. 76 individuals have self limiting HCV infection, and 543 individuals have persistent infection.

Haplotype	Self-limiting	Persistent	OR	95%C.I.	p-value
1/1	24	206	0.76	0.43 - 1.29	0.2826
1/2	14	112	0.87	0.43 - 1.64	0.6548
1/3	5	53	0.65	0.20 - 1.70	0.3727
1/4	12	90	0.94	0.45 - 1.86	0.8628
1/5	0	8	0.41	0.02 - 7.21	0.5306
2/2	6	15	3.02	1.10 - 8.0	0.0206
2/3	3	12	1.82	0.32 - 6.95	0.3562
2/4	6	10	4.57	1.32 - 14.32	0.0018
2/5	0	2	1.42	0.07 - 29.77	0.8223
3/3	0	9	0.37	0.02 - 6.38	0.4745
3/4	4	13	2.26	0.52 - 7.58	0.1521*
4/4	1	8	0.89	0.02 - 6.81	1.0*
4/5	1	4	1.80	0.04 - 18.45	0.5973
5/5	0	1	2.36	0.10 - 58.55	0.5884

*Fisher's exact 2-tail.

Table 6.3.7. Association of *NOS2A* haplotypes (as genotypes) with initial response (IR) and non-response (NR) to therapy among 314 Caucasian patients in the HENCORE Study. 214 patients achieved initial response, while 100 were non-responders (NR) to interferon monotherapy.

Haplotype	IR	NR	OR	95%C.I.	p-value
1/1	75	38	0.88	0.52 - 1.49	0.6155
1/2	42	25	0.73	0.40 - 1.35	0.2789
1/3	21	15	0.62	0.29 - 1.35	0.1789
1/4	40	9	2.32	1.05 - 5.68	0.0275
1/5	0	2	0.09	0.01 - 1.93	0.0556
2/2	5	2	1.17	0.19 - 12.50	1.0*
2/3	5	1	2.37	0.26 - 113.12	0.4203
2/4	8	0	8.27	0.47 - 144.78	0.0853
2/5	1	0	1.41	0.06 - 34.97	0.8325
3/3	3	5	0.27	0.04 - 1.43	0.0594
3/4	8	2	1.90	0.37 - 18.69	0.4138
4/4	3	1	1.41	0.11 - 74.62	0.7674
4/5	2	0	2.36	0.11 - 49.71	0.5687
5/5	1	0	1.41	0.06 - 34.97	0.8325

*Fisher's exact 2-tail.

Table 6.3.8. Association of NOS2A haplotypes (as genotypes) with initial response (SR) and non-response (NR) to therapy among 314 Caucasian patients in the HENCORE Study. 112 were sustained responders (SR), while 100 were non-responders (NR) to interferon monotherapy.

Haplotype	SR	NR	OR	95%C.I.	p-value
1/1	39	38	0.87	0.48 - 1.59	0.6309
1/2	25	25	0.86	0.43 - 1.71	0.6465
1/3	13	15	0.74	0.31 - 1.78	0.4664
1/4	16	9	1.69	0.66 - 4.55	0.2336
1/5	0	2	0.18	0.01 - 3.69	0.2078
2/2	4	2	1.81	0.25 - 20.42	0.6861*
2/3	2	1	1.80	0.09 - 107.24	1.0*
2/4	2	0	4.55	0.22 - 95.86	0.2872
2/5	0	0	---	---	---
3/3	1	5	0.17	0.003 - 1.58	0.1024*
3/4	7	2	3.27	0.60 - 32.80	0.1716*
4/4	2	1	1.8	0.09 - 107.24	1.0*
4/5	0	0	---	---	---
5/5	1	0	2.70	0.11 - 67.13	0.5285

*Fisher's exact 2-tail.

CTLA4

Cohort characteristics

Patients in the present study were quite representative of the entire UAB cohort in their characteristics at enrollment (Table 6.3.9). SRs and NRs differed as expected in age, gender, viral load, viral genotype and ALT—all characteristics previously reported to influence response to treatment. Among those with genotype 1 and non-1 infections, 39 and 40 were SRs while 73 and 6 were NRs, respectively. Only one individual who was positive at week 12 achieved sustained response.

Distribution of CTLA4 promoter and exon 1 allelic variants and haplotypes

The frequencies of the *CTLA4* promoter and exon 1 genotypes in Caucasians approximated Hardy-Weinberg equilibrium and were comparable to those reported elsewhere (for the promoter: C/C: 83.6%; C/T: 14.6%; T/T: 1.8%; for exon 1: G/G: 10.4%; G/A: 44.8%; A/A: 44.8%).[54, 64] This study observed strong linkage disequilibrium between exon 1 position 49 and promoter -318 alleles, and the haplotypes observed are comparable to those reported in other populations.[54, 64]

In univariate analysis of Caucasian SRs with genotype-1 infections, 49G allele carriers and 49G/G genotype homozygotes were significantly more frequent among SRs {(OR=2.3; p=0.042) and (OR=5.2; p=0.049), respectively} (Table 6.3.10). Carriers of the promoter –318C variant were frequent among both SRs and NRs, but more so among SRs. As measured by allele frequency based on 2N chromosomes, a two-fold higher likelihood of response was seen with both the 49G (OR=2.1, p=0.017) and the –318C variant. Carriage of the tightly linked -318C-49G haplotype also conferred a two-fold higher likelihood of response (OR=2.4; p=0.030). The magnitude of the association of SR with homozygosity for the haplotype was even higher (OR=5.2; p=0.04). The nearly exclusive haplotype pairing of 49G with –318C prevented separate analysis of 49G alone; every effect seen with 49G also occurred with the –318C-49G haplotype at the same magnitude. The dimorphic nature of the –318 and 49 SNPs produced reciprocal effects (inhibition of response) for the –318T and 49A variants and carrier frequencies. Neither the 49G nor the haplotype effect was observed in patients infected with non-1 genotype viruses (p> 0.25 for all).

Table 6.3.9. Baseline characteristics of Caucasians with hepatitis C infection enrolled in trials of interferon and ribavirin in the UAB cohort.

Characteristics	A. Comparison of the entire Caucasian cohort with patients selected for the present study		B. Comparison of selected sustained responders (SRs) and non-responders (NRs)	
	Entire Cohort (N=312)	Selected Study group (N=158)	SRs (N=79)	NRs (N=79)
baseline viral load (mean ± SD)	$2.82 \times 10^6 \pm 1.96 \times 10^6$	$2.97 \times 10^6 \pm 1.98 \times 10^6$	$2.61 \times 10^6 \pm 2.05 \times 10^6$	$3.32 \times 10^6 \pm 1.84 \times 10^6$
[International Units ± SD][a]	$[1.17 \times 10^6 \pm 8.34 \times 10^5]$	$[1.23 \times 10^6 \pm 8.42 \times 10^5]$	$[1.09 \times 10^6 \pm 8.70 \times 10^5]$	$[1.37 \times 10^6 \pm 7.86 \times 10^5]$
baseline ALT (mean ± SD)	105 ± 81.9	105.2 ± 82.8	110.6 ± 93.6	99.4 ± 71.6
Age at treatment (mean ± SD)	45 ± 8.8	44.5 ± 8.1	43.5 ± 7.9	45.2 ± 8.1
HCV genotype (type-1 (%) / non-1 (%))	73.4 / 26.6	70.9 / 29.1	49.4 / 50.6	92.4 / 7.6
Male (%) / female (%)	55.3 / 44.7	57.3 / 42.7	51.3 / 48.7	63 / 37

[a] International Units approximated using a conversion factor. (Konnick 2002)

Table 6.3.10. Differential distribution of allele frequency, allele carriage and genotype for the -318 SNP, the 49 SNP and the -318-49 haplotype among 39 Caucasian sustained responders (SR) and 73 non-responders (NR) infected with genotype-1 virus infection.

MARKER	SR (%) (N=39)	NR (%)[a] (N=73)	OR	95% C.I.	P-VALUE
Allele/haplotype (carriage)[b]					
-318C	38 (97.4)	70 (95.9)	3.8	0.19 – 75.9	0.350
-318T	4 (10.3)	15 (20.5)	0.45	0.14 – 1.48	0.186
49G	25 (64.1)	32 (43.8)	2.3	1.0 - 5.1	0.042
49A	34 (87.2)	71 (97.2)	0.2	0.04 - 0.9	0.049
-318C-49G	25 (64.1)	31 (20.5)	2.4	1.1 - 5.4	0.030
Genotype					
49G/G	5 (12.8)	2 (2.7)	5.2	1.0 - 28.3	0.049
49A/A	14 (35.9)	41 (56.2)	0.4	0.2 - 1.0	0.042
-318C-49G / -318C-49G	5 (12.8)	2 (2.7)	5.2	1.0 - 29.2	0.049
-318C-49A / -318C-49A	12 (30.8)	33 (45.2)	0.5	0.3 - 1.3	0.139

[a] NRs served as the referent group for all calculations.
[b] Analyses performed using allele frequencies based on 2N chromosomes (SR=78 and NR = 146) showed comparable ORs and p values).

Multivariable analyses

The associations of the 49G variant and the corresponding –318C+49G haplotype persisted after multivariable adjustment for baseline characteristics {gender, age at treatment, interaction between age at treatment and gender, baseline viral load, baseline ALT and possession of the *IL10* (108) TCATA haplotype} (Table 6.3.11) in both the group of 158 patients and in the subgroup with genotype 1 infections. Control for effect of homozygosity for the *IL10* (108) TCATA haplotype previously reported to be associated with SR to IFN+R was precluded by the small number of individuals with this haplotype (N=5).[62] However, when considered jointly with the *CTLA4* variant, carriage of a single *IL10* (108) TCATA haplotype, also previously associated with SR, actually demonstrated a stronger association (OR=3.0 when adjusted for 49G carriage or –318C+49G carriage; p=0.02) than was originally reported (OR=1.6; p=0.30).[62] High level (multiplicative) interaction due to carriage of 49G or its haplotype plus carriage of *IL10* (108)TCATA was not detected.

Longitudinal analyses of viral dynamics

In an analysis of the effect of these markers on viral dynamics during the initial 12 weeks of therapy among the entire group of 158, 49G carriers and non-carriers showed significant differences in viral load (Figure 6.3.1). Viral RNA concentration declined more rapidly in 49G carriers (p=0.0095); lower levels were seen in carriers at each successive point, with the greatest difference occurring by week 4 (p=0.0053) with the difference being less pronounced by week 12 (p=0.0718) (Figure 6.3.1). Both the apparent acceleration in response among 49G carriers and the interaction between 49G carriage and time on viral dynamics were significant. Patients with the –318C+49G

Table 6.3.11. Multivariable analyses of the effects of 49G allele and -318C-49G haplotype carriage on sustained response to IFN+R in 158 Caucasian patients

A. MULTIVARIABLE MODEL FOR 49G	OR	95% C.I.	P-VALUE
49-(G) allele carriers	2.0	1.02 - 3.97	0.04
Baseline viral load	1.7	0.85 - 3.40	0.14
Baseline ALT	0.98	0.487 - 1.96	0.95
Gender	0.04	0.002 - 0.91	0.04
Age at treatment	0.27	0.03 - 2.7	0.26
IL-10 (108)-TCATA allele carriage	3.0	1.2 - 7.4	0.02
Interaction between sex and age	4.1	0.77 - 22.2	0.1

B. MULTIVARIABLE MODEL FOR -318C-49G	OR	95% C.I.	P-VALUE
-318-(C) + 49-(G) haplotype carriers	2.0	1.0 - 3.9	0.04
Baseline viral load	1.7	0.87 - 3.5	0.12
Baseline ALT	1.0	0.49 - 2.0	0.95
Gender	0.04	0.002 - 0.9	0.04
Age at treatment	0.26	0.025 - 2.65	0.25
IL-10 (108)-TCATA allele carriage	3.0	1.2 - 7.3	0.02
Interaction between sex and age	4.2	0.77 - 22.3	0.1

C. MULTIVARIABLE MODEL FOR 49G AMONG THOSE WITH GENOTYPE-1 INFECTIONS ONLY	OR	95% C.I.	P-VALUE
49-(G) allele carriers	2.5	1.1 - 6.0	0.04
Baseline viral load	0.50	0.21 - 1.2	0.12
Baseline ALT	1.4	0.58 - 3.6	0.43
Gender	2.5	0.82 - 7.6	0.11
Age at treatment	0.41	0.02 - 7.0	0.54
IL-10 (108)-TCATA allele carriage	2.6	0.81 - 8.5	0.11
Interaction between sex and age	1.1	0.18 - 6.4	0.94

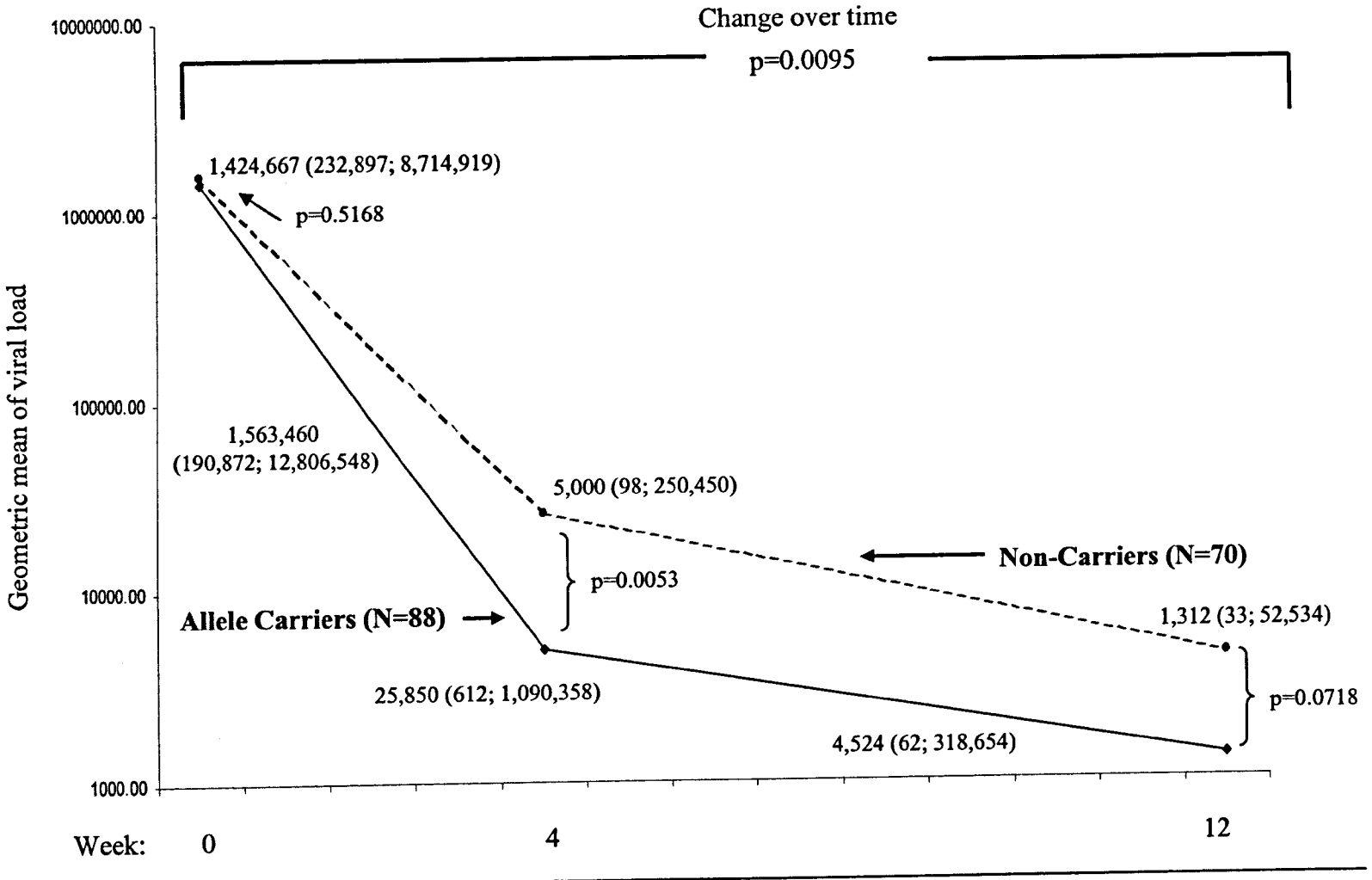

Figure 6.3.1. Effects of CTLA4G variant carriage on viral dynamics during the first 12 weeks of therapy among 158 Caucasian individuals in the study. The geometric mean of HCVRNA levels (copies/mL) among 88G carriers and 70 non-G carriers Are plotted on a log scale against the duration of therapy. Measurements (mean ± SD) are shown for weeks 0, 4, 12.

haplotype and with HCV genotype 1 only showed comparable longitudinal effects (data not shown).

6.4 Conclusions

Interferon-stimulated genes

These observations suggest that polymorphisms in the interferon-induced genes may be involved in determining the outcome of hepatitis C virus infection. With respect to the *MxA* gene, the TT and the GT genotypes at the –88 locus were associated with self limiting infection, suggesting that the T allele conferred an advantage in HCV infection. Furthermore the GT genotype was found more frequently in those with an initial response or sustained response to interferon therapy. This observation is consistent with a previous report in a Japanese population in which the GG genotype was associated with non-response to interferon.[60, 68] Interestingly the allele frequency of the T allele is much lower in our Caucasian population than in the Japanese population (14.5% for Caucasians and 29% for Japanese). The apparent effect of the T-allele is supported by *in vitro* functional work suggesting that this variant has higher transcriptional activity than the T allele when stimulated with interferon-α.[68] This is due to the fact that the polymorphism at position -88 lies within a sequence element similar to an interferon sensitivity response element (ISRE), and the T-allele increases this similarity.[60] Consequently, patients with the G/G genotype at position –88 may produce a sub-optimal MxA response when given interferon. Clinical studies have also suggested that those who respond to interferon treatment express increased amounts of MxA mRNA during treatment.[12]

There is an association between the 3'-UTR OAS-(G/G) genotype with self-limiting infection, but not with response to therapy. This observation may be explained by studies suggesting that OAS levels do not change with the administration of interferon or that levels may elevate and quickly dimish.[69, 70]While levels of OAS protein expression have been reported to be elevated in chronic HCV infection, administration of exogenous interferon does not appear to have a modulatory effect on its expression.[69, 71-73] It is quite possible that the natural induction of interferon by an infectious agent such as HCV is

different from the administration of exogenous interferon or that persistent induction of OAS through chronic HCV infection reduces sensitivity to exogenously supplied interferon. Thus, it is possible that OAS plays a greater role in mediating self-limiting versus persistent HCV infections rather than during the stage after viral persistence is established.

The length of the alleles in the PKR promoter trinucleotide repeat polymorphism influenced the outcome of infection. Patients who possessed one short allele were approximately two fold more likely to spontaneously clear HCV whereas those with two short alleles were estimated to have 18 fold increased chance of self limiting infection although this fails to reach statistical significance. Variation of the number of repeats in this PKR microsatellite may alter binding capabilities of transcriptional factors and affect PKR expression during interferon therapy. Expansion of the number of trinucleotide repeats in a promoter regions has been correlated with transcriptional silencing as in the FMR1 gene and Fragile X syndrome.[73] While the number of repeats in the FRM1 gene expands from 10-50 to 52-2000, the present study only found a maximum number of 10 repeats in the 5' untranslated region of the *PKR* gene, and the effect of the smaller number of repeats remains unknown.

Our observed differences in the association between the long/long repeat and response to therapy and lack of association between this genotype and self-limiting infection may be due to, at least in part, differential effects from endogenously derived interferon (as with self-limiting infection) and exogenous interferon (as with anti-viral therapy) or differences in the level of interferon between natural infections and therapies. While PKR expression occurs at a low level under most physiological conditions, its expression is greatly enhanced within a few hours of interferon treatment.[6] The large dose of interferon (3 million IU) given during interferon therapy may strongly increase PKR expression and the increased quantity of PKR may facilitate enhanced clearance of HCV.

Our study also suggests that the SNP at position –168 in the *PKR* gene may also be important in the outcome of HCV. However, in this case it appears that heterozygosity confers an advantage for spontaneous elimination. This observation may be explained in a number of ways. Firstly, heterozygosity may in fact confer a beneficial effect. Such observations have been reported initially for hemaglobinopathies in falciparum malaria and

more recently for MHC class II genes for hepatitis B virus infection.[74, 75] Second, the association may have arisen through a systematic genotyping error. This is unlikely as the genotyping was performed by sequence analysis and for the cohort as a whole the genotypes conform to Hardy Weinberg equilibrium. Thirdly this may arise through latent ethnic stratification as the cohort was recruited in nine different European centres. Again this is explanation is not tenable as the allele frequencies of this polymorphisms did not vary significantly between the centres. Finally, the difference in genotype frequencies may have occurred by chance.

The present study highlights the important role of interferon-induced genes and the outcome of HCV. In addition it identifies important polymorphisms that may be further examined for potential use as genetic markers of HCV disease outcome.

NOS2A haplotypes and hepatitis C

The observations suggest that *NOS2A* gene haplotypes may be involved in determining the outcome of HCV infection. Homozygosity for haplotype 2 and the combination of haplotypes 2/4 were associated with self-limiting infection. Stratification by gender suggested that the effect of the homozygous haplotype 4 was stronger among males than females. These differences by gender are not surprising, as the natural history of HCV has been observed to differ with respect to gender. Specifically, with respect to self-limiting infection, some studies suggest that females are more likely to clear HCV viraemia naturally than men.[76, 77] It is plausible that *NOS2A* polymorphisms exert a differential biologic effect in males and females.

There is also an association of the homozygous haplotype 3 with non-response (in a comparison with initial responders). Unfortunately, the frequency of haplotype 3 homozygotes is low. Consequently stratified analysis was precluded. The combination of haplotypes 1 and 4 was associated with initial response to interferon along with haplotypes 1 and 5. The relatively low numbers of individuals with these haplotypes also precluded meaningful stratified analyses.

Determination of whether effect underlying our observed associations are due to the haplotype as a whole or the individual loci must await functional studies. It is also plausible that these observed associations are due to linkage disequilibrium with another as yet unidentified marker, and that the observed haplotypes merely reflect linkage with another locus. Genes in this region include kinase suppressor of RAS (*KSR*) and *NOS2c* on the centromeric side and 2 genes of unknown function on the telomeric side.[78] Nevertheless, these observations are important in highlighting the potential importance of the inducible nitric oxide synthase pathway and the region surrounding the *NOS2A* gene, in the outcome of HCV infection. Future studies should also address the functionality of *NOS2A* haplotypes. In addition, future studies are needed to replicate these findings and it is important that these studies also be sufficiently powered to not only accommodate simultaneous adjustment for potential confounding variables through multivariate analyses, but the analysis of host-pathogen interactions, such as the possible interaction of gender with host genetics.

CTLA4 and HCV Therapy

Polymorphisms in *CTLA4* may augment the current array of predictors of therapeutic response to IFN+R. For genotype-1 infection in Caucasians with sustained response the present study observed associations of the allelic variants and the homozygous genotypes consisting of the exon 1 49G and the linked promoter SNP –318C. Tight linkage disequilibrium of 49G with –318C accounted for the apparent advantage for SRs due to each marker individually and to the corresponding –318C+49G haplotype; the 49G and the haplotype effects could not be distinguished from each other. The relationships of carriage of these markers were independent of previously described cofactors for response (e.g. age, gender, baseline viral load and the *IL10* (108)-TCATA polymorphism). The finding that treated carriers of those *CTLA4* variants reached lower levels of viraemia more rapidly than treated persons not carrying them further attests to the likely biologic/clinical significance of the association.

This study population, the apparent advantage of the one or both alleles, or the corresponding haplotype, was confined to infection with genotype-1 virus in Caucasians.

Genotype-1 viruses may differ intrinsically from the others in the magnitude or quality of the T-cell response they elicit. In a study of the differential effect of interferon (IFN) on antiviral T-cell responses, those infected with non-1 virus had stronger IFN-induced T-cell responses to HCV core antigen.[79]

Any of several mechanisms may account for these observations. First, the polymorphisms, either individually or as a haplotype, may confer a difference with respect to gene expression. Carriage of the –318T allele has been associated with increased levels of CTLA4 mRNA compared with –318C/-318C homozygotes.[56] Similarly, 49G has been shown to have a reduced effect on CTLA4 up-regulation.[55] Another study of the functional effects of the haplotypes formed by these two loci demonstrated increased expression of CTLA4 by the –318T-49A haplotype.[54] Thus, investigative efforts to date consistently suggest that –318C, 49G, or the –318C-49G haplotype plays a role in down-regulating CTLA4, presumably thereby amplifying the T-cell response in some individuals. Alternatively, since CTLA4 ligands B7-1 (CD80) and B7-2 (CD86) may play a role in the Th1/Th2 developmental pathways, polymorphism in *CTLA4* could shift the Th1/Th2 balance.[80-82] A third possibility is that these SNPs are in linkage disequilibrium with an adjacent marker that, by itself or as part of the haplotype, may alter CTLA4 expression and/or function. Such a marker could instead account for the association by some other mechanism that enhances the T-cell responses believed to govern both natural acute viral clearance and equilibrium concentration of virus during IFN+R therapy.[27, 83]

This study had certain limitations. First, the small number of patients infected with non-1 HCV genotypes precluded meaningful analysis of associations between *CTLA* polymorphisms and response to therapy in those individuals. Second, the study was conducted before assays for plasma HCV concentration were standardized or results were reported in international units (IU).[84] An HCV-RNA copy has not been officially defined, measurement variability between assays still exists, and the correlation of RNA copies with IUs is uncertain.[84-90] The factor used to convert measured levels of HCV-RNA into IUs only approximates the true value. Future studies attempting to replicate our observed epidemiologic associations may be more informative if they measure viral loads directly in IUs. Third, this study was conducted in a single Caucasian population; the associations of

CTLA4 polymorphism with response to interferon therapy should be examined in other ethnic groups and populations.

In summary, successful viral clearance during therapy for HCV infection appears to depend on robust T-cell responses facilitated by the reduction of CTLA4 levels, and diminished i*n vitro* expression of CTLA4 has been found in the presence of *CTLA4* polymorphisms associated with improved viral clearance. [53, 54] With further clinical validation of this phenomenon, it will remain to be determined precisely how the *CTLA4* alleles/haplotype, along with the *IL10* (108)-TCATA haplotype, influence the pathogenesis of HCV infection and its prognosis following antiviral therapy. Meanwhile, beyond the implication that the co-stimulatory pathway involving CTLA4 affects the outcome of therapy for HCV infection, there is the prospect that the *CTLA4* gene or its product might represent a separate target for therapeutic intervention.

6.5 Chapter 6 References

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Chapter 7

Conclusions, Public Health Impact and Future Directions

7.1 Summary

In this thesis, I have addressed several important aspects relating to chronic hepatitis C virus (HCV) infection: co-morbid conditions in HCV infection (namely the formation of anti-nuclear autoantibodies) and the identification of genetic polymorphisms that may affect HCV natural history. Below, I will summarise the major findings and discuss their implications, as well as offer some ideas for future research directions.

7.2 Host Genetics

Host genetic factors that may affect HCV outcome have been recognised as an important aspect of HCV research.[1] While there has been a substantial body of literature produced aimed at identifying genetic markers for HCV outcome, there have been mixed results. A more positive example is the reproduced association of DQB1*0301 with self-limiting infection. In contrast, studies such as those of the haemochromatosis gene (*HFE*) and the progression of liver fibrosis have been inconsistent.

The present study used the candidate gene approach to study correlations between several genes and the outcome of HCV infection. The study examined whether polymorphisms in several interferon-stimulated genes (*PKR*, *OAS*, and *MxA*) are associated with HCV outcome. Several associations were observed. Unfortunately, the relative frequency of these variants in the Caucasian population studied would preclude its successful and cost-effective use as a screening marker to assist in determining an individual's chances of responding to therapy. Nevertheless, this study implicates the importance of the interferon-stimulated genes pathway in the outcome of HCV infection.

Similarly, we examined whether haplotypes in the *NOS2A* gene were associated with HCV outcome. Although we observed several relationships, the relative frequency of these haplotypes in the Caucasian population precludes its effective use as a screening marker for prognostic tests of disease outcome. However, our study highlights the importance of the inducible nitric oxide synthase pathway in HCV infection. The identification of these pathways also may be a target for future therapeutic strategies. We also observed a variant of the *CTLA4* gene, and its corresponding haplotype associated with responsiveness to interferon+ribavirin therapy. The frequency of the variants in this gene occur at a relatively high frequency. If future larger-scale studies confirm the associations reported in the present study, then research should explore combining these, along with other genetic variants that may be associated with therapeutic outcome in HCV, with existing factors known to be predictive of response such as viral genotypes and patient race and gender. By "screening" for those individuals who would most likely not ultimately response to interferon therapy, needless suffering from the multiple and often debilitating side effects of interferon therapy may be avoided. In addition, the costs of interferon therapy may be better spent on an individual who would have a higher chance of responding. Future research will be needed exploring new combinations of algorithms that incorporate genetic markers with existing prognosticators of response.

Another future direction for drug therapy concerns the custom tailoring of drug regiments to a person's specific genetic makeup.[2] For example if a person has variant A at a specific locus that is known to be highly correlated with response to a specific regimen, that person will be given a drug cocktail that includes the specific drug, while those with variant B are given another.[2]

The public health impact of genetic prognosticators is high. Identification of successful markers for screening of individuals who would likely not benefit from expensive and debilitating therapies like those involving interferon will prevent needless suffering in patients who will ultimately not benefit from these therapies, as well as direct limited financial resources towards those who will more likely benefit from these expensive therapies.

There is also an urgent need for large-scale, properly-designed epidemiologic studies to allow for the systematic study of genetic factors in HCV. As mentioned in chapter 2 (systematic review of genetic factors and HCV natural history), it is important for future studies to be sufficiently powered to detect meaningful relationships with respect to genetic factors and HCV outcome.

7.3 Autoimmunity in HCV: Antinuclear antibodies

In the study of antinuclear antibodies (ANA) and HCV outcome, no adverse effects of ANA positivity on the response to interferon therapy was detected. This observation has important ramifications with respect to HCV treatment, as it suggests that the estimated 4%-41% of individuals with low titre ANA should not be precluded from treatment with interferon. Interestingly, we detected an almost two-fold higher association of ANA positivity with faster progression of HCV-related fibrosis. Although this association did not reach statistical significance, the strength of association suggests that it may be worthwhile to explore this relationship further. If future larger studies, are able to replicate this association, then it may warrant the further exploration of the biological mechanisms behind this epidemiologic observation. Such studies may facilitate the understanding of fibrogenesis in general as well.

The present study identified an association between female gender and a higher likelihood of developing ANA, which is consistent with the association of female gender with autoimmunity in general. I also observed an association between latitude and increased prevalence of ANA, similar to the reports of increased cryoglobulins in southern Europe compared to northern Europe. Further research employing ANA positivity in HCV as a paradigm for autoimmunity may lead to a better understanding of autoimmunity in general.

7.4 Concluding thoughts

In this dissertation I have addressed several important aspects concerning the clinical outcomes of HCV infection. In addition to exploring genetic predictors of HCV outcome, I have also examined the effect of comorbidities on the clinical outcome of hepatitis C. In addition to identifying important clinical observations such as the important lack of a negative effect of ANA positivity on HCV outcome, I have identified important avenues for future research, such as the identification of genetic associations to be replicated in further studies as well as pathways that may be further explored in basic research. Collectively, the research included in this thesis offers a piece to the hepatitis C puzzle.

Chapter 7 References

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Appendices

Appendix 1

List of key demographic variables in the St. Mary's Hospital/HENCORE database

List of key demographic variables in the St. Mary's Hospital/HENCORE database

- Patient ID
- DNA ID Number
- HENCORE Study number (if part of HENCORE)
- Centre (if part of HENCORE)
- Ethnic Origin
- Gender
- Route of Infection, if known
- Year of HCV infection, if known
- Did they receive anti-viral therapy
- Anti-viral therapy regimen type
- Response to anti-viral treatment
- HCV viral genotype
- Date of first liver biopsy
- Fibrosis of biopsy #1 (Metavir)
- Fibrosis of biopsy #1 (HAI)
- Inflamamtory score biopsy #1
- Date of biopsy #2, if done
- Fibrosis of biopsy #2 (Metavir)
- Fibrosis of biopsy #2 (HAI)
- Inflamamtory score biopsy #2
- Current alcohol intake
- Previous alcohol intake
- HBV co-infection

Appendix 2

List of key demographic and clinical variables in the Alabama hepatitis C cohort database

List of key demographic and clinical variables in the Alabama hepatitis C cohort database:

- Patient ID
- DNA ID Number
- Centre (Birmingham or Mobile)
- Race
- Gender
- Level of education
- Route of Infection, if known
- Year of infection, if known
- HCV viral genotype
- Baseline viral load
- Week 4 viral load
- Week 12 viral load
- Week 24 viral load
- Week 48 viral load
- 6 month follow-up viral load
- Baseline liver function tests (ALT and AST)
- Week 4 liver function tests (ALT and AST)
- Week 12 liver function tests (ALT and AST)
- Week 24 liver function tests (ALT and AST)
- Week 48 liver function tests (ALT and AST)
- 6 month follow-up liver function tests (ALT and AST)
- Baseline platelet
- Week 4 platelet
- Week 12 platelet
- Week 24 platelet
- Week 48 platelet
- 6 month follow-up platelet
- Baseline neutrophils
- Week 4 neutrophils
- Week 12 neutrophils
- Week 24 neutrophils
- Week 48 neutrophils
- 6 month follow-up neutrophils
- Previous alcohol intake

Appendix 3

Laboratory Protocol for Antinuclear Antibody (ANA) Detection by Indirect Immunofluorescence

ANA Laboratory Protocol:

Detection of antinuclear autoantibodies (ANA) by indirect immunofluorescence on Hep-2 cell substrates

Prepared by LJY on 21 February 2002

Reagents needed:

Reagent	Catalogue number	Company
Hep-2 substrate slides	FS001.2	Binding Site
Goat anti-human Ig FITC-conjugate	FA003	Binding Site
(at working dilution 1:100 or 1:50—see manufactuer's recommendations)		
DABCO (1,4-diazabicyclo-(2,2,2) octane)	D 2522	Sigma
PBS Tablets	P4417	Sigma
Glycerol	G5516	Sigma
Clear Nail varnish (polish)	---	Any store
Positive and Negative control sera	Pooled patient sera	Immunology Lab

Other supplies needed:

Serum samples

3 plastic boxes (for washes and staining slides)

curvettes or microfuge tubes (for dilution of samples)

box or rack to hold samples and curvettes during dilution stage

cotton swabs ("Q-tips")

slide cover slips

towels for drying backs of slides

Pasteur (serologic) pipettes

Pipettors with corresponding tips

Preparation of Solutions needed:

PBS

1) As usual.

Mounting solution:

1) Dissolve 5g of DABCO in 20mL PBS using a stirrer (will take a while to dissolve).

2) Add this 20mL solution to 380 mL of glycerol.

3) Mix well.

Brief Theory

Hep-2 cells are used to express substrate antigen for binding of anti-nuclear autoantibodies (ANA) present in positive sera. Excess sera is then washed off. Anti-Ig with FITC (fluorescein isothyocyanate) is then applied to bind to any human antibodies that may have bound to the substrate and excess washed off. Samples with positive ANA will then bind this secondary antibody. If positive, the wells will light up in a green colour when examined under a fluorescent microscope. The pattern of immunofluorescence can then be read as well.

Procedure:

Part A: Preparation of samples:

1) Place samples in a rack, along with empty curvettes in front.
2) Label curvettes with appropriate number for identification of patient number.
3) First 6 wells on the first slide in each batch are for controls (Well 1 for neg control and 2-6 for positive control dilutions)
   NOTE: dilutions are done at 1:40, 1:80, 1:160, 1:320, and 1:640.
4) Last 5 tubes are for dilutions of previous positive samples (although this set-up may vary)
5) Place serum samples in tray analogous to slide set-up illustration on the accompanying page, skipping rows. Place the curvettes in front of the respective serum. Typically a 3-slide configuration is used; however, more may be added—but be sure to include the first slide as is, as it has all the controls.

SUMMARY OF SERUM QUANTITIES NEEDED:

10μL serum + 390 μL PBS for samples and 1:40 dilution

200 μL of the previous dilution (i.e. 1:40) + 200μL PBS for subsequent ones

6) Add 390 μL PBS to all curvettes for the following samples: negative control, positive control 1:40 dilution only, samples, and 1:40 sample of dilutions only.
7) Add 200 μL PBS to the curvettes for the following dilutions on the positive controls as well as the samples undergoing dilution: 1:80, 1:160, 1:320, 1:640.
8) Place 10μL negative control sera in appropriate (first) curvette.
9) Place 10μL sera into the 1:40 dilution curvettes and patient samples only.
10) Do doubling dilutions by taking 200μL 1:40 mixture and adding to the 1:80 mixture.

11) After mixing well, take 200 µL of this mixture and add to the 1:160 mixture and so forth, so do sequential doubling dilutions.

Part B: Sample substrate binding:

1) Remove slides with pre-fixed Hep-2 cells from freezer, remembering to log the batch number down onto sample sheets and filling in the other appropriate information, such as sample identification numbers.
2) Label each slide at the top with "ANA" and slide number. Number wells, place the date at bottom of slide.
3) Place slides in the "damp box." This is a box with wet towels at the bottom. Make sure they are level.
4) Apply 20µL of each sample to the appropriate well. Be careful to be neat and make sure the edge of the meniscus reaches the borders of the well.
5) Stand in the damp box for 30 minutes to bind at room temperature.
6) THAW CONJUGATE at this time.

Part C: First wash to remove unbound antibodies:

1) Prepare the wash basin by filling about 2 cm deep with PBS.
2) Take slides and a wash bottle with PBS and tilt the slide to the side and carefully spray off the sample over the catch basin, being careful not to let samples run over other wells and contaminate.
3) Place slides in wash basin, submerging it in the PBS.
4) Repeat for all slides.
5) Place at room temperature for 20 minutes. It is not necessary to have it shaking.
6) Centrifuge conjugate for a couple of minutes at this time to shoot particulates to the bottom of the tube.

Part D: Application of FITC-conjugate:

1) Remove slide from PBS solution and give a few good flicks to remove excess PBS.
2) Since the slide is already wet, add just 10 µL conjugate to each well.
3) Incubate in the wet box for 30 minutes at room temperature.
4) NEVER let the wells dry-out.

NEVER EVER USE THE REMAINING DREGS OF CONJUGATE!!!

This contains particulates that might ruin your final product or even appear like speckled pattern ANA!

Part E: Final wash of conjugate:

1) Over the catch basin, squirt PBS over the slide. Since all wells are covered by the same conjugate, you don't have to be as careful with run-over this time.
2) Place slides in wash basin with the PBS (can use same PBS from previous wash) for 20 minutes at room temperature.

Add Chlorox bleach to the solution in the basin when finished and mix and sit for a while. Then dispose of as you would normal contaminated waste.

Part F: Mounting of slides:

1) Flick the slides a few times to rid it of excess PBS.
2) Put a drop of the DABCO/PBS solution over each well.
3) Place the cover slip on a towel.
4) Invert the slide and gently touch to cover slip starting from one side, to not catch any bubbles.
5) After making sure there are no bubbles in the wells.
6) Seal the cover slip to the slide using fingernail varnish along the sides, being careful not to put too much or cover the wells to obscure reading.
7) Wipe any excess glycerol solution off, especially from the back side so that it doesn't stain the microscope.

Slides are now ready for scoring. They should be kept in the refrigerator at 4°C and should be read within 48-72 hours.

Appendix 4

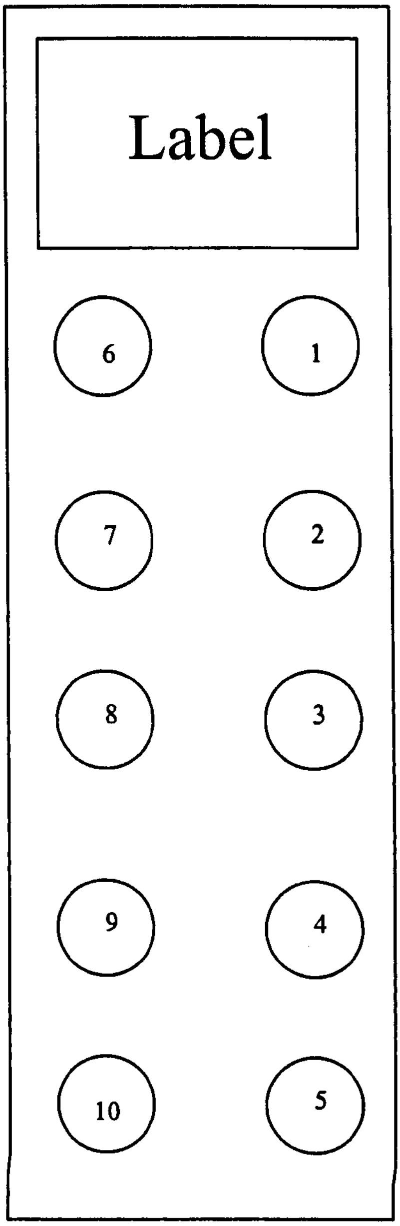
Antinuclear Antibody (ANA) Data Collection/Entry Sheet

Data Entry Completed: ☐ Double Entry Completed: ☐

ANA Slide Set-Up Record Sheet

Date Run: _____/_____/_____ **Slide No.: ________**

Slide Batch No.: _________



ID: ____________________
Pos. Neg Titre:______
HMG Speck. Nucl Other

ID: ____________________
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Appendix 5

Data Dictionary for Antinuclear antibodies (ANA) in Hepatitis C Study Dataset

DATA DICTIONARY

Anti-nuclear antibody (ANA) dataset

Variable name	Explanation	Coding
ID	Unique ID number for each patient	6-digit number
DNAID	Unique ID number to identify the DNA sample in the laboratory	1-digit alpha followed by a 1-3 digit number
CENTRE	The study center the patient is from	1= London, United Kingdom 2= Padova, Italy 3= Lund, Sweden
SERUM	Unique ID number of serum	5-digit number
YOB	Year of the patient's birth	4-digit number
RACE	Patient's race	1=Northern European 2=Southern European 3=Indian/Indian subcontinent 4=Black African 5=North African 6=Eastern European 7=Afro-Caribbean 8=Oriental 9=South American 10=Middle eastern
AGE	Age of patient in 2003	2-digit number
YOI	Estimated year of acquisition of HCV infection, if known	4-digit number
DUR	Estimated duration of infection as of 2003. Based on YOI variable as above	1 to 2-digit number
AGEINF	Estimated age at infection Based on variables above (AGEINF= YOI – YOB). For individuals infected during their first year of life, 1 is entered rather than 0.	1 to 2-digit number

ROUTE	most probable route of HCV acquisition	1= injection drug use 2= blood products 3= sex 4= occupational exposure
GENDER	Patient's gender	1= male 2= female
POS	Whether sample is ANA positive	0= negative 1=positive
PATTERN	For positive samples, the pattern of ANA staining	1= homogenous 2= speckled 3= nucleolar 4= other undefined pattern
COMMENTS	Comments about the pattern, (for unusual patterns, if any)	alpha characters
TITRE	For positive samples, the highest positive titre	1= 1: 40 2= 1: 80 3= 1: 160 4= 1: 320 5= 1: 640
YRBX1	The year of the patient's first liver biopsy	4-digit numberic
BXNI	necro-inflammatory score for the biopsy	1-8 ordinal categorical
FSCORE	The Knodell fibrosis score for the biopsy	1-6 ordinal
RESP	The patient's response to anti-viral therapy	1= non-responder 2= sustained responder 3= relapsed responder
RXYEAR	Year of anti-viral therapy	4-digit
ETOH	Level of previous alcohol use	1= none 2= minimal 3= moderate 4= heavy
VGENO	HCV viral genotype	1= genotype 1 2= genotype 2 3= genotype 3 4= genotype 4 88= mixed genotypes 1 and 2

Appendix 6

List of Thesis-Related Publications

Appendix 6

Thesis-Related Publications

1. Thursz MR and Yee LJ. Immunogenetics of HCV Chronicity and Response to Therapy. (in press: to be published in the Supplement for the 2003 Annual Meeting of the European Association for the Study of the Liver (EASL)).

2. Yee LJ and Thursz MT. Genetic diversity in the major histocompatability complex and the immune response to infectious diseases. In: Susceptibility to infectious diseases: the importance of host genetics. Richard Bellamy, editor. (in press)

3. Yee LJ, Hall AJ, and Thursz MR. Comorbid conditions in chronic hepatitis C virus infection: the role of autoimmunity. (In press: Proceedings of the 11th International Symposium on Viral Hepatitis and Liver Disease)

4. Yee LJ, Perez KJ, Tang J. et al. Association of *CTLA4* polymorphisms with sustained response to interferon + ribavirin therapy for chronic hepatitis C virus infection. *Journal of Infectious Diseases* (2003) 187 (8); 1264-1271.

5. Knapp S, Yee LJ, Frodsham AJ, et al. Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of *MxA*, *OAS-1* and *PKR*. *Genes and Immunity* (in press).

6. Yee LJ, Kelleher PK, Goldin RD, et al. Antinuclear antibody positivity in chronic hepatitis C virus infection: correlates of positivity and clinical relevance (in review)

7. Yee LJ, Knapp S, Burgner D, et al. Inducible nitric oxide synthase (*NOS2A*) haplotypes and the outcome of hepatitis C virus infection (in review)

8. Yee LJ. Host genetic diversity and the outcome of hepatitis C virus infection: a systematic review and meta-analysis. (in preparation)