T-Lymphocyte Senescence and Hepatitis C virus infection

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This dissertation is submitted for the degree of Doctor of Philosophy

For my wife, Sue

DECLARATION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text or the acknowledgements.

This thesis has not been submitted, either wholly or in part, for a degree, diploma or other qualification at any other university.

Matthew Hoare

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Thesis abstract

Hepatitis C virus (HCV) infection is a leading cause of cirrhosis and hepatocellular carcinoma. The degree of fibrosis progression and treatment-related outcomes are critically dependent on the age of the infected individual. Progressive ageing is associated with a decline in the efficacy of adaptive immune system function. T-lymphocytes from aged subjects demonstrate multiple phenotypic and functional changes, including telomere shortening. Short telomeres are associated with poor proliferative capacity, pro-inflammatory responses and increased mortality in clinical studies.

This research aimed to study telomere length changes in T-lymphocytes in chronic HCV infection and its relationship to clinical endpoints. Further, the intracellular signalling changes in T-lymphocytes with short telomeres were studied in subjects with chronic HCV.

Short CD4+ T-lymphocyte telomeres were associated with the presence of severe hepatic fibrosis independent of other known factors. Telomere length was associated with blood markers of hepatic damage and dysfunction as well as histological markers of inflammation and fibrosis. Further, on prospective follow-up, short CD4+ telomere length at enrolment predicted progression to clinical endpoints of hepatic decompensation, development of hepatocellular carcinoma and death. Short CD4+ telomere length predicted a failure to respond to anti-viral treatment for HCV infection.

Unexpectedly, subjects with non-viraemic HCV had short CD8+ telomere length. Liver biopsy tissue from a cohort of subjects with non-viraemic HCV was studied and demonstrated significant inflammation or fibrosis in most.

To study the IFN-α signalling pathway in cells with short telomeres, I utilised the phospho-histone γ-H2AX, a downstream signal from short telomeres. CD8+ T-lymphocytes expressing γ-H2AX had the form and function of cells with end-stage differentiation. γ-H2AX+ cells had a pro-inflammatory cytokine secretion profile with high expression of IFN-γ and low IL-2. Further γ-H2AX+ cells were unable to respond to exogenous IFN-α by phosphorylating Stat1. This failure was attributable to a post-receptor defect.

T-lymphocyte telomere length changes in HCV may underpin the effect of age on clinical and treatment-related outcome. Short telomeres are associated with intracellular signalling defects which may explain the failure to respond to anti-viral therapy.

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List of abbreviations

AAF	Interferon-α activated factor
ALT	Alanine transaminase
AUROC	Area under receiver operator characteristic curve
BMI	Body mass index
CMV	Cytomegalovirus
CT	Computed tomography
DKC	Dyskeratosis congenita
DM	Diabetes Mellitus
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSB	Double strand breaks
EBV	Epstein Barr virus
ER	Endoplasmic reticulum
EVR	Early virological response
FISH	Fluorescence in-situ hybridisation
HAART	Highly active anti-retroviral therapy
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPA	Health Protection Agency
HPV	Human papilloma virus
HR	Hazard ratio
HSC	Hepatic stellate cells
IDU	Intravenous drug usage
IFN	Interferon
IQR	Inter-quartile range
IRF	Interferon regulatory factor
KIR	Killer-cell immunoglobulin-like receptor
LCMV	Lymphocytic choriomeningitic virus
LR	Logistic regression
MCM	Mini chromosome maintenance protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility antigen
MMP	Matrix metalloproteinase
NAFLD	Non-alcoholic fatty liver disease
NK / NKT	Natural Killer / Natural Killer T-cells
OAS	Oligoadenylate synthetase
PBC	Primary biliary cirrhosis
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PKR	Protein kinase R

PPAR	Peroxisome proliferator activated receptor
PPD	Purified protein derivative
PT	Prothrombin time
RBV	Ribavirin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute 1640 medium
SCID	Severe combined immune deficiency
SD	Standard deviation
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
Stat	Signal transducer and activators of transcription
SVR	Sustained virological response
TGF	Transforming growth factor
TRAIL	TNF-related apoptosis inducing ligand
TRAP	Telomere repeat amplification protocol
UAPMP	Unlinked anonymous prevalence monitoring programme
UTR	Untranslated region
VZV	Varicella zoster virus
β-GAL	Beta-galactosidase

Chapter 1 - Introduction

Hepatitis C (HCV) is a blood-borne viral infection which, by current WHO (World Health Organisation) estimates, infects around 170 million people or 3% of the world population. After acute exposure, chronic infection of the individual occurs in around 80% of cases, leading to chronic hepatitis, with the attendant risk of cirrhosis, progressive liver failure and the development of hepatocellular carcinoma (HCC).

Hepatitis C virus infection

Epidemiology

The 2008 UK Health Protection Agency (HPA) report estimates that there are 142,000 cases of HCV infection in the UK [1], albeit with wide 95% confidence intervals (90,000 – 231,000) reflecting the lack of certainty and difficulty in measuring the total disease burden [2]. Measuring the prevalence of HCV infection is fraught with problems given that infection is often asymptomatic. HCV is transmitted largely through exposure to infected blood and blood-products. The predominant risk factor in most new cases of HCV in developed countries currently, including the UK, is intravenous drug usage (IDU) [1]. IDU was identified as the risk factor for acquisition in 92% of new infections with HCV [1] (Table 1).

A variety of epidemiological studies have been performed including the Unlinked Anonymous Prevalence Monitoring Programme (UAPMP), which screens patients attending drug or genitourinary services. The UAPMP suggests that around 40% of active IDUs are seropositive for HCV infection. Worryingly, the acquisition of HCV infection now occurs earlier in the episode of intravenous drug usage. Analysis of IDUs who started injecting within the last three years found that the seroprevalence for HCV was 22%. Clearly, prospective study of IDUs with serial testing for anti-HCV would be the ideal methodology to determine disease incidence. However, IDUs who are prepared to attend serial follow-up are unlikely to be representative of the IDU population as a whole.

Risk factor (where reported)	Number of reports	Percentage
Injecting drug use	10651	92.5%
Transfusion	180	1.6%
Blood product recipient	107	0.9%
Sexual exposure	164	1.4%
Renal failure	73	0.6%
Vertical (mother to baby) or household	36	0.3%
Occupational	13	0.1%
Other	296	2.6%
Total	11520	100%

Table 1. Risk factor information for new laboratory reports of HCV infection in England between 1996 and 2007. Source: (1).

Historically, there was a large cohort of cases who had acquired HCV from blood administered as part of health care provision before the introduction of universal screening for the virus in 1991, following its discovery in 1989 [1]. Many haemophiliacs, given infected factor VIII or IX preparations to treat their coagulopathy, became either singly infected with HCV or multiply infected with HCV and either hepatitis B virus (HBV) and/or human immunodeficiency virus (HIV) [1]. Similarly, there are a number of cohorts of individuals who acquired HCV infection through a single source of either infected plasma or infected anti-Rhesus D immunoglobulin preparations. These cohorts represent a failure of health care provision, but represent good opportunities to study the natural history of HCV infection retrospectively/prospectively from the known time of infection and thus to determine the rate of disease progression accurately [3, 4].

In other countries the prevalence and risk factors for infection differ (Figure 1 & Table 2). Egypt has one of the highest seroprevalence rates for HCV in the world approaching 10% in some regions [5]. This relates to the usage of intravenous tartar emetic in a community wide effort to control *Schistosomiasis sp*. infection between the 1950s and the 1980s. This programme utilised re-usable

needles leading to huge numbers of cases of HCV infection. This was compounded as subjects became re-infected with Schistosomiasis, a known cofactor in accelerating HCV-related fibrosis progression [5, 6] (see factors influencing disease progression later).

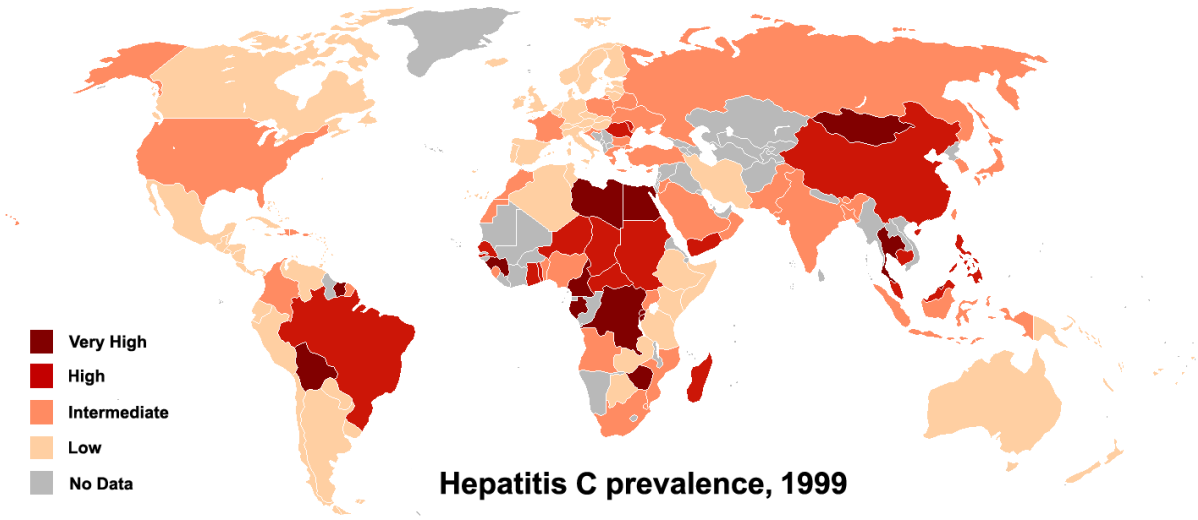

Figure 1. Geographic distribution of Hepatitis C prevalence, 1999. Very High: Prevalence >5 %; High: 2.5-5 %; Intermediate: 1-2.5 %; Low: <1%. Source: (9).

The WHO estimates the global burden of HCV infection to be around 170 million cases [7] (Table 2). Due to global imbalances in health care provision, lack of education about reducing risk and the relatively large cost of currently available anti-viral therapeutic regimes this burden is unlikely to be controlled or reduced in the foreseeable future. Recent data from the HPA has suggested that, in the UK, education and needle-exchange programmes resulted in a decline in the incidence of new infections [1] (Figure 2); at the same time increased immigration from areas of high seroprevalence may change the demography of HCV infection, as demonstrated in the United States over the last 2 decades [8].

WHO Region	Total Population (Millions)	Hepatitis C prevalence %	Infected Population (Millions)	Number-of countries where data are not available
Africa	602	5.3	31.9	12
Americas	785	1.7	13.1	7
Eastern Mediterranean	466	4.6	21.3	7
Europe	858	1.03	8.9	19
South-East Asia	1 500	2.15	32.3	3
Western Pacific	1 600	3.9	62.2	11
Total	**5 811**	**3.1**	**169.7**	**57**

Table 2. Estimated seroprevalence and total numbers of individuals infected with HCV infection by continent. Reference Source: Weekly Epidemiological Record. N° 49, 10 December 1999, WHO.

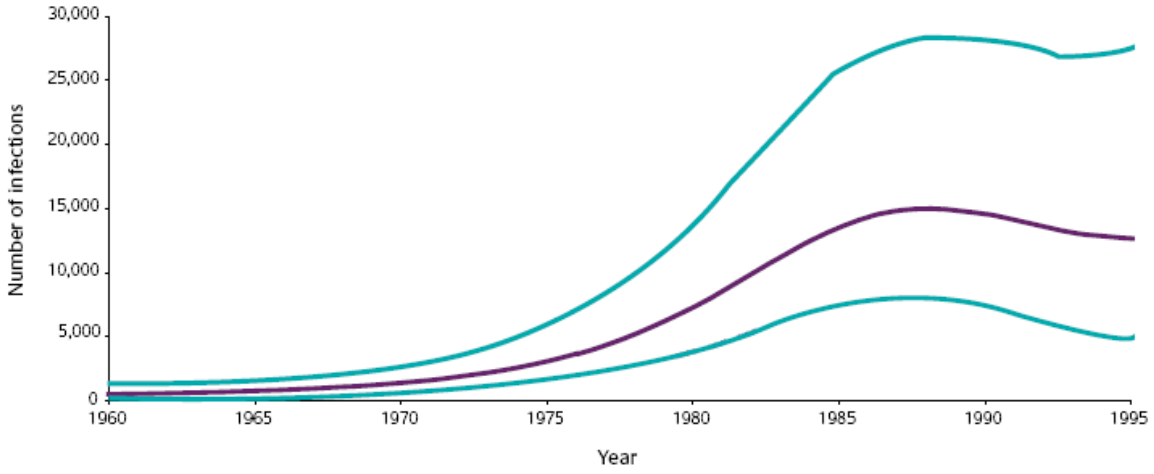

Figure 2. Annual estimated incidence of new HCV infections in England between 1960 and 1995. Dark blue line represents mean and light blue lines 95% confidence intervals. Source: The Health Protection Agency. Source: (1).

Acquisition of HCV is associated with IDU in the indigenous population of the UK and is thus a disease of males between 18 and 40 (Figure 3). However, as the disease is often asymptomatic for many years, patients may present for testing or with disease complications at a much later age.

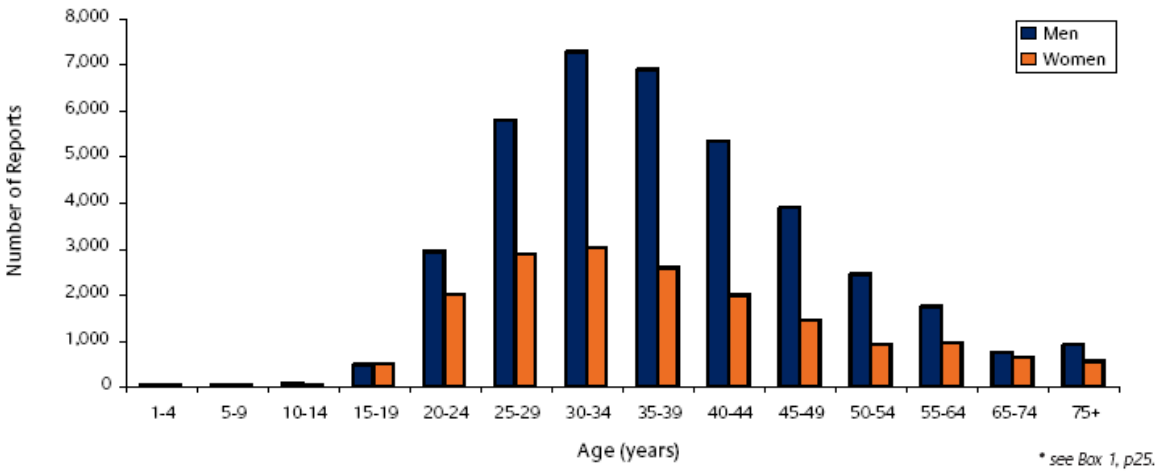

Figure 3. Age and sex distribution of new laboratory reports of HCV infection in England between 1996 and 2007. Source: The Health Protection Agency. Source: (1).

Diagnosis of HCV infection

Anti-HCV testing

The diagnosis of HCV infection rests upon two methods [9]. The first is the demonstration of antibody to HCV in serum; a positive result demonstrates either past or present infection and has a high positive predictive value for exposure to HCV. Enzyme immunoassays utilise antigens from either the HCV core protein or non-structural proteins, which bind to serum antibody. Initial positive results are confirmed with either a second enzyme immunoassay, or a line assay that utilises multiple HCV antigens impregnated onto strips. Confirmatory tests now include testing for both HCV antigens as well as anti-HCV. The Bio-Rad MONOLISA HCV Ag-Ab ultra kit utilises recombinant antigens from HCV NS3 and NS4 as well as monoclonal antibodies directed against HCV capsid antigens immobilised on a microplate.

False negative tests can occur soon after acquisition of HCV, before humoral responses develop [10]. Alter et al demonstrated that detection of anti-HCV was delayed a median 21 weeks after transfusion of infected blood products [11]. False negative results can also occur in subjects with an impaired humoral immunity including immune compromise [12] and haemodialysis for chronic renal impairment [13], itself a risk factor for acquisition of HCV. In addition, some individuals lose anti-

HCV over time; subjects treated for HCV who undergo successful anti-viral therapy may become anti-HCV negative [14], while in the study of Irish women that acquired HCV from infected anti-D immunoglobulin a proportion that were HCV RNA negative have become anti-HCV negative.

HCV RNA testing

Direct demonstration of viral ribonucleic acid (RNA) in serum relies on different polymerase chain reaction assays which are commercially available. Detection of HCV RNA in peripheral blood is the definitive marker of current HCV infection. The simpler assays are qualitative, indicating whether viral RNA is present or not. The more complex but less sensitive quantitative assays are utilised to determine the viral load and genotype, which have become an integral part of the clinical management of subjects with HCV infection.

Quantitation of HCV viral load has become increasingly important in antiviral therapy for HCV. Pre-treatment viral load is an important predictor of treatment success with higher pre-treatment loads associated with poorer response to therapy [15, 16]. Further, the change in viral load by 12 weeks of therapy predicts treatment outcome and is an important part of therapeutic decision making in subjects undergoing antiviral therapy [15]. Quantifying HCV viral is problematic as the range of viral loads encountered covers 10 log copies. Further, differences between commercially available assays prevented comparison of viral loads obtained from different laboratories. This issue has been resolved through introduction of World Health Organisation HCV international standard for Nucleic Acid Amplification Technology Assays in 1999 [17] and subsequently updated standard in 2005 [18].

All the commercial quantitation assays rely on RT-PCR to amplify viral RNA before hybridisation to specific oligonucleotide probes. Detection of hybridisation varies between assays; the Bayer bDNA assay (VERSANT HCV RNA 3.0 assay, Bayer Diagnostics UK) relies on detection of enzyme-conjugated hybridised oligonucleotides, whereas the Roche Cobas Amplicor HCV monitor 2.0 assay relies on colorimetric determination of hybridised oligonucleotides.

HCV genotype has also become integral to the management of subjects during antiviral therapy. Most prospective analyses have demonstrated that subjects infected with HCV genotypes 2 or 3 have a higher chance of clearing viraemia during therapy when compared to subjects with genotype 1 or 4 [15, 16, 19]. Classification of HCV genotypes is based upon sequencing of the NS5b protein [20], but high variability in this region reduces assay sensitivity and therefore makes it impractical for routine clinical testing.

Most commercial assays therefore rely on PCR amplification of the HCV 5' un-translated region (UTR). This area is highly conserved, yet contains a number of genotype-specific motifs. The assay most commonly used assay for genotype determination (VERSANT HCV genotype assay, Siemens Medical Diagnostics) utilises PCR amplification of the 5' UTR, followed by hybridisation to a large number of genotype-specific probes immobilised on a nitrocellulose membrane [21].

Natural history of chronic HCV infection

Acute infection

Upon exposure to HCV most individuals undergo an asymptomatic acute hepatitis around 6 weeks after exposure [22]. Symptoms occur in just 15% and can be difficult to diagnose as anti-HCV antibody may be undetectable until a median of 21 weeks after infection [11]; one third of individuals do not have detectable antibody at the onset of symptoms [22].

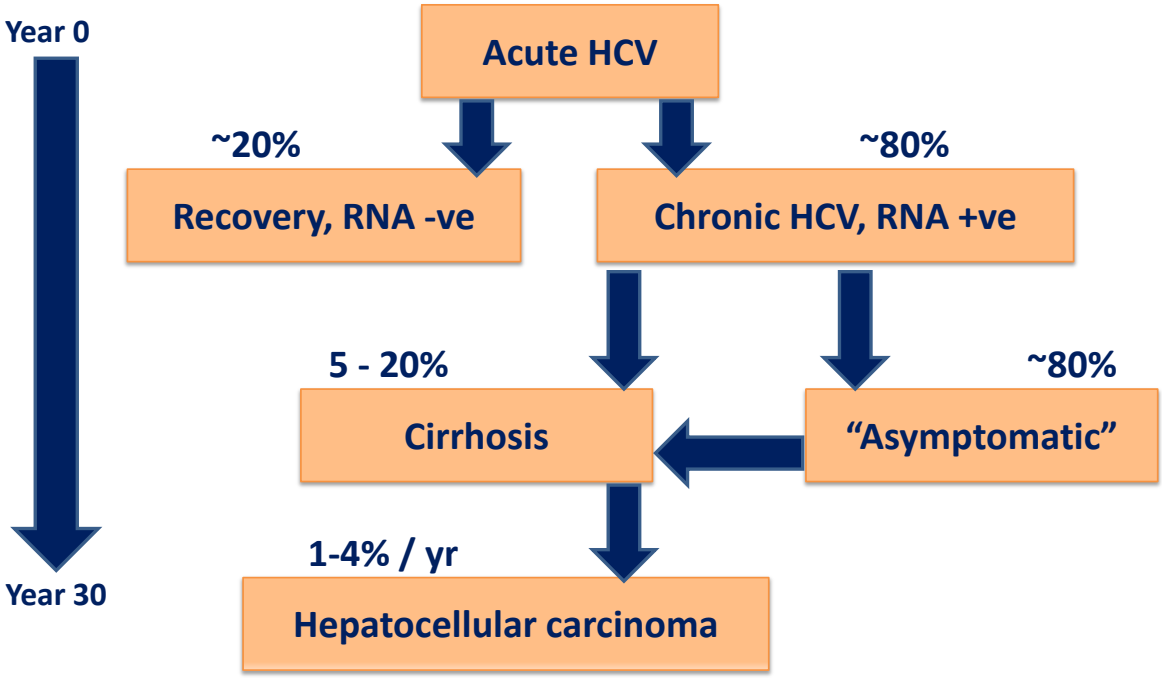

Figure 4. Flow-chart schema of the natural history of HCV infection following acute exposure.

During this stage as many as 20% of subjects [23] develop strong and multi-specific T-lymphocyte responses [24, 25] and remain non-viraemic. These individuals retain anti-HCV antibody, the marker of exposure and a humoral immune response, but are HCV RNA negative by PCR in serum.

Immunology of acute HCV infection

1. Innate immunity

The ability of HCV to subvert the immune system extends to both innate and adaptive immunity and occurs early in the development of infection. Several groups have now demonstrated the effect of HCV infection upon the various components of the innate immune system.

Della et al demonstrated that peripheral blood dendritic cells from subjects with viraemic HCV have reduced IL-12 secretion and increased IL-10 secretion when compared to healthy controls [26]. Dolganiuc et al demonstrated that the HCV core and NS3 proteins were able to induce IL-10 secretion by both dendritic cells as well as macrophages [27]. Further, they demonstrated that the

same proteins inhibited differentiation of peripheral dendritic cells. Lai et al have extended these findings to demonstrate an increase in plasmacytoid dendritic cells within the HCV-infected liver, with increased expression of the regulatory receptor BDCA-2 [28]. Subsequent data by Amjad et al has demonstrated that the HCV protein NS5 impairs the differentiation of plasmacytoid dendritic cells , their ability to produce IFN-α and the ability to stimulate T-lymphocytes in response to viral infection [29].

Data regarding the role of NK cells and invariant-NKT cells has been mixed. Radaeva et al demonstrated that NK cells were activated in HCV infection and had anti-fibrotic properties, through their killing of activated intrahepatic stellate cells [30]. Lucas et al demonstrated a reduced frequency of Vα24 NKT cells in HCV-infected individuals, but these cells demonstrated evidence of in vivo activation [31]. Golden-Mason et al prospectively followed 22 subjects through an episode of acute HCV infection demonstrating that individuals with NKT cells with a more activated phenotype were more likely to resolve HCV viraemia. Further, that the ability of NKT cells to secrete IL-2 and IL-13 during acute HCV infection was associated with clearance of HCV viraemia at the acute stage [32].

2. Adaptive immunity

Because acute HCV infection is often asymptomatic [22] and occurs in subjects involved in high-risk behaviours, study of the pathogenesis has relied on acute HCV in the chimpanzee, the only available animal model. Two studies have conducted gene array studies of acute HCV infection and demonstrated that there is up-regulation of type I interferon responses within the liver within one week of infection [33, 34].

The development of detectable immune responses is delayed despite evidence of early anti-viral responses within hepatocytes [23, 35, 36]. Cooper et al demonstrated that animals which cleared infection at the acute stage developed strong Th1 responses against multiple epitopes [37]. The two chimpanzees developed HCV-specific CD8+ responses between 6 and 12 weeks after inoculation

with HCV and the clearance of peripheral viraemia was contemporaneous with infiltration of the liver by HCV-specific CD8+ cells. However, animals which developed chronic infection developed weak responses to small numbers of epitopes [37].

Similar findings have been demonstrated in humans undergoing acute HCV infection. Gerlach et al studied thirty-eight subjects with acute HCV and demonstrated that those individuals who subsequently cleared HCV infection developed greater CD4+ proliferative and cytokine responses to HCV proteins within the first 6 months of infection when compared to subjects with developed chronic infection [38]. Of the 18 patients who developed chronic infection, six generated strong CD4+ responses initially leading to undetectable HCV RNA levels in serum. However, as this response disappeared, HCV viraemia recurred at a median of 6 months after initial infection, leading to chronic infection [38].

Not only is the immune response different quantitatively in those who successfully clear the virus, but the response also differs qualitatively with skewing of the response toward Th1 cytokines. Tsai et al demonstrated that individuals with acute HCV who successfully cleared infection had strong Th1 responses with high levels of interferon-γ and IL-2 in response to HCV proteins. In distinction, those individuals with chronic evolution developed very poor or undetectable Th1 responses, but had detectable Th2 responses with IL-4 and regulatory responses, reflected by antigen-induced secretion of IL-10 [39].

Data linking chronic infection to impairment of CD8+ lymphocyte function are not as robust. Urbani et al demonstrated that CD4+ responses were important in the acute stage, whereas the scale or quality of CD8+ responses did not predict subsequent outcome of infection [40]. Cox et al demonstrated that initial CD8+ responses were lost during the acute stage in those with chronic evolution of infection. Further, that despite chronic viraemia, these responses were not replaced by responses to other epitopes [23].

More recent work has shed light on the role of CD8+ T-lymphocytes in the pathogenesis of chronic HCV infection. Jo et al have studied the ability of CD8+ cells to inhibit HCV replication in vitro by transfection of human HLA-A2 into cell lines supporting HCV replication in culture. CD8+ T-lymphocytes were able to inhibit HCV replication by 95% at ratios as low as 100 targets per effector. This effect did not involve cytolysis; inhibition was observed in transwell experiments and neutralization antibodies demonstrated that the effect was mediated mostly through interferon-γ [41].

The ability of the adaptive immune system to generate long-lasting immunity against HCV after clearance at the acute hepatitis stage is critical in determining the feasibility of generating an effective HCV vaccine in the future. Most research in this area is derived from the chimpanzee model; this model is the only animal model allowing study of viral-immune system interactions [42, 43]. However, the natural history of HCV in chimpanzees is different to that in humans with far higher rates of viral clearance at the acute stage.

Data from the chimpanzee model of HCV regarding protection against re-infection are inconsistent. Shoukry et al demonstrated that duration of viraemia after second infection with HCV was significantly shorter than after initial infection in two chimpanzees. Viraemia persisted for 14 days after second infection after a prior clearance of HCV after a median of 4 months [44]. Further, they demonstrated the importance of CD8+ T-lymphocytes by depleting this subset before infecting these animals for a third time with HCV resulting in chronic viraemia.

Grakoui et al extended these findings by depleting CD4+ T-lymphocytes from two chimpanzees with previous acute hepatitis C. Despite the presence of functional anti-HCV CD8+ cells within the liver, both animals developed persistent viraemia [45]. Nascimbeni et al demonstrated that virological outcome was correlated with the functional activity of T-lymphocytes during re-challenge of HCV-recovered animals. The animal which developed strong CD4+ and CD8+ T-cell cytokine and

proliferation responses cleared viraemia, whereas the animal that did not develop such responses developed chronic viraemia [46]. Further, several studies have demonstrated that neutralizing antibody responses against HCV antigens are not developed during re-challenge suggesting that antibody-based protection from re-infection is less important than cellular immunity [45, 47].

Data from human observational studies has suggested that previous clearance of HCV reduces the risk of chronicity from a subsequent re-infection from 21% to 12% [48]. However, other studies have found no reduction in viral persistence rate between HCV-naive individuals and subjects with previous acute HCV [49].

Non-viraemic HCV

Between 15 and 50% of individuals are reported to clear HCV RNA from serum at the stage of acute infection [22, 50]. Studies in the two largest cohorts infected by blood products suggest that 45% of individuals clear viraemia at the acute stage [4, 51], although these patients may not reflect the majority of HCV-exposed individuals. The long-term prognosis of this group is good [22, 52, 53]. However, an increasing body of work has questioned whether these individuals have indeed cleared infection or whether HCV lies latent within the liver [53].

Castillo et al described a cohort of HCV-seronegative patients with persistently raised transaminases without clear cause. They demonstrated positive and negative strand viral RNA from liver biopsy specimens in 57% of cases, even though the individuals were negative for both anti-HCV and serum HCV RNA by routine clinical tests [54]. The same group went on to demonstrate that viral RNA was detectable in liver tissue in 83% of anti-HCV positive, non-viraemic individuals [55].

The issue of whether such patients represent latent infection [53, 56] or chronic HCV infection with viral replication below the current limit of detection of PCR assays [57] remains contentious and unresolved.

Development of chronic infection

For the remaining 80% of subjects, the initial immune response fails to control the viral infection and the subject develops chronic infection. These individuals retain both detectable anti-HCV antibody and viral RNA in serum (Figure 4) and are at risk of developing long-term complications of HCV infection, usually related to progressive hepatic fibrosis.

Immunology of chronic HCV infection

Chronic HCV infection has been associated with defects of both innate and adaptive immune systems. There are demonstrable defects in the number or function of CD4+ T-lymphocytes [36], CD8+ T-lymphocytes [35], NK cells [58, 59], invariate NKT cells [31, 58, 60, 61] and dendritic cells [28, 62].

In particular, the failure of T-lymphocyte responses in chronic HCV infection and the mechanisms underlying this failure have been the subject of intense research. Possible mechanisms that have been postulated include [35]:

1. Primary T-cell failure or exhaustion

There is evidence that T-cells undergo exhaustion during the primary immune response. Cox et al demonstrated waning responsiveness to certain CD8+ epitopes [23]. Other groups suggested that HCV-mediated dysfunction of dendritic cell antigen presentation and co-stimulation fails to prime CD8+ and helper CD4+ responses, preventing maturation of the immune response [28, 62].

Certainly it is known that HCV-specific lymphocytes fail to undergo in vivo maturation and differentiation despite the continued presence of antigen. Appay et al demonstrated that CD8+ T-lymphocytes specific for a range of chronic infections had widely divergent cell-surface phenotypes described by the presence or absence or CD27 and CD28. When compared to CD8+ cells specific for CMV, EBV and HIV, HCV-specific cells had the highest surface expression of the co-stimulatory molecules CD27 and CD28 [63]. It is also known that HCV infection is able to retard the normal

maturation of both HCV-specific and non-HCV-specific cells. Lucas et al demonstrated that CMV-specific cells which normally have a highly differentiated phenotype in CMV mono-infected subjects had higher expression of CD27, CD28 and CD45RA in subjects also infected with HCV [64]. This suggests that HCV infection is able to prevent the normal differentiation pathway in the whole CD8+ T-lymphocyte pool and not just HCV-specific cells. Spangenberg et al demonstrated a failure of intrahepatic HCV-specific CD8+ lymphocytes to express IFN-γ in response to HCV peptides. The same was not true for flu-specific CD8+ cells in the same patients which had maintained antigen-specific IFN-γ secretion [65].

Other groups have studied the failure of HCV-specific CD4+ lymphocytes in chronic HCV infection. It is known that these cells are very rare in patients with chronic HCV infection, ranging between 1:1000 and 1:100,000 despite chronic antigenaemia [66]. Further, despite having a restricted T cell receptor repertoire suggesting expansion of a limited number of clones these cells had a CD27+ CCR7+ phenotype suggesting a failure to mature [66]. Functionally HCV-specific CD4+ T-lymphocytes have a skewed cytokine secretion pattern with a complete failure to secrete IL-2, but maintained IFN-γ secretion [67] attendant with a failure to proliferate [68].

Yao et al suggest that HCV core protein may inhibit T-lymphocyte priming through interaction with the complement receptor gC1qR, inhibiting activation of the Akt intracellular messenger pathway [69, 70].

Further, whilst there are few HCV specific lymphocytes present in peripheral blood of subjects with chronic HCV infection they can be expanded *ex vivo*. Chang et al demonstrated that HCV-specific cytolytic CD8+ memory T cells were undetectable ex vivo but could be expanded with in vitro stimulation [71]. Interestingly, similar cells from subjects with non-viraemic HCV could not be expanded, perhaps suggesting the need for cognate antigen to preserve memory responses and the lack of long-term protective T-lymphocyte response even after apparent viral clearance.

2. *Suppression by regulatory T-lymphocytes*

HCV is associated with an accumulation of CD4+CD25+FoxP3+ regulatory T –cells which inhibit HCV-specific responses [72, 73]. These cells are concentrated within the liver [74] and *in vitro* depletion leads to an increase in proliferative and cytotoxic responses [73].

Similarly a regulatory CD8+ T-lymphocyte population that secretes IL-10 has been identified as important in the pathogenesis of chronic HCV infection. Accapezzato et al identified CD8+ regulatory cells which were specific for HCV epitopes present within the HCV-infected liver. These cells suppressed other T-cell responses; suppression was reversible through the addition of anti-IL-10 [75].

3. *Inhibitory receptors*

There is increasing interest in lymphocytic expression of inhibitory receptors and in particular, PD-1, a cell-surface receptor of the CD28 superfamily, that when bound to its ligand PD-L1 or PD-L2 generates an inhibitory signal that prevents further immune activation [76]. It is believed that this system evolved to prevent exuberant immune reactions that might lead to host damage.

In a mouse model of Lymphocytic Chorio-Meningitis virus (LCMV) infection, Barber et al demonstrated that viral infection was associated with increased expression of PD-1 on LCMV-specific cells which displayed features of exhaustion. Further, the administration of antibody inhibiting interaction of PD-1 with PD-L1 increased LCMV-specific cell proliferation, cytokine secretion, cell killing and reduced viral load [77].

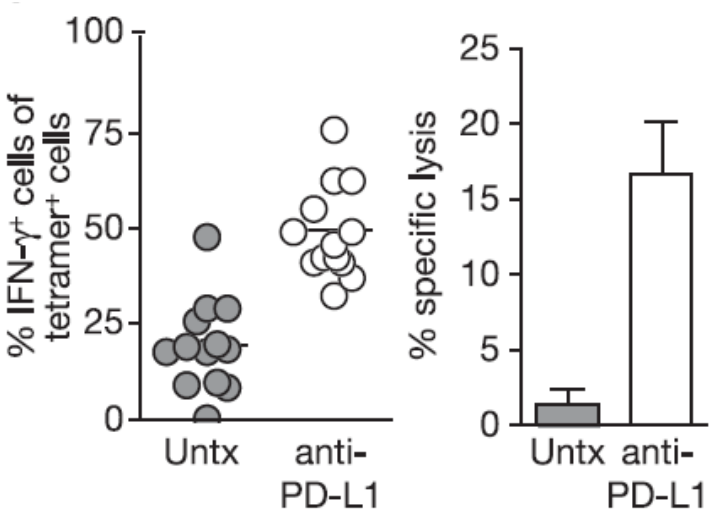

Figure 5. Increase in LCMV-specific CD8+ lymphocyte function after administration of anti-PD-L1. Mice infected with LCMV were injected with irrelevant antibody or anti-PD-L1, which led to an increase in the proportion of viral-specific CD8+ lymphocytes. Cells able to secrete IFN-γ after specific stimulation (left panel) and the ability of CD8+ lymphocytes to lyse target cells bearing LCMV antigens (right panel). Source: [77].

Since this study there has been a rapid advance in the study of this receptor system in the field of HCV infection. Urbani et al demonstrated that CD8+ lymphocytes maintained high levels of PD-1 expression beyond the acute phase in those individuals with evolving chronic infection [78]. In distinction, those subjects with acute clearance of infection had rapid down-regulation of PD-1, commensurate with the decline in viral load. Both Golden-Mason et al and Penna et al have demonstrated that PD-1 is up-regulated on HCV-specific CD8+ T-lymphocytes and that administration of blocking antibodies is associated with a restoration of viral-specific secretion of IFN-γ and IL-2 [79, 80]. Yao et al demonstrated that interaction of HCV core protein with gC1qR leads to up-regulation of PD-1 on viral-specific lymphocytes [81].

The importance of both the identification of both active regulation and the activity of inhibitory receptors is the potential for therapeutic manipulation to reverse the immune defects seen in chronic HCV infection.

4. Viral quasi-species and escape mutants

The HCV RNA-dependent RNA polymerase that is responsible for the replication of the viral genome lacks proofreading capability. HCV infection generates an estimated 10^{10} virion particles per day and the process of viral replication leads to large numbers of viral mutations. These mutants can evade the adaptive immune response due to sequence changes and therefore viral epitope modification [82, 83]. Erickson et al demonstrated that HCV infection in chimpanzees led to the development of viral mutations which impair epitope binding to MHC class 1 or recognition by CD8+ lymphocytes [82]. Several studies have demonstrated escape mutants in human HCV infection which pre-date the development of chronic infection [84, 85].

Mechanisms of HCV-induced liver injury

Direct cytopathic effects and immune-mediated liver injury are both likely to be involved in the pathogenesis of HCV-related liver damage. Whilst immune-mediated liver injury during chronic HCV infection is felt to represent the predominant mechanism of damage the evidence base for this hypothesis is not strong [86]. The evidence for direct cytopathy comes from individuals who are immune deficient because of HIV infection [87, 88] or receiving immunosuppressive drugs after organ transplantation [89] who have increased rates of disease progression when compared to immune competent individuals. The evidence for immune-mediated liver damage comes from chimpanzee experiments and human observation where liver damage is contemporaneous with liver infiltration by CD8+ cells, not HCV replication.

1) Direct cytopathic effects

a. Modulation of hepatocyte apoptosis

Several studies have linked HCV infection of hepatocytes to up-regulation of apoptosis. Bantel et al identified high levels of Cytokeratin-18 cleavage products in the serum of subjects with HCV infection, which correlated with the degree of elevation of transaminases [90]. CK-18 is the major cytokeratin of hepatocytes [90, 91]; increased

serum concentrations may reflect either necrosis or apoptosis. Joyce et al demonstrated that human hepatocytes in a SCID mouse model demonstrated increased levels of oxidative stress and increased apoptosis by TUNEL staining [92]. Walters et al demonstrated that HCV infection of Huh-7.5 cells in culture was associated with increased levels of caspases 3, an enzyme involved in apoptosis. Further, peak of viral replication was contemporaneous with the peak of caspases 3 expression [93]. Zhu et al demonstrated similar findings demonstrating that HCV induced apoptosis of an in vitro hepatocyte cell line utilising tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) as the marker of apoptosis. Further, they demonstrated that IFN-α protected hepatocytes from apoptosis [94].

Whilst the in vitro data linking HCV infection to hepatocyte apoptosis is suggestive, studies investigating in vivo hepatocyte apoptosis in HCV-infected liver are equivocal. Safraz el al demonstrated there is minimal expression of caspase 3 in the hepatocytes in chronic HCV infection using immunohistochemistry on HCV-infected liver tissue [95]; whereas sinusoidal lining-cells did express caspases 3. Other studies investigating both TRAIL and caspase 3 by immunohistochemistry have demonstrated that TRAIL is maximally expressed in livers with low stage fibrosis, whereas caspases 3 is maximally expressed in livers with high stage fibrosis [96].

Further, expression of CD95 (Fas) on the surface of hepatocytes is up-regulated in subjects with HCV infection [97]. CD95 is a surface receptor for the external apoptotic pathway, which when ligated by Fas-ligand causes activation of intracellular caspase-8 and ultimately apoptosis [98]. Therefore, whilst up-regulation of CD95 increases the susceptibility of a cell to apoptosis triggered externally, it does not equate with apoptosis directly.

b. Hepatic steatosis and insulin resistance

The prevalence of the metabolic syndrome in patients with chronic HCV infection is far higher than expected allowing for obesity and other known risk factors [99]. An increasing body of work has linked HCV infection as a causative agent to insulin resistance and dyslipidaemia. HCV, particularly genotype 3 infection, is known to be associated with the development of both hepatic steatosis and insulin resistance. It is known that both conditions are linked to mitochondrial toxicity in the hepatocyte and increased generation of reactive oxygen species (ROS) [99].

Both structural and non-structural HCV proteins affect the cellular redox environment and insulin signalling pathways. Gong et al demonstrated that the HCV NS5 protein led to the release of calcium from the endoplasmic reticulum (ER) of hepatocytes [100]. The interaction of HCV and NS5 in particular with the ER is thought to lead to overload of chaperone proteins, responsible for ensuring correct protein folding. This overload leads to an unfolded protein response which leads to ER dysfunction and ultimately cell death [101].

HCV core protein has been demonstrated to induce insulin resistance in three separate ways: induction of mitochondrial permeability transition; overload of the ER; and inhibition of Peroxisome proliferator-activated receptor-α (PPAR) [99]. Core protein is known to bind to the outer membrane of mitochondria leading to the mitochondrial permeability transition. This leads to mitochondrial uptake of calcium and activation of the electron transport chain leading to increased generation of ROS [102]. Moriya et al developed two separate mice lines that expressed HCV core protein. These mice develop progressive hepatic steatosis, linking the core protein to steatosis in the absence of other HCV components [103]. Naas et al extended these findings to demonstrate that viral protein production increased with increasing age in a transgenic

mouse model and further, that core, E1 and E2 proteins were all able to induce hepatic steatosis [104].

PPAR-α is involved in the intracellular handling of lipids and in particular triglyceride. HCV core protein has been demonstrated to inhibit both PPAR-α and PPAR-γ, leading to hepatocyte accumulation of triglyceride and increased oxidative stress [105].

Recent data has revealed a potential mechanism underlying the increased insulin resistance seen in HCV infection. Kasai et al have demonstrated that infection of Huh-7.5 cells with a HCV sub-genomic replicon led to a reduced expression of cell surface glucose transporters GLUT1 and GLUT2 and subsequent reduced cellular glucose uptake [106]. This down-regulation could be reversed by treating the cells with IFN-α. They further demonstrated an in vivo reduction of GLUT2 on hepatocytes from HCV-infected individuals [106].

In addition to direct effects upon hepatocytes by HCV proteins, insulin resistance can also arise due to the increases levels of intrahepatic immune effectors and attendant cytokine release. HCV infection leads to Kupffer cell activation within the liver and subsequent release of pro-inflammatory cytokines [107] such as TNF-α and IL-6 [99].

TNF-α leads to down-regulation of hepatocyte surface membrane glucose transporters reducing the hepatic uptake and promoting hyperglycaemia [108], in addition to down-regulating lipoprotein lipase leading to increased triglyceride levels [109].

c. Activation of hepatic stellate cells (HSC)

Hepatic stellate cells are present within both healthy and diseased liver. Normally they are quiescent, but upon activation they are able to produce large quantities of extracellular matrix proteins including fibrous tissue.

Bataller et al demonstrated that transfection of the HCV-proteins core, NS3 and NS5 into quiescent HSCs led to expression of pro-collagen 1 and secretion of the pro-fibrogenic cytokine TGF-β [110]. Further, Mazzocca et al demonstrated that exposure of HSCs to the HCV protein E2 led to the secretion of matrix metalloproteinase 2 (MMP2) which led to the destruction of the normal extracellular matrix and the deposition of dense type 1 collagen [111].

Incubation of HSCs with the conditioned media from HCV-infected cell lines leads to the up-regulation of the pro-fibrogenic response and the suppression of the fibrinolytic response [112]. Schulze-Krebs et al demonstrated that conditioned media from HCV-replicon infected Huh-7.5 cells led to human and rat HSCs secreting increased quantities of pro-collagen alpha1(I) and pro-collagen alpha1(III) and down-regulating fibrolytic matrix metalloproteinases [112]. Inhibition of TGF-β led to a 50% reduction in pro-collagen release.

2) Immune-mediated liver injury

As discussed previously there are a number of induced immune defects consequent upon HCV infection. Much of the liver damage is felt to represent prolonged, unsuccessful attempts by the immune system to eliminate virally-infected hepatocytes. However, the evidence to support this hypothesis in humans rather than animal or cell-culture models is only observational.

a. Cytotoxic T-lymphocytes

Intracellular viral peptides are processed in the context of MHC class I and presented on the cell surface of hepatocytes and presented to CD8+ T-lymphocytes in chronic HCV infection [24]. In acute HCV infection the appearance of HCV-specific CD8+ T-lymphocytes in peripheral blood is contemporaneous with the decline in viral load [44,

113]. Further, the expression of pro-inflammatory Th1-associated genes within the liver is correlated with the degree of liver injury [114]. Urbani et al studied eight subjects with HCV infection and found that two with severe hepatitis had strong CD8+ responses to a epitope within the HCV NS3 region, which those with less severe hepatitis did not [115]. Further, they demonstrated that there was significant cross-reactivity between these NS3-specific cells and an epitope in the influenza neuraminidase protein.

b. Natural Killer (NK) cells

NK cells respond to perceived threats in a non-antigen specific fashion. They are able to detect reductions in MHC class I expression on the surface of cells, which can be associated with and utilised by viral infections to reduce antigen-presentation to CD8+ T-lymphocytes [86].

In a mouse model of viral hepatitis, Liu et al demonstrated that intrahepatic NK cells were necessary for correct priming of CD8+ T-lymphocytes and ultimately viral clearance through hepatocyte killing [116]. However, other groups have demonstrated that NK cells may actually be anti-fibrogenic. In a carbon tetrachloride mouse model, NK cells were responsible for killing activated stellate cells and reducing the level of fibrosis after liver injury [30].

c. Natural Killer T (NKT) cells

This subset of NK cells which also express the T-cell receptor CD3, recognise glycolipid antigens when presented in the context of CD1d [117]. They are enriched within the HCV-infected liver and are able upon activation to secrete large quantities of the Th1 cytokine IFN-γ [118]. Other groups have demonstrated that NKT cells from HCV-infected liver display an activated phenotype [31], but there has been no study directly linking this cell type to liver damage in HCV.

Interferon-α signalling in HCV infection

The interferons were first discovered in studies on anti-viral agents in the 1950's [119]. They are a multi-gene family of inducible cytokines that elicit multiple changes in both cellular metabolism and gene transcription [120]. They are divided into two subgroups: type 1 interferons include 13 different subtypes of IFN-α and IFN-β, produced by dendritic cells in response to viral infection and IFN-γ, the sole type 2 interferon, induced by mitogenic or antigenic stimulation of lymphocytes [120]. Interferon-α is the anti-viral cytokine of particular importance in both acute and chronic HCV infection and forms the basis of anti-viral treatment for HCV infection [19]. Experiments have suggested that CD8+ T-lymphocyte derived IFN-γ has a significant role in the inhibition of HCV replication [41]. However, attempts at administering IFN-γ as a therapeutic anti-viral agent have been disappointing [121].

Type 1 interferons exert their actions by binding to their cell-surface receptor which consists of two subunits IFNAR-1 and IFNAR-2 [122, 123]. IFN-α binds to IFNAR-2 which causes it to associate with IFNAR-1, leading to phosphorylation of the janus tyrosine kinases Jak1 and Tyk2 associated with the intracellular tail of IFNAR-1 (Figure 6) [122].

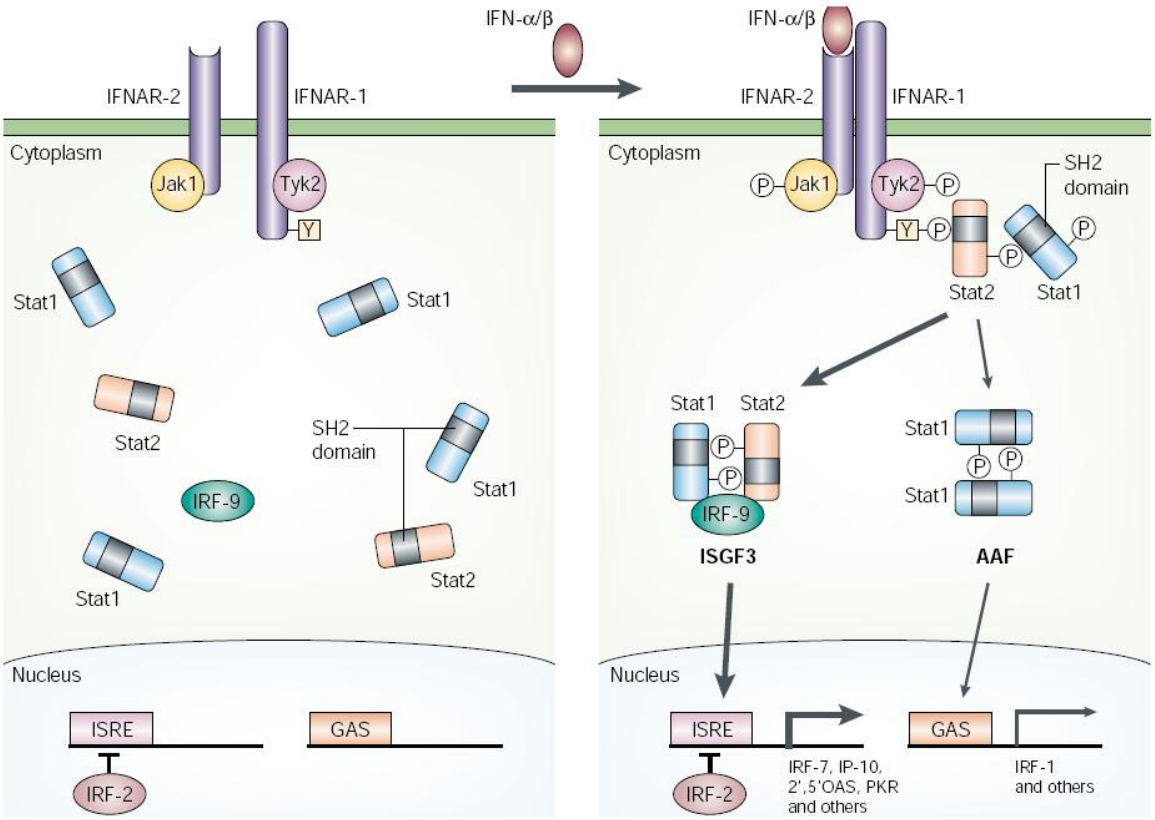

Figure 6. Cartoon of the signalling pathway of Interferon-α (IFN-α). IFN-α associates with IFNAR-2, leading to dimerisation with IFNAR-1. This leads to phosphorylation of the intracellular kinases Jak1 and Tyk2 and subsequently phosphorylation of Stat1 and Stat2. This leads to the formation of the complexes ISGF3 and AAF, which are able to act on gene promoters and inhibitors. Source: [122].

Phosphorylation of Jak1 and Tyk2 leads to the phosphorylation of signal transducer and activator of transcription (STAT) 1 and 2. Once phosphorylated at tyrosine 701 (Y701) Stat1 is able to form homodimers termed Interferon-α activated factor (AAF) or larger complexes with phospho-Stat2 and interferon regulatory factor 9 (IRF-9) termed interferon-stimulated gene factor 3 (ISGF3). These two second messengers mediate changes in the transcription of a number of genes termed interferon-stimulated genes (ISG) through binding to gene promoters and inhibitors.

Through the use of gene or mRNA array technology it is possible to study the pattern of ISG expression in different disease stages. Sarasin et al demonstrated that high level ISG expression prior to treatment with IFN-α therapy for HCV was associated with failure to respond to therapy. Conversely, individuals with significant increments in ISG in response to IFN-α had successful

responses to anti-viral therapy [124]. Lalle et al demonstrated that the ISG response of peripheral lymphocytes to IFN-α in vitro prior to anti-viral therapy predict patients ultimate virological response [125]. Similarly, Aceti et al demonstrated that failure to phosphorylate Stat1 in peripheral lymphocytes in response to IFN-α therapy was associated with a failure to respond to therapy [126].

HCV has evolved a number of mechanisms of interfering with the IFN-α / Stat1 pathway, abrogating anti-viral signalling. A number of groups have demonstrated that the HCV core protein suppresses signalling through Stat1 [127, 128]. Yao et al demonstrated that core protein prevents Stat1 phosphorylation in addition to down-regulating suppressor of cytokine signalling 1 (SOCS) expression in peripheral T-lymphocytes [129]. Gong et al demonstrated that the HCV NS5A protein reduces Stat1 phosphorylation and nuclear translocation in response to IFN-α in vitro [130].

Increased age may diminish the effectiveness of the IFN-α signalling pathway. Rytel et al demonstrated that aged mice injected with either Coxsackie or the non-replicating Newcastle Disease Virus had significantly less IFN-α secretion than younger animals [131].

IFN-α induces a number of proteins important in cellular anti-viral defence. Two of the most important are protein kinase R (PKR) and 2'-5' oligoadenylate synthetase (OAS). PKR is activated by auto-phosphorylation following detection of double stranded RNA molecules which occur during intracellular viral infection. Once activated PKR phosphorylates a number of cytoplasmic targets preventing RNA translation and thereby viral replication [120]. Activation of OAS leads to degradation of RNA within the cytoplasm.

IFN-α therapy for chronic HCV infection

The current gold standard of therapy for chronic HCV infection comprises IFN-α and ribavirin (RBV) [19]; IFN-α is conjugated to a polyethylene-glycol (PEG) tail in order to increase the half life. Patients who have developed or are at risk of developing progressive fibrosis secondary to chronic HCV infection are commenced on once weekly subcutaneous PEG-IFN-α and twice daily oral RBV.

Patients with genotype 1 and 4 infections are treated for 12 weeks and then testing for viral load is performed. Those who are non-viraemic at this stage have achieved an early virological response (EVR); therapy is continued for a total of 48 weeks in those whose viral load has reduced by 2 $\log_{10}$. For those who remain viraemic, with less than a 2$\log_{10}$ drop in viral load, the likelihood of achieving viral clearance is less than 2% and therapy is stopped [19]. For individuals with genotype 2 or 3 infection therapy is continued for a total of 24 weeks (Figure 7).

For all subjects viral load is then repeated 6 months after cessation of therapy. Subjects who are non-viraemic at this stage have achieved sustained virological response (SVR) and remain non-viraemic in the long-term in over 95% of cases [19, 132].

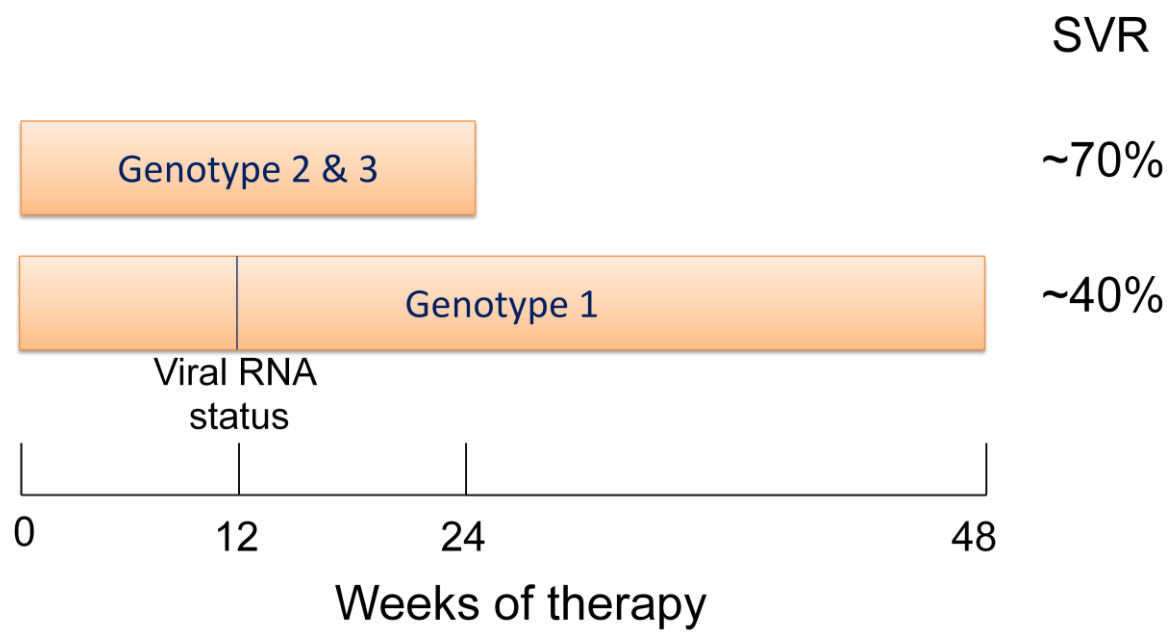

Figure 7. Schematic of current treatment time-course in different genotypes of chronic HCV infection.

Whilst rates of SVR achieved in the context of clinical trials are reportedly higher, 'real-world' rates of achievement of SVR are 40% for genotype 1 or 4 and 70% for genotypes 2 or 3 (Tracy Woodall & Graeme Alexander, Cambridge 2009, personal communication).

Side effects are one of the main drawbacks of therapy with IFN-α leading to premature withdrawal from therapy in 10 – 14% of subjects [133]. Further, symptoms or complications of therapy result in dose-reductions in either IFN-α or RBV in around one third of cases [133], leading to reduction in the efficacy of treatment translating into reduced rates of SVR [19, 133]. Common side-effects include fatigue, pyrexia, malaise and depression. However, therapy can be associated with the development of significant auto-immune phenomena which may not abate after cessation of therapy [133].

Fibrosis progression in chronic HCV infection

As HCV was only discovered in 1989, true prospective studies to investigate the rate at which HCV infection leads to the development of hepatic fibrosis currently lack the long-term follow-up required. In their place a number of retrospective-prospective studies with patients already infected but at a known time point yield the strongest evidence. A number of single time point cohorts exist around the world who acquired HCV through single sources including recipients of infected anti-rhesus D immunoglobulin [4] and recipients of infected human plasma [3]. Several groups have followed cohorts of non-iatrogenically infected individuals; thus most were infected with HCV through IDU, where single time point of infection cannot be established [134].

HCV is an indolent disease in the majority of sufferers, with progressive fibrosis in a minority usually associated with secondary factors now known to significantly impact upon the prognosis of HCV infection.

Hepatic pathology

In common with other liver diseases chronic inflammation of the liver can lead to the development of hepatic fibrosis (Figure 8). In HCV infection, the majority of inflammation resides within the portal tract, with some spill over into the hepatic lobule (Figure 9).

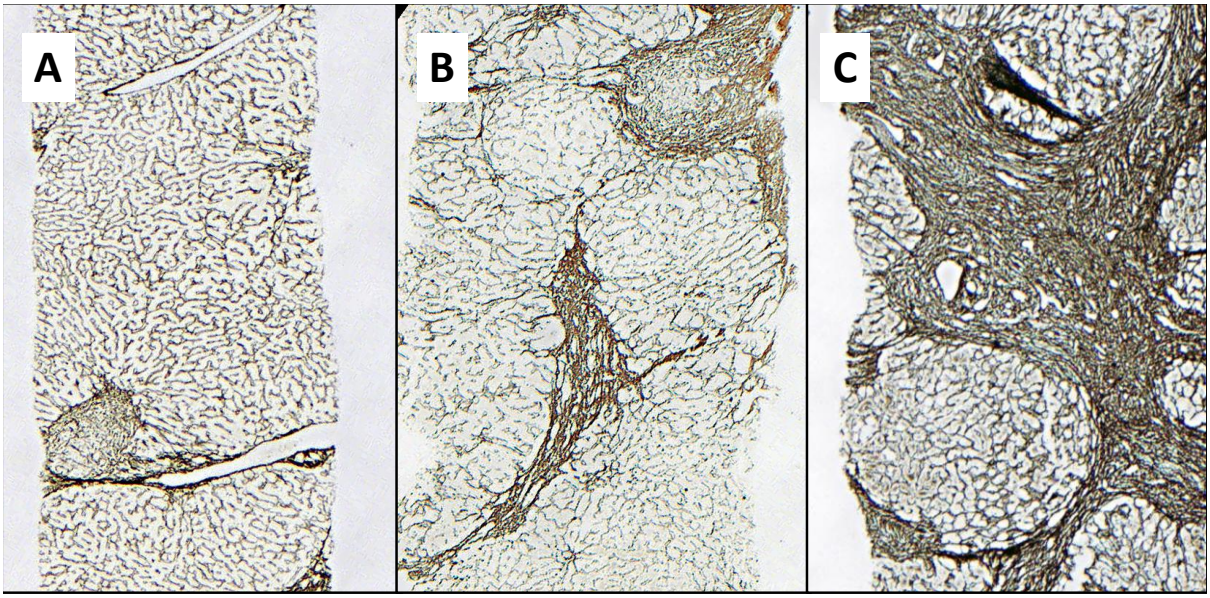

Figure 8. HCV-related hepatic fibrosis. Three representative photomicrographs of liver biopsy specimens taken from subjects with mild fibrosis (Panel A), moderate to severe fibrosis (Panel B) and cirrhosis (Panel C) stained for reticulin.

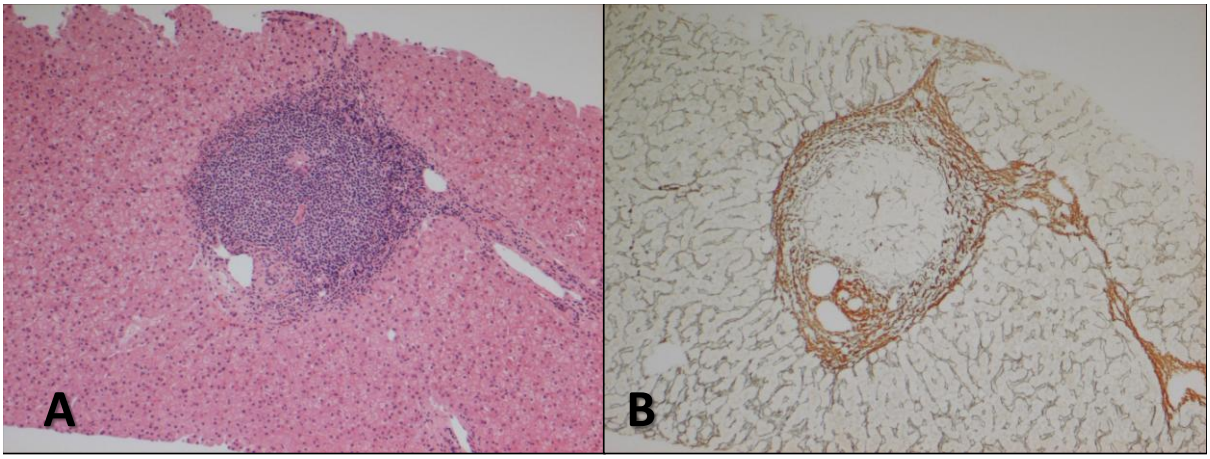

Figure 9. Photomicrograph of liver biopsy obtained from HCV infected subject. Section A stained with haematoxylin and eosin demonstrating representative liver pathology in HCV infection; section B shows next level stained for reticulin demonstrating hepatic fibrosis There is a significant lymphoid infiltrate into the portal tract including a lymphoid follicle. Lymphoid infiltration is mostly limited to the portal tract; there is relatively little lobular inflammation.

Hepatic fibrosis usually develops in a porto-central distribution. A number of scoring systems for HCV-infected liver biopsies have been developed in order to standardise histopathological reporting for both routine clinical practice and research studies. The most widely used was developed by Ishak et al [135], a modification of the previous Knodell scoring system [136]. The Ishak system was

originally devised in 1995 as a basis for scoring liver biopsies from subjects with any form of chronic hepatitis, whether virus mediated or autoimmune. The grading score consists of various components describing the infiltration of both portal and lobular areas by lymphocytes as well as describing various types of hepatocyte damage, whether necrosis or apoptosis, all scored 0 to 4.

The staging score consists of an assessment of the degree of fibrosis present, ranging from 0 to 6; a score of 0 represents no fibrosis with 6 being definite cirrhosis [135]. Both the stage and grade components of the Ishak score are subjective and non-linear variables. Therefore, correlations involving components of the Ishak score require ranked correlations rather than linear regression. Other scoring systems include the METAVIR score, popular in continental Europe [137].

Natural history studies of HCV progression have concerned the rate at which hepatic fibrosis develops. Sweeting et al reviewed the data from three HCV infected cohorts in order to estimate fibrosis progression rates in the UK [138]. After adjustment for confounding variables, they found that the probability of developing cirrhosis after 20 years of HCV infection varied between 6% for a post-transfusion cohort (Figure 10b), 12% for a cohort derived from secondary care (Figure 10a), to 23% in a cohort at St Mary's Hospital in London, a tertiary referral centre (Figure 10c). Clearly there was significant ascertainment bias inherent to these cohorts; patients with HCV-related complications and rapid disease progression will be over-represented in a cohort derived from tertiary care. Further, work from Fu et al has demonstrated that referral bias and deriving cohort data from subjects with known HCV in the hospital setting leads to an overestimate of the fibrosis progression rate in the context of chronic HCV infection [139]. They found that subjects with a cluster of other factors including harmful alcohol usage were more likely to have come to clinical attention and to be referred to a hospital setting, therefore leading to an over-representation of subjects with high fibrosis stages in hospital-derived cohorts.

The ability to predict fibrosis progression accurately is important in the clinical management of subjects with HCV given that HCV-related complications are very unusual before the development of cirrhosis [140]. Further, predicting long-term outcome allows planning of future health care needs based on current incidence and seroprevalence of HCV.

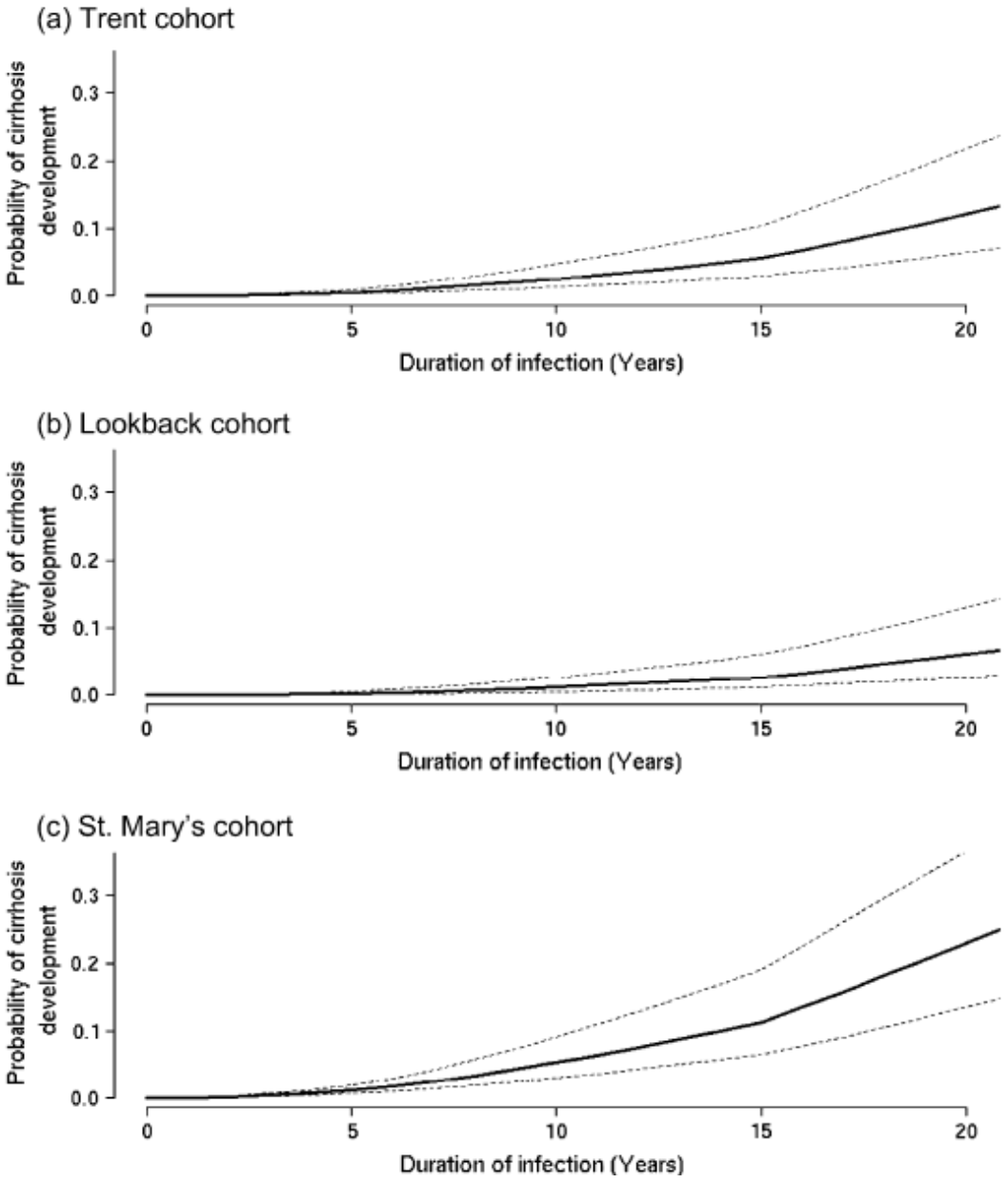

Figure 10. Cumulative probability of developing cirrhosis due to HCV infection from 3 UK cohorts of HCV patients. A) Trent cohort derived from community and secondary care, b) The 'look back' cohort derived from post-transfusion HCV patients and c) St Mary's Hospital cohort. Solid line, mean with 95% confidence intervals dotted lines. Source: [138].

Factors influencing disease progression

From these natural history studies it has become apparent that there is significant disparity in the rate of fibrosis development in HCV [6]. The identification of host, environmental or virological factors that are amenable to modification in order to improve the long-term prognosis or to prevent the development of cirrhosis are important in the clinical management of HCV but also in informing the mechanisms underlying disease progression. The most important risk factors demonstrated to be associated with significant increased rates of fibrosis progression in the context of HCV are age at infection, misuse of alcohol and male gender [6, 141].

Non-modifiable risk factors for progression

1. Age at acquisition and duration of infection

Even after controlling for duration of infection, the age at which patients acquire HCV infection has been shown to hugely influence the subsequent rate of disease progression [6]. Poynard et al demonstrated that individuals infected with HCV before the age of 20 had a twenty year probability of developing cirrhosis of 2% compared to 63% in those individuals infected after the age of 50 (Figure 11).

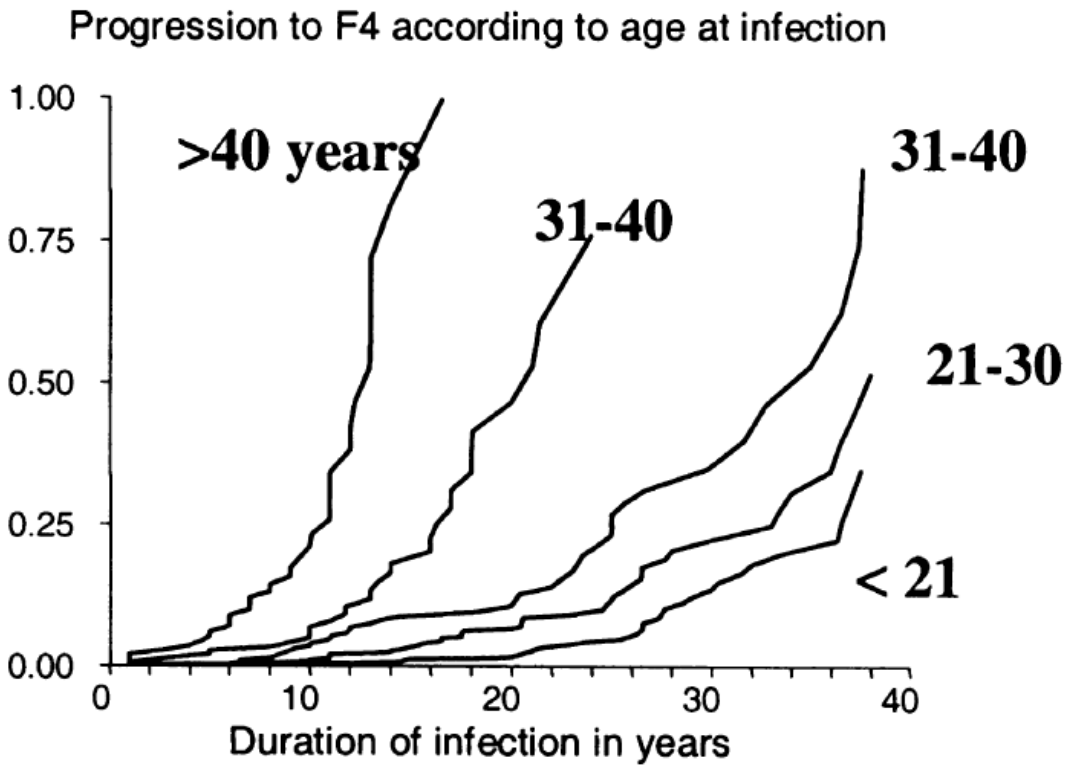

Figure 11. Cumulative probability of developing cirrhosis (METAVIR F4) according to age at acquisition of HCV infection. Source: [142].

Minola et al demonstrated that the time to 50% cumulative probability of developing cirrhosis was 33 years for those infected between 21 and 30, but only 16 years in those infected with HCV after the age of 40 [143]. Poynard et al [141] demonstrated that fibrosis progression rates also increased with increasing age. However, the underlying pathogenesis of the relationship of subsequent fibrosis progression rate and age of patient has not yet been elucidated. Similar findings were demonstrated by Pradat et al who demonstrated increased rates of fibrosis progression with increasing age at infection [144]. Kao et al demonstrated that increasing age was correlated with increasing viral load in viraemic HCV infection [145]. Sweeting et al performed a meta-analysis of three UK based cohorts of patients with HCV infection demonstrating that duration of infection independent of age at infection was significantly associated with increasing hepatic fibrosis [138].

2. Gender

Multiple studies have demonstrated that males have a significantly worse prognosis when infected with HCV [142, 146]. Both Poynard et al and Wright et al have determined that the hazard ratio (HR) for fibrosis progression attributable to male sex is around 2 independent of age and alcohol intake [142, 146].

3. Subject ethnicity

Patients from an Afro-Caribbean background are less likely to develop significant fibrosis in the context of HCV infection [147, 148]. However, Afro-Caribbean individuals are less likely to respond to anti-viral therapy [149] and once cirrhosis has developed are more likely to develop HCC [150].

4. Host genetic factors

Genetic studies to determine the risk attributable to different genetic backgrounds on the course of HCV infection have fallen into two groups: hypothesis-driven studies of genes involved in immune function and hypothesis-free genome-wide association studies.

Asti et al determined that whilst age and sex were the predominant determinants of HCV progression, the presence of DRB11 or DRB3 was associated with higher degrees of hepatic fibrosis [151]. DRB3 was associated with significant fibrosis with an odds ratio of 16, albeit with wide confidence intervals. However, Godkin et al demonstrated that DRB11 was associated with increased rates of viral clearance at the acute stage. Further, on comparing cleared and chronically infected DRB11 positive individuals, they identified 4 restricted epitopes which induced a strong IFN-γ response in those who had cleared HCV, but no response in those with chronic HCV. They suggest this is evidence for the role of CD4+ responses and DRB11 restricted responses in the successful clearance of HCV [152].

Khakoo et al studied killer-cell immunoglobulin-like receptor (KIR) expression patterns in chronic HCV infection and determined that polymorphisms in KIR2DL3 or its ligand HLA-C1 were associated

with increased rates of clearance or HCV infection, but only when the source of HCV infection was not blood transfusion [153]. Their interpretation was that at low infecting viral titres these alleles may allow the immune system to respond to HCV more effectively, but that if HCV was acquired from infected blood transfusion then the infecting viral load was so enormous as to render these alleles unimportant.

Huang et al conducted a genome-wide association scan on 916 HCV-infected individuals, and identified a single nucleotide polymorphism (SNP) in the DEAD box polypeptide 5 (DDX5) gene (p68), an RNA helicase involved in secondary RNA structure [154]. In two further validation cohorts, the presence of this mis-sense SNP was associated with more advanced fibrosis with a HR of between 1.8 and 2.2 [155]. Goh et al had previously demonstrated that DDX5 binds to the HCV polymerase NS5B and that over expression reduces HCV RNA replication in vitro [156].

Ge at al have also performed a genome-wide association scan to identify genetic determinants of successful treatment response to IFN-α. They have identified a polymorphism in the IFN-λ-3 gene that was associated with a two-fold change in the likelihood of successful treatment response. Further, they found that this polymorphism was more common in Caucasian than Afro-Caribbean subjects and accounted for half of the difference in treatment responsiveness between these two ethnic groups [157].

5. Viral factors

The impact of viral factors such as genotype and viral load upon fibrosis progression remains controversial. Most studies in this area suggest that they have no significant contribution [141, 146, 148, 158], and the few positive studies have been criticised for their methodology [159].

Further, the studies demonstrating an effect of genotype on fibrosis progression have conflicting results. Wright et al demonstrated in a cohort of 917 subjects with chronic HCV that non-1 genotypes were associated with increased rates of fibrosis progression independent of age, sex and

alcohol consumption [146]. However, Kobayashi et al demonstrated that Japanese patients with genotype 1 infection were 50% more likely to develop progressive fibrosis on follow-up when compared to genotype 2 infected subjects [159]. Harris et al demonstrated that genotype 1 infection was associated with increased fibrosis progression rates in a UK cohort [160].

What is still not clear is whether the rates of viral clearance at the acute hepatitis stage are different between genotypes. A previous study by Lehman et al has suggested that individuals infected with genotype 3 HCV have higher rates of spontaneous clearance than those infected with non-3 [50]. However, Harris et al suggest that rates of spontaneous clearance may be higher with genotype 1 infection [160].

Modifiable risk factors for progression

1. Alcohol consumption

Misuse of alcohol has been demonstrated to have a significant effect in accelerating the rate of fibrosis progression in the context of HCV infection. Poynard et al demonstrated that patients who consumed greater than 50g of alcohol per day were significantly more likely to develop progressive hepatic fibrosis (Figure 12) [141]. Ostapowicz et al demonstrated that HCV cirrhotic subjects had greater lifetime alcohol intake and greater intake of alcohol during the period of HCV infection than non-cirrhotic subjects [158]. Wiley et al determined the HR for developing cirrhosis attributable to excess alcohol consumption was between 2 and 3 [161].

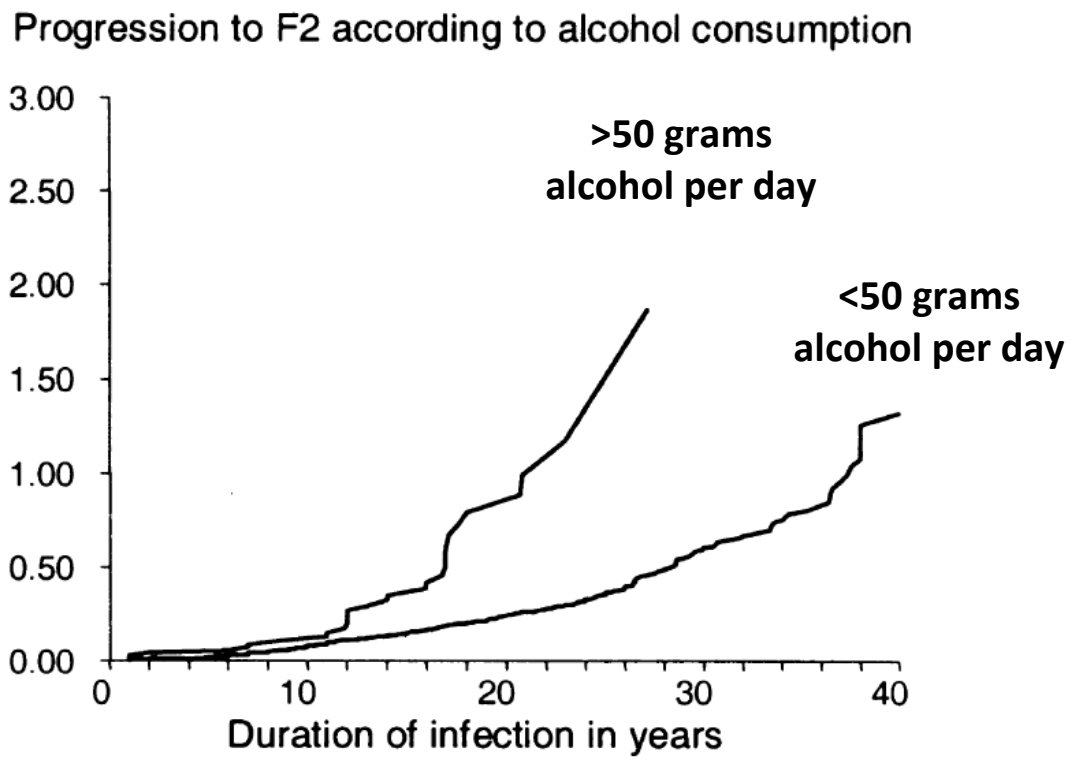

Figure 12. Cumulative probability of developing moderate fibrosis (METAVIR F2) in the context of HCV infection and relationship to alcohol intake. The cohort of 2234 patients was divided based on daily alcohol intake of more or less than 50g alcohol per day. The curves were significantly different by Log-Rank test ($p<0.05$). Source: [141].

Further, other groups have demonstrated that excess alcohol consumption led to a significant increase in mortality compared to HCV infected subjects who were abstinent or drank within recommended limits [162]. Through prospective follow-up of a cohort of 924 HCV-infected individuals over sixteen years, Harris et al identified thirty-four who had died as a direct result of liver disease. In ten cases (29%) excess alcohol was a co-factor in their death [162]. Compared to drinkers of 1 – 20 units of alcohol per week, survival was worse for those consuming greater than twenty units per week (HR 1.15, 95% CI: 0.8 – 1.6).

In a meta-analysis Hutchinson et al demonstrated the relative risk of developing cirrhosis in the context of HCV infection attributable to excess alcohol usage was 2.33 (95% confidence interval, 1.67-3.26) [163].

2. *Iron metabolism*

The deposition of iron on liver biopsy occurs in around one third of patients with chronic HCV infection [164, 165]. This may occur due to chronic inflammation [166] or to increased absorption of dietary iron. Iron absorption is suppressed by hepcidin, released from the liver to act upon small bowel enterocytes. Fujita et al demonstrated that subjects with HCV infection have lower hepcidin levels than healthy control subjects [167]. Furutani have investigated this further with a mouse model expressing the HCV polyprotein. These mice develop hepatic iron loading and have low hepcidin levels [168].

Several studies have linked the presence of iron deposition with increased rates of fibrosis progression [166, 169, 170] and reduced rates of response to IFN-α based anti-viral therapy [171]. However, when studying the effect of HFE mutations, the commonest cause of hepatic iron accumulation in a Caucasian population, there is no effect of either hetero- or homozygous mutations on fibrosis progression rates in HCV infection [166], suggesting that iron accumulation in HCV infected liver is independent of the mechanisms leading to iron loading in genetic haemochromatosis.

Hepatic iron depletion, through repeated venesection, is associated with a reduced rate of fibrosis progression [172], lower rates of development of hepatocellular carcinoma [173], and increased response to anti-viral therapy [174].

3. *Inflammatory activity and ALT*

Several studies have linked the severity of inflammation on initial biopsy to subsequent development of fibrosis [144, 175-177]. However, other studies have not [141, 146]. Pradat et al demonstrated in their cohort that those individuals with increased ALT, a surrogate marker of hepatic inflammation and hepatocyte necrosis, had significantly increased rates of subsequent fibrosis progression compared to those with lower levels of ALT [144].

Similarly, several studies have investigated whether subjects with persistently normal ALT, an indirect marker of hepatic inflammation and hepatocyte necrosis, have improved prognosis. Kyrlagkitsis et al demonstrated that those with persistently normal ALT had reduced rates of subsequent fibrosis [178]. However, given that around 15% of their study cohort with persistently normal ALT had significant inflammation or fibrosis, they recommended not using ALT levels to guide decisions regarding clinical management.

4. Obesity and Insulin resistance

Studying the relationship between HCV, insulin resistance and hepatic steatosis is complicated as both HCV and the development of cirrhosis have been linked independently to the subsequent development of insulin resistance and the metabolic syndrome [99, 179]. Further, it is clear that infection with genotype 3 HCV is associated with the development of hepatic steatosis [99].

Determining whether individuals with insulin resistance are at increased risk of fibrosis progression due to HCV infection is fraught with confounding variables. Several studies have demonstrated that those with steatosis on biopsy have increased levels of fibrosis [180, 181]. Moucari et al studied 500 patients with HCV infection prospectively and demonstrated that insulin resistance was associated with genotype 1 infection and viral load, but was independent of the degree of steatosis on liver biopsy [182]. Further, Tanaka et al demonstrated that insulin resistance improved when receiving interferon-α therapy [183]. However, given that some degree of anorexia occurs in most patients receiving IFN-α, a study of insulin resistance in those achieving SVR compared to those who do not is needed to answer this question.

Similarly, investigating the relationship of obesity independent of insulin resistance and steatosis on the prognosis of chronic HCV is problematic. Obesity is known to be associated with increased rates of HCC in the context of HCV [184] and reduced rates of response to IFN-α therapy [185]. Further,

weight loss in subjects with HCV is associated with improvements in ALT levels and insulin resistance [186].

Recent data from the multi-centre HALT-C trial has demonstrated that baseline features of metabolic syndrome such as increased weight or insulin resistance are associated with an increased likelihood of fibrosis progression in a subsequent liver biopsy [187]. Specifically they found that each quartile increase in insulin resistance as defined by the HOMA-IR2 had a HR for fibrosis progression on second liver biopsy of 1.26.

5. Co-infection with other pathogens

a. Hepatitis B virus (HBV) infection

Because of the shared risk factors for the acquisition of both HCV and HBV, particularly IDU, co-infection is not uncommon. From a clinical point of view several studies have demonstrated that co-infection with HBV and HCV leads to a worse clinical outcome when compared to individuals infected with either single virus [188, 189]. HBV / HCV co-infection leads to increased rates of fibrosis progression [190], increased rates of development of HCC [191], but similar rates of treatment response compared to singly-infected patients [192]. Amin et al demonstrated in a large community-based study that the standardised mortality ration for liver-related death was 12.2 for HBV, 16.8 for HCV and 32.9 for HBV / HCV co-infection [193].

Although the clinical outcome for co-infection is worse than singly infected patients, significant in vivo interaction between the two viruses occurs leading to an inverse relationship in the replicative level of the two viruses [188, 189]. Liaw et al demonstrated that acute HCV infection in patients previously infected with HBV led to a worse clinical outcome, but suppression of HBV replication [191]. Similarly, Sagnelli et al demonstrated that acute HBV infection in subjects with chronic HCV infection led to suppression of HCV viral replication [194]. Further, suppression of replication of one of the viruses can lead to the re-emergence of the other. Liu et al demonstrated that successful IFN-

α therapy for HCV could lead to the re-emergence of HBV viraemia in patients with previously undetectable HBV DNA [192].

Occult HBV infection has been described recently, where individuals lack serological evidence of HBV infection, but have HBV DNA in serum [195]. Current evidence suggests occult HBV infection is not associated with increased rates of fibrosis progression [196], but is associated with an increased risk of HCC [197, 198].

Anti-HBc in the absence of HBsAg, indicative of previous acute HBV infection, may have a negative impact upon HCV infection. In a cross-sectional study Carvalho-Filho et al demonstrated that HCV infected individuals with detectable anti-HBc had higher levels of both inflammation and fibrosis independent of confounding variables [199]. However, other studies have not demonstrated a link between previous acute HBV infection and fibrosis progression in HCV [200].

Relatively few studies have investigated the mechanisms underlying the interaction between HBV and HCV viruses. Bellecave et al utilised the Huh-7 cell line stably expressing HBV virus and demonstrated that subsequent transduction of the HCV replicon led to successful replication of both viruses within the same cell [201]. Further specific inhibition of the replication of either virus had no effect upon the replication of the other [201].

b. Human immunodeficiency virus (HIV) infection

Similar to HCV/HBV co-infection, the risk factor of IDU makes HCV/HIV co-infection common amongst high-risk groups. In the current era of Highly Active Anti-Retroviral Therapy (HAART), liver disease and in particular HCV-related liver disease has emerged as one of the main causes of morbidity and mortality in patients with HIV infection [202].

Eyster et al demonstrated in prospective study that HIV infection was associated with a log increase in HCV viral load, associated with declining immune function [203].

HIV infection is associated with an increased rate of chronic infection following acute HCV [48] and increased rates of fibrosis progression in chronic HCV infection [87, 88]. In a meta-analysis of eight separate cohorts, Graham et al demonstrated that HIV/HCV co-infection was associated with a hazard ratio of around two for the development of cirrhosis compared to HCV mono-infection [87].

HAART may improve the prognosis of HCV-related liver disease in the context of HIV infection. Qurishi et al demonstrated that HAART therapy was associated with a odds-ratio of 0.11 (0.02 - 0.56) for HCV-related mortality when compared to untreated or historical controls treated with less effective anti-viral regimes [204]. However, HCV infection is associated with higher rates of HAART-related liver toxicity compared to HIV mono-infected subjects [205].

c. Co-infection with *Schistosomiasis Mansoni*

In many parts of the developing world chronic infection with Schistosomiasis is endemic [206]. *S.Mansoni* infection not only leads to portal fibrosis and non-cirrhotic portal hypertension [207], but the helminth skews the immune response to subsequent viral infections [208, 209]. Schistosomiasis is associated with a reduction of Th1 responses and an increase in both Th2 and regulatory responses [210, 211]. It has been established that co-infection with HCV and *S.Mansoni* is associated with reduced rates of viral clearance [212], increased rates of fibrosis progression [213] and reduced rates of response to anti-viral therapy [214].

Ageing and the liver

The mechanisms of normal human ageing are little understood but recognised increasingly as important in a number of disease processes [215]. Clearly patients of the same age can be very different in terms of physiological reserve and susceptibility to disease in the absence of co-morbidity. However, the processes involved and correlates that are easily identifiable or measurable are lacking. Studies are hampered by the increased prevalence of pathology with age, confounding what constitutes healthy change with age and what constitutes pathological change.

Studies of healthy human ageing have relied on the study of healthy elderly individuals or by studying younger individuals afflicted with one of the Progeria syndromes associated with accelerated and premature ageing [216]. However, both are affected by methodological problems. The former, by 'survivor bias' [217], i.e. those individuals who have survived to old age may show retarded features of biological ageing compared to those who have died; the latter, by a lack of understanding of the underlying pathophysiology and its relevance to normal human ageing.

With an ever increasing geriatric population in most developed countries and a disproportionate consumption of healthcare resources, increasing effort needs to be channelled into understanding the mechanisms of ageing and possible avenues for therapeutic intervention [215].

Interest in the role of ageing within the sphere of hepatology has increased with the recognition of its importance in predicting outcome in chronic HCV infection [218] and donor age as a critical predictor of post-transplant outcome [219]. Furthermore, liver-related death in the elderly is increased when compared to younger individuals [220].

Macroscopic changes of the liver with age

Post-mortem studies have demonstrated a reduction in liver mass with advancing age in humans [221, 222] which has been corroborated by *in vivo* liver volume measurements with ultrasonography [223-225]. This change may relate to reduced hepatic blood flow; Sherlock et al described a fall in splanchnic blood flow with increasing age [226]. Other groups have confirmed progressive loss of liver volume, associated with a 35% reduction of hepatic blood flow in subjects over 65 years when compared to those less than 40 years [227].

Wakabayashi et al [228] utilised radio-labelled galactosyl-albumin to demonstrate that while total hepatic mass was not diminished, functional hepatocyte mass decreased in relation to age, whilst others have demonstrated a decline in hepatic clearance of plasma galactose with age [229]. Meier et al utilising computed tomography (CT) and CT-positron emission tomography (PET) found no

decrease in liver volume with age; however a significant negative correlation between increasing age and attenuation and a significant positive correlation between age and metabolic activity were demonstrated [230].

Microscopic changes of the liver with age

Profound microscopic changes occur in the liver with ageing. Ageing in rats, baboons and humans is associated with a 50% increase in the thickness [231-234] and defenestration of the sinusoidal epithelium measured by reduced porosity. Ageing is also associated with deposition of collagen and formation of basement membrane within the space of Disse [235]. Radio-labelled phosphate spectroscopy indicated hepatocyte hypoxia, leading to the suggestion that with reduced hepatic blood-flow and an increased barrier to diffusion, that impairment of liver function with age may be consequent to intra-hepatic hypoxia [235].

The underlying pathogenesis of these changes is unclear. Portal venous transported gut-derived toxins, such as alcohol, endotoxin [236] and oxidants [237] are known to produce significant changes to the sinusoidal endothelium. Ex vivo rat liver, perfused via the portal vein with hydrogen peroxide, demonstrated significant sinusoidal endothelial damage, Kupffer cell activation and increased space of Disse volume [237]. Chronic exposure to toxins transported in the portal vein may be important in age-related changes seen in humans.

Hepatocyte changes with age

Hepatocyte structure also changes with age. The volume of hepatocytes increases with development and maturation but declines with senescence [238, 239]. The relative volumes of hepatocyte organelles also change during ageing. The most common change on diagnostic liver biopsy specimens is age-related cytoplasmic accumulation of highly oxidised insoluble proteins, known as lipofuscin [240, 241].

These accumulations of highly cross-linked protein are thought to relate to chronic oxidative stress and a failure to degrade damaged and denatured proteins [241]. Increasing evidence suggests that lipofuscin interferes with cellular pathways due to its ability to trap metallic cations and facilitate further free radical formation [242].

Other sub-cellular hepatocyte changes with age are less well described, with most data arising from animal studies. There is a marked decline in smooth endoplasmic reticulum surface area with age [239, 243] which correlates with decreased hepatic microsomal protein concentrations and enzymatic activity such as Glucose-6-Phosphatase [243]. Recent data have suggested there is an age-related decline in autophagy, the cellular pathway leading to degradation of molecules and sub-cellular organelles. In a mouse model associated with age-related decline in liver function, restoration of autophagy was associated with maintenance of liver-function [244].

There are also data suggesting that there is a change in hepatocyte nuclear morphology with increasing age. It is known there is increased variation in nuclear size [245], associated with increasing incidence of polyploidy of hepatocytes [246]. After the age of 85, around 27% of human hepatocytes demonstrate polyploidy [247].

Change in liver function with age

Drug metabolism declines with ageing, manifest as increased levels of toxicity and increased levels of adverse drug reactions within the elderly population [248, 249]. Age related susceptibility has been shown for some drugs, of which isoniazid [250], halothane [251] Flucloxacillin and Co-amoxiclav [252-254] are the most notable.

Age-related decline of drug-metabolizing enzyme activity has been shown in animals but has been replicated in very few human studies. Animal studies have shown decreased hepatocyte microsomal content of cytochrome P-450 in older mice [255] and reduced activity of alcohol dehydrogenase in aged rats [256]. One human study has demonstrated a progressive decline in microsomal P-450

concentration from liver biopsy specimens in subjects after the age of 40. Similarly, the plasma clearance rate for antipyrine declined progressively after 40 years of age [257]. However other studies have only demonstrated non-significant trends in declining liver metabolic activity with age, possibly due to high inter-individual variability [258].

Commensurate with possible declining metabolic activity rat studies have demonstrated that there may be a decrease in bile acid secretion or flow rate with progressive ageing [259].

Change in the liver's response to injury with age

One of the most important age-related changes in liver function is the significant decrease in regenerative capacity of the liver [260-262]. In rat studies utilising the partial hepatectomy model, complete hepatic restoration occurs in older animals, but at a slower rate than in younger animals [263]. Liver regeneration in both young and old animals was complete by day 7 after 70% hepatectomy, but at day 1 younger animals had significantly increased liver mass and increased intrahepatic mitotic activity [264].

Clinical outcome in human acute liver injury partly relies on hepatic regenerative potential. Older people have a higher incidence of acute liver failure and a higher mortality with acute hepatitis A [265, 266]. In a cohort of patients with acute liver failure secondary to viral hepatitis, age was an independent predictor of a poor outcome with those over 50 faring worst [267].

Ageing and chronic liver diseases

Increasing age has now been recognised as a significant predictor of poor outcome in a number of chronic liver disorders. Perhaps the best studied is chronic HCV infection. Poynard et al demonstrated that age at infection was a major risk factor for subsequent fibrosis progression [218]. Other groups have subsequently confirmed these findings; individuals older than 37 at time of infection have accelerated fibrosis compared to younger individuals [6, 144, 268].

Studies of other liver diseases have demonstrated similar findings. Age is an independent risk factor for poor outcome in primary biliary cirrhosis (PBC) in addition to the presence of portal hypertension and impaired liver function [269]. Similarly, age independent of bilirubin, prothrombin time and renal function predicted outcome in patients with alcoholic hepatitis [270].

There is some evidence that age may influence disease progression in non-alcoholic fatty liver disease (NAFLD). Studies from France and the USA have shown that increasing age, increasing body mass index and type-2 diabetes were associated independently with cirrhosis in patients with NAFLD [271, 272]. Being older than 50 years gave an odds ratio of 14 for the presence of at least severe fibrosis [271].

Work by Wali et al [273] and Berenguer et al [274] indicate an association between advancing allograft donor age and more rapid histologic progression after HCV graft infection. If the liver donor was younger than 40 years, the median interval to post-transplant cirrhosis was 10 years compared to only 2.2 years when the donor was aged 50 years or more [273]. Berenguer et al studied 522 patients undergoing liver transplantation between 1991 and 2000, including 283 (54%) infected with HCV. Patient survival was lower in HCV positive recipients and they suggest that the increasing age of donor organs was contributing to decreased patient survival in recent years [274].

Mechanisms of ageing and senescence in the liver

There has been considerable interest in elucidating mechanisms that regulate the capacity for cell division and regeneration in the context of ageing. The accumulation of nuclear DNA damage and the cellular response to this damage is crucial in determining the response of whole organs and organisms to chronic diseases.

Telomeres

Substantial attention has focussed on the telomere / telomerase system as a mediator of replicative capacity [275]. Telomeres are repeating hexanucleotide sequences which, with their associated

protein complexes, function to protect chromosomes against events such as chromosomal end-end fusion and non-reciprocal translocations. Dysfunctional telomeres cannot retain chromosomal integrity by progressive attrition or uncapping associated with loss of peri-telomeric protein components of the shelterin complex including telomere repeat-binding factor 2 (TRF2) [276].

In the absence of compensatory mechanisms, telomere DNA shortens with each division, reflecting incomplete synthesis of telomere termini during chromosomal replication [277]. With repeated cell division a point of critical telomere shortening is reached and a growth arrest signal is developed, preventing further cell division.

Short telomeres are detected as double-strand DNA breaks [276]. With progressive loss of telomeres, there is a commensurate loss of inhibitors of DNA damage response elements such as TRF2 [278]. Loss of this inhibition leads to recruitment of the PI3-kinases ATM and ATR, phosphorylation of Histone 2A at serine 139 (γ-H2AX) and stabilisation of p53 [277, 279, 280] and possibly p16 [281, 282]. This state is characterised *in vitro* by growth arrest, inability to respond to external mitogens and altered cell morphology termed replicative senescence.

Accelerated telomere shortening has been demonstrated in conditions associated with inflammation and accelerated cell turnover [283], leading to the concern that in chronic liver diseases chronic hepatocyte turnover may lead to a telomere mediated loss of replicative ability. Further, known risk factors for ill-health such as obesity and smoking have been associated with accelerated loss of telomere length [284, 285].

Telomerase

Telomerase is a reverse transcriptase consisting of enzymatic (TERT), RNA template (TERC) and several other protein components including heat-shock protein 90 (hsp90) and dyskerin [286]. It can maintain telomere length by adding TTAGGG repeats, but its expression is tightly controlled outside stem-cell populations [287, 288]. Ectopic expression of telomerase led to lengthening of telomeres,

continued cell division and extension of *in vitro* lifespan [289]. Re-expression in non-stem cell populations in life is unusual and telomerase is expressed in around 80% of human carcinomas, including hepatocellular carcinoma [290].

Recent evidence has suggested that telomerase may have a role in several cellular pathways beyond its known role in elongation of telomeres and replicative ageing. Regulation of apoptosis, gene expression and chromatin structure during the cell-cycle have been ascribed to telomerase activity [291].

Genetic evidence for a role of telomere dysfunction in human ageing comes from the discovery that germ-line mutations of the telomerase complex cause the Progeroid syndrome Dyskeratosis Congenita [292]. In the field of hepatology, there is a growing body of evidence for the role of hepatocyte telomere shortening and a relation to clinical outcome. Reduction in hepatocyte telomeres occurs with normal ageing and may give an indication of residual proliferative potential [293, 294].

Telomeres in chronic liver disease

Both Kitada et al and Urabe et al showed that those with chronic viral hepatitis had shorter hepatic telomeres than healthy controls and that increasing fibrosis was associated with shorter telomere lengths [295, 296]. However, utilising liver biopsy homogenates and restriction fragment length analysis precluded identifying the cell types that underwent telomere shortening. Wiemann et al demonstrated that hepatic telomere length was shortened in cirrhosis irrespective of the primary disease aetiology and suggested that the shortening was limited to hepatocytes [297]. Rudolf et al demonstrated the importance of this system in chronic liver diseases. Telomere dysfunction in mice was associated with the onset of cirrhosis in the context of chronic liver injury [298]. Through adenoviral reintroduction of telomerase activity it was possible to prevent critical telomere shortening, deterioration in liver function and cirrhosis [298].

Telomeres in HCC

Intact telomeric signalling has also been demonstrated to be important in the development of hepatocellular carcinoma (HCC). The beneficial effects of the intact telomere / telomerase system have been demonstrated with a suppression of the development of HCC in a mouse model [299].

Telomeres within HCC were demonstrated to be shorter than surrounding non-cancerous liver [300], suggesting that HCC had escaped from the cell-cycle arrest signal generated from short telomeres. Interestingly, short telomeres or telomere dysfunction appears permissive for the development of early stage neoplasia, but inhibitory to later stage and more anaplastic lesions [301]. The role of the telomere / telomerase system in the pathogenesis of HCC have led some to suggest that therapeutic manipulation may hold the promise for future therapies [287].

Telomeres: ageing versus cancer

Through the use of the telomere system of replicative ageing, organisms may be paying the price of continued tumour suppression [299, 302]. In mice, driven to senescence and critically short telomeres, through germ-line lesions of telomerase [303], a multi-organ phenotype of senescence develops with functional impairment of tissues requiring high levels of cell replication such as skin, bone marrow, and reproductive organs [303]. Cells from these mice demonstrate multiple features of telomere-induced senescence with aneuploidy, chromosomal end-to-end fusions, high p53 expression and limited number of cell divisions in culture.

These features of cellular and organ senescence can be reversed through a subsequent lesion of p53, thereby preventing telomere dysfunction to signal through to cell-cycle arrest, allowing further *in vitro* cell division and *in vivo* preservation of organ function [304]. Telomere shortening in the telomerase deficient mouse line was associated with increased expression of p53, growth arrest and increased apoptosis. Simultaneous lesions of telomerase and p53 prevented testicular atrophy and cells had reduced levels of apoptosis with higher rates of passage through the cell cycle [304].

However, whilst these mice have a partial reversal of their aged phenotype, there is an increased risk of *in vitro* cellular transformation and *in vivo* development of skin, breast and gastrointestinal carcinoma [305]. In contrast to telomerase deficient mice, compound telomerase and p53 deficient mice have a high rate of development of epithelial carcinomas which are associated with complex non-reciprocal translocations as a consequence of loss of telomeric protection of chromosomal ends. Therefore these data suggest that telomeric attrition in tissues with a high turnover rate leads to the development of a DNA damage signal through p53 and subsequent senescence before loss of telomeric chromosomal protection can lead to the development of pro-carcinogenic genetic translocations [305].

More recent studies on a similar mouse model by Choudury et al have shed further light on the downstream mechanisms of telomere dysfunction [306]. In late generation telomerase-deficient mice, a subsequent lesion of the cell-cycle inhibitor p21 prevented the organ dysfunction and accelerated ageing phenotype associated with dysfunctional telomeres but did not lead to an increase in chromosomal instability or increase in tumour formation demonstrated in the p53 deficient mouse.

Davoli et al have utilised p53 deficient cells to demonstrate that persistent DNA damage signal from deprotected telomeres led to a bypass of mitosis and second progression through S phase [307]. This allows tetraploidisation, an early step in cellular transformation.

These studies suggest that p21 may mediate cellular senescence down-stream of both dysfunctional telomeres p53. Further, that p53 has a wider role in tumour suppression and prevention of cellular transformation as well as cellular senescence.

Viruses and the telomere / telomerase system

A number of viruses causing human disease have evolved mechanisms for manipulating telomere signalling or telomerase to promote replication and transmission [308]. EBV and HPV encode

proteins that up-regulate telomerase activity, which may play a role in the human cancers associated with these viruses.

HBV is associated with a high incidence of HCC. Several groups have reported a role of HBV genome integration and up-regulation of telomerase activity. In particular the HBV X protein disrupts p53 signalling as well as other cellular signalling pathways including protein kinase C and NF-kappa B [309]. Several groups have demonstrated a direct role of HBV X protein in the up-regulation of telomerase activity [310, 311]. Others have also demonstrated that other HBV associated proteins also up-regulate telomerase activity [312]. This ability to functionally bypass telomere control of replication might predispose to HCC formation. However, some groups have demonstrated *suppression* of telomerase activity [313].

Oxidative stress

Senescence can occur in the absence of telomere shortening due to oxidative stress or DNA damage distant to the telomere [281, 314]. Zhang et al [315] compared hepatocyte injury in response to hypothermic challenge, leading to increased oxidative stress, in young and old rats and concluded that old rats were more susceptible. There is evidence for both a decline in DNA repair and increase in DNA or cell sensitivity to oxidative stress with increasing age. Intano [316] reported a 50% age related decline in DNA base excision repair in old age; Hamilton [317] attributed the increased levels of oxidative damaged suffered by DNA in senescent mice and rat livers to increased DNA or cell sensitivity to oxidative stress. How cell sensitivity to oxidative stress is mediated is unclear. This may be mediated by NF-κB [318] which has increased activity with ageing and which may induce target genes including Haemoxygenase. Up-regulation of pro-apoptotic genes, such as Gadd153, which increase cellular sensitivity to oxidative stress may also play a role [319].

Oxidative stress and telomeres

Recent evidence suggests that oxidative stress accelerates telomere shortening [320, 321]. It has long been known that low ambient oxygen conditions can extend the lifespan of cells in culture [322]; however the underlying mechanism of this effect was uncertain. Studies have demonstrated that when cultured cells are protected from oxidative stress through low ambient oxygen tension, addition of anti-oxidants or over-expression of antioxidant enzymes, telomeric decline is slowed [323-325].

Due to their high guanine content, telomeres are highly susceptible to oxidative-induced DNA damage [326]; a human telomeric sequence inserted into a plasmid suffered 7 times more DNA damage due to hydrogen peroxide induced oxidative damage than a control sequence [327]. Reactive oxygen species cause single-strand DNA breaks and telomeres lack the surveillance mechanisms for single-strand breaks that are employed in the rest of the genetic material [328]. Induced single-strand breaks in non-telomeric DNA were repaired within one day, whereas DNA repair within the telomere was delayed and ultimately incomplete [328]. The mechanism linking accumulation of oxidative DNA damage and accelerated telomere shortening is currently uncertain [320]. One possibility is the extrusion of telomerase from the nucleus of cells suffering oxidative stress, thus preventing the usual intra-nuclear role of telomere preservation. Once in the cytoplasm, telomerase co-localises with mitochondria [329]. In cells with over-expression of telomerase, mitochondrial DNA is protected from oxidative damage and cellular levels of reactive oxygen species are lower than control cells [329].

Further data has demonstrated the important interaction between telomere-induced senescence and oxidative stress. Passos et al demonstrated that cells driven to either irradiation- or telomere-induced senescence developed increased mitochondrial ROS production [330]. Further, they demonstrated that knockdown of either p53 or p21 prior to induction of senescence prevented the increased mitochondrial ROS production. They demonstrated that activation of p21, or downstream

signalling through TGF-β, was able to induce further DNA damage foci and subsequent DDR, leading to the conclusion of a positive feedback loop [330]. Senescence leads to the development of oxidative stress which reinforces the senescent state of the cell and causes further oxidative stress.

Whether the combination of telomere shortening and oxidative stress represents the double hit required to accelerate progression of liver diseases is uncertain. Certainly the combination of two concurrent liver diseases leads to accelerated disease progression and a worse outcome. For example, both diabetes mellitus (DM) and alcohol misuse accelerate the fibrosis progression of HCV infection [6, 218, 331, 332]. It has been described that HCV is associated with shortened intrahepatic telomeres [297] and both DM [333] and alcohol [334] are known to cause oxidative stress through mitochondrial dysfunction. In their study of hepatocyte telomeres Sekoguchi et al investigated the relationship between hepatocyte telomeres, markers of cell-turnover and oxidative stress in chronic HCV infected patients [335]. They found that hepatocyte telomeres were shortened progressively with increasing hepatocyte fibrosis stage and with increasing evidence of hepatocyte oxidative stress as measured by 8 deoxy-guanosine [335].

Bypassing the telomere system

Whether all cell types are affected equally by telomere signalling and telomerase is not known. Data from the mouse suggested that hepatocytes may bypass the growth inhibitory effects of short or dysfunctional telomeres [336]. TRF2 is a component of the shelterin complex, a group of peri-telomeric proteins that protect telomeres from strand-breaks and regulate access of telomerase to the telomere [337]. Denchi et al, using a conditional knockout of TRF2 demonstrated evidence of telomere dysfunction with telomere fusions and cellular responses to DNA damage with increased γ-H2AX. However, despite telomere dysfunction, there was no increase in p53 expression, no decrement in hepatic regeneration following partial hepatectomy, but cell replication did not occur. Instead, the liver was reconstituted by an increase in the size and ploidy of the remaining

hepatocytes (Figure 13) [336]. Whether similar mechanisms operate within the human liver is unknown.

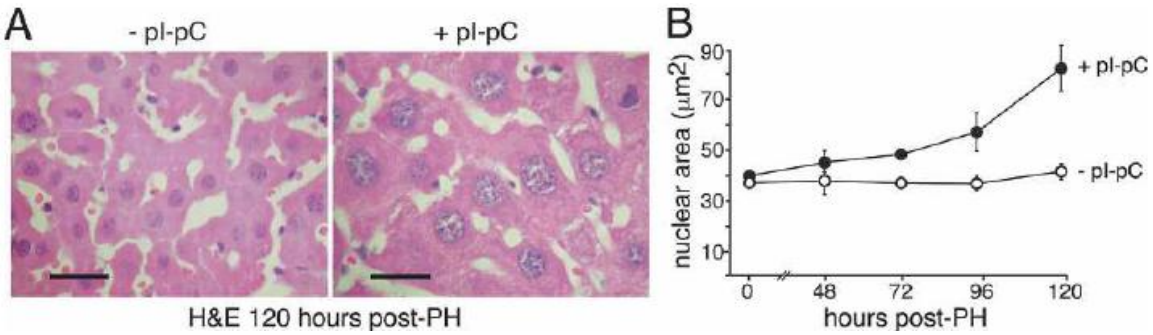

Figure 13. Photomicrograph of H&E stained mouse liver sections following partial hepatectomy (PH) taken from a conditional knock-out of TRF2. When treated with pI-pC, the hepatocytes develop telomere dysfunction, thereby preventing cell proliferation. After knockout of TRF2, the hepatocytes responded to partial hepatectomy by increasing nuclear area (panel B), and ploidy (not shown). Source: [336].

Other markers in senescence

The presence of senescence can be inferred by other markers. In particular senescence associated β-galactosidase (β-GAL) is associated with replicative senescence. In a study of liver tissue from subjects with chronic HCV infection, the presence of β-GAL positive cells was associated with increasing age and increasing levels of fibrosis [338]. Further, analysing the role of senescent cells in donor allografts has demonstrated a significant relationship between β-GAL positive cells on the reperfusion biopsy and the subsequent rate of fibrosis progression of post-transplant HCV recurrence [339].

Senescence of other cell types within the liver

The evidence that hepatocytes may be resistant to the effects of replicative ageing and telomere shortening is intriguing [336]. Given the evidence linking chronic liver disease with telomere shortening and the rescuing of experimentally induced cirrhosis by telomerase gene delivery [340], this throws up the possibility of senescence affecting other cell types within the liver other than hepatocytes.

A recent study by Krizhanovsky et al has demonstrated the crucial role of stellate cell senescence in hepatic fibrosis progression in a mouse model [341]. In a CCl_4 model they demonstrated an accumulation of stellate cells bearing markers of senescence such as β-GAL; that these cells had a reduced capacity to synthesise extracellular matrix components; and that in mice with knockouts of senescence associated genes, the failure of stellate cells to develop senescence was associated with enhanced hepatic fibrosis production. This is perhaps unexpected when cirrhosis is associated with hepatocyte senescence, that fibrosis progression was associated with a failure of stellate cells to senesce.

Therefore, the precise interplay of differential ageing of different intrahepatic cell types in different hepatic diseases may be far more complex than first imagined.

Form and function of senescent cells

There is an increasing body of work demonstrating the role of senescence in vivo. With the onset of cellular senescence cells can remain viable within tissues for long periods; resistance to apoptosis is a characteristic of senescent cells [338, 342]. These cells have a significant change in their form and function.

Induction of senescence of fibroblasts *in vitro* leads to a change to larger, flatter cells with increasing irregularity of shape [277]. An increasing body of work describes the change in the cellular secretosome. This senescence-associated secretory phenotype (SASP) has been demonstrated in other systems to be pro-inflammatory (figure 7). Kuilman et al demonstrated that senescent cells secrete large quantities of IL-6 among other factors such as IL-8 [343]. They demonstrated that IL-6 acted in a paracrine manner to cause other cells to develop senescence. Depletion of IL-6 prevented this 'infectious senescence' [343].

This raises the possibility that once hepatocyte senescence develops, the change in the tissue micro-environment wrought by the SASP could lead to other hepatocytes becoming senescent.

Cell cycle arrest

Whether cells undergo senescence due to telomere shortening or oxidative stress or a combination of the two, cell cycle arrest supervenes and prevents further cell division. Cell cycle arrest has been demonstrated in chronic liver diseases. Marshall et al [344] have shown chronic HCV infection is associated with increased levels of hepatocyte cell-cycle arrest, which correlated with stage of hepatic fibrosis.

Increased fibrosis is related to p21 expression and senescence is associated with expression of p21 [341, 345]. Cell senescence has been linked to carcinogenesis; senescent fibroblasts promote proliferation of epithelial cells in co-culture [346]. It is possible that senescent hepatocytes promote oncogenic mutations in neighbouring hepatocytes pre-disposing to HCC.

Recent data suggest that viral induced cell cycle arrest is advantageous to the virus. In a p53 knockout fibroblast line, CMV replication was noted to be significantly less efficient when p53 was not present [347], suggesting that some viruses induce a state of cell cycle arrest to aid replication and spread. This effect has also been demonstrated for hepatitis B virus replication. Huang et al demonstrated that four differing methods of inducing cell-cycle arrest in the host cell line all led to increased HBV replication [348].

Ageing and the immune system

Normal human ageing is associated with a number of changes within the immune system. However, these changes may be maladaptive; with increasing age humans suffer increased rates of mortality when developing varied infections such as pneumonia [349], meningitis [350] and influenza [351]. Evidence suggests that the decline in immune function with age is particularly marked within the T-lymphocyte compartment. Clinical tests of T-lymphocyte function such as the delayed-type hypersensitivity reaction elicited by the Mantoux test for immunity to Purified Protein Derivative

(PPD) of tuberculosis suggest that elderly individuals lose T-lymphocyte mediated immunity to antigens they were previously reactive to [352].

Elderly individuals have oligoclonal accumulations of T-lymphocytes, many of which are specific for latent and persistent viruses such as EBV and CMV [353, 354] (Figure 14). Further, in healthy elderly subjects there are a number of immune parameters known to be associated with increased levels of subsequent morbidity and mortality. Ferguson et al identified poor non-specific T-cell proliferative responses, high numbers of peripheral CD8+ T-lymphocytes and low numbers of CD4+ lymphocytes associated with subsequent poor outcome [355]. Olsson et al extended these findings demonstrating that CD8+ T-lymphocytes bearing the mature or antigen-experienced phenotype of CD28- and CD57+ were increased in those who went on to die during the follow-up period [353]. CMV infection was also more common in those subjects who died compared to subsequent survivors [353].

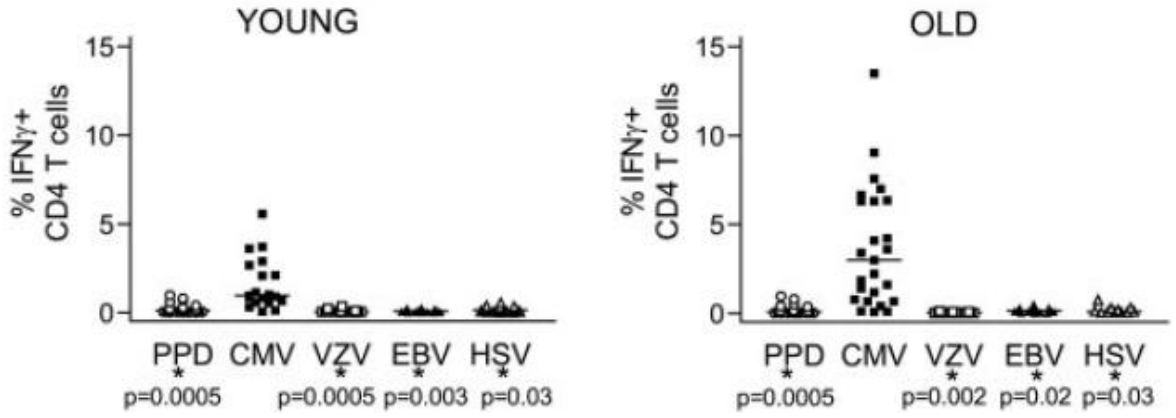

Figure 14. Accumulation of CD4+ T-lymphocytes specific for CMV in elderly individuals. Peripheral CD4+ cells were stimulated with a variety of antigens and identified by staining for intracellular interferon-γ. Source: [356].

T-lymphocyte maturation and differentiation

Prior to antigen-experience naive T-lymphocytes express various surface markers such as CD27, CD28, CD45RA and CD127 (the interleukin-7 receptor). Interleukin-7 signalling via CD127 is required for homeostatic maintenance of the naive T-lymphocyte pool. With antigen experience, cells lose

these markers and express CD45RO, CD57 and KLRG1 [357, 358]. It is known that latent infection, particularly with CMV, drives the accumulation of cells bearing a more advanced or mature phenotype within the peripheral immune compartment [359, 360]. Effros et al demonstrated that cultured lymphocytes driven to senescence more than 99% of the cells were CD28- [361]

Upon studying elderly subjects with and without CMV infection it is apparent that not only does CMV lead to increasingly mature phenotype of CMV-specific lymphocytes but also increasingly more mature phenotype of cells specific for other antigens such as PPD or EBV [356] (Figure 15). In this experiment cells specific for antigens such as EBV and PPD were more likely to display a highly differentiated phenotype of CD27-CD28- if the individual was CMV positive (Figure 15, right panel).

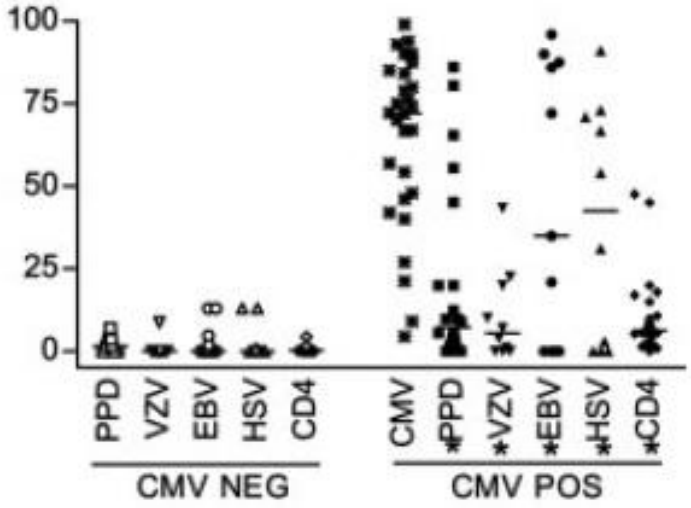

Figure 15. CMV infection leads to change in phenotype of both CMV-specific and non-specific CD4+ lymphocytes. Cells from elderly CMV negative (left panel) and CMV positive individuals (right panel) were stimulated with a variety of antigens and identified by intracellular staining for interferon-γ. The proportions (%) of cells displaying a mature CD27-CD28- phenotype (y axis) specific for different antigens are plotted. Source:[356].

These experiments indicate that there is accumulation of highly differentiated memory T-lymphocytes in elderly individuals which are approaching terminal differentiation and that these changes are in part driven by latent viral infections such as CMV [356, 359, 360, 362].

Commensurate with this change in phenotype is a decline in the function of T-lymphocytes in the older subject. Lymphocytes from elderly people have impaired proliferative ability and sometimes poor cytokine production capacity when compared to lymphocytes from younger individuals [363]. Voehringer et al demonstrated in a study of LCMV infection of mice that infection was associated with large numbers of viral-specific cells within older animals with maintained cytokine secretion but severely impaired proliferative ability [364]. In a study of elderly human subjects with CMV infection Vescovini et al demonstrated that CMV-specific CD4+ and CD8+ lymphocytes had maintained cytokine secretory abilities but severely impaired proliferation [365].

In a series of elegant mouse experiments Ennis et al utilised an adoptive transfer of lymphocytes from young or aged donors into nude (athymic) recipient mice to study the response to primary influenza infection [366]. 16% of younger lymphocytes developed cytotoxicity as compared to 2% of elderly lymphocytes in response to influenza and the peak of response was delayed from day 5 to day 7 with older donor lymphocytes.

By the age of 60 the thymic output of new T-lymphocytes is negligible and therefore the peripheral compartment has to be maintained through replication of pre-existing cells rather than renewal by new cells [363]. However, many of these peripheral lymphocytes have a restricted ability to proliferate due to terminal differentiation and telomere shortening due to large numbers of rounds of previous cell division. Replicative senescence is reached when further replication by a cell is not possible and was originally defined by Hayflick et al [367]. The most accurate correlate available is telomere length which shortens with progressive cell division and can prevent further cell division when critically short telomere attrition is achieved, through signalling through γ-H2AX, p53 and subsequent cell-cycle arrest [282, 368].

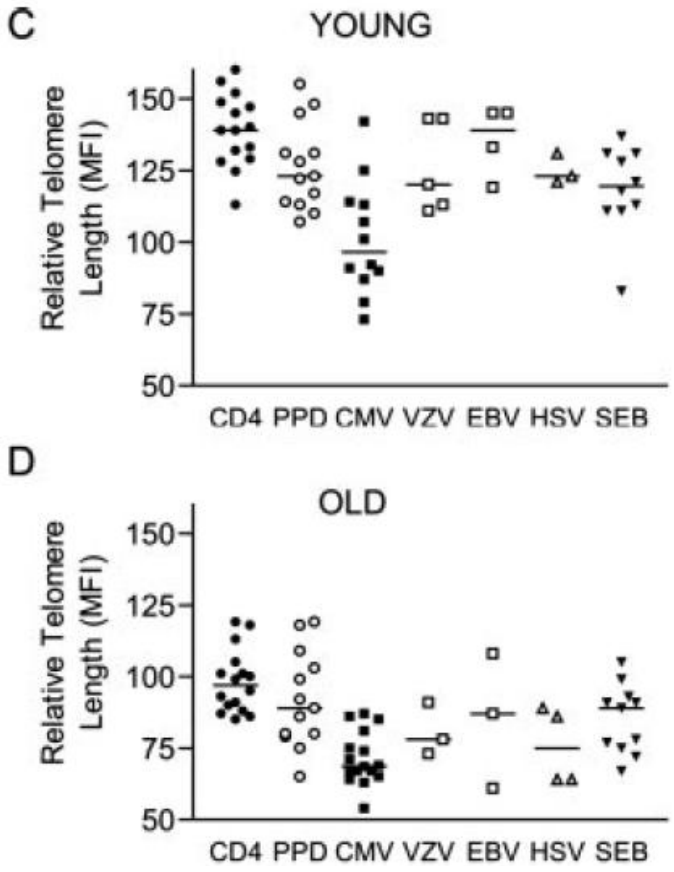

Figure 16. Telomere length of CD4+ lymphocytes specific for different antigens in young (upper panel) and elderly (lower panel). Telomere length is measured as mean fluorescence intensity. Elderly individuals have shorter telomere length in all subsets compared to younger individuals and cells specific for latent or persistent viral infections have shorter telomeres than cells specific for non-persistent antigens. Source: [356].

Lymphocytic telomere length declines with age (Figure 16), but cells specific for persistent antigens undergo particular shortening, presumably due to persistent stimulation and cell turnover. Because immune responses are dependent on the ability of relatively few memory lymphocytes to replicate and develop a population of effector cells on each exposure to antigen, responses are dependent upon either development of telomerase activity to maintain telomere length or ultimately specific responses will develop replicative senescence [369].

Telomerase activity in T-lymphocytes

Lymphocytes are able to up-regulate telomerase in certain circumstances in responses to stimulation [370]. With increasing differentiation and maturation the ability of T-lymphocytes to up-regulate

telomerase is curtailed and therefore stimulation and proliferation lead to unopposed telomere shortening [356, 371]. Valenzuela et al demonstrated that after activation by antigen or mitogen telomerase activity peaks at day 5, before declining to baseline 3 weeks after stimulation [372].

Telomerase activity is low or absent in resting lymphocytes, but can be induced readily by *in vitro* stimulation [356]. This effect of stimulation has also been demonstrated *in vivo* during acute EBV infection. Maini et al demonstrated that acute EBV infection led to up-regulation of telomerase activity and therefore preservation of telomere length despite proliferation [373]. Soares et al confirmed that both peripheral and tonsillar lymphocytes were able to up-regulate telomerase during acute EBV [374]. This up-regulation and resulting telomere preservation serves to maintain the replicative capacity of cells from the primary response that are destined to enter the memory pool.

Lymphocytes lose the ability to up-regulate telomerase activity with repeated stimulation and therefore telomere shortening occurs [375]. On analysis of increasingly mature lymphocyte subsets, Plunkett et al demonstrated that as lymphocytes differentiate and lose the surface receptors CD27 and CD28, they lose the ability to up-regulate telomerase to non-specific stimulation *in vitro* [376]. The underlying defect seems to be an inability to phosphorylate intracellular messenger Akt [376]. Valenzuela et al demonstrated that telomerase activity can be induced after second exposure to antigen, but not after third or subsequent exposure [372]. Therefore in most memory responses proliferation will directly lead to telomere attrition due to the lack of telomerase activity.

Several cytokines have been demonstrated to inhibit telomerase activity. Reed et al demonstrated that IFN-α induced a reversible inhibition of telomerase [377], which has relevance for the study of the telomere – telomerase system in HCV infection. Further, TGF-β, a pro-fibrogenic cytokine, known to be increased in concentration in the HCV-infected liver [378], has also been demonstrated to inhibit telomerase gene transcription [379].

What remains unclear in various chronic infections is the relative effect of chronic antigenic stimulation, the effect of long-term pro-inflammatory cytokines or direct infection of lymphocytes upon the T-cell failure that develops. Bucks et al utilised the murine influenza A virus which does not lead to high viral titres, cytokine responses or lymphocyte infection and demonstrated lymphocytic exhaustion related to chronic antigen exposure [380]. They demonstrated that removal of the chronic antigen can reverse the exhaustion from antigen-specific cells, and that whilst PD-1 was up-regulated by antigen-specific cells it was not involved in development or resolution of exhaustion.

Methods of determining telomere length

Telomere length can be determined by several different methodologies which each have advantages and drawbacks:

1. Restriction fragment length (RFL) and blotting

Cells are homogenised and DNA extracted, before digestion with restriction enzymes. Blots are hybridised with a radio-labelled consensus sequence to several telomere repeats [381]. This method has the advantage of generating absolute telomere length, but requires large numbers of cells and cannot be used easily to study cell subsets. The technique has been superseded by the PCR based technique.

2. Flow-cytometric FISH

This technique takes whole fixed cells which can be stained for other markers and utilises a heat step to interpose a fluorochrome-labelled telomere consensus sequence between DNA strands. This technique allows study of whole cells and through the use of other fluorochromes, telomere lengths in different cell subsets. However, the telomere length is expressed as mean fluorescence intensity and is expressed relative to a control sample [356, 382, 383].

3. *Quantitative FISH*

This FISH technique allows telomere length to be calculated from fresh-frozen or paraffin – embedded tissue. Again a fluorochrome-labelled telomere consensus sequence is used, and other markers can be investigated simultaneously. However, the technique is labour intensive, the results are relative to an internal control and relatively low numbers of cells have been studied to date [384].

4. *Polymerase chain reaction*

Quantitative real-time PCR can be utilised to calculate absolute telomere length, but similar to the RFL technique, cell subsets require sorting before analysis [385].

Peripheral lymphocyte telomere length as predictor of disease outcome

Numerous studies in different disease models have investigated the association of peripheral lymphocyte telomere length and clinical outcome. Cawthon et al investigated the prognostic importance of peripheral telomere length in healthy individuals. They measured telomere length by restriction fragment length analysis in 143 healthy subjects older than 60 years of age and found that telomere length was predictive of mortality on prospective follow-up. Subjects with telomere length shorter than the population mean had a worse survival due to a 3.5 fold increase in rates of death due to cardiovascular disease and 8.5 fold increase in the rates of death due to infection [386] (Figure 17)

Figure 17. Kaplan-Meier survival curves of healthy elderly subjects grouped according to telomere length. The cohort was divided into 2 based on relationship of telomere length to population means. Subjects with telomere length shorter than population mean had a significantly worse survival over fifteen years of follow-up. Source: [386].

Further studies have demonstrated similar relationships between telomere length and outcome in cerebrovascular disease [387], cardiovascular disease [388, 389], respiratory disease [285, 390], inflammatory disease [391-393] and infectious disease [394]. Clearly in all these studies telomere length has been established within the peripheral blood lymphocyte compartment. Rather than lymphocyte telomere length having particular prognostic information, it is likely that there is a significant correlation between lymphocyte telomere length and telomere length in particular organs and tissues. There are no current studies demonstrating preferential or solitary telomere length changes in one particular tissue but not others in the context of chronic disease. However, given the ease of obtaining peripheral blood lymphocytes and the numbers of subjects needed to adequately power studies assessing the correlation between telomere length and outcome it may be very difficult to assess similar relationships between telomere lengths in different tissues.

Telomeres and telomerase –The Progeria syndromes

Much of our knowledge regarding ageing and accelerated ageing has been derived from the studies of Progeria syndromes. These rare syndromes cause the affected individual to age at a faster rate

than usual. Dyskeratosis Congenita (DKC) involves a mutation within the telomerase gene and subjects are unable to generate telomerase activity in any tissue. As a consequence they undergo telomere attrition within tissues with a high cellular turnover rate. Ultimately, most subjects die of infection due to accelerated immune senescence and bone marrow failure [395]. Combined with the results of the Cawthon studies [386], it is apparent that telomere shortening has an impact upon survival due to impaired immunity.

Werner syndrome is associated with mutations in a DNA helicase leading to defects in both DNA repair and telomere length maintenance [396]. Hutchinson-Gilford Progeria syndrome, where few children live beyond the age of 13, is associated with a mutation in the Lamin A gene on chromosome 1 , which leads to accelerated telomere loss and the development of premature ageing [397].

Evidence for telomere shortening in chronic viral disease

Most evidence for the role of telomere attrition in infectious disease occurs in HIV infection. Palmer et al analysed the peripheral lymphocyte telomere length in pairs of HIV-discordant monozygotic twins [394]. Studying 7 pairs of twins they found that peripheral CD8+ T-lymphocyte telomere length was significantly shorter in the HIV positive twin, whereas CD4+ T-lymphocyte telomere length was significantly longer in the HIV positive twin.

Fauce et al demonstrated that peripheral CD8+ lymphocytes from HIV infected individuals could be treated with a small molecule telomerase activator TAT2 which led to up-regulation of telomerase, improved proliferative ability and restoration of cytokine production [398] (Figure 18).

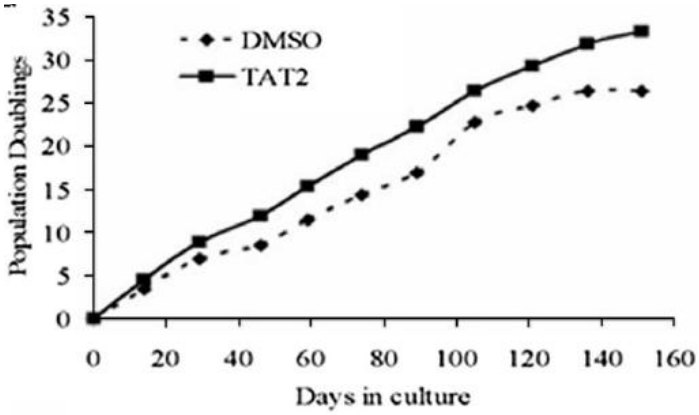

Figure 18. Improvement in proliferative ability of peripheral CD8+ T-lymphocytes taken from HIV+ individuals when treated with the telomerase activator TAT2. Cells were maintained in culture and stimulated with CD3/CD28 coated microbeads. Source: [398].

Lichterfeld et al demonstrated that HIV-specific CD8+ T-lymphocytes had shorter telomeres compared to EBV-specific cells from the same individual. Furthermore, blockade of the PD-1/PD-L1 system led to up-regulation of telomerase activity and stabilisation of telomere length [385]. These results raise the possibility that the process of senescence is not irreversible and that therapeutic manipulation is possible, of relevance to both HIV and possibly HCV infection.

HCV infection and immune senescence

Very little is known of the immunological basis of the marked effect of ageing upon the prognosis of HCV infection. Manfras et al studied 28 subjects with HCV infection and identified increased oligoclonality and expression of the end-stage marker CD57 on peripheral lymphocytes as associated with increased levels of hepatic fibrosis and reduced rates of response to interferon-α therapy [399]. CD57 is known to be a marker of end-stage differentiation of CD8+ T-lymphocytes and its presence is associated with lack of proliferative ability, low secretion of IL-2 and short telomeres [359, 400].

CMV infection is associated with increasing markers of maturation and differentiation on both CMV- and non-CMV-specific cells; in contrast, HCV infection is associated with a retardation of the normal maturation pathway in lymphocytes. Appay et al studied CD8+ T-lymphocytes specific for a variety

of antigens and found that HCV-specific CD8+ lymphocytes had higher expression of both CD27 and CD28 than cells specific for HIV, CMV and EBV [63] (Figure 19).

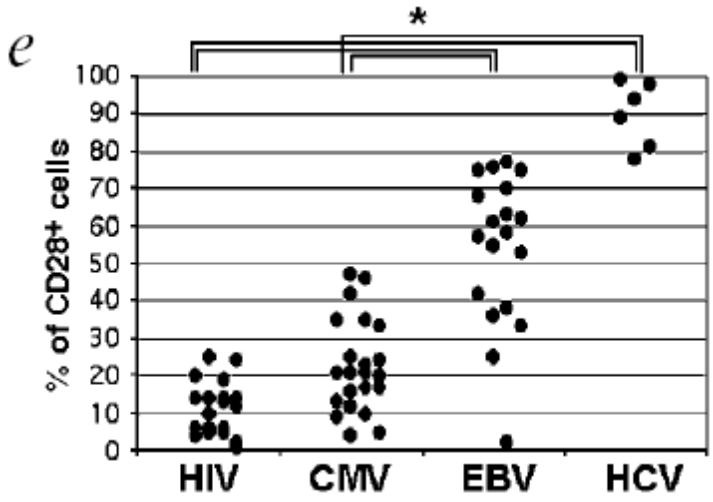

Figure 19. Expression of CD28 on CD8+ lymphocytes specific for a variety of latent and persistent antigens. Source: [63].

Similarly, Lucas et al studied the phenotype and function of CMV specific CD8+ T-lymphocytes from both HCV- and HCV+ individuals. They found that the usual advanced phenotype of CMV-specific CD8+ lymphocytes was retarded in the presence of HCV infection, with an increase in markers associated with early lymphocyte differentiation CCR7 and CD62L. Conversely, there was a reduction in the expression of markers associated with cytotoxicity such as perforin and CD95 [64] (Figure 20).

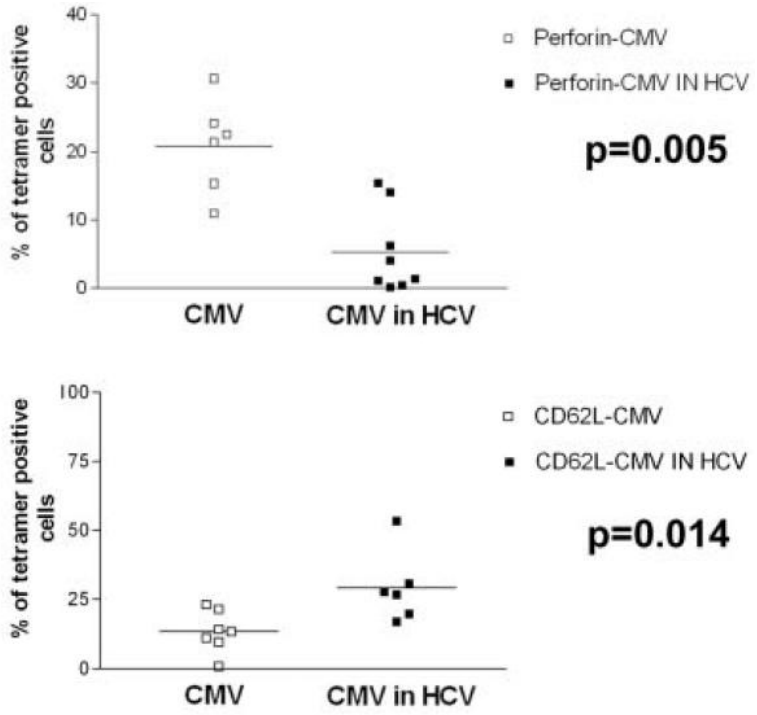

Figure 20. Phenotype of CMV-specific CD8+ lymphocytes in the absence (left column) or presence (right column) of HCV infection. CMV-specific cells had a reduction in effector markers such as perforin (upper panel) and an increase in early stage lymphocyte markers such as CD62L (lower panel). Source: [64].

Therefore, whilst HCV seems to retard the normal maturation process of lymphocytes, the presence of peripheral changes associated with immune senescence and CMV infection seems to lead to a worse prognosis in HCV infection.

Recent work has demonstrated that viraemic HCV infection is associated with shorter telomeres in peripheral lymphocytes than in healthy controls, albeit in just 22 patients and 22 healthy controls [401]; Kitay-Cohen et al demonstrated peripheral telomere shortening but not the relationship between length and patient outcome.

Summary

1. HCV infection is an important public health problem, with a high rate of chronic infection. It can lead to cirrhosis and HCC and has a poor response to current anti-viral therapy.

2. There are a number of risk factors for progression of chronic HCV infection, some of which are modifiable. One of the most important risk factors identified is age at time of acquisition. There are few data to explain this effect.
3. One of the most important anti-viral cytokines is IFN-α which achieves its effects through the intracellular messenger phospho-Stat1, leading to alterations in gene transcription.
4. Increasing age is associated with changes in liver structure, blood flow and function. Increasing age is associated with reduced response to injury, impaired regeneration and increased mortality in acute liver disease.
5. Age has been identified as a co-factor in several chronic liver diseases including chronic viral hepatitis.
6. Telomeres are DNA structures located at the end of each chromosome, which protect the underlying coding DNA from breaks and fusions. Telomeres shorten with age. Cell proliferation leads to progressive telomere shortening, due to the inability of DNA polymerase to transcribe to the tip of the chromosome.
7. Telomere shortening leads ultimately to a double strand DNA break signal through γ-H2AX which leads to cell cycle arrest and a state of replicative senescence.
8. Telomerase is able to prevent telomere attrition by synthesising new telomere repeats but its expression is tightly controlled due to its oncogenic potential.
9. With increasing age, the function of the immune system declines manifest as increased incidence of and increased mortality from infectious disease.
10. Features of immune ageing, telomere shortening in particular, are associated with increased mortality in prospective studies of healthy individuals.
11. Persistent infections lead to chronic cell turnover and increased evidence of immune ageing and lymphocyte telomere shortening in particular. Most evidence exists for CMV and HIV infection in humans; no data exist for chronic HCV infection.

12. Changes of immune ageing may be reversible. Re-introduction of telomerase by adenoviral transfection into mice prevented the development of cirrhosis and small molecule activation of telomerase activity in human lymphocytes in vitro led to an improvement of proliferative potential.

Hypotheses

1. Subjects with chronic HCV infection and progressive fibrosis have enhanced features of immune senescence including short lymphocyte telomeres.
2. HCV-infected subjects with features of immune senescence will have a worse clinical and treatment outcome than non-senescent HCV infected individuals.
3. Lymphocytes with features of accelerated ageing will have poor activity against HCV virus infection, respond poorly to anti-viral cytokines such as IFN-α and may be pro-fibrogenic.

Chapter 2 - Materials and methods

Experimental methodology

Lymphocyte preparation

Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation of citrated blood over Lymphoprep (Nycomed, Roskilde, Denmark) and analysed immediately or cryopreserved at -80^{0}C in 80% foetal calf serum (Biosera, East Sussex, UK), 10% RPMI-1640 (Gibco, Paisley, UK) and 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Gillingham, UK).

Circulating and intrahepatic lymphocytes were obtained simultaneously whenever possible from HCV infected patients undergoing diagnostic percutaneous liver biopsy, liver resection or liver transplantation. Liver biopsies were performed with a 1.9mm diameter Menghini needle (Steriseal, Redditch, UK). Liver tissue was disaggregated mechanically in RPMI-1640, passed through a 70µm nylon filter before layering over Lymphoprep. Controls for these experiments comprised HCV negative patients undergoing hepatic resection for colorectal metastases or hepatic adenoma; circulating and intrahepatic lymphocytes were obtained in the same fashion.

Cell surface phenotype by flow cytometry

Flow-cytometric analysis of T-lymphocytes was performed [377] using combinations of the following: CD4-biotin (Beckman Coulter, Fullerton, CA), CD4-PE-Cy5, CD8-biotin, CD8 PE-Cy5, CD27-APC, CD57-biotin (all BD, San Diego, CA), CD45RO-biotin (Ebiosciences, San Diego, CA), CD45RO-FITC (Dako, Glostrup, Denmark). Biotinylated antibodies were followed by streptavidin-Cy3 (Cedarlane laboratories, Ontario, Canada); Alexa-488 conjugated anti-KLRG1 (a kind gift, Dr Hanspeter Pircher, University of Freiburg). All cytometry was performed on a FACScalibur analyser (BD) unless otherwise stated; data were analysed with FCSpress software (www.fcspress.com).

Lymphocyte separation

CD4+ and CD8+ T-cells were purified from PBMCs by negative selection using CD4+ or CD8+ separation kits, through MS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' instructions. Purity exceeded 90%.

Telomere length by flow cytometry

Telomere length of CD4+ or CD8+ T-cells was measured using 3-colour flow-FISH assay as described [377, 382]. PBMCs were stained with CD4-Biotin (Immunotech) or CD8-biotin (BD) and CD45RO-FITC (Dako) followed by streptavadin-Cy3 (Cedarlane laboratories, Ontario, Canada) after which samples were fixed and permeabilised (Fix and Perm cell permeabilisation kit, CALTAG laboratories, Burlingame, California, USA). After washing in hybridization buffer (containing 70% formamide (VWR), 1% bovine serum albumin, 150mM NaCl, 20mM Tris-HCl) cells were incubated with 0.75µg/ml Cy5-conjugated telomere probe ($(C_3TA_2)_3$, Applied Biosystems, Warrington, UK). Samples were placed at 82^0C for 10 minutes, followed by rapid cooling on ice and hybridization in the dark at room temperature for 60 minutes. Samples were then washed in post-hybridisation buffer (70% formamide, 10mM Tris-HCl, 150mM NaCl, 0.1%BSA, 0.1% Tween 20) and analysed immediately in triplicate by flow cytometry. CD4+ PBMCs from the same healthy individual were analysed in every experiment as an internal control. Results are expressed as mean fluorescence intensity (MFI).

Flow cytometry for Ki67

PBMCs were stained with CD8-FITC and CD4-APC (BD) before fixation and permeabilisation in CALTAG A and B. Cells were then stained with Ki67-PE (BD) or isotype control. Cytometry was performed on a FACSCanto II (BD).

Flow cytometry for γ-H2AX and p53

PBMCs were stained with combinations of IFN-AR1-FITC, IFN-AR2-FITC (PBL biomedical laboratories), CD45RO-biotin (Abcam), CD57-biotin (BD), CD27-APC (BD), CD8-Qdot605 and CD4-Qdot655

(Invitrogen, Paisley, UK) before fixation in CALTAG medium A. Cells were permeabilised in 90% methanol (VWR) and stained with combinations of Alexa Fluor 488 conjugated anti-human γ-H2AX (ser-139), Alexa Fluor 647 conjugated anti-human γ-H2AX (ser-139), Alexa Fluor 647 conjugated anti-human Stat1 (BD Biosciences), Alexa Fluor 647 conjugated anti-human p53 or appropriate isotype controls (all Cell Signaling, MA, USA). A positive control for γ-H2AX (PBMCs irradiated with 50Gy) was included on every run. Cytometry was performed on a FACSCanto II (BD).

To examine phospho-Jak1 and phospho-Tyk2 expression, CD8+ cells were negatively separated from whole lymphocytes with the CD8+ lymphocyte isolation kit II (Miltenyi Biotec). CD8+ purity routinely exceeded 90% in each assay. The isolated CD8+ cells were incubated with 1000 iu/ml IFN-α2b for 20 minutes. Cells were then fixed in CALTAG A solution, prior to permeabilisation in ice cold methanol and stained with either rabbit anti-phospho-Jak1 or rabbit anti-phospho-Tyk2 (both Santa Cruz Biotech) and mouse anti- γ-H2AX (Abcam). Cells were then washed twice, blocked in goat serum, prior to incubation with goat anti-rabbit Alexa Fluor-488 and goat anti-mouse Alexa-Fluor 647, before washing twice and analysis on a FACSCanto II cytometer.

Liver immunohistochemistry

Paraffin-embedded formalin-fixed liver tissue was cut as 5 μm sections to polylysine coated slides. Slides were processed for immunohistochemistry as described previously [402]. Antigen retrieval was achieved by pressure-cooking for 3 minutes in citrate buffer (pH 6.0). Mouse monoclonal antibodies were used: anti-Mcm-2 (generated as reported previously [403]), anti-CD3, anti-CD4, anti-CD8 and anti-perforin (Novocastra, Newcastle, England). Mcm-2, a marker of cell cycle re-entry, is expressed throughout the cell cycle but not in quiescent cells. CD3 is a T-lymphocyte marker. CD4 is expressed on helper T-lymphocytes and CD8 on cytotoxic T-lymphocytes. Perforin expression denotes a T-lymphocyte with cytotoxic potential. Biotinylated goat-anti-mouse immunoglobulin was applied as a secondary antibody. Tonsil was used as a positive control and appropriate primary antibody isotype served as a negative control on each run.

A streptavidin-horseradish peroxidase system (DAKO, Denmark) with the substrate diaminobenzidine was used to develop staining. Slides were counterstained with Harris haematoxylin, dehydrated in an ethanol series and cleared in xylene. Cover slips were applied with DEPEX mounting medium (BDH, United Kingdom).

A novel approach was used to quantify the results of immunohistochemistry in an objective fashion. This technique was developed by Dr. Will Gelson, Department of Medicine, University of Cambridge. A high definition image was taken at 3.5 x magnification using the Olympus Dotslide system (Olympus Microscopes, UK) (Figure 21a). Consecutive sections were used for each antibody and the same field was selected on each occasion based on a reproducibly identifiable feature e.g. a portal tract or central vein. Immunohistochemistry was assessed using the public domain ImageJ software [404] (U.S. National Institutes of Health, http://rsb.info.nih.gov/ij). The operator defines the scale and areas of interest which in this series comprised the lobule and the portal tract (Figure 21b). Images were transformed into 'black and white' and a threshold was established to educate the programme to identify positive staining of either nuclei or membrane with each antibody specifically.

Positive nuclei are identified readily by size and shape. To separate overlapping nuclei a 'watershed' was applied (Figure 21c). The results are expressed as the number of positive nuclei/mm^2 of either lobule or portal tract.

Interpretation of membranous staining can be difficult in sections where cell density is high (in the past leading to a number of semi-quantitative and subjective scoring systems). Thus, for assessment of membrane staining the results are expressed as a proportion; in this study the numerator was the area of cells detected as positive membranous staining by immunohistochemistry (Figure 21c) and the denominator was the total area of interest (lobule or portal tract) (Figure 21d). The proportion of lymphocytes positive for membrane staining for CD3, CD4 or CD8 was assessed according to

either a lobular or portal distribution. Perforin staining was discrete and cytoplasmic and the results are expressed as number of cells positive per mm^2 of either lobule or portal tract.

Immunohistochemistry for Ki67 or γ-H2AX

Paraffin-embedded formalin-fixed liver tissue was cut as 5 μm sections to polylysine coated slides. Antigen retrieval was achieved through incubation in epitope retrieval solution 2 at pH 8.8 (Leica) before endogenous peroxidases were quenched with hydrogen peroxide. Anti-human Ki67 (Dako) or anti-human γ-H2AX antibody (Cell Signaling, MA, USA) was applied before a poly-peroxidase anti-mouse/anti-rabbit reagent (Leica) and then nickel-enhanced diaminobenzidine to develop the staining. Slides were counterstained with Harris haematoxylin, dehydrated in an ethanol series and cleared in xylene. Cover slips were applied with DEPEX mounting medium (BDH, United Kingdom).

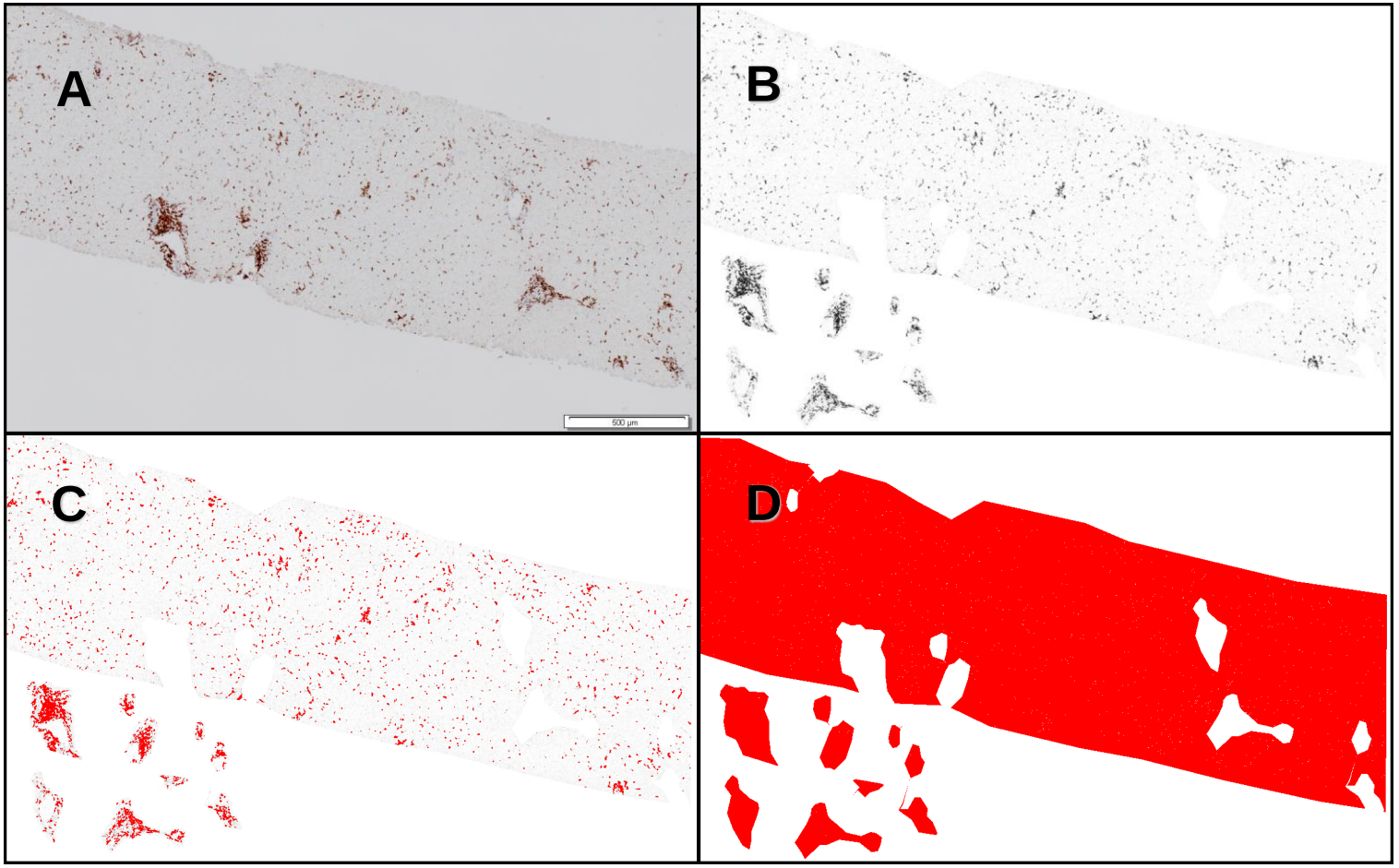

Figure 21. Immunohistochemical analysis using ImageJ software. Panel A: a representative image obtained of CD3 immunohistochemistry using the Olympus Dotslide system. A scale bar (Panels A, 500µm) allows absolute areas to be calculated. Panel B, C and D demonstrate the analysis process for membranous staining. Panel B: an ImageJ-enhanced 8-bit "black and white" image with portal tracts "cut out" to allow separate analysis of both lobular and portal regions. Panel C: positive immunohistochemistry defined in red using a primary antibody-dependant standardised threshold, the area of

which provides the numerator for positive immunohistochemical staining. Panel D: a threshold that gives a total area for both portal tract and lobular regions; the denominator. Analysis of nuclear staining is identical to membranous immunohistochemical staining, except that the immunohistochemical numerator is the number of positive cells. After a "watershed" is applied to separate overlapping cells, ImageJ calculates the number of positively stained cells using operator determined shape and size characteristics.

Telomerase assay

Telomerase activity was measured using the telomere repeat amplification protocol (TRAP) according to the manufacturers' instructions (Roche Applied Sciences). CD8+ PBMCs were obtained with the CD8+ isolation kit II (Miltenyi Biotec). Autologous non-CD8+ cells, irradiated with 40Gy, served as antigen presenting cells (APCs). CD8+ PBMCs and APCs were plated in a 1:1 ratio in 96-well round-bottom plates pre-coated with anti-CD3 (1ug/ml) (BD Biosciences) and incubated at 37^0C in 5% CO_2. After 5-days cells were harvested, snap-frozen in liquid nitrogen and stored at -80^0C until analysis. Absolute numbers of proliferating T-cells were enumerated using Tru-COUNT tubes with Tritest and staining for CD8 and intracellular Ki67. The volume of cell extract was adjusted to 500 CD8+Ki67+ cells per TRAP reaction to correct for different rates of proliferation [356]. An internal standard was included in each reaction to calculate relative telomerase activity.

Serum interferon-α by ELISA

Serum was stored at -20^oC. IFN-α was assayed by ELISA according to the manufacturers' instructions (Chemicon, USA). Serum was incubated in a flat-bottomed 96-well plate pre-coated with anti-human IFN-α, washed and incubated with further anti-human IFN-α. Anti-mouse immunoglobulin conjugated to horseradish peroxidase was added prior to final incubation with tetramethyl-benzidine. The reaction products were read in a plate reader at 405nm.

T-cell receptor directed stimulation

Cells were cultured in RPMI-1640 medium (Gibco) supplemented with 2 mM L-glutamine, 10% foetal calf serum (Biosera, UK), 100iu/ml penicillin and 0.1mg/ml streptomycin. 1ug/ml plate-bound anti-CD3 and 4ug/ml soluble anti-CD28 (BD, San Diego, CA) were used to stimulate the cells. PBMC were stimulated and then left with paired (control) unstimulated samples in a humidified 5% CO_2 atmosphere for 15 hours. After 2 hours Brefeldin A (BD biosciences, San Diego, CA) was added according to the manufacturer's instructions.

Effect of exogenous Interferon-α

Cells were cultured in supplemented RPMI-1640 medium as above with variable concentrations of Interferon-α2b (PBL biomedical laboratories) from 10iu/ml to 3000iu/ml. After incubation with IFN-α or medium alone, cells were surface stained, fixed in CALTAG A medium, permeabilised in 90% methanol before staining with Alexa-fluor 488-conjugated anti-human p-Stat1 (tyrosine 701) and Alexa-fluor 647-conjugated anti-human γ-H2AX (ser-139) or appropriate isotype control (Cell Signaling). Cells were then washed twice before cytometry on a FACSCanto II (BD). Surface staining for IFNAR-1 was achieved with specific antibody conjugated to fluorescein (PBL biomedical laboratories).

Scoring of liver biopsy sections

Liver biopsies were performed with a 1.9mm diameter Menghini needle. Biopsy specimens were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. 4μm sections were stained with Meyer's haematoxylin & eosin, periodic acid–Schiff with diastase pre-treatment, Prussian Blue, a trichrome stain (van Gieson or chromotrope alanine blue), and Gomori's reticulin stain. All biopsies were examined by a single specialist liver histopathologist blinded to the lymphocyte studies or outcome (Dr Susan Davies, Department of Histopathology, Addenbrooke's Hospital, Cambridge). Biopsies were scored according to modified Ishak criteria [135] after assessing adequacy of specimen. Histological activity index represented the sum of interface hepatitis (0 - 4), confluent necrosis (0 - 6), lobular inflammation (0 - 4), and portal inflammation (0 - 4). Fibrosis was scored 0 (absent) to 6 (cirrhosis) and steatosis was scored 0 - 3. Features of steatohepatitis were recorded.

Viral serology and PCR work

All routine serology and PCR work was performed by the Department of Virology, Addenbrooke's Hospital, Cambridge. Anti-HCV IgG was sought by ADVIA Centaur sandwich immunoassay (Bayer,

Tarrytown, NY). IgG antibody against CMV was sought by chemiluminescent immunoassay (Diasorin, Saluggia, Italy).

The same PCR assay was used both for HCV RNA detection (qualitative) and determining HCV viral load (quantitative). This is a real-time Taqman PCR assay which targets the conserved 5' non-coding region on a Rotor-gene™ 3000 instrument (Corbett Lifescience, Cambridge, UK). HCV quantitation is carried out using a serial dilution of recombinant plasmid standards, incorporating the HCV target site, as an external calibration system. These standards are included in each run alongside the samples to be tested. A standard curve is generated by the Rotor-Gene by plotting the threshold cycle (Ct) versus the concentration of the standards. The HCV RNA quantity of each unknown sample is determined by locating its Ct on the standard curve. Probit analysis (Stats Direct, www.statsdirect.com) revealed a lower limit of detection of 25 IU/ml (6.3-38.6, 95% confidence intervals).

Experimental development and validation

Flow-FISH assay

The Flow-cytometric FISH assay for telomere length was developed by Lansdorp's group in Vancouver and first described in 1998, where they demonstrated that there was a direct correlation between telomere length by restriction fragment length electrophoresis and telomere length by flow-FISH (Figure 22) [405, 406]. In a separate report in 2002, the same group reported various methods of optimising the technique. Baerlocher et al found the optimal conditions for hybridisation of DNA with the fluorochrome-labelled probe, in terms of optimal temperature and duration for hybridisation [382].

I undertook a number of experiments to validate the technique before embarking on patient studies.

Multiple aliquots of lymphocytes from the same individual taken on the same occasion were analysed for telomere length to investigate the intra-experimental variability of telomere length for

a single sample. Three separate lymphocyte samples had a CD4+ telomere length of 136.2, 137.4 and 135.9 (standard deviation of 0.73) and CD8+ telomere lengths of 140.1, 141.3 and 139.8 (SD 0.83), demonstrating minimal variability in telomere length due to experimental variance.

To investigate the inter-experimental variability samples from the same individual were analysed on different experimental runs. Three separate lymphocyte samples had a CD4+ telomere length of 117.0, 119.3 and 113.5 (SD 2.9) and CD8+ telomere lengths of 135.2, 144.0 and 140.4 (SD 4.4) on separate experimental runs.

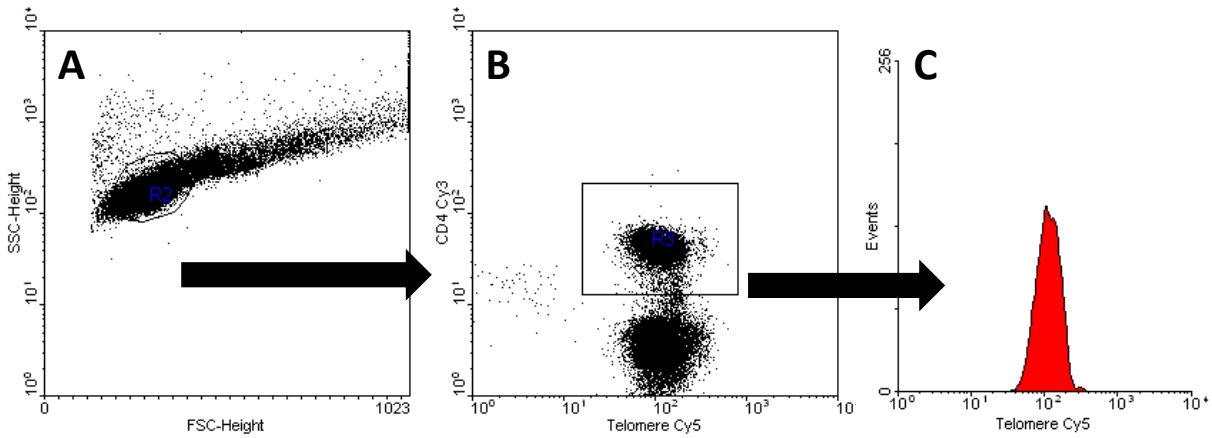

Figure 22. Example flow-cytometry plots and gating strategy for analysis of telomere length from flow-FISH assay. Panel A: Live lymphocyte gate by light-scatter characteristics. Panel B: CD4+ cells are identified. Panel C: Histogram of CD4+ lymphocyte telomere length is constructed with median telomere length calculated by cytometry software.

A second concern was the effect of freezing and thawing upon lymphocyte telomere length. As samples were analysed in batches of two or four patients, lymphocytes were frozen for up to twelve months prior to analysis. I collected lymphocyte samples from the same individual at three monthly intervals over the period of a year. From the subsequent data (see chapter three), the expected reduction of telomere length over the course of one year was 0.7 MFI units, similar to the inter-experimental variability. Therefore, one would expect that the telomere length of the samples would appear to have the same telomere length by Flow-FISH assay, if there were no telomere length changes attributable to the freezing or thawing process.

CD8+ T-lymphocyte telomere length frozen for 0, 3, 6, 9 and 12 months was 131.8, 132.1, 132.5, 135.1 and 133.0 respectively (Mean 132.9, SD 1.3). CD4+ T-lymphocyte telomere length frozen for 0, 3, 6, 9 and 12 months was 128.2, 125.8, 125.2, 126.2, and 128.9 (Mean 126.9, SD 1.6) (Figure 23). The technique had low inter-experimental variability and low artefactual telomere length changes attributable to the storage of lymphocytes at -80^0 C.

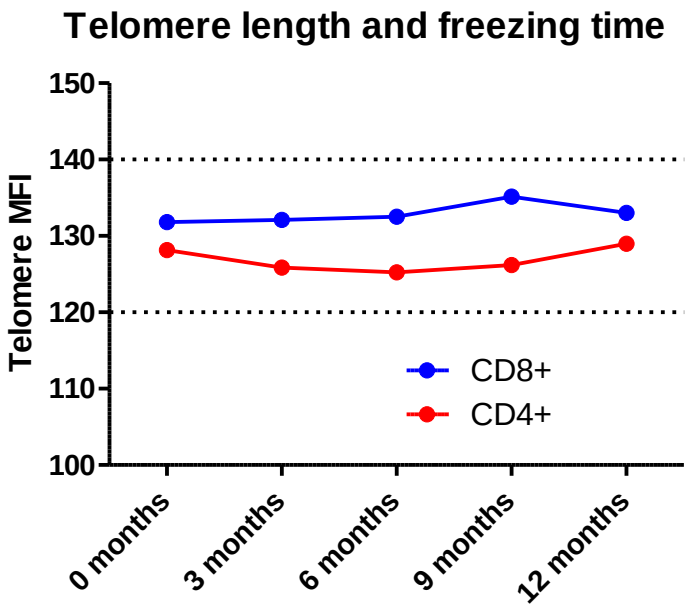

Figure 23. Effect of prolonged freezing at -80^{0}C upon lymphocyte telomere length by Flow-FISH. Lymphocytes from the same individual were collected at 3 month intervals over a year and then analysed on the same assay.

Antigen-specific telomere length

One of the early aims of the project was to analyse the telomere length of CD8+ and CD4+ T-lymphocytes specific for HCV peptides and proteins respectively. This was likely to be technically difficult due to the low circulating frequency of these cells demonstrated in previous studies [23, 63, 71, 407].

Previous work in the laboratory of collaborators at UCL in London demonstrated the feasibility of analysing telomere length of CD8+ and CD4+ cells specific for CMV, EBV, VZV and PPD [356, 383]. CD8+ cells were incubated overnight with immunodominant peptides, such as the CMV pp65

peptide, before identifying pp65-specific CD8+ T-cells the following day through intracellular staining for IFN-γ. Antigen-specific CD4+ cells were identified more readily though incubation of whole lymphocytes with viral proteins or lysed cell extracts from infected cell lines before identifying antigen-specific cells through intracellular IFN-γ staining.

Attempts to identify HCV-specific CD8+ or CD4+ T-lymphocytes were mostly unsuccessful. I attempted to develop the technique in HCV-specific CD4+ T-cells; although the circulating frequency of these cells may be lower than HCV-specific CD8+ T-cells, the ability to stimulate cells with whole proteins and therefore to elicit a polyclonal multi-epitope response seemed most logical. CMV-specific and PPD-specific CD4+ responses were elicited readily using CMV-infected cell lysate (East Coast Biologics) or Tuberculin (Statens Serum Institute) before intracellular cytokine staining for IFN-γ. These cells were the subjected to the Flow-FISH assay to examine telomere length (Figure 24).

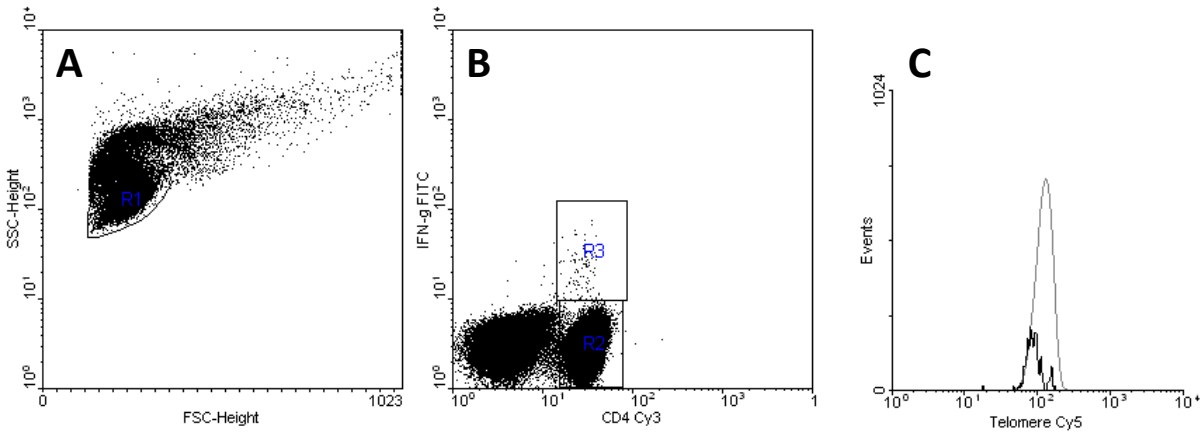

Figure 24. Example cytometry plots and gating strategy for measurement of antigen-specific telomere length. Whole PBMCs were incubated overnight with lysate from a CMV-infected cell line and Brefeldin A to prevent cytokine secretion. The cells were then surface-stained before fixation, permeabilisation and staining for intracellular IFN-γ. Panel A: live lymphocytes were selected based on light-scatter characteristics. Panel B: IFN-γ+ CMV-specific CD4+ lymphocytes were gated (R3) as well as IFN-γ- control whole CD4+ cells (R2). Panel C: Bold histogram demonstrates telomere length of CMV-specific CD4+ cells previously derived from the R3 gate, with whole CD4+ cells in the pale histogram (R2 gate).

HCV-specific CD4+ T-cells were sought in 19 subjects with viraemic HCV infection with varying degrees of fibrosis. A cocktail of HCV proteins including NS3, NS5 and core proteins was used (Mikrogen, Germany). Previous studies had examined CD4+ responses successfully using the same

recombinant proteins at a concentration of 1ug/ml, albeit in Elispot assays and thymidine incorporation studies [67, 68, 408]. Whole lymphocytes were therefore incubated with the cocktail of HCV proteins at this concentration. No HCV-specific CD4+ IFN-γ responses above 0.05% of the total CD4+ population were elicited in any subject. Repeated experiments at higher concentrations of the peptide cocktail, up to 8ug/ml of each peptide, similarly could not demonstrate antigen-specific responses.

It is possible that intracellular IFN-γ staining may be insufficiently bright due to fluorochrome extinguishing during the heat step, insufficient fixation or insufficient production of IFN-γ. Therefore I proceeded to utilise a cytokine capture assay for IFN-γ. This combines an antibody directed against CD45, present on all peripheral lymphocytes with an antibody against IFN-γ; the antibody would bind lymphocytes and then trap any IFN-γ released from the cell, allowing surface staining for IFN-γ rather than permeabilisation and intracellular staining. However, no HCV-specific CD4+ cells could be detected by surface IFN-γ staining after incubation with the NS3, NS5 and core protein cocktail.

One further approach would have been to re-stimulate whole lymphocytes over a period of 5 days with HCV proteins to expand the HCV-specific cell pool, but this would involve several rounds of cell division and the potential for telomere shortening, introducing experimental artefact.

HCV-specific CD4+ T-cells could be detected with intracellular cytokine staining in subjects with non-viraemic HCV. These were present at low frequency (between 0.05% and 0.15%) and were detected in eight of the eleven (73%) subjects analysed (Figure 25).

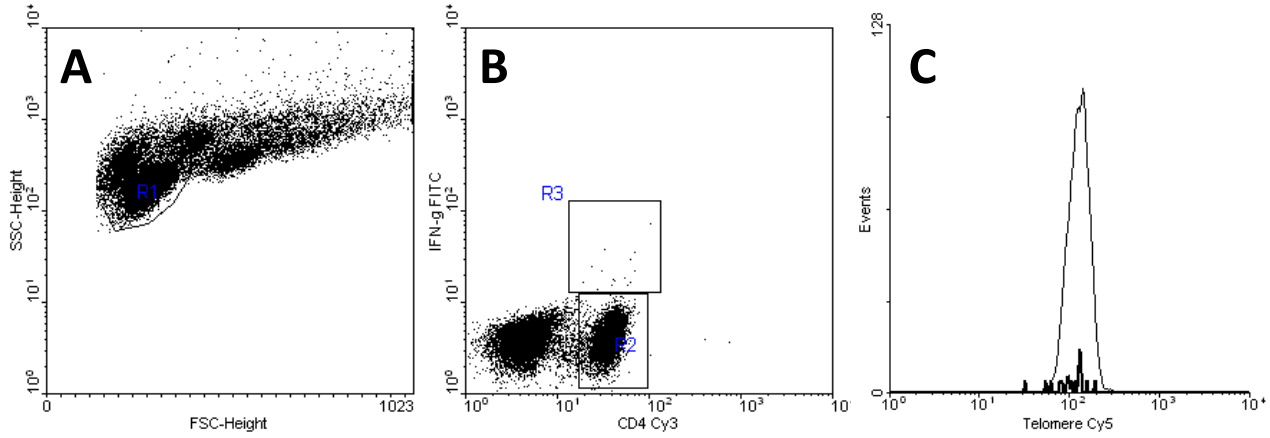

Figure 25. HCV-specific CD4+ T-lymphocyte telomere length. Whole PBMCs were incubated overnight with a cocktail of HCV core, NS3 and NS5 proteins and Brefeldin A to prevent cytokine secretion. As before, live lymphocytes (Panel A) were then divided into IFN-γ+ HCV-specific CD4+ lymphocytes (Panel B) (R3) as well as IFN-γ- control whole CD4+ cells (R2). Panel C: Bold histogram demonstrates telomere length of HCV-specific CD4+ cells with whole CD4+ cells in the pale histogram (R2 gate).

In these subjects it was possible to compare the relative telomere lengths of CD4+ cells specific for HCV, CMV and PPD with control groups of whole CD4+ cells and IFN-γ positive cells after non-specific stimulation with anti-CD3 and anti-CD28. Median telomere length in the whole CD4+ subset was 145.3 (IQR: 122.6 – 151.1) compared to 127.3 (107.3 – 143.4) for CD3 / CD28 stimulated IFN-γ positive CD4+ cells, 132.4 (107.1 – 147.5) for PPD-specific CD4+ cells, 122.4 (107.4 – 135) for CMV-specific CD4+ cells and 138.2 (122.4 – 150.2) for HCV-specific CD4+ cells (Figure 26A). CMV-specific CD4+ cells had shorter telomeres than HCV-specific CD4+ cells in the same individual (Wilcoxon signed rank test, p = 0.031) (Figure 26B).

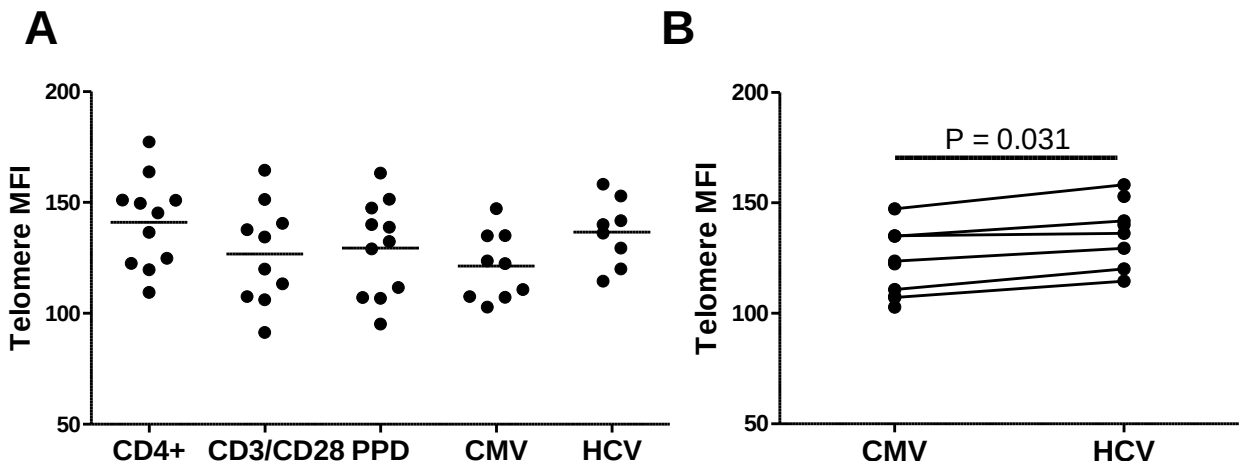

Figure 26. Antigen-specific CD4+ telomere length in subjects with non-viraemic HCV infection (n = 11). Whole PBMCs were left unstimulated, stimulated with plate bound anti-CD3 and CD28, or stimulated with PPD, CMV lysate or cocktail of HCV proteins. After overnight stimulation cells were surface-stained, fixed, permeabilised and then subject to flow-FISH analysis for telomere length. Panel A: telomere length of CD4+ cells specific for different antigens; horizontal bars demonstrate median. Panel B: comparison of CMV- and HCV-specific CD4+ telomere length from the same individuals; Wilcoxon signed rank test.

Attempts were then made to investigate HCV-specific CD8+ telomere length. Autologous irradiated PBMCs pulsed with the cocktail of NS3, NS5 and core proteins were used to circumvent the problem of unknown epitope specificity in co-culture with bead-separated CD8+ lymphocytes (Miltenyi Biotec, Germany). This should allow internalisation of protein by APCs within the PBMCs, prior to lysosomal processing and presentation in the context of MHC class 1 to CD8+ lymphocytes. Cells were separated before the non-CD8+ cells were irradiated with 40Gy and then incubated with the protein cocktail for one hour. After this cells were washed before being plated in a one to one ratio with CD8+ lymphocytes and left overnight. Cells were then surface stained, fixed, permeabilised and then stained for intracellular IFN-γ. Utilising this method no HCV-specific CD8+ T-cells could be demonstrated in subjects with viraemic HCV.

Much recent work investigating the role of HCV-specific CD8+ cells involves the use of MHC class one tetramers or multimers. These molecules consist of several recombinant MHC class one molecules containing the peptide epitope of interest. These are tagged to a fluorochrome to allow visualisation of epitope-specific cells through flow-cytometry [63, 407]. I was able to demonstrate

HCV-specific CD8+ cells through the use of MHC class I tetramers in a limited number of HLA-A2 positive individuals (Figure 27).

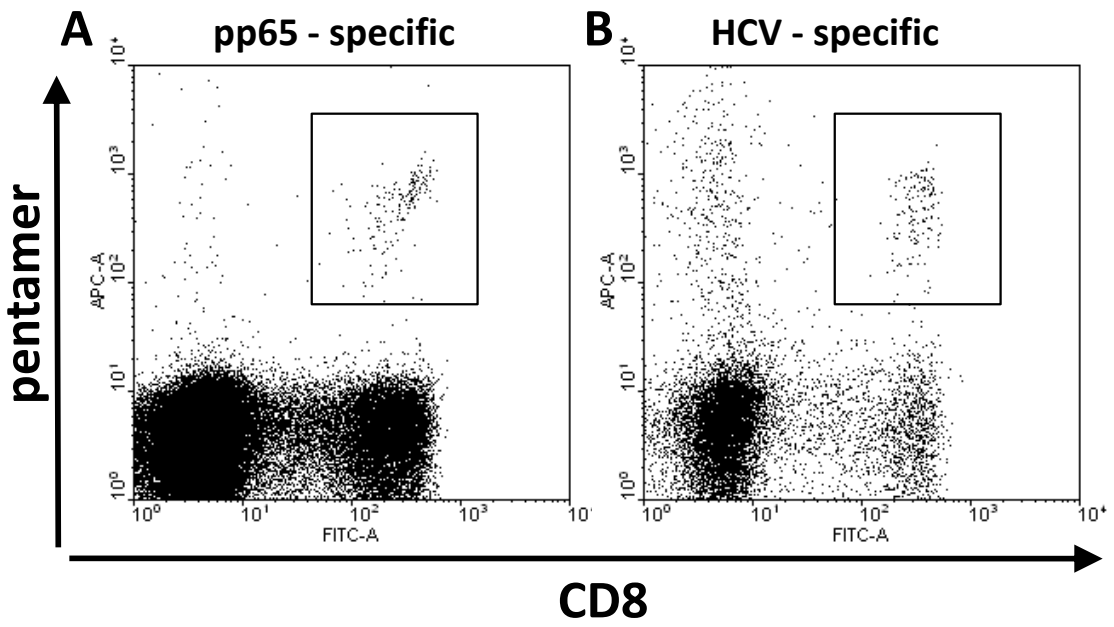

Figure 27. Example cytometry plots demonstrating CMV pp65-specific (NLVPMVATV) (Panel A) and HCV NS3-specific (KLVALGINAV) (Panel B) CD8+ cells. Cells were previously gated on the live lymphocytes by light scatter characteristics.

This allowed analysis of cell-surface phenotype of CD8+ T-lymphocytes specific for three HCV peptides (DLMGYIPAV, HCV core 132 - 140; CINGVCWTV; HCV NS3 1073 – 1081; KLVALGINAV, HCV NS3 1406 – 1415) and to analyse the change in cell-surface phenotype over IFN-α therapy. In common with previous investigations [409] HCV-specific CD8+ lymphocytes had a similar phenotype in viraemic (n = 13) and non-viraemic HCV infection (n = 8), except for increased expression of CD127 on HCV-specific CD8+ lymphocytes in non-viraemic HCV (Figure 28).

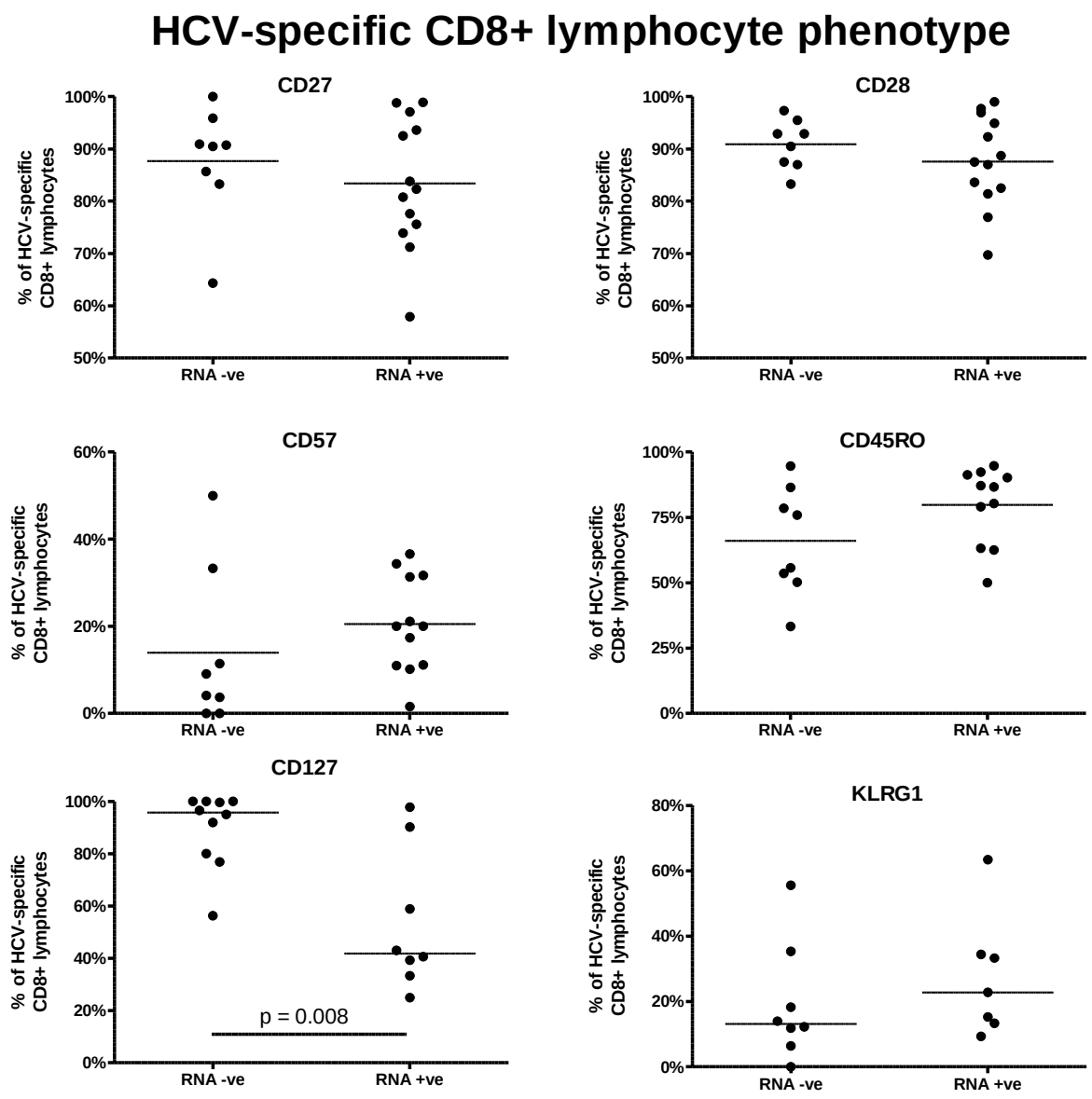

Figure 28. Phenotype of HCV-specific CD8+ cells identified by HCV-specific MHC class I pentamers in non-viraemic (n = 8) and viraemic HCV (n = 11) infection. Cells were co-stained for CD27, CD28, CD57, CD45RO, CD127 and KLRG1. HCV-specific CD8+ cells had significantly higher expression of the IL-7 receptor CD127 in non-viraemic compared to viraemic HCV subjects. Analysis by Mann-Whitney U test.

Expression of CD27, CD28 and CD57 by both pp65- (NLVPMVATV, CMV pp65 495-504) and HCV-specific CD8+ cells over IFN-α therapy was analysed in relation to antiviral therapy outcome (Figure 29).

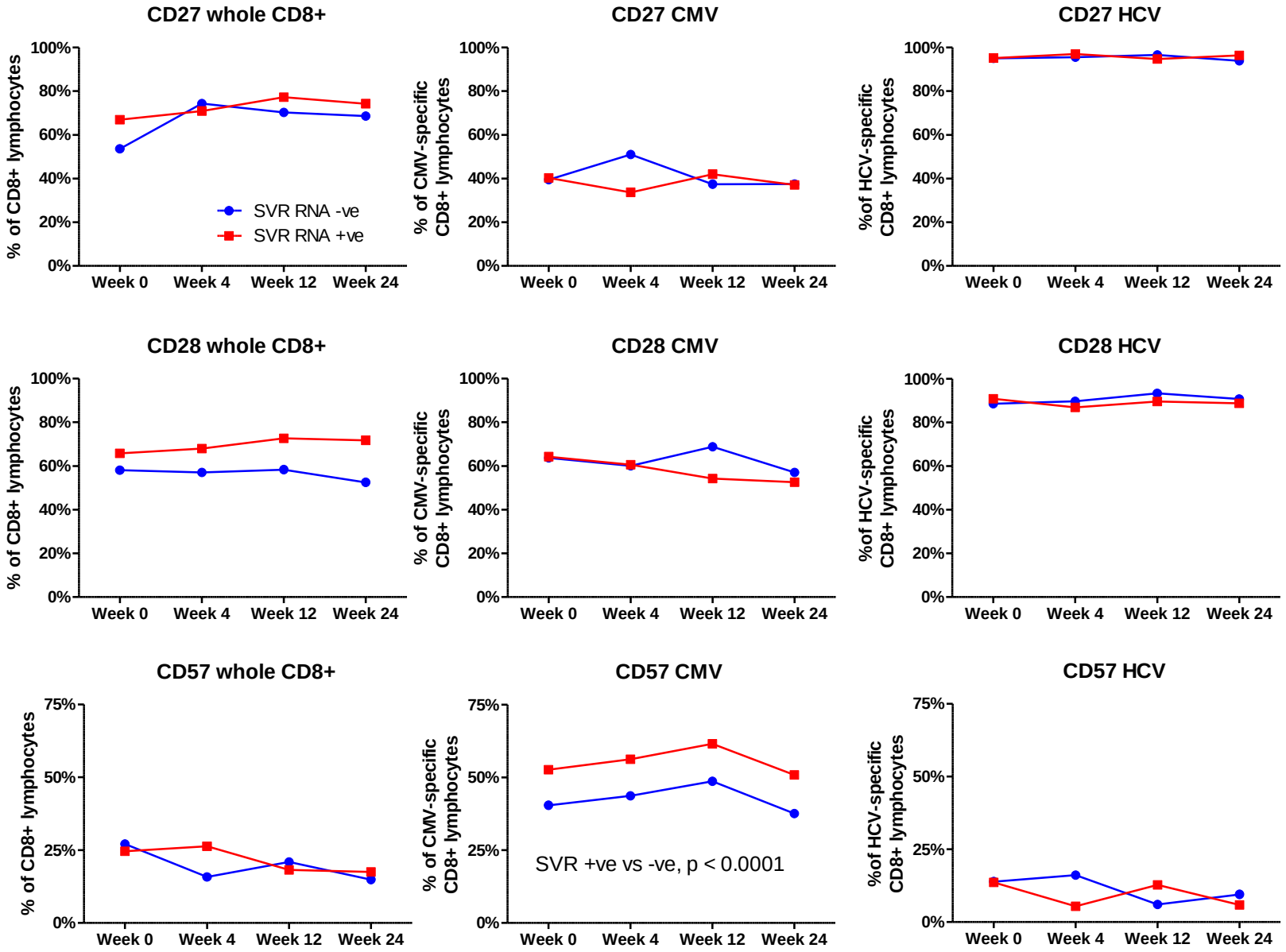
CD27 whole CD8+
% of CD8+ lymphocytes
100%
80%
60%
40%
20%
0%
Week 0
Week 4
Week 12
Week 24
SVR RNA -ve
SVR RNA +ve
CD27 CMV
% of CMV-specific CD8+ lymphocytes
CD27 HCV
%of HCV-specific CD8+ lymphocytes
CD28 whole CD8+
CD28 CMV
CD28 HCV
CD57 whole CD8+
75%
50%
25%
CD57 CMV
SVR +ve vs -ve, p < 0.0001
CD57 HCV

Figure 29. Cell-surface phenotype of whole CD8+ (left column), CMV-specific (centre column) and HCV-specific (right column) CD8+ cells over 24 weeks of IFN-α therapy for HCV infection. Subjects were stratified into those who responded to treatment and were non-viraemic (SVR, n = 10 blue lines and symbols) and those who failed to respond to therapy and remained viraemic (n = 13, red lines and symbols). Cells were analysed for co-expression of CD27 (top row), CD28 (middle row) and CD57 (bottom row). Analysis by 2-way ANOVA revealed the only significant differences between treatment responders and non-responders was expression of CD57 by CMV-specific CD8+ cells.

The only significant differences between those subjects who responded to IFN-α therapy (n = 10) and those who failed to respond (n=13) was lower expression of CD57, a marker of end-stage lymphocyte differentiation, on pp65-specific CD8+ lymphocytes (2-way ANOVA, $p < 0.0001$) (Figure 29).

It proved impossible to combine tetramer staining with the Flow-FISH telomere assay to demonstrate antigen-specific CD8+ telomere length; MHC multimers are fragile and did not survive the hybridisation heat step. This was attempted with both CMV-specific and HCV-specific cells identified by pentamers. Further, identification of cells through biotin-conjugated pentamers with streptavidin-Cy3, already known to be heat-stable, could not identify any antigen-specific cells through the flow-FISH assay.

Chapter 3 - Global T-lymphocyte telomere length is related to clinical outcome in chronic hepatitis C virus (HCV) infection.

Abstract

Background. Increasing age is associated generally with impaired immune function and in chronic HCV infection specifically, is related to the stage of fibrosis, liver failure, HCC and impaired responses to therapy. The relationship between T-lymphocyte telomere length, an objective measure of immune senescence and clinical outcome in patients with chronic HCV infection was investigated.

Methods. Circulating T-lymphocyte telomere length was measured by Flow-FISH in 135 HCV-RNA positive treatment-naïve patients undergoing liver biopsy, 32 HCV-antibody-positive, HCV-RNA negative subjects and 41 age-matched healthy controls. Telomerase activity was analysed by telomere repeat amplification protocol and PCR – ELISA. IFN-α levels were measured by ELISA.

Results. CD8+ and CD4+ T-lymphocyte telomeres were shorter in viraemic patients compared to controls after correction for confounding factors, including age ($p = 0.006$ and $p = 0.015$ respectively). There were inverse correlations between CD8+CD45RO+ or CD4+CD45RO+ telomere length and fibrosis stage ($p = 0.0003$ and $p < 0.0001$ respectively), portal tract inflammatory grade ($p = 0.027$ and $p = 0.035$), prothrombin time ($p = 0.004$ and $p = 0.001$) and bilirubin ($p = 0.003$ and $p = 0.001$).

CD8+CD45RO+ telomeres, but not CD4+CD45RO+ telomeres, were particularly short in non-viraemic HCV-exposed individuals ($p = 0.002$).

124 viraemic individuals were followed prospectively to a composite endpoint of death, hepatic decompensation or HCC. Baseline CD4+CD45RO+ telomere length was predictive of the development of clinical endpoints independent of age and fibrosis stage. Those with shorter CD4+CD45RO+ telomere length were less likely to be complication free after 2-years than those with

longer telomeres (83.4% versus 100%, p = 0.009) with an age-adjusted hazard ratio of 0.93 (0.90 – 0.96).

Conclusion. T-lymphocyte telomere length was inversely correlated with all biochemical and histological markers of HCV infection in a cross-sectional cohort. CD4+CD45RO+ T-lymphocyte telomere length, independent of age, was predictive of all measures of clinical outcome in patients with chronic HCV infection.

Introduction

Prospective studies of HCV-infected cohorts demonstrate that the majority of viraemic individuals never develop severe hepatic fibrosis [134, 177]; factors associated with progressive fibrosis include male sex, obesity, concurrent alcohol misuse [6] and particularly, older age at acquisition [6, 142, 144].

Normal human ageing is associated with changes in the adaptive immune system [363, 410] with reduced numbers of naïve cells, increased numbers of antigen-experienced cells and oligoclonal expansion of CD8+ T-lymphocytes. Progressive shortening of lymphocyte telomeres is characteristic of immune senescence and may underpin the changes of lymphocyte function [357].

Immune senescence is also associated with poor outcome in cardiovascular disease, cerebrovascular disease and obstructive airways disease [285, 388, 389]. Smoking and obesity, both linked to increased risk of fibrosis in chronic HCV infection, [179, 331, 411] are associated independently with accelerated attrition of lymphocytic telomeres [285], linking high-risk activities, immune senescence and subsequent ill-health.

Telomere length shortens with each cell division, leading eventually to a DNA damage signal mediated by γ-H2AX, p53 and $p16^{INK4A}$ [368, 412] that prevents further cell division [413], a point defined as replicative senescence. Telomeres are maintained and less often elongated by telomerase, an enzyme comprising reverse transcriptase (TERT) and RNA template (TERC) [414]. Expression is controlled tightly in normal somatic cells; introduction of exogenous telomerase activity to human somatic cells maintains telomere length and can prevent senescence [415]. Telomerase undergoes post-transcriptional modification within different tissues in order to generate functional activity; thus many tissues contain telomerase mRNA or protein, but telomerase activity is restricted [416, 417].

Chronic viral infections accelerate immune senescence and declining immune function [374, 383]. Cytomegalovirus (CMV) infection is implicated particularly in accelerated immune senescence within both CMV-specific and non-specific lymphocyte subsets [356]. CMV-specific CD4+ and CD8+ lymphocytes possess an 'advanced' cell-surface phenotype, short telomeres and low telomerase activity [63, 356]. CMV is associated with marked oligoclonal expansion of CD4+ and CD8+ effector cells within an elderly population [365].

Less is known of the relation between HCV and immune senescence. Non HCV-specific T-cells have an 'advanced' phenotype in CMV infection, but this is retarded in HCV infected subjects [64], suggesting that HCV infection may impair normal lymphocyte maturation. Intrahepatic CD8+ lymphocytes in HCV infection have increased expression of markers of antigen-experience and differentiation [409, 418].

Few data explain the marked effect of age at acquisition of HCV on either the natural clinical course or the response to antiviral therapy [419, 420]. This study addresses the relation between immune senescence (measured as lymphocyte telomere length), age and clinical outcome in chronic HCV infection.

Patients and methods

Subjects (Table 3)

Patients recruited at Addenbrooke's Hospital, Cambridge gave written informed consent with approved of the Local Research Ethics Committee. Patients co-infected with HIV, HBV or with other chronic liver disease identified by history, blood tests or liver biopsy were excluded. Lymphocytes from healthy controls were obtained from local volunteers; none gave a history of chronic illness or intravenous drug usage. Study subject groups were defined as: healthy controls; non-viraemic HCV-exposed; viraemic with mild disease (Ishak fibrosis 0 - 3); or viraemic with severe disease (Ishak fibrosis 4 - 6).

	Healthy Controls	HCV-RNA -	HCV-RNA + mild	HCV-RNA + severe	P value†
N	41	32	73	62	
Age (years, mean ± SD)	47.0 ± 11.1	50.6 ± 10.1	49.4 ± 11.2	53.2 ± 7.2	0.005
Sex (% male)	51.2%	68.8%	71.2%	77.4%	0.23[1]
BMI (mean ± SD)	26.8 ± 3.5	25.6 ± 5.2	25.6 ± 4.3	27.2 ± 5.6	0.20
Source of HCV: IDU % Blood products % Unknown %	-	 68.8% 18.8% 12.4%	 58.9% 15.1% 26.0%	 67.7% 8.1% 24.2%	0.33[1]
% IgG anti-HBc positive	12.2%	36.3%	26.0%	32.3%	0.0002[1]
% CMV antibody positive	26.8%	53.1%	49.3%	58.1%	0.02[1]
Biochemical indices					
Bilirubin (µmol/L, mean ± SD)	-	8.1 ± 3.6	9.9 ± 5.1	14.3 ± 10.1	0.0003
ALT (iu/L, mean ± SD)	-	32.7 ± 19.6	95.5 ± 88.7	109.1 ± 66.4	<0.0001
PT (seconds, mean ± SD)	-	12.7 ± 1.1	12.5 ± 0.8	13.8 ± 1.7	<0.0001
Ishak score					
Interface hepatitis (0 - 4)	-	-	1.1 ± 0.8	1.9 ± 0.6	<0.0001
Confluent necrosis (0 - 6)	-	-	0.0 ± 0.2	0.0 ± 0.2	0.9
Lobular hepatitis (0 - 4)	-	-	1.8 ± 0.6	2.2 ± 0.6	0.007
Portal inflammation (0 - 4)	-	-	1.8 ± 0.7	2.3 ± 0.5	0.0001
Fibrosis (0 - 6)	-	-	1.8 ± 0.9	4.8 ± 0.7	<0.0001
Steatosis (0 - 3)	-	-	0.5 ± 0.7	0.9 ± 0.9	0.002
Telomere lengths (MFI)					
CD8+CD45RO+ telomere median	125.2	111.7	123.8	116.1	
CD8+CD45RO+ telomere IQR	118.6 – 137.1	101.5 – 126.8	108.8 – 137.0	105.0 – 127.3	
CD4+CD45RO+ telomere median	117.2	113.7	115.0	107.7	
CD4+CD45RO+ telomere IQR	109.7 – 131.1	103.0 – 122.7	103.7 – 125.0	99.58 – 117.6	

Table 3. Demographic characteristics of subjects in the four study groups. † Kruskal Wallis unless otherwise stated. [1]Chi-squared.

Patients with anti-HCV were defined as viraemic if HCV-RNA was detected on all 3 occasions at 6-month intervals; only those with a recent liver biopsy were included. Those who were negative for HCV-RNA on a minimum of 3 tests at annual intervals were defined as non-viraemic, but did not undergo liver biopsy. Those with inconsistent HCV-RNA results were excluded. At study entry all subjects were treatment naïve and none had evidence of HCC or previous hepatic decompensation.

A composite end-point was used for outcome analysis: outcome events were death, first episode of hepatic decompensation (new rise in bilirubin to twice the upper limit of normal, new onset of ascites, encephalopathy, or portal hypertensive haemorrhage) or the development of hepatocellular carcinoma. Outcome was determined from study entry and survivors were censored at time of last clinic appointment.

Statistics

Lymphocyte telomere length within an individual is expressed as mean fluorescence intensity (MFI). Population data were subjected to non-parametric analysis, with lymphocyte surface phenotype, γ-H2AX staining, telomerase activity and serum IFN-α analysed by Kruskal-Wallis, lymphocyte subset telomere length by repeated measures 2-way ANOVA, peripheral and intrahepatic telomere length by Wilcoxon signed rank test and portal tract Ki67 expression by Mann Whitney U test. Ishak scores are non-linear variables and therefore associative data were analysed by Spearman's Rank correlation coefficient. Univariate analysis of survival was performed by the Kaplan-Meier method; curves were compared with the log-rank method and hazard ratios constructed from a Cox regression analysis (Prism 5.0, Graphpad, San Diego, CA).

Backward stepwise multinomial regression analysis was performed to identify predictors of severe fibrosis using SPSS 15.0 for Windows, with allocation to the severe fibrosis group (Ishak fibrosis stage

4-6) as outcome. Input variables were gender, IgG anti-CMV status, age, BMI, IgG anti-HBc status, CD4+CD45RO+ telomere length and CD8+CD45RO+ telomere length.

Multi-variate analyses were performed by Ms. Sarah Vowler, Centre for Applied Medical Statistics, Department of Public Health and Primary Care, University of Cambridge.

Results

T-lymphocyte telomere length, viraemia and fibrosis stage (Figure 30a, b & c)

Increasing age was associated with shortened lymphocytic telomeres in healthy subjects and patient study groups. There was no evidence of difference in the slopes of the age versus telomere distribution in the four study groups by multiple linear regression ($p = 0.578$) (Figure 30B).

Study subjects were recruited according to HCV status and hepatic fibrosis stage, so there were predictable differences between patient groups and controls with respect to CMV and IgG anti-HBc status (Table 3) and within patient groups for age. Therefore, a backward stepwise multinomial regression model was constructed to determine which factors were predictive of severe fibrosis. Input variables into the model were gender, IgG anti-CMV status, age, BMI, IgG anti-HBc status, CD4+CD45RO+ telomere length and CD8+CD45RO+ telomere length.

Median telomere length was similar in CD4+CD45RO+ lymphocytes from healthy controls and viraemic patients with mild disease. However, median telomere length in CD4+CD45RO+ lymphocytes from viraemic patients with severe fibrosis was shortened. Backward stepwise multinomial regression demonstrated that male sex (OR; 95% CI; p value) (2.84 (1.07, 7.53); $p = 0.04$), CMV positivity (4.18 (1.63, 10.73); $p = 0.003$), anti-HBc positivity (4.74 (1.53, 14.72); $p = 0.007$) and decreasing CD4+CD45RO+ telomere length (1.05 (1.02, 1.78); $p = 0.003$) were independently associated with severe HCV-related fibrosis (Table 4).

	Simple multinomial regression					Backward stepwise multinomial regression				
Variable	β	SE	df	OR (95% CI)	p	β	SE	df	OR (95% CI)	p
Male gender	0.89	0.44	1	2.43 (1.03, 5.74)	**0.043**	1.04	0.50	1	2.84 (1.07, 7.53)	**0.04**
CMV +	1.33	0.44	1	3.78 (1.61, 8.83)	**0.002**	1.43	0.48	1	4.18 (1.63, 10.73)	**0.003**
Age	0.07	0.02	1	1.08 (1.03, 1.12)	**0.001**					
BMI	0.02	0.04	1	1.02 (0.94, 1.10)	0.71					
Anti-HBc +	1.65	0.54	1	5.20 (1.80, 15.1)	**0.002**	1.58	0.58	1	4.74 (1.53, 14.72)	**0.007**
CD4+CD45RO+ telomere length	0.05	0.01	1	1.05 (1.02, 1.08)	**0.001**	0.04	0.01	1	1.05 (1.02, 1.78)	**0.003**
CD8+CD45RO+ telomere length	0.03	0.01	1	1.03 (1.01, 1.06)	**0.002**					

Table 4. Predictors of severe fibrosis (Ishak fibrosis 4 – 6) by backward stepwise multinomial regression. Input variables were gender, previous CMV or HBV, age, BMI and CD4+CD45RO+ telomere length and CD8+CD45RO+ telomere length. Variables associated with severe fibrosis ($p < 0.1$) in simple linear regression were included in a multiple regression analysis.

CD8+CD45RO+ telomere length showed significant association on simple regression analysis with severe fibrosis, but not independent association in backward stepwise regression analysis.

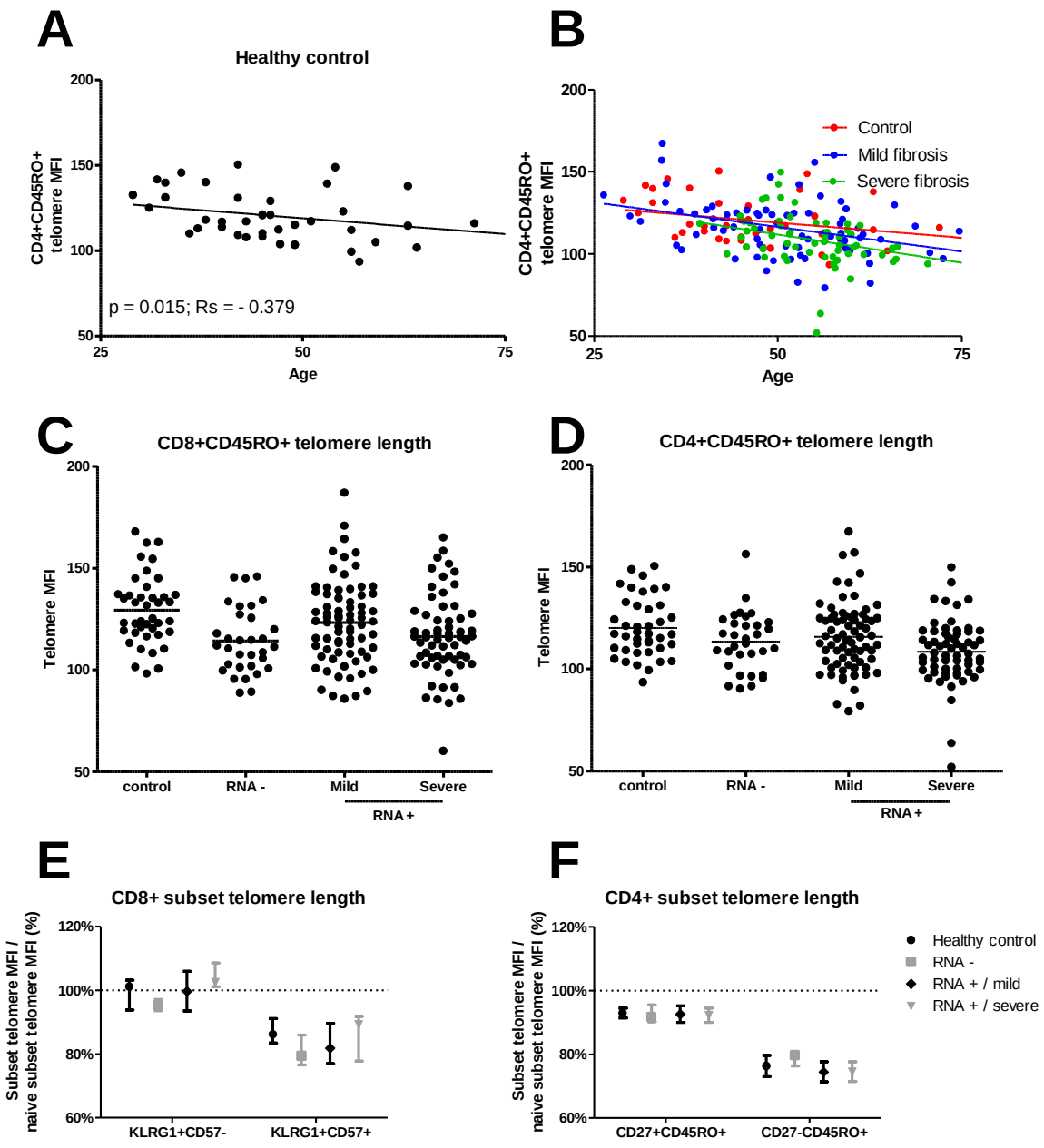

Figure 30. Telomere length of CD8+ and CD4+ T-cells from healthy controls and HCV-infected subjects.

A & B: The correlation between age and telomere length in circulating CD4+CD45RO+ lymphocytes from 41 healthy controls (Panel A) (p = 0.015; Rs = - 0.379) and 135 viraemic individuals (Panel B) (p < 0.001; Rs = -0.414).

C: Telomere length of circulating CD8+CD45RO+ lymphocytes in 41 healthy controls, 32 non-viraemic HCV-exposed cases, 73 viraemic patients with mild fibrosis and 61 viraemic patients with severe fibrosis. The horizontal bar represents the median.

D: Telomere length of circulating CD4+CD45RO+ lymphocytes in 41 healthy controls, 29 non-viraemic HCV-exposed cases, 73 viraemic patients with mild fibrosis and 62 viraemic patients with severe fibrosis.

E: Telomere length of circulating CD8+ lymphocyte subsets defined by KLRG1 and CD57 expression in 7 healthy controls, 5 non-viraemic HCV-exposed cases, 6 viraemic patients with mild fibrosis and 6 viraemic patients with severe fibrosis; results were normalised to CD8+KLRG1-CD57- telomere length. Symbol and whiskers represent median and interquartile range.

F: Telomere length of circulating CD4+ lymphocyte subsets defined by CD27 and CD45RO expression in 10 healthy controls, 6 non-viraemic HCV-exposed cases, 9 viraemic patients with mild fibrosis and 9 viraemic patients with severe fibrosis; results were normalised to CD4+CD27+CD45RO- telomere length. Symbol and bars represent median and interquartile range.

Telomere length and cells with 'advanced phenotype'

There was no evidence of differences between the four groups in the proportion of CD8+ T-cells that were CD27+KLRG1-CD57- (Kruskal-Wallis, p = 0.21), KLRG1+CD57- (p = 0.48), or KLRG1+CD57+ (p = 0.58). Nor was there evidence of differences in the proportion of CD4+T-cells that were CD27+CD45RO- (p = 0.07), CD27+CD45RO+ (p = 0.14) or CD27-CD45RO+ populations (p = 0.44). Thus, the telomere length changes demonstrated were not attributable to accumulation of lymphocytes with an 'advanced phenotype'.

Telomere length and naïve or antigen-experienced lymphocytes (Figure 30d & e)

Normally lymphocyte telomere length shortens with differentiation and maturation [356, 376]. To determine whether this was true in HCV, or whether there was global shortening of all T-cell subsets (both naïve and antigen-experienced), CD8+ and CD4+ lymphocyte subset telomere lengths were measured. Subset telomere length was normalised to the length in the most naïve subset to correct for inter-individual variation and confounding factors.

There was no statistical evidence of differences in median telomere length between the study groups for CD8+KLRG1+CD57- or CD8+KLRG1+CD57+ subsets (2-way ANOVA, p = 0.12); advanced phenotype generally was associated with shortened telomere length ($p < 0.0001$). Thus, telomere length changes in HCV were unrelated to accelerated shortening in a particular T-cell subset. Nor was there evidence of differences in median telomere length between study groups for CD4+CD27+CD45RO+ or CD4+CD27-CD45RO+ subsets (p = 0.55); advanced phenotype generally was associated with shortened telomere length ($p < 0.0001$).

Circulating lymphocyte telomere length, fibrosis and portal inflammation (Figure 31)

CD8+CD45RO+ lymphocyte telomere length in 133 viraemic subjects correlated with fibrosis stage (Spearman's Rank correlation, p = 0.0003), portal tract inflammatory grade (p = 0.027) and confluent necrosis (p = 0.039), but showed no evidence of correlation with interface hepatitis (p = 0.125),

lobular hepatitis (p = 0.504) or steatosis (p = 0.706). CD4+CD45RO+ lymphocyte telomere length also correlated with fibrosis stage (p<0.0001, Figure 31b), portal tract inflammation grade (p = 0.035) and confluent necrosis (p = 0.036) (data not shown), but there was no evidence of correlation with interface hepatitis (p = 0.343), lobular hepatitis (p = 0.839) or steatosis (p = 0.755).

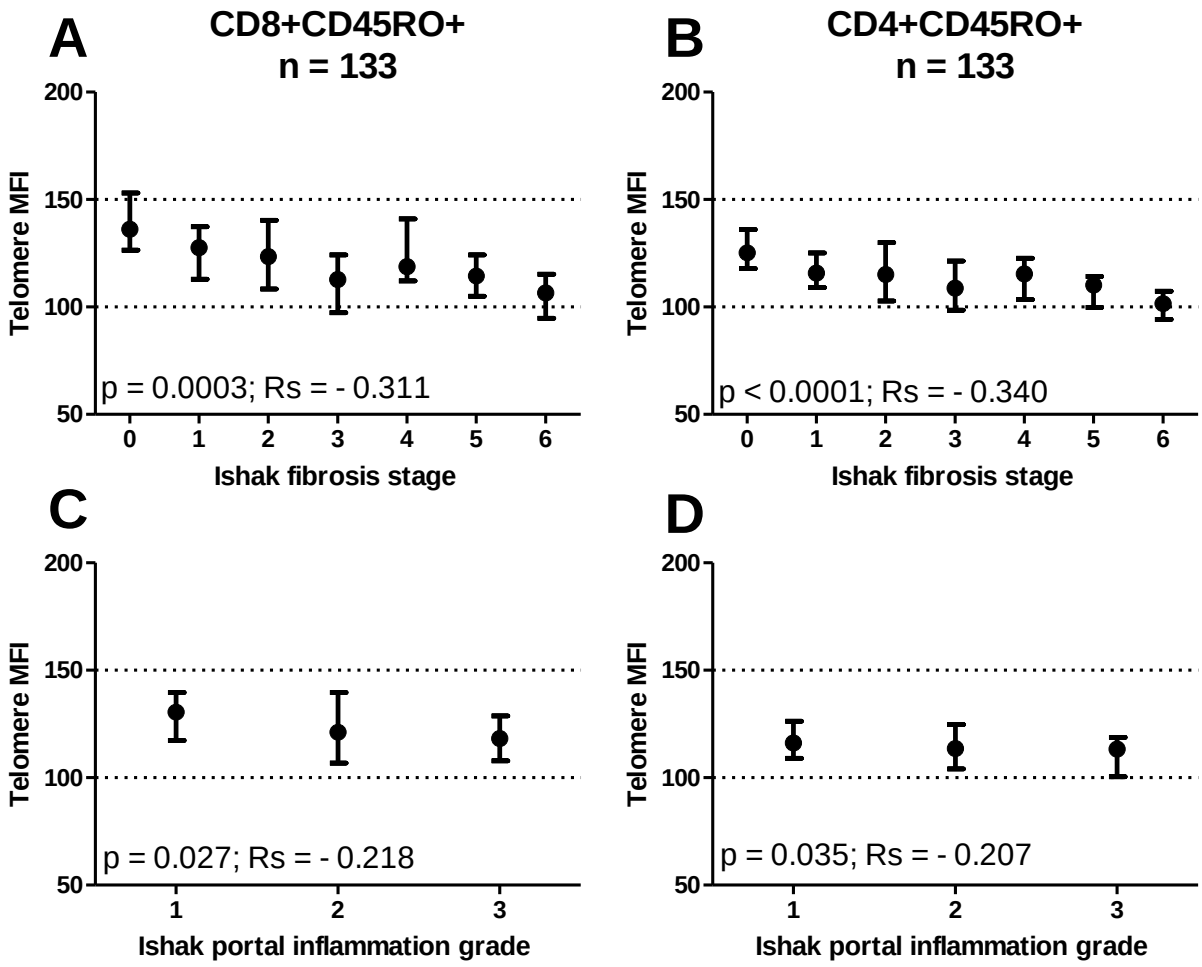

Figure 31. Correlation between telomere length of circulating CD8+CD45RO+ lymphocytes (A & C) or CD4+CD45RO+ lymphocytes (B & D) from 133 viraemic HCV subjects and fibrosis stage (A & B) or portal tract inflammation grade (C & D). Symbols and bars represent median and interquartile range; correlation by Spearman's Rank.

Lymphocyte telomere length and clinical parameters (Figure 32)

The relation between CD4+ or CD8+ lymphocyte telomere length and clinical severity was investigated in 133 viraemic patients. There was a correlation between CD8+CD45RO+ telomere length and serum bilirubin (p = 0.003), prothrombin time (p= 0.004), but not with serum ALT (p =

0.115). There was also a correlation between CD4+CD45RO+ telomere length and bilirubin ($p = 0.001$), prothrombin time ($p = 0.001$) but not ALT ($p = 0.08$). The significant correlations, detailed above, remained even after exclusion of outlying values.

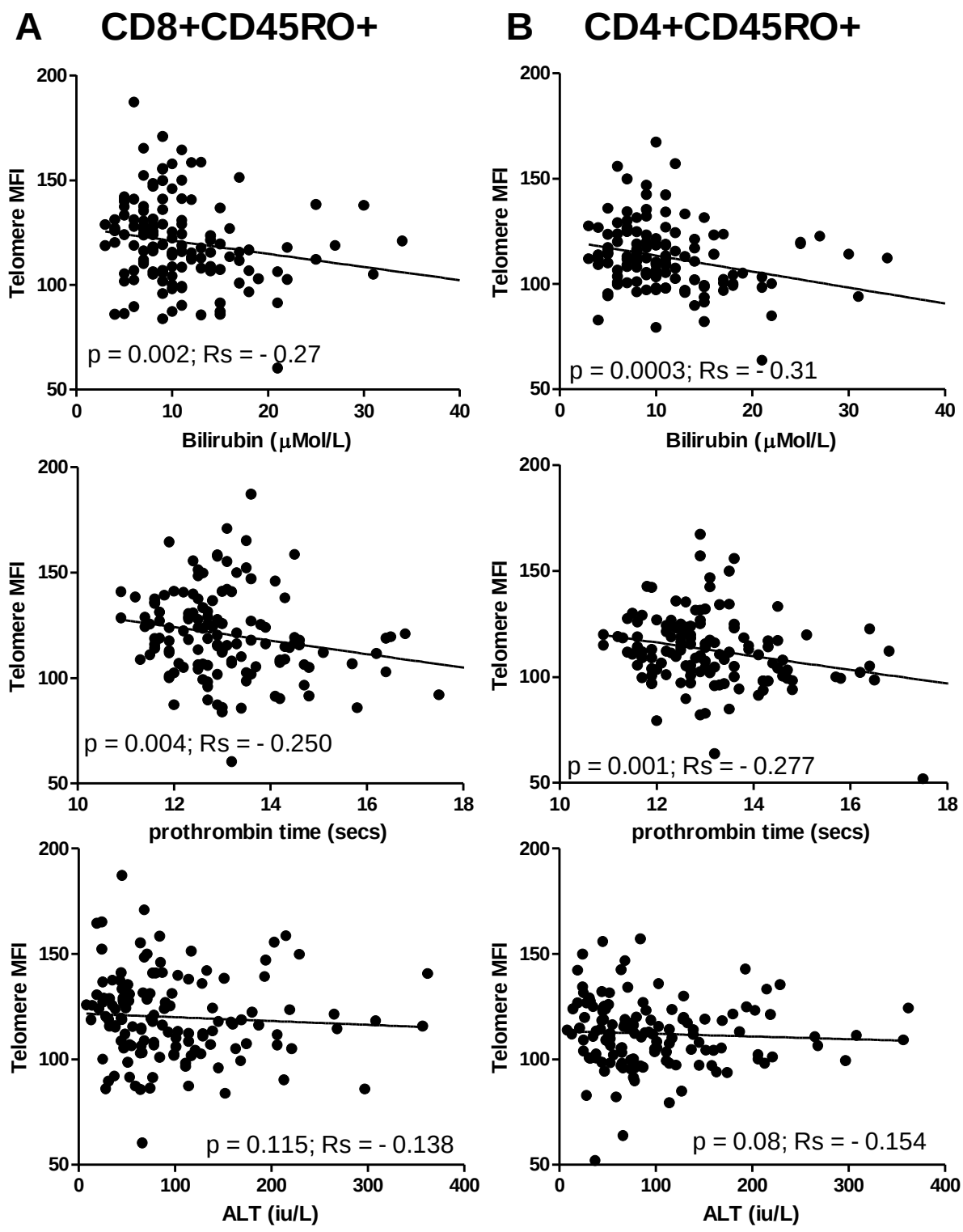

Figure 32. Correlation between telomere length of circulating CD8+CD45RO+ lymphocytes (A) or CD4+CD45RO+ lymphocytes (B) from 133 viraemic HCV patients with measures of the severity of liver disease: upper row, serum total bilirubin (µM/L); middle row, prothrombin time (seconds); and lower row, serum alanine transaminase (ALT) (IU/L).

Short CD4+ lymphocyte telomere length and poor clinical outcome (Figure 33 and Table 5)

11 viraemic patients were lost to follow-up after enrolment. Therefore, 124 viraemic subjects without previous decompensation or elevated bilirubin were followed prospectively for a median of 724 days (IQR: 533-906). The cohort was divided into telomere length that was longer or shorter than the cohort median (118.8 for CD8+CD45RO+ and 111.9 for CD4+CD45RO+).

The proportion free of complication at 2 years was 93.9% and 87.9% for longer and shorter CD8+CD45RO+ telomeres respectively ($p = 0.039$) and 95.4% and 86.2% for longer and shorter CD4+CD45RO+ telomeres respectively ($p = 0.009$). Utilising a proportional hazards model to investigate telomere length as a predictor of outcome independent of age demonstrated that a single point increase in CD8+CD45RO+ telomere MFI was associated with a HR of 0.96 (95% CI: 0.93 - 0.99) and 0.93 (0.91 - 0.96) in the CD4+CD45RO+ subset.

Restricting analysis to viraemic subjects with severe fibrosis ($n = 55$, median follow-up 742 days (333 - 913 days) divided by cohort medians of 116.1 and 108.3 respectively, revealed the proportion free of complications at 2 years was 86.3% and 79.8% for longer and shorter CD8+CD45RO+ telomeres respectively ($p = 0.27$) and 96.6% and 69.9% for longer and shorter CD4+CD45RO+ telomeres respectively ($p = 0.0009$). The age-adjusted HRs for CD8+CD45RO+ and CD4+CD45RO+ telomeres and the development of the composite outcome were 0.96 (0.92 - 0.99) ($p < 0.001$) and 0.94 (0.91 - 0.96) ($p = 0.02$) respectively.

Analysis of each outcome independently in those with severe fibrosis ($n = 55$) revealed that increased CD4+CD45RO+ telomere length was associated with reduced evolution to HCC ($p = 0.003$, age-adjusted HR 0.92 (0.87 - 0.97) and fewer episodes of decompensation ($p = 0.003$, HR 0.93 (0.89 - 0.98). No separate outcome was associated with shorter CD8+CD45RO+ telomere length (HCC ($p = 0.1$), decompensation ($p = 0.05$), death ($p = 0.95$)) in those with severe fibrosis (data not shown).

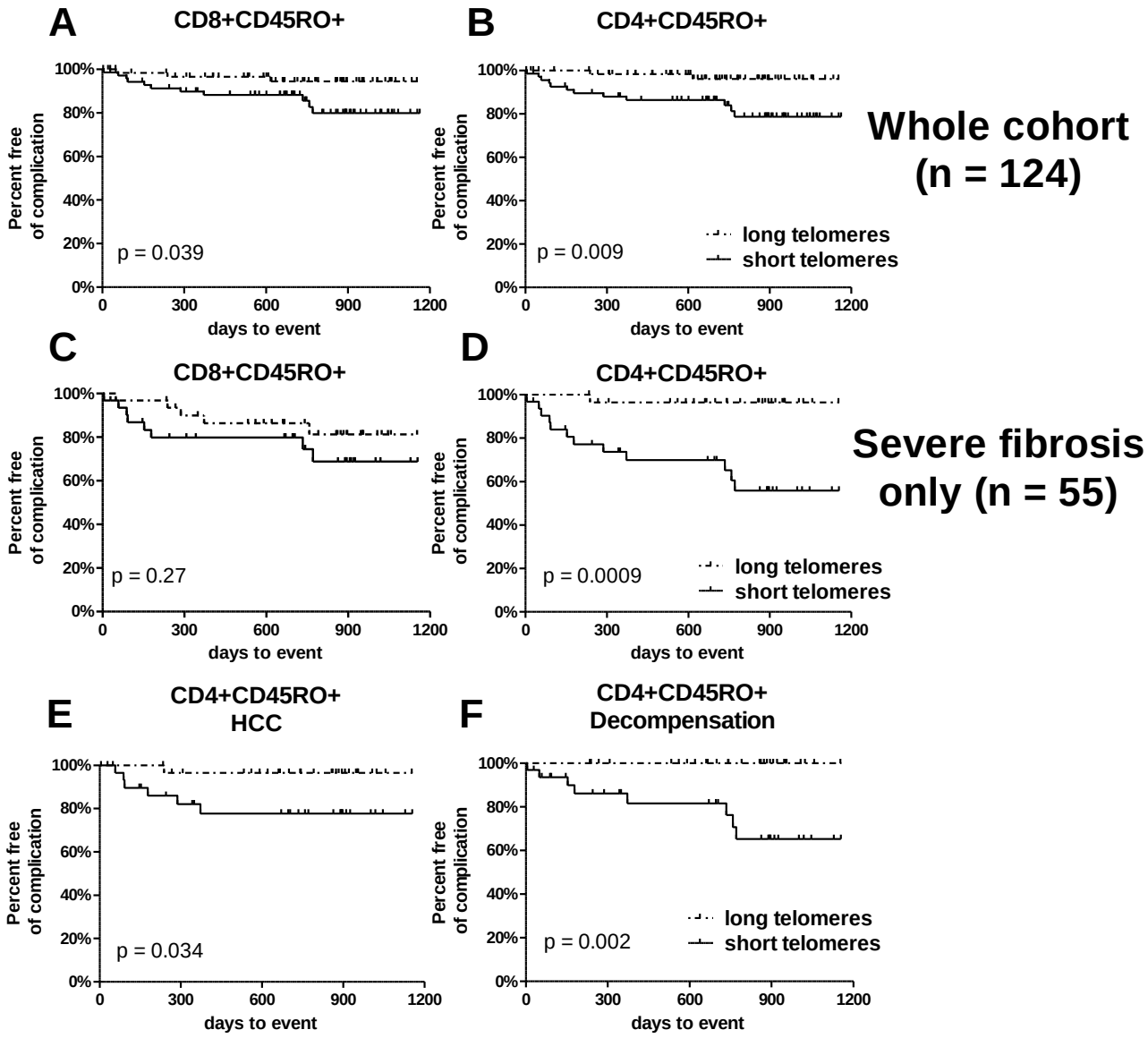

Figure 33. Clinical outcome of all viraemic subjects (n = 124) (A & B) or viraemic patients with severe fibrosis only (n = 55) (C & D) divided into those with telomeres longer (dashed line) or shorter (solid line) than the median. Kaplan-Meier analysis from study entry to outcome or censor date by log rank test: A & C: CD8+CD45RO+ telomere length and B & D: CD4+CD45RO+ telomere length. CD4+CD45RO+ telomere length from subjects with severe fibrosis (n = 55) analysed with each outcome independently: E, Development of HCC; F, Episode of decompensation.

Cohort	Endpoint	Unadjusted HR (95% CI)						Age-adjusted HR (95% CI)			
		CD8+ CD45RO+ telomere	p	CD4+ CD45RO+ telomere	p	Age (years)	p	CD8+ CD45RO+ telomere	p	CD4+ CD45RO+ telomere	p
Overall (n = 124)	Composite	0.96 (0.93 - 0.99)	**<0.001**	0.93 (0.91 - 0.96)	**0.008**	1.10 (1.04 - 1.15)	**<0.001**	0.96 (0.93 - 1.00)	**<0.001**	0.93 (0.90 - 0.96)	**0.03**
Severe fibrosis (n = 55)	Composite	0.96 (0.92 - 0.99)	**<0.001**	0.94 (0.91 - 0.96)	**0.01**	1.11 (1.04 - 1.19)	**0.002**	0.96 (0.93 - 0.99)	**<0.001**	0.93 (0.90 - 0.96)	**0.02**
	De-compensation	0.95 (0.91-1.00)	**0.04**	0.93 (0.89 - 0.98)	**0.003**	1.05 (0.94 - 1.16)	0.4	0.95 (0.91-1.00)	0.05	0.93 (0.89 - 0.98)	**0.003**
	HCC	0.97 (0.92-1.01)	0.2	0.94 (0.90 - 0.98)	**0.003**	1.16 (1.07 - 1.26)	**<0.001**	0.97 (0.92-1.01)	0.1	0.92 (0.87 - 0.97)	**0.003**

Table 5. Unadjusted and adjusted Hazard Ratios (and 95% CI) for decompensation, development of HCC and a composite endpoint of death, decompensation or development of HCC by CD8+CD45RO+ and CD4+CD45RO+ T-lymphocyte telomere length and age among the whole viraemic cohort (n = 124) or restricted to those with severe fibrosis (n = 55).

Therefore, shorter CD4+CD45RO+ telomere length was significantly associated with the development of clinical endpoints associated with HCV viraemia over a follow-up period of around 2 years, independent of age or fibrosis stage.

To investigate the underlying mechanism for the changes in telomere length, peripheral and intrahepatic lymphocyte proliferation and the ability of peripheral lymphocytes to develop telomerase activity were studied.

Peripheral and intrahepatic lymphocyte telomere lengths (Figure 34 & Table 6)

The relative maturation status of peripheral and intrahepatic lymphocytes by cell surface phenotype was investigated prior to investigating telomere length in both peripheral and intrahepatic compartments. Cells from liver biopsy, resection specimen or liver transplantation were utilised and peripheral lymphocytes taken contemporaneously were the comparator.

The proportion of CD8+ lymphocytes with an advanced cell surface phenotype as judged by CD57 and KLRG1 expression are increased in the intrahepatic compartment when compared to peripheral lymphocytes (2-way ANOVA, p = 0.0002). Similarly, CD4+ lymphocytes with an advanced cell surface phenotype, as judged by CD27 and CD45RO expression, are increased within the intrahepatic compartment in HCV-infected subjects (p = 0.036).

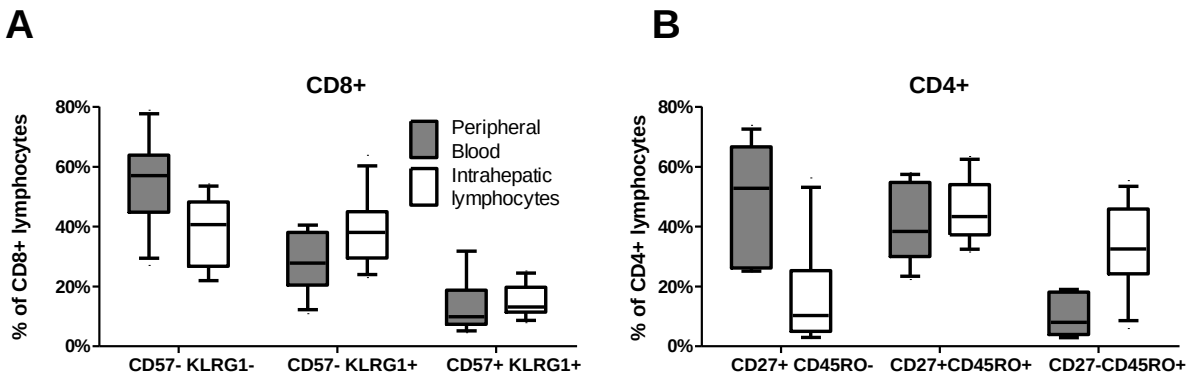

Figure 34. Cell surface phenotype of peripheral and intrahepatic CD8+ (Panel A) and CD4+ (Panel B) lymphocytes from HCV-infected subjects (n = 12). CD8+ lymphocytes are differentiated on the

basis of CD57 and KLRG1 expression; the more mature phenotype to the right. Similarly, CD4+ lymphocytes are differentiated by CD27 and CD45RO expression; the more mature phenotype to the right.

There were no differences between intrahepatic and circulating lymphocyte telomere length in any individual, whether control (n = 12) or viraemic HCV infected patient (n = 6) for either CD4+ or CD8+ cells.

Group		CD8+CD45RO+			CD4+CD45RO+		
		PBMC	IHL	Wilcoxon signed rank	PBMC	IHL	Wilcoxon signed rank
Healthy control (n = 12)	Median	108.0	108.8	p = 0.85	106.8	112.0	p = 0.47
	IQR	101.8 - 125.5	103.9 - 121.5		102.1 - 121.0	107.8 - 118.0	
HCVRNA+ (n = 6)	Median	117.0	119.4	p = 0.84	116.2	116.8	p = 0.69
	IQR	110.6 - 131.9	117.5 - 129.9		97.88 - 136.2	107.6 - 132.4	

Table 6. Telomere length of peripheral (PBMC) and intrahepatic lymphocytes (IHL) from 12 'healthy' controls undergoing liver resection for hepatic adenoma or colorectal metastases and 6 HCV viraemic individuals undergoing liver transplantation.

Therefore, whilst there is a concentration of cells with an advanced cell –surface phenotype within the intrahepatic compartment, the cells within the antigen experienced CD45RO+ subset do not show any preferential shortening within the liver.

Telomerase (Figure 35)

To investigate whether the peripheral telomere length changes were attributable to a failure to induce telomerase, CD8+ lymphocytes were stimulated with anti-CD3/CD28 to assess their ability to generate telomerase activity via the Telomere repeat amplification protocol (TRAP) assay. Induction of telomerase, after correction for cell proliferation by Ki67 expression, was similar in CD8+ lymphocytes in all four groups (p = 0.16). Changes in telomere length are unlikely to be linked to a failure of lymphocytes to up-regulate telomerase activity. However, the sensitivity of the TRAP assay for small changes in telomerase activity is low and therefore complete exclusion of a change in telomerase is not possible.

Serum interferon-α (Figure 35)

IFN-α has been demonstrated to inhibit telomerase activity in vitro and in vivo [356], but serum IFN-α levels were similar in all four groups (p = 0.86).

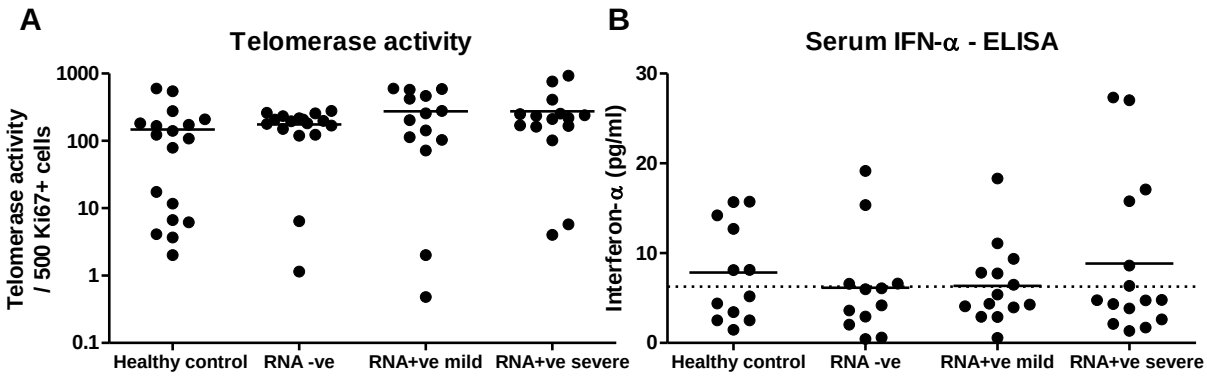

Figure 35. In vitro induction of telomerase activity in CD8+ PBMCs. Panel A: Telomerase expression in CD8+ PBMCs after 5-days non-specific stimulation in 18 healthy controls, 17 non-viraemic HCV-exposed cases, 14 viraemic patients with mild fibrosis and 15 viraemic patients with severe fibrosis was similar (p = 0.16). Horizontal bar represents the median. Panel B: Serum IFN-α concentrations in 12 healthy controls, 12 non-viraemic HCV-exposed cases, 14 viraemic patients with mild fibrosis and 15 viraemic patients with severe fibrosis were similar (p = 0.863). Horizontal bar represents the median; the dotted line represents the lower limit of detection (6.25 pg/ml).

Peripheral and intrahepatic lymphocyte proliferation (Figure 36)

To investigate whether the telomere length changes were attributable to higher levels of in vivo proliferation I examined Ki67 as a marker of lymphocyte proliferation. Peripheral lymphocytes from healthy controls (n = 23), non-viraemic HCV-exposed (n = 22), viraemic with mild disease (n = 42) and viraemic with severe disease (n = 28) were stained for Ki67 without stimulation. There was no significant difference in the level of Ki67 expression in either CD8+ (Figure 36a) or CD4+ (Figure 36b) between the four groups.

Intrahepatic lymphocyte proliferation in viraemic subjects with severe liver disease known to have shortened peripheral lymphocyte telomeres was investigated, given that antigenic exposure of lymphocytes in HCV infection is likely to occur within the liver. Immunohistochemistry was undertaken in subgroups of viraemic patients with mild disease (n = 10) and viraemic patients with

severe disease (n = 11) (Figure 36c). There was significantly greater portal tract area positive for Ki67 in subjects with mild disease (median (IQR); 0.93% (0.72% – 1.4%)) compared to severe disease (0.67% (0.53% - 0.85%) (p = 0.02) (Figure 36d).

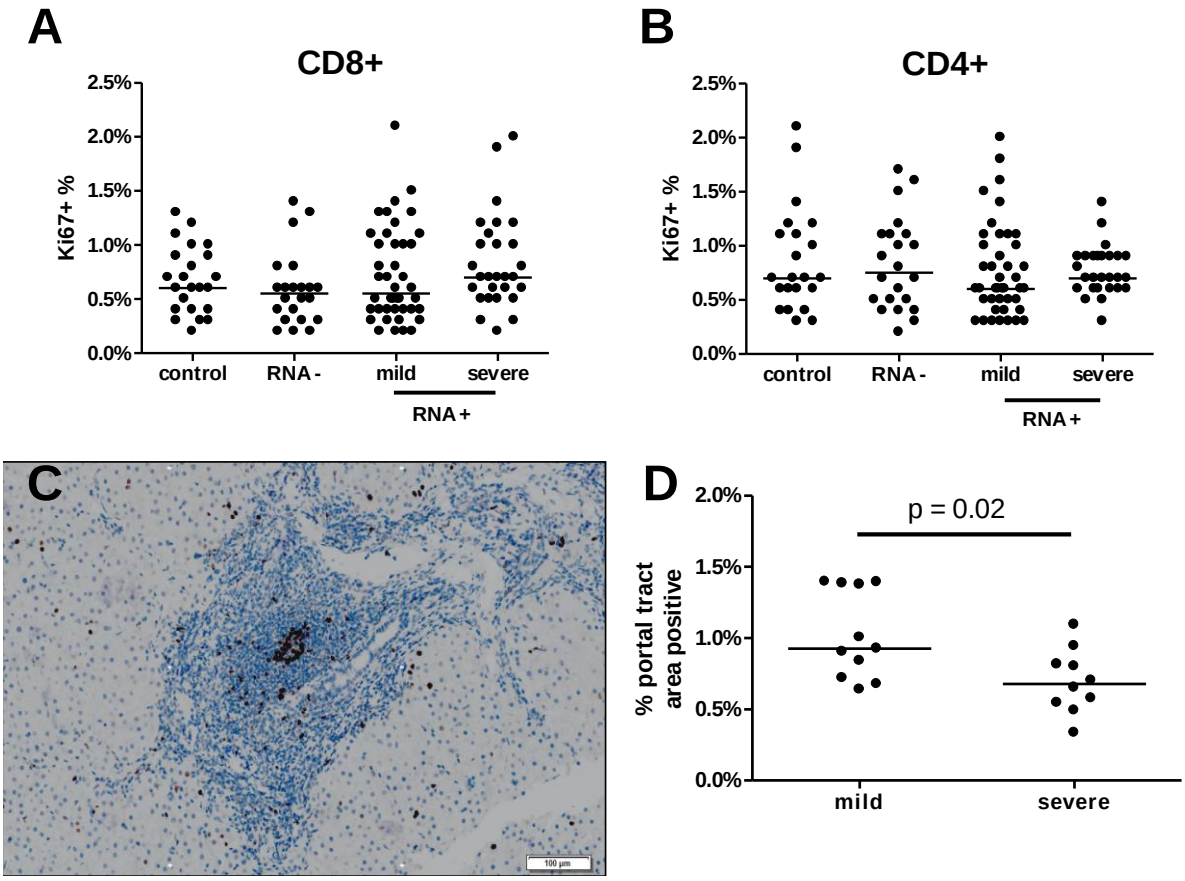

Figure 36. Intrahepatic lymphocyte proliferation is impaired in groups with short lymphocytic telomere length. Ex vivo CD8+ (A) and CD4+ (B) lymphocyte expression of Ki-67 by flow-cytometry in healthy controls (n = 23), non-viraemic HCV-exposed cases (n = 22), viraemic patients with mild fibrosis (n = 42) and viraemic patients with severe fibrosis (n = 28). Panel C: photomicrograph of portal tract demonstrating lymphocytic expression of Ki-67 by immunohistochemistry; scale bar 100µm. Panel D: proportion of portal tract area expressing Ki-67 in viraemic patients with mild fibrosis (n = 11) and severe fibrosis (n = 10). Analysis by Mann-Whitney U test.

Further, there was significant correlation between portal tract area positive for Ki67 and peripheral CD4+CD45RO+ telomere length (p = 0.03, r^2 = 0.24) (Figure 37a). There was no evidence of a correlation between Ki67 expression and peripheral CD8+CD45RO+ telomere length (p = 0.16).

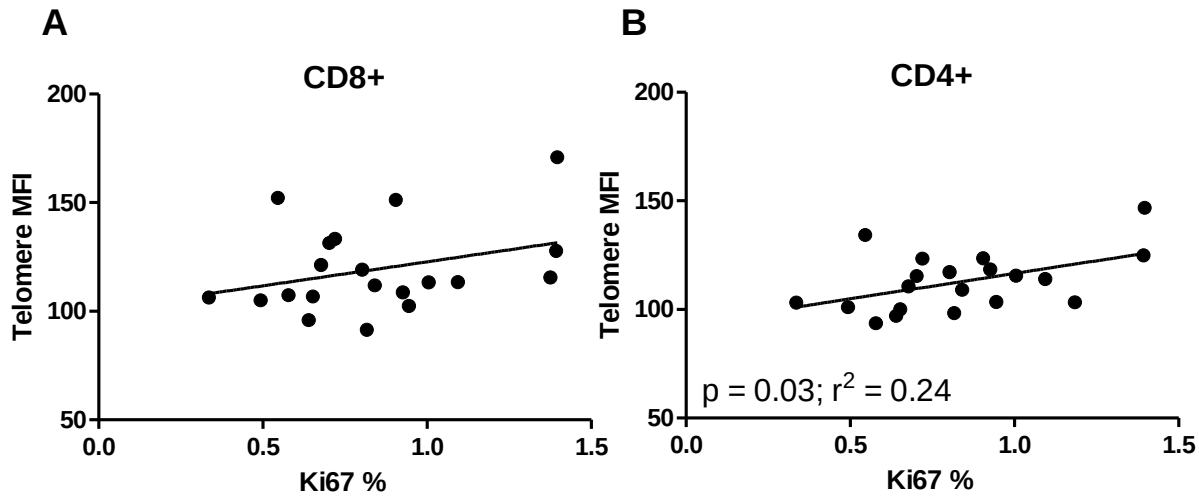

Figure 37. Relationship between portal tract area positive for Ki67 staining by immunohistochemistry and peripheral CD8+CD45RO+ telomere length (panel A) or CD4+CD45RO+ telomere length (panel B) in subjects with viraemic HCV infection (n = 21).

Those subjects with longer telomeres have enhanced intrahepatic lymphocyte proliferation in vivo. Further, there was no evidence that the telomere length changes demonstrated earlier were attributable to either excess intrahepatic or peripheral proliferation, failure to induce telomerase or peripheral inhibition of telomerase by IFN-α.

Discussion

T-lymphocyte telomeres shortened with age in all study groups, as anticipated [357]. T-cells from patients exposed to HCV had telomeres that were shorter than healthy controls with an overall difference in telomere length between the two groups equivalent to 10-years additional ageing. The changes in T-cell telomere length in viraemic patients were more marked in those with severe fibrosis, equivalent to 15-years additional ageing. The findings were independent of factors known to influence immune senescence, including age, sex and CMV status. The changes were similar in both CD4+ and CD8+ T-lymphocytes but the important association was between CD4+ telomere length and severe fibrosis. Unexpectedly, the changes in telomere length affected all T-cell subsets and were just as marked in antigen experienced as they were in naïve T-cells; thus the findings cannot be explained readily by accumulation of T-cells with a particular phenotype, as seen in other

settings [356, 410]. Alternative explanations for these findings, including a failure to induce telomerase or an effect of IFN-α on telomerase induction, seem improbable based on subsequent findings in this study.

An important relation between immune senescence and clinical outcome was supported by the findings that T-lymphocyte telomere length in viraemic patients correlated closely with increased fibrosis stage, increased grade of portal tract inflammation, prolonged prothrombin time and increased bilirubin, all factors that predict morbidity and mortality in patients with chronic HCV infection [6]. The duration of follow-up remains short, yet patients with shorter telomeres already have outcomes inferior to those with longer telomeres with more frequent progression to the composite endpoint of death, first episode of hepatic decompensation or hepatocellular carcinoma. 'Survivor bias' [217], whereby those with shorter telomeres in any population die at an earlier age of varied causes, so that survivors have longer telomeres, could account for some of these findings. Prospective study will answer this issue; however, more marked liver disease in cross sectional analysis and evolving liver failure in short term follow-up in those with the shortest telomeres suggests that even if this was the case then liver injury due to HCV is the likely cause of any differences.

This cross-sectional study could not address whether HCV infection causes accelerated telomere shortening, whether individuals with shorter T-cell telomeres are pre-disposed to cirrhosis or whether lifestyles associated with HCV infection affect telomere length. Longitudinal follow-up is underway and may address some of these issues.

One explanation for shortened T-cell telomeres in patients with chronic HCV infection is accelerated lymphocyte turnover, but the findings are inconsistent with that view. The observed T-cell telomere shortening was global; there were similar changes in antigen-naïve and antigen-experienced T-cells; there was no evidence of a relationship to an advanced phenotype and there was no excess

peripheral or intrahepatic proliferation in groups demonstrated to have short lymphocyte telomeres. Indeed, those individuals with shorter telomeres had evidence of reduced intrahepatic lymphocyte proliferation. It is probable that changes in telomere length were determined long before the study, since exposure to HCV predated the study by many years in most cases and such changes may be manifest shortly after exposure. Since replicative senescence cannot explain all of the findings it appears that senescence might arise by an alternative, or additional, pathway. Telomere shortening is accelerated following oxidative stress [321, 421]. HCV infection is known to be associated with increased oxidative stress within the liver [99].

The finding that lymphocytic telomere length is similar in peripheral and intrahepatic compartments is perhaps unexpected. Previous studies have demonstrated that the intrahepatic lymphocyte compartment is enriched in cells that bear a more advanced cell-surface phenotype [418, 422]. Heydtmann et al demonstrated that there was almost a complete absence of naive T-cells within the intrahepatic compartment [418]. However, I measured the telomere length of CD45RO+ CD8+ and CD4+ lymphocytes. This allowed correction for the different proportions of lymphocyte subsets in the two compartments. Therefore, despite enrichment of mature cells within the liver there was no preferential shortening of telomere length in antigen-experienced lymphocytes attendant with liver infiltration.

Unexpectedly, non viraemic HCV-exposed cases, a group described variously as resolved, cleared or occult infection [25, 56, 423], had very short CD8+CD45RO+ telomeres, equivalent to around 20-years additional ageing. These ‘patients’ are considered to have had a successful immune response to HCV at exposure with an excellent long term outcome. However, a small proportion become viraemic with extended follow up and a significant proportion have intrahepatic inflammation consistent with latent viral replication (Hoare et al [424] & chapter five), while other studies suggest that some have low level viral replication [55, 425]. The changes demonstrated in this series suggest that either this group had a sudden decline in CD8+ telomere length at exposure to HCV (which

seems unlikely given that most episodes of acute HCV are asymptomatic and unlikely to involve massive lymphocyte turnover and cytokine release) or that there is chronic low grade lymphocyte turnover related to successful immune control of HCV, particularly in CD8+ T-cells, or that lymphocytes may become senescent consequent to long term antigenic exposure. Shortened T-lymphocyte telomeres, rather than specific subset decline, to the degree seen in the non-viraemic HCV exposed cases in this series has been associated with a considerable increase in morbidity and mortality from infections, vascular disease and cancer in studies outside the field of hepatology [285, 388, 389, 426]. There are no published studies of the long-term natural history of non-viraemic HCV infection and it is unclear if this group suffer an increased risk of premature mortality.

Chapter 4 - Short T-lymphocyte telomere length compromises sustained virological response with pegylated interferon-α and ribavirin in chronic hepatitis C virus infection.

Abstract

Background: Ageing is associated with impaired immunity and features of immune senescence, including shortened lymphocyte telomeres. In patients with chronic HCV increasing age is associated with higher fibrosis stage and a failure to respond to antiviral therapy. There are strong correlations between measures of clinical outcome and lymphocyte telomere length in chronic HCV infection, suggesting accelerated immune senescence in chronic HCV infection. In this study I assessed whether immune senescence was also related to the response to antiviral therapy in chronic HCV.

Methods: Telomere length was measured before, during and on completion of antiviral therapy for HCV infection in peripheral CD8+ and CD4+ T-cells from 85 patients (75% male, 45% genotype 1, mean Ishak fibrosis stage 3.6) by Flow-FISH. Patients were followed for 6 months after completion of treatment. Results were analysed by multiple logistic regression (LR); age, sex, Ishak fibrosis stage, viral genotype, viral load, BMI, CD8+CD45RO+ and CD4+CD45RO+ telomere length were input variables.

Results: Baseline median (IQR) CD8+CD45RO+ (128.1 (113.4 - 142.3) and CD4+CD45RO+ (119.0 (107.8 - 126.8) telomere lengths were longer in the 38 patients (44.7%) who achieved SVR than those who did not (112.5 (104.5 - 118.2)($p = 0.0006$) and 105.5 (99.4 - 114.2)($p = 0.0003$) respectively. CD8+CD45RO+ and CD4+CD45RO+ telomere lengths were unaffected by therapy.

By univariate LR viral load ($p = 0.02$), CD4+CD45RO+ telomere length ($p = 0.1$) and severe fibrosis ($p = 0.08$) were associated with SVR; in this model age ($p = 0.4$), male sex ($p = 0.47$), genotype ($p = 0.17$), BMI ($p = 0.93$) and CD8+CD45RO+ telomere length ($p = 0.76$) were not associated with SVR.

Utilising 'backwards stepwise regression' to remove non-significant variables viral load (p = 0.02) and CD4+CD45RO+ telomere length (p = 0.001) were associated independently with SVR. Each single point increase in CD4+CD45RO+ telomere length was associated with an OR of achieving SVR of 1.08 (95% CI: 1.03 – 1.13).

Conclusion: Short CD4+CD45RO+ T-lymphocyte telomere length predicted a failure to respond to antiviral therapy for HCV infection, independent of other known factors.

Introduction

Pegylated interferon-α (peg-IFN-α) in combination with ribavirin (RBV) is the most effective available therapy for viraemic HCV infection; both the duration of therapy and the response rate are related to HCV genotype. Permanent loss of HCV-RNA from serum following therapy is reported in around 40% of those with genotype 1 infection and 75% in genotypes 2 & 3, but responses are lower in those with more advanced disease [6, 19].

Increased age at exposure is a critical determinant of outcome following infection with HCV in terms of progressive fibrosis, the risk of hepatocellular carcinoma and the probability of responding to combination antiviral therapy [6, 142, 144, 419, 420, 427]. Healthy human ageing is associated with a progressive impairment of immune performance and shortened telomere length. The extent of such changes are clearly important since they predict clinical outcomes, including mortality, in large scale prospective studies in man [353, 355, 357].

Telomeres shorten naturally with increasing age. Studies in diverse clinical scenarios indicate that telomere length predicts outcome including mortality [388-390]. In patients with chronic HCV infection T-lymphocyte telomere length is related to clinical outcome; it is correlated with hepatic fibrosis stage and those with severe fibrosis and shorter CD4+CD45RO+ telomeres are more likely to develop hepatic decompensation, hepatocellular carcinoma and death (See chapter three). Based on telomere length T-cells from patients with chronic HCV infection are around 10 years older than their chronological age.

Successful therapy with peg-IFN-α in combination with RBV is associated with loss of HCV-RNA for at six months after cessation of treatment; this appears permanent in many cases [428, 429]. The mode of action however, is unknown. One hypothesis is that therapy enhances immune responses generally, which might underlie the onset of autoimmune disorders during combination antiviral therapy.

I have investigated first whether T-lymphocyte telomere length was related to treatment response after pegylated IFN-α in combination with RBV and second, whether treatment, successful or otherwise, was associated with a change in telomere length.

Materials and methods

Subjects

Patients recruited at Addenbrooke's Hospital, Cambridge gave written informed consent with approval of the Local Research Ethics Committee. Patients co-infected with human immunodeficiency virus or hepatitis B virus were excluded. Those patients with liver disease of mixed aetiology (based on history, blood tests or liver biopsy) were also excluded. All the patients underwent liver biopsy within 24 months of starting treatment; all were HCV-antibody positive and were also HCV-RNA positive in serum on at least three occasions. None had undergone previous antiviral therapy. None had hepatocellular carcinoma.

Telomere length was measured immediately before treatment in all cases and after 4, 12 and 24 weeks therapy in a randomly selected subgroup of the whole cohort. Patients were treated with peg-IFN-α2a and RBV (Roche, Welwyn Garden City, UK). To begin with, all patients received 180mcg of peg-IFN-α2a sub-cutaneously once weekly, with subsequent dose-alterations dictated by clinical and laboratory parameters. RBV was given at 800mg for those with genotype 2 or 3 infection and weight-based between 1000mg - 1200mg for those with genotype 1 or 4 infection. Patients with genotype 1 or 4 infection were treated for 12 weeks and then for a further 36 weeks if they were HCV-RNA negative or had undergone a 2 $\log_{10}$ drop in viral load compared to baseline (early virological response (EVR)). Those with genotype 2 or 3 infection were treated for 24 weeks. All subjects underwent HCV RNA testing six months after cessation of therapy to determine if they had achieved a sustained virological response (SVR), as defined conventionally. No patient received

growth factors to support haematological parameters. Patients who were unable to tolerate therapy with peg-IFN-α and RBV on symptomatic grounds were excluded from further analysis.

Statistics

Lymphocyte telomere length within an individual is expressed as mean fluorescence intensity (MFI). Population data were subjected to non-parametric analysis conducted in Prism 5.0 for Windows (Graphpad, San Diego, CA). Associative data were analysed by Spearman's Rank correlation coefficient and telomere length over IFN-α therapy by repeated measures 2-way ANOVA.

Multi-variate analyses were performed by Ms. Sarah Vowler, Centre for Applied Medical Statistics, Department of Public Health and Primary Care, University of Cambridge. Multiple regression analysis was performed using SPSS 15.0 for Windows, with SVR as outcome. Input variables were age, sex, CMV antibody status, HCV genotype, Ishak fibrosis score, body mass index (BMI) and either CD8+CD45RO+ or CD4CD45RO+ telomere length. As Ishak fibrosis score is a non-linear variable, scores were grouped into categorical mild (Ishak 0 – 3) and severe (Ishak 4 – 6) groups. Only variables with a p value of <0.10 on univariate analysis were subjected to multiple regression analysis. P values of <0.05 were considered significant.

Results

Peripheral blood lymphocytes were obtained from 91 patients immediately prior to commencing antiviral therapy. 6 patients subsequently failed to complete the course of IFN / RBV therapy because of intolerable symptoms and were excluded from further analysis. The remaining 85 patients underwent a full course of anti-viral therapy and were followed-up to 6 months after cessation of therapy. The demographic characteristics are detailed in Table 7.

	Whole cohort	Did not achieve SVR	Achieved SVR	P value†
N	85	47	38	
Age (years, mean ± SD)	52.3 ± 8.8	55.0 ± 8.9	49.7 ± 7.6	0.01
Sex (% male)	75.3%	72.3%	78.9%	0.48[1]
BMI	26.6 ± 5.3	27.5 ± 5.7	25.5 ± 4.5	0.12
Bilirubin (µmol/L, mean ± SD)	12.3 ± 6.6	12.2 ± 6.1	11.5 ± 6.8	0.34
ALT (iu/L, mean ± SD)	113.2 ± 81.9	112.7 ± 83.2	117.1 ± 85.7	0.96
PT (seconds, mean ± SD)	13.2 ± 1.4	13.2 ± 1.1	13.2 ± 1.7	0.41
Genotype 1 2 3 4	45.9% 11.8% 41.2% 1.1%	53.2% 10.6% 36.2% 0%	36.8% 13.2% 47.4% 2.6%	0.36[1]
Viral Load (iu/ml) (mean ± SD)	$1.35 \times 10^6 \pm 6.99 \times 10^6$	$2.31 \times 10^6 \pm 9.34 \times 10^6$	$1.67 \times 10^5 \pm 2.54 \times 10^5$	0.003
% CMV antibody positive	51.0%	51.1%	50.0%	0.77[1]
Ishak score				
Interface hepatitis (0 - 4)	1.8 ± 0.6	1.7 ± 0.5	1.8 ± 0.7	0.61
Confluent necrosis (0 - 6)	0.1 ± 0.2	0.1 ± 0.3	0.0 ± 0.2	0.31
Lobular hepatitis (0 - 4)	2.2 ± 0.6	1.0 ± 0.5	2.3 ± 0.6	0.08
Portal inflammation (0 - 4)	2.2 ± 0.6	2.2 ± 0.6	2.2 ± 0.6	0.86
Fibrosis (0 - 6)	3.6 ± 1.5	3.9 ± 1.4	3.4 ± 1.5	0.09
Steatosis (0 - 3)	0.8 ± 0.8	1.0 ± 0.8	0.6 ± 0.8	0.02

Table 7. Demographic characteristics of study participants. † Achieved SVR vs did not achieve SVR, Mann-Whitney U test unless otherwise stated. [1]Chi-squared test.

Baseline T-lymphocyte telomere length and clinical parameters (Figure 38)

There was a significant inverse correlation between age and T-cell telomere length for both CD8+CD45RO+ and CD4+CD45RO+ subsets at commencement of therapy as anticipated (Figure 38 A, B) and described previously (see chapter three). There was also a significant inverse correlation

between CD4+CD45RO+ telomere length and fibrosis stage, but not CD8+CD45RO+ telomere length and fibrosis (Figure 38 C, D). However, there was no relation between CD8+CD45RO+ or CD4+CD45RO+ telomere length and viral load Rs = -0.03, p = 0.77 and Rs = -0.005, p = 0.96 respectively). Nor was there a relation between CD8+CD45RO+ or CD4+CD45RO+ telomere length and genotype (Figure 38 G, H).

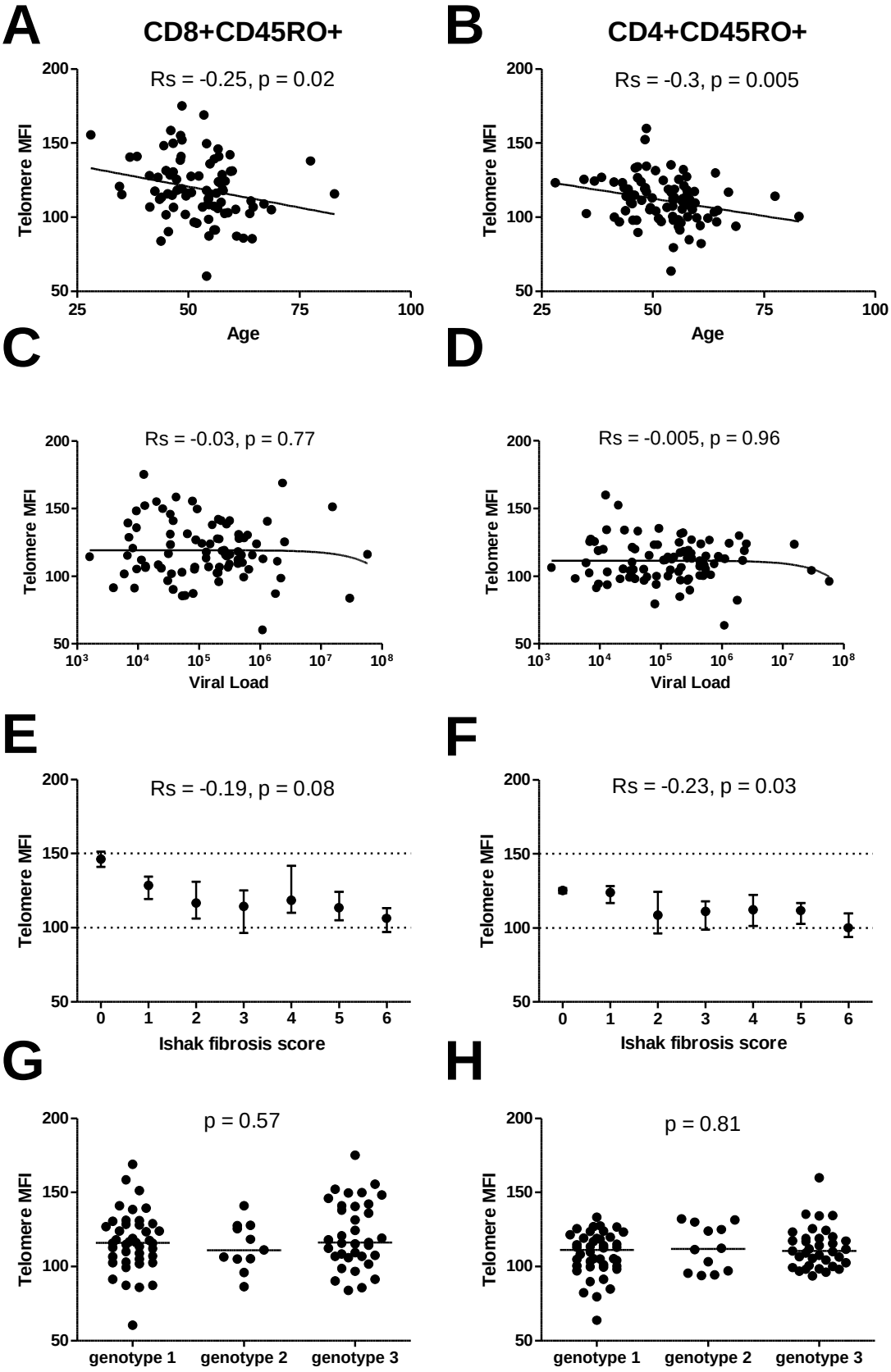
A
CD8+CD45RO+
Rs = -0.25, p = 0.02
Telomere MFI
Age
B
CD4+CD45RO+
Rs = -0.3, p = 0.005
Telomere MFI
Age
C
Rs = -0.03, p = 0.77
Telomere MFI
Viral Load
D
Rs = -0.005, p = 0.96
Telomere MFI
Viral Load
E
Rs = -0.19, p = 0.08
Telomere MFI
Ishak fibrosis score
F
Rs = -0.23, p = 0.03
Telomere MFI
Ishak fibrosis score
G
p = 0.57
Telomere MFI
genotype 1
genotype 2
genotype 3
H
p = 0.81
Telomere MFI
genotype 1
genotype 2
genotype 3

Figure 38. Baseline T-lymphocyte telomere length, expressed as mean fluorescence intensity, in (A, C & E) CD8+CD45RO+ and (B, D & F) CD4+CD45RO+ subsets and relationship to age (A & B), Ishak fibrosis score (C & D), viral load (iu/ml) (E & F) and genotype (G & H, genotype 4 (n = 1) not shown) in viraemic HCV infection (n = 85). Analysis by Spearman's Rank correlation and Kruskal-Wallis test.

Baseline T-lymphocyte telomere length was unrelated to early virological response (EVR) (Figure 39)

45 individuals with genotype 1 or 4 HCV infection were followed to 12 weeks of treatment, at which point 29 were HCV-RNA negative (64.4%), 6 individuals (13.3%) had undergone a 2 $\log_{10}$ drop in viral load and 10 (22.2%) remained HCV-RNA positive. There was no evidence of a difference in CD8+CD45RO+ lymphocyte telomere length in the three groups (median (IQR)): 118.8 (109.7 - 140.2), 115.9 (105.7 - 128.8) and 110.2 (97.8 - 117.0) respectively (Kruskal-Wallis, p = 0.12); nor was there evidence of a difference in CD4+CD45RO+ lymphocyte telomere length in the three groups: 115.0 (99.3 - 120.6); 102.8 (97.17 - 115.7); 103.8 (99.9 - 106.1) respectively (p = 0.07). All individuals who underwent a greater than 2 $\log_{10}$ drop in viral load but remained viraemic, subsequently failed to achieve SVR.

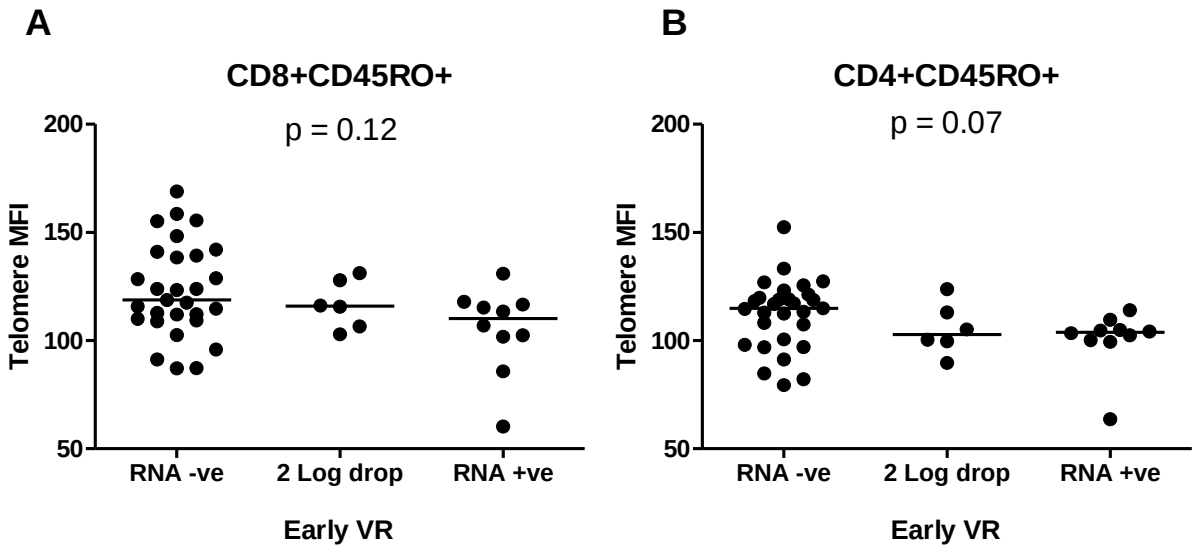

Figure 39. Baseline T-lymphocyte telomere length in (A) CD8+CD45RO+ and (B) CD4+CD45RO+ subsets in relation to EVR in subjects with genotype 1 or 4 HCV infection (n = 45). Subjects were divided by HCV-RNA status after 12 weeks peg-IFN-α/RBV therapy. Analysis by Kruskal Wallis test.

Baseline T-lymphocyte telomere length was related to SVR (Figure 40 and Table 8)

85 individuals provided samples 6 months after the end of treatment. 38 subjects (44.7%) achieved SVR; the remaining 47 (55.3%) were HCV-RNA positive. CD8+CD45RO+ telomere length at baseline was significantly longer in those individuals achieving SVR compared to those who did not: 128.1 (113.4 - 142.3) and 112.5 (104.5 - 118.2) respectively (Mann Whitney, p = 0.0006).

Similarly, CD4+CD45RO+ lymphocyte telomere length was longer in those individuals achieving SVR compared to those who remained viraemic: 119.0 (107.8 - 126.8) and 105.5 (99.4 - 114.2) respectively (p = 0.0003).

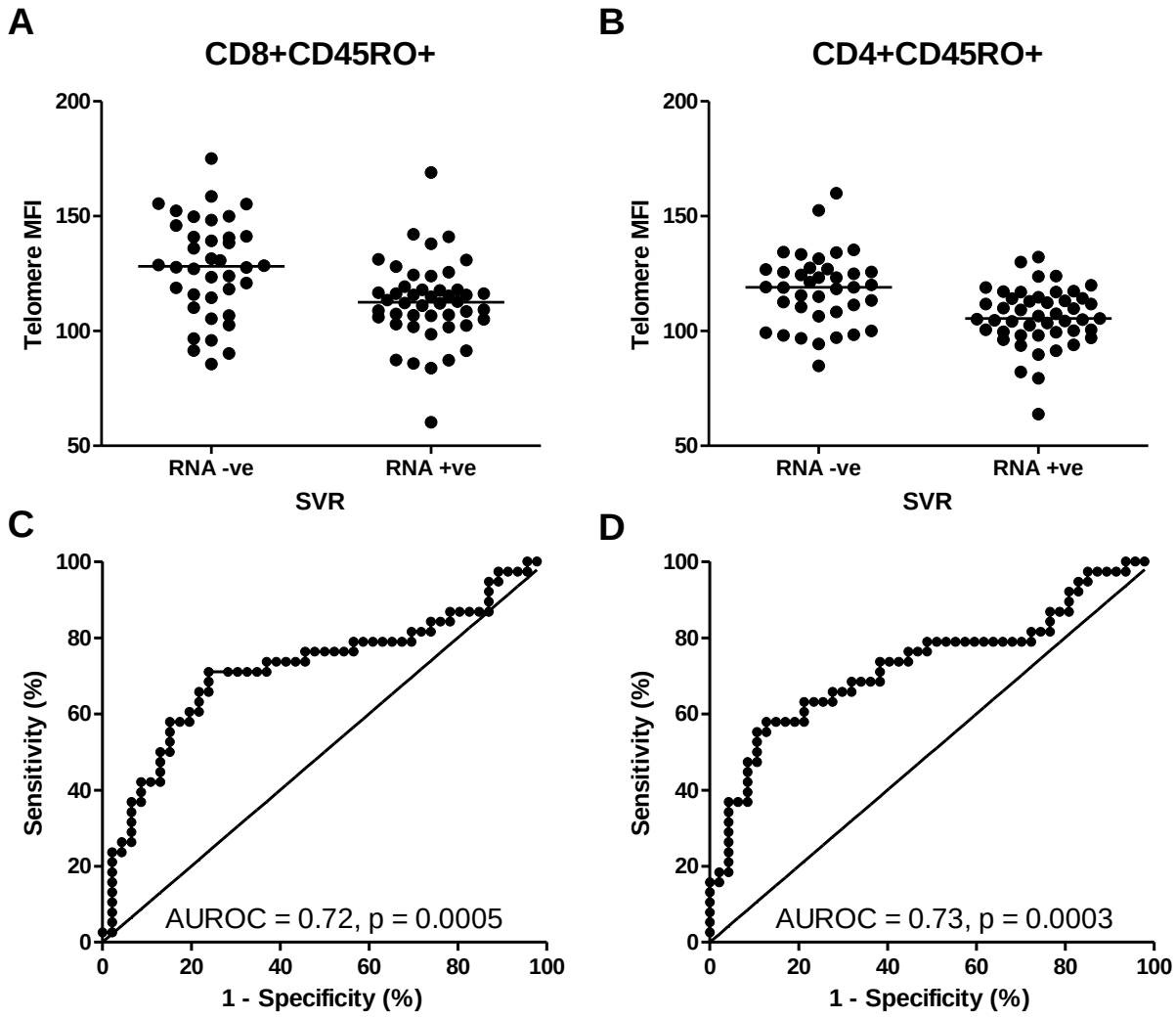

Figure 40. Baseline T-lymphocyte telomere length in (A & C) CD8+CD45RO+ and (B & D) CD4+CD45RO+ subsets in relation to SVR (n = 85) following peg-IFN-α/RBV. ROC curves showing

the sensitivity and specificity of different telomere lengths for prediction of SVR by (C) CD8+CD45RO+ telomere length or (D) CD4+CD45RO+ telomere length.

To investigate whether baseline T-lymphocyte telomere length predicted SVR independent of other factors known to be associated with treatment success, a multi-variate model was constructed. Input variables included age, sex, HCV viral load, HCV genotype, Ishak fibrosis score, body mass index (BMI) and either CD8+CD45RO+ or CD4CD45RO+ telomere length. The outcome measure was SVR.

By logistic regression viral load ($p = 0.02$) and CD8+CD45RO+ telomere length ($p = 0.01$) were associated with SVR on univariate analysis; in this model age ($p = 0.19$), male sex ($p = 0.51$), presence of severe fibrosis ($p = 0.11$), genotype ($p = 0.18$) and BMI ($p = 0.94$) were not associated with SVR. Utilising backwards stepwise regression to remove non-significant variables age ($p = 0.048$), viral load ($p = 0.02$) and CD8+CD45RO+ telomere length ($p = 0.007$) were independently associated with achievement of SVR.

The telomere length of CD4+CD45RO+ lymphocytes ($p = 0.006$) and viral load ($p = 0.02$) were associated with SVR on univariate analysis; in this model age ($p = 0.37$), male sex ($p = 0.48$), presence of severe fibrosis ($p = 0.07$) genotype ($p = 0.19$) and BMI ($p = 0.89$) were not associated with SVR. Utilising backwards stepwise regression to remove non-significant variables viral load ($p = 0.02$) and CD4+CD45RO+ telomere length ($p = 0.001$) were independently associated with achievement of SVR.

For each one point increase in telomere MFI the odds ratio of achieving SVR was 1.05 (95% CI, 1.01 - 1.08) and 1.08 (1.03 - 1.13) in the CD8+CD45RO+ and CD4+CD45RO+ lymphocyte subsets respectively.

To investigate the sensitivity and specificity of different telomere lengths to discriminate between successful and unsuccessful anti-viral therapy, receiver-operator characteristic (ROC) curves were constructed (Figure 40 C, D). The area under the ROC curve for CD8+CD45RO+ telomere length was 0.72 ($p = 0.0005$) and 0.73 ($p = 0.0003$) for CD4+CD45RO+ telomere length. In comparison, viral

load, the only other variable consistently associated with SVR through multiple regression analysis had an area under the ROC curve of 0.70 ($p = 0.001$).

A. Predictors of SVR by logistic regression and backward LR stepwise regression including CD8+CD45RO+ telomere length

	Logistic regression					Backward LR stepwise regression				
Variable	Comparison	Wald	DF	OR (95% CI)	p	Comparison	Wald	DF	OR (95% CI)	p
Age	-	1.76	1	0.95 (0.88, 1.03)	0.19	-	3.92	1	0.94 (0.88, 0.99)	**0.048**
Sex	Male vs. female	0.44	1	1.61 (0.39, 6.67)	0.51					
Fibrosis	Severe vs. Mild	2.55	1	0.23 (0.04, 1.40)	0.11					
Genotype	2/3 vs. 1/4	1.80	1	0.47 (0.16, 1.42)	0.18					
Viral load	-	5.13	1	1.00 (1.00, 1.00)	**0.02**	-	5.35	1	1.00 (1.00, 1.00)	**0.02**
BMI	-	0.005	1	1.00 (0.89, 1.13)	0.94					
CD8+45RO+telomere MFI	-	6.29	1	1.04 (1.01, 1.08)	**0.01**	-	7.20	1	1.05 (1.01, 1.08)	**0.007**

B. Predictors of SVR by logistic regression and backward LR stepwise regression including CD4+CD45RO+ telomere length

	Logistic regression					Backward LR stepwise regression				
Variable	Comparison	Wald	DF	OR (95% CI)	p	Comparison	Wald	DF	OR (95% CI)	p
Age	-	0.79	1	0.96 (0.89, 1.05)	0.37					
Sex	Male vs. female	0.50	1	1.70 (0.39, 7.45)	0.48					
Fibrosis	Severe vs. Mild	3.38	1	0.17 (0.03, 1.13)	0.07					
Genotype	2/3 vs. 1/4	1.75	1	0.47 (0.15, 1.45)	0.19					
Viral load	-	5.86	1	1.00 (1.00, 1.00)	**0.02**	-	5.45	1	1.00 (1.00, 1.00)	**0.02**
BMI	-	0.02	1	0.99 (0.88, 1.12)	0.89					
CD4+45RO+telomere MFI	-	7.51	1	1.07 (1.02, 1.12)	**0.006**	-	11.23	1	1.08 (1.03, 1.13)	**0.001**

Table 8. Predictors of SVR by multiple logistic regression analysis. Input variables were age, gender, fibrosis group (mild (0 – 3), severe (4 – 6)), viral genotype (2 & 3 vs 1 & 4), viral load, BMI and CD8+CD45RO+ lymphocyte telomere length (Panel A) or CD4+CD45RO+ lymphocyte telomere length (Panel B). Non-significant variables were removed by backward stepwise regression.

A single multiple linear regression model was constructed utilising both CD8+CD45RO+ and CD4+CD45RO+ telomere length in addition to age, sex, fibrosis stage, viral genotype, viral load and BMI. By univariate LR viral load (p = 0.02), CD4+CD45RO+ telomere length (p = 0.1) and severe fibrosis (p = 0.08) were associated with SVR; in this model age (p = 0.4), male sex (p = 0.47), genotype (p = 0.17), BMI (p = 0.93) and CD8+CD45RO+ telomere length (p = 0.76) were not associated with SVR.

The only variables significantly and independently associated with SVR were viral load (p = 0.02) and CD4+CD45RO+ telomere length (p = 0.001) (Table 9). In this model CD8+CD45RO+ telomere length was not independently associated with SVR (p = 0.76) and therefore may derive predictive weight in the previous model from its significant association with CD4+CD45RO+ telomere length.

Backward LR stepwise regression					
Variable	**Comparison**	**Wald**	**DF**	**OR (95% CI)**	**p**
Viral load	-	5.58	1	1.00 (1.00, 1.00)	**0.02**
CD4+45RO+ telomere MFI	-	11.28	1	1.08 (1.03, 1.13)	**0.001**

Table 9. Predictors of SVR by multiple logistic regression analysis. Input variables were as for Table 8, but included both CD8+CD45RO+ and CD4+CD45RO+ telomere length. Only multiple logistic regression results are shown.

T-lymphocyte telomere length does not change during IFN-α therapy (Figure 41)

CD8+CD45RO+ and CD4+CD45RO+ telomere lengths were measured at baseline and after 4, 12 and 24 weeks treatment in a subgroup of 16 subjects who failed ultimately to respond to antiviral therapy (remained viraemic) and 18 subjects who achieved SVR to ascertain whether telomere length changed during antiviral therapy. Results were analysed by 2-way ANOVA with duration of treatment and treatment outcome as variables. There was no relation between duration of treatment and telomere length in either CD8+CD45RO+ (p=0.26) or CD4+CD45RO+ lymphocyte subsets (p=0.49).

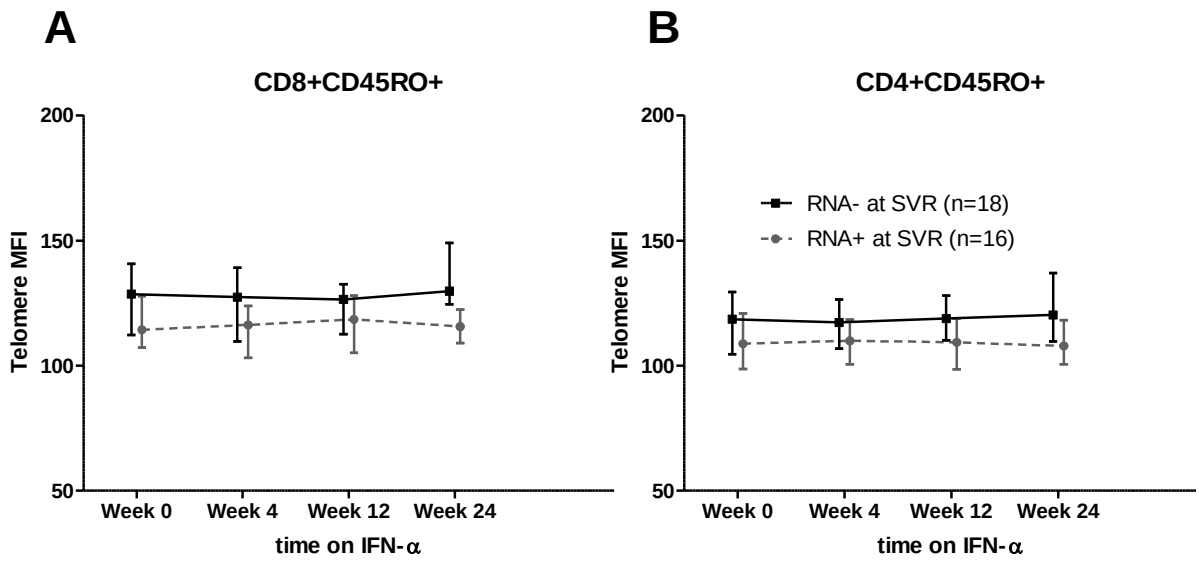

Figure 41. T-lymphocyte telomere length in (A) CD8+CD45RO+ and (B) CD4+CD45RO+ subsets in relation to subsequent RNA status at SVR and time after starting peg-IFN-α/RBV. Telomere length was analysed on 4 occasions (baseline, week 4, week 12 and week 24) only in those cases that remained on therapy. 16 individuals failed treatment and were viraemic 6 months after therapy (dashed grey symbols and bars) while 18 subjects achieved SVR (black symbols and bars). Symbols and whiskers represent the median and interquartile ranges.

Discussion

Prospective study revealed that CD4+CD45RO+ lymphocyte telomere length predicted response to antiviral therapy in chronic HCV infection; through multi-variate analysis, this was found to be independent of other known predictors of SVR. Previous studies have repeatedly demonstrated the importance of age, sex, HCV genotype, BMI and hepatic fibrosis as predictors of response to. antiviral therapy [19, 419, 430]

In chapter three lymphocyte telomere length was associated with a number of clinical, biochemical, and histological features of chronic HCV infection. These data extend these findings and demonstrate that CD4 T-lymphocyte telomere length is not only associated with clinical outcome of chronic HCV infection, but also treatment outcome.

Other groups have demonstrated the importance of age as a prognostic factor in both clinical [142, 144] and treatment-related [419, 420, 427] outcome in HCV infection. Hayashi identified 39 years of age as a critical point whereby older patients had a significantly lower likelihood of responding to IFN-α based therapy for HCV [427]. Elefsiniotis et al investigated the interaction of age with other known predictors of successful treatment outcome [420]. For younger patients viral genotype was the best predictive pre-treatment factor, whereas for subjects older than 55 years, fibrosis stage was more important. They found that with increasing age the factors most useful in identifying treatment outcome changed. However, there have been few studies linking the biology of ageing or accelerated ageing with this effect.

Oligoclonality of the peripheral lymphocyte compartment has been associated with premature ageing of the immune system and poor outcome [410]. Manfras et al utilised Vβ-spectratyping to demonstrate that increased oligoclonality of the peripheral lymphocyte compartment was associated with poor response to IFN-α therapy in chronic HCV [399]. They also demonstrated that

those individuals with large populations of CD8+ lymphocytes bearing the terminally differentiated phenotype of CD28-CD57+ had significantly lower response rates to antiviral therapy [399].

Further studies have demonstrated the importance of lymphocyte exhaustion or senescence in predicting responses to antiviral therapy in chronic HCV. Golden-Mason et al have recently demonstrated that chronic HCV infection is associated with increased programmed-death 1 (PD-1) expression on total peripheral CD8+ and CD4+ T-cells [431]. Further, increased levels of PD-1 expression on HCV-specific CD8+ cells was associated with a failure to achieve SVR in African-Americans [431]. Penna et al have extended these findings to demonstrated that the low proliferative ability and poor cytotoxic potential of HCV-specific CD8+ cells can be reversed through the blockade of the PD-1 / PD-L1 system [80].

Many groups have demonstrated that HCV-specific CD8+ cells have an 'early' phenotype when compared to cells specific for other chronic viral infections [63, 64, 409]. Further, it has been demonstrated that despite this phenotype, the cells display the functional behaviour of exhaustion or senescence depending on the interpretation [35, 64, 80, 422]. Little attention has focused on the effect of chronic HCV infection upon the total lymphocyte compartment. Lucas et al demonstrated that HCV infection had an apparent retarding effect upon the differentiation phenotype of CMV-specific CD8+ cells in HCV [64].

Despite the significance of the association to successful treatment outcome it is unlikely that CD4+ T-lymphocyte telomere length by itself imparts prognostic information for a given individual. Lymphocytic telomere length varies with a number of variables including age, sex, CMV status [285, 357, 386], chronic inflammation or infection [432]. The degree of overlap between those who achieve and do not achieve SVR was large, suggesting that single point values by themselves will not develop into a clinically significant tool. The integration of lymphocyte telomere length into existing panels of fibrosis markers or into algorithms incorporating known prognostic variables would

certainly be more feasible, but would require careful work to establish telomere lengths in local populations.

These results demonstrate that there is no change of global telomere length in subjects treated with antiviral therapy during the first 6 months of therapy. Obviously there may be extensive proliferation within the HCV-specific T-cell population, which would remain undetected. Previous work has demonstrated that IFN-α is a potent inhibitor of telomerase [356, 373], the enzyme which protects telomeres and can lead to their elongation in certain circumstances. On the flip side, IFN- α promotes the survival of T-cells and prevents activation induced cell death [433].

Without intervention, telomere length usually changes very slowly [285]. Therefore, it is perhaps not surprising that global telomere length changes could not be demonstrated. I do not believe that extending the surveillance period would have helped. Previous groups have demonstrated that IFN-α therapy leads to an up-regulation of T-cell reactivity and proliferation within the first 8 weeks of therapy [434]. Barnes et al demonstrated that increased HCV-specific T-cell proliferative responses were only sustained whilst the patient was on IFN-α therapy and were not sustained after cessation [408].

It was important to establish whether lymphocyte telomere lengths changed commensurate with a change in HCV RNA status. Earlier data (see chapter three) demonstrated that individuals who were HCV RNA negative spontaneously had very short CD8+ T cell telomeres. Patients rendered HCV RNA negative with antiviral therapy did not exhibit shortening of CD8+ T cell telomeres, suggesting that the previously demonstrated telomere length changes in the non-viraemic cohort may not have occurred at the time of viral clearance.

Chapter 5 - Histological changes in HCV antibody positive, HCV RNA negative subjects suggest persistent virus infection.

Abstract

Background: It is unclear whether HCV has been eradicated or persists at low level in HCV-antibody positive HCV-RNA negative individuals; the natural history and liver histology are not well characterised.

Methods: 172 HCV-antibody positive, serum HCV-RNA negative patients underwent diagnostic liver biopsy between 1992 and 2000 and were followed a median 7-years (range 5-12). Patients with any possible cause of liver injury other than HCV were excluded. A single histopathologist scored sections using Ishak criteria. Characterisation of the inflammatory infiltrate in selected cases used a novel semi-quantitative technique and compared with HCV-RNA positive patients and healthy controls.

Results: 102 patients were excluded because of a risk factor for liver injury other than HCV. 70 patients met study criteria; 4 (5.7%) became HCV-RNA positive during follow-up. 66 cases remained HCV-RNA negative; 5 (7.5%) had a normal liver biopsy; 54 (82%) had fibrosis (stage 2 or 3 in 16 (24%)). Non-viraemic cases revealed expanded portal tracts ($p < 0.05$), with fewer CD4+ ($p < 0.05$) and more CD8+ cells ($p < 0.05$) than healthy controls, but were indistinguishable from HCV-RNA positive cases for these parameters. Lobular CD4 staining, absent in healthy controls, was noted in both HCV-RNA negative and positive cases and was more marked in the latter ($p < 0.05$) with a sinusoidal lining cell distribution.

Conclusions: Non-viraemic HCV-antibody positive patients have a liver biopsy that is usually abnormal. Fibrosis was present in most with similar inflammatory infiltrate to viraemic cases. The

presence of a CD8+ rich inflammatory infiltrate suggests an ongoing immune response in the liver, supporting the view that HCV may persist in the liver in the majority of HCV-RNA negative cases.

Introduction

Hepatitis C virus (HCV) infection has a prevalence of 0.5% to 2% in Western countries, with sustained viraemia in 50 - 90% of exposed individuals [132]. Between 5 and 20% of those with viraemia develop cirrhosis eventually [134, 139] and are then at risk of chronic hepatic failure and hepatocellular carcinoma (HCC). The gold-standard for investigation of HCV-related disease remains liver biopsy. Sequential liver biopsies demonstrate progressive liver fibrosis in more than 50% of subjects with chronic viraemia [134, 177, 435].

A number of studies describe the association of strong peripheral T-cell responses with resolution of viraemia immediately after acute HCV infection [25, 113, 423], which contrasts with the weak, narrow T-cell response in viraemic HCV carriers [24, 436]. There have been fewer studies of the intrahepatic lymphocyte compartment in individuals long after spontaneous resolution of viraemia. There has been resurgent interest in this particular group following the demonstration of intrahepatic negative strand HCV-RNA, suggesting continued viral replication [55], leading to the suggestion that such patients have occult or alternatively, low level HCV replication [56], but the effect of immune responses on viral turnover is uncertain.

The natural history of HCV infected patients without viraemia is believed to be excellent but is less well characterised and histological abnormalities have been described in only a limited number of studies [52]. A proportion of non-viraemic HCV subjects continue to be identified in screening programmes, but at present their optimal management remains undefined. Until 2000 the practice in this centre was to offer full clinical assessment including liver biopsy, due to uncertainty of the natural history of non-viraemic subjects.

In this series the liver biopsy features in a cohort of HCV antibody positive, HCV-RNA negative patients followed in a single centre for at least 5 years are described. Other causes of liver injury had been excluded carefully and the recognition that hepatic inflammation was a common feature in such patients led to further study to characterise the infiltrate in a subset of cases. Using immunohistochemistry I compared the inflammatory infiltrate in a subset of HCV antibody positive, viraemic and non-viraemic subjects and healthy controls.

Patients and methods

A retrospective analysis of patients known to remain HCV antibody positive but persistently HCV-RNA negative (non-viraemic) that had undergone percutaneous liver biopsy in our centre between July 1992 and December 2000. During this period all patients who were anti-HCV antibody positive were offered liver biopsy irrespective of RNA status.

Case inclusion was defined strictly to ensure that exposure to HCV was the only recognised cause of liver injury. All were HCV RNA negative at presentation, and none had undergone therapy with interferon. Patients that consumed more than the recommended amount of alcohol per week (>21U per week in men and >14U per week in females) were excluded. Patients infected with Human Immunodeficiency virus (HIV) or Hepatitis B virus (HBV) and those with other recognised causes of chronic liver disease identified on blood tests or liver biopsy were also excluded. Thus, all had a body mass index < 30 without risk factors for insulin resistance; were negative for anti-mitochondrial, anti-nuclear and anti–smooth muscle antibodies with normal serum immunoglobulins; had no evidence of iron overload; had normal serum α1-antitrypsin, copper and caeruloplasmin levels. Patients were analysed according to age, sex and risk factors for acquisition.

All study patients were followed for a minimum of 5 years (median 7 years, range up to 12) with annual clinical assessment supported by laboratory tests including liver function tests, HCV antibody and HCV-RNA.

The study was carried out with the approval of the Local Research Ethics Committee.

PCR for HCV-RNA

Prior to 2003, a nested blocked based reverse transcriptase-PCR assay was used to detect HCV-RNA. After 2003, HCV-RNA was sought using a real-time taqman PCR assay, targeting the conserved 5' non-coding region of the HCV genome and carried out on a Rotor-gene™ 3000 instrument (Corbett Lifescience, Sydney, Australia). Through previous internal audit at the Department of Virology the detection limit of the nested reverse transcriptase PCR assay was not significantly different to the later real-time assay (data not shown). Patients were only included in this study if a minimum of 5 (up to 12) separate tests at 12 month intervals had failed to detect HCV-RNA.

Routine liver histology

To further characterise the inflammation that was demonstrated at routine histology, the inflammatory infiltrate was investigated by immunohistochemistry in a subgroup of cases. A group of 12 non-viraemic patients selected randomly from the original cohort with portal or lobular inflammation between Ishak 1 and 3, was compared with a group of 13 viraemic patients and 18 controls. Liver tissue from viraemic HCV subjects (n = 13) was matched carefully for age, fibrosis stage and inflammation grade with the non-viraemic patients; these patients also met the strict entry criteria for the study group, except for the presence of HCV-RNA in serum and served as a comparison group. The age and biopsy features of the 2 groups were the same except for increased interface activity in the viraemic cohort (Table 11).

Eighteen liver biopsy specimens assessed as within normal histological limits by a specialist liver histopathologist (Dr Susan Davies, Department of Histopathology, Addenbrooke's Hospital, Cambridge) served as controls. In particular there was no increase in the portal cell infiltrate. The clinical indication for liver biopsy in that group was investigation of asymptomatic abnormal liver enzymes. All were negative for HCV antibody; negative for hepatitis B virus surface antigen; had a

body mass index < 30 without risk factors for insulin resistance; were negative for anti-mitochondrial, anti-nuclear and anti–smooth muscle antibodies with normal serum immunoglobulins; had no evidence of iron overload; had normal serum α1-antitrypsin, copper and caeruloplasmin levels.

Statistics

Immunohistochemistry results were analysed using Prism 5.0 for Windows (Graphpad, San Diego, CA). Multiple groups were analysed with the Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. Biopsy Ishak scores were analysed with Mann-Whitney U test. A p value < 0.05 was regarded as significant.

Results

Patients

172 subjects positive for HCV antibody but without HCV-RNA in serum (by PCR) underwent liver biopsy in our centre between 1992 and 2000. 102 subjects were excluded from the study because of evidence of a further risk factor for liver injury other than HCV exposure. Current or previous excessive alcohol intake, risk factors for insulin resistance and concomitant liver disease, including steatohepatitis, accounted for the majority of those excluded. The remaining 70 subjects were followed for a median of 7 years (range: 5 - 12). All subjects retained anti-HCV antibody. However, during prolonged follow-up, 9 of the 70 cases became HCV-RNA positive. In 5 the result was positive on only one occasion and was determined subsequently to be a false positive reaction. However, 4 (5.7%) patients were confirmed repeatedly to be HCV-RNA positive and were excluded from further analysis. Detection of HCV-RNA in these 4 cases was not associated with intercurrent illness, immune suppression, or further exposure to HCV as far as could be determined and may reflect either *de novo* infection or reactivation of previously quiescent HCV infection.

Thus, HCV exposure remained the only identified risk factor for liver injury in the remaining 66 subjects, in accordance with the strict study criteria. Demographic details are described in Table 10. Seven (10.6%) acquired HCV through contaminated blood products, 46 (69.7%) through injecting drug use (IDU) and in the remaining 13 (19.7%) the source of HCV infection was undetermined. 10 subjects (15.2%) had ALT levels that were elevated at some time during the study period, but all other laboratory parameters including alkaline phosphatase, gamma GT, bilirubin and platelet counts were within the normal range consistently in all patients.

Non-viraemic, HCV antibody positive (n = 66)	Mean ± SD	Range
Age (years)	37.6 ± 8.6	21.2 - 65.7
Male: Female (%)	41:25 (62%:38%)	
Follow-up (years)	7	5 - 12
Number of HCV-RNA assays per subject	7	5 - 12
ALT median (normal range) IU/L	31 (< 40)	IQR: 22.25 – 38.75 range 8 - 213
Lobular activity (0 - 4)	0.82 ± 0.65	0 - 2
Portal activity (0 - 4)	0.66 ± 0.60	0 - 2
Fibrosis (0 - 6)	1.1 ± 0.73	0 - 3
Interface activity (0 - 4)	0.11 ± 0.30	0 - 1
Confluent necrosis (0 - 6)	0.05 ± 0.20	0 - 1
Steatosis (0 - 3)	0.27 ± 0.57	0 - 3

Table 10. The demographic characteristics of 66 non-viraemic, HCV antibody positive subjects. IQR: interquartile range.

Hepatic fibrosis and inflammation (Figure 42 and Figure 43)

Only 5 of 66 (7.5%) patients had a normal liver biopsy; 54 of 66 (81.8%) patients had fibrosis.

Stage 0 fibrosis was present in 12 of 66 patients studied (18.2%); these included 4 (33.3%) with grade 1 portal tract inflammation and 5 (41.6%) with grade 1 lobular inflammation.

Stage 1 fibrosis was present in 38 patients (57.6%); 63.2% and 7.9% had grade 1 or 2 portal tract inflammation respectively; 60.5% and 13.2% had grade 1 or 2 had lobular inflammation respectively; 13.2% had grade 1 interface hepatitis.

Stage 2 or 3 fibrosis was present in 16 patients (24.24%); 93.7% had portal tract inflammation scored as grade 1 and 2 in 75% and 18.7% respectively; 81.3% had lobular inflammation scored as grade 1 and 2 in 56.3% and 25% respectively; 18.8% had grade 1 interface hepatitis.

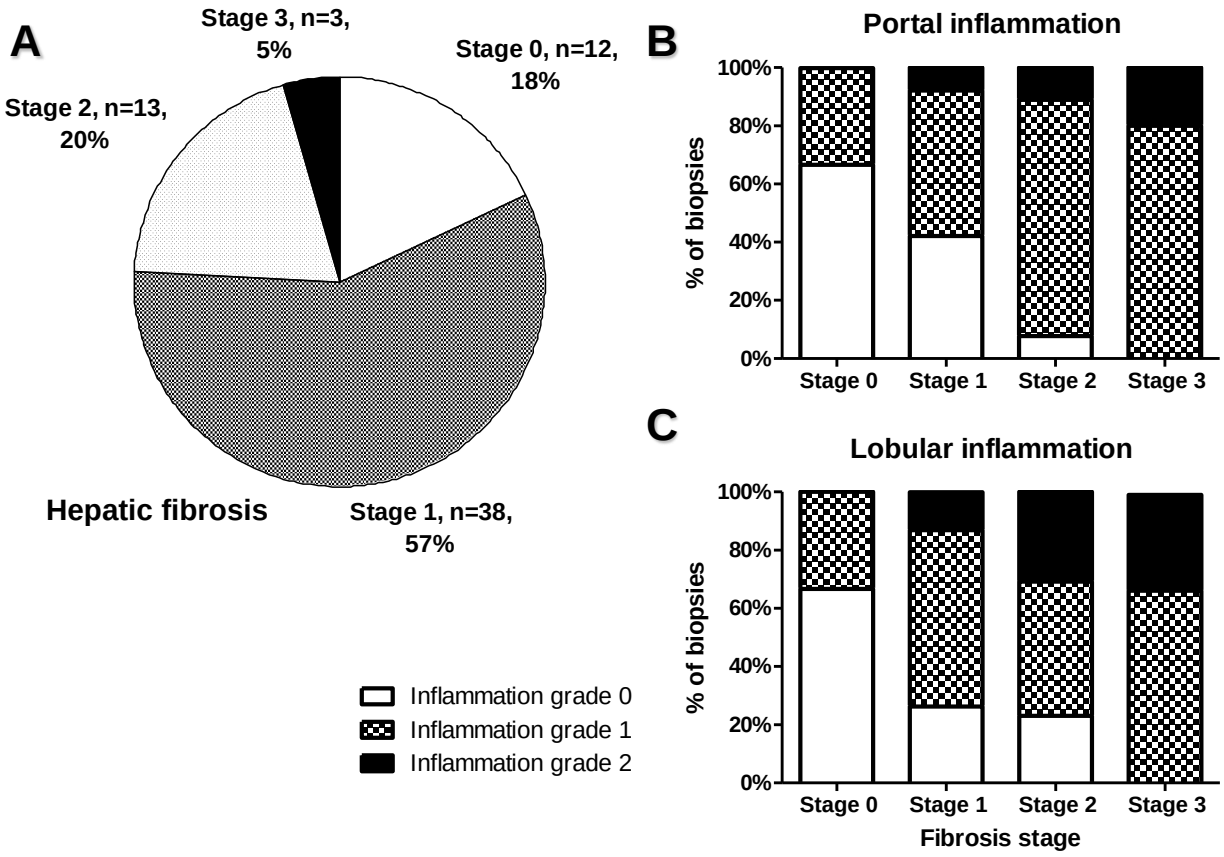

Figure 42. Fibrosis stage and inflammation grade in HCV antibody positive, HCV-RNA negative subjects (n = 66). Panel A: pie chart representation of fibrosis stage by modified Ishak criteria (0 - 6). Panel B: Portal tract and panel C: lobular inflammation according to stage of fibrosis.

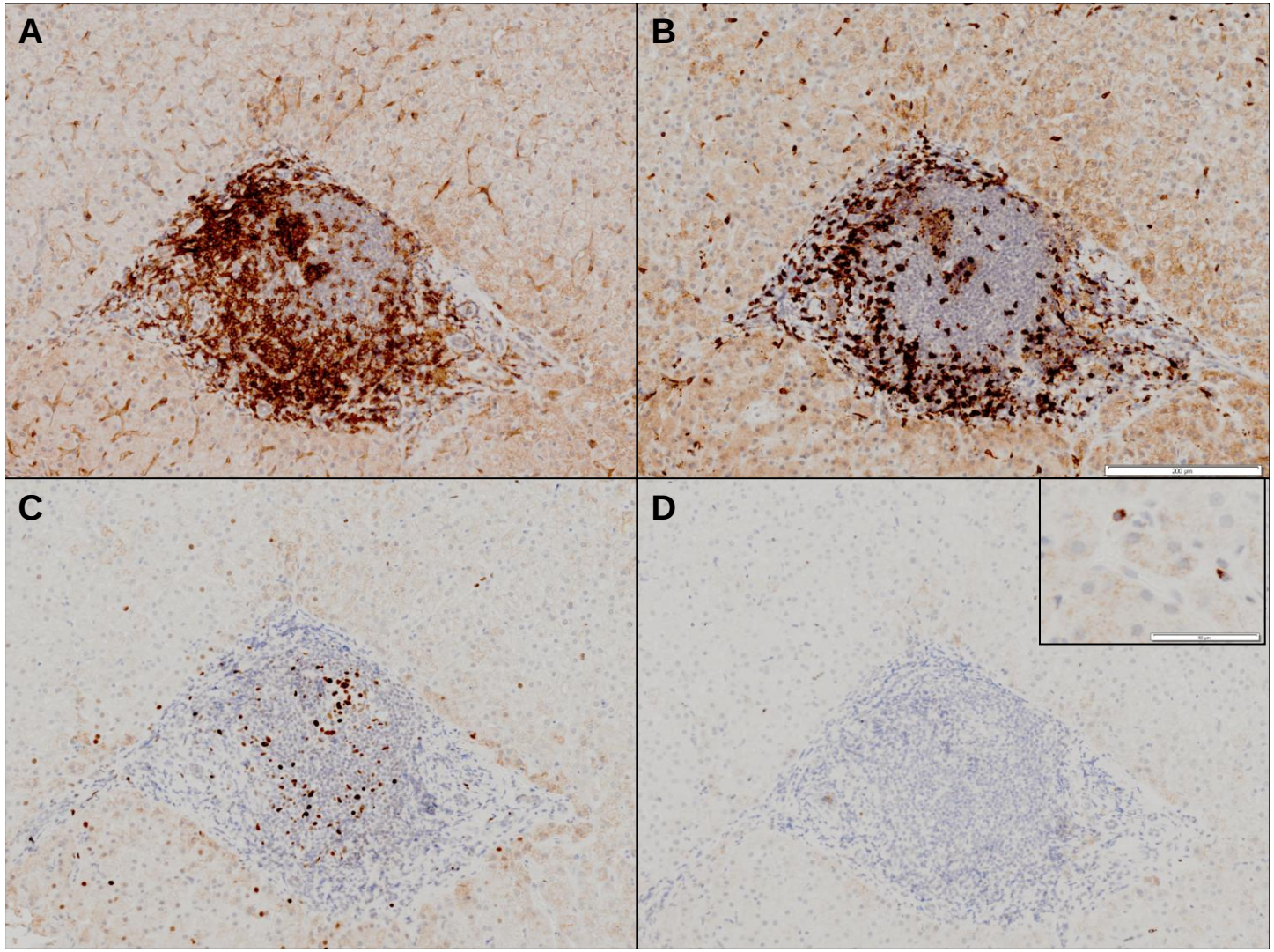
A
B
C
D

Figure 43. Immunohistochemistry of the portal inflammatory infiltrate from a subject with anti-HCV antibody but negative for HCV-RNA. Formalin-fixed, paraffin-embedded tissue was stained for CD4 (A), CD8 (B), Mcm-2 (C) and perforin (D and inset). Scale bars included in C (200µm) and inset (50µm). Portal tracts are rich in CD3 positive cells (not shown), which are more often CD4 positive than CD8 positive. These cells express Mcm-2 and perforin rarely.

Bile duct damage and steatohepatitis

Neither bile duct damage nor steatohepatitis were observed. Confluent necrosis was present in 3 biopsies (4%), never exceeding grade 1 (0 - 6). There was no histological evidence of covert alcohol consumption, consistent with the strict definition of the study group.

The inflammatory infiltrate

The inflammatory infiltrate was investigated further by immunohistochemistry in 12 non-viraemic subjects and compared with 2 control groups (see above): liver tissue from 13 viraemic HCV subjects matched with the non-viraemic HCV antibody positive group and 18 healthy controls. The demographic and liver biopsy characteristics of the groups are detailed in Table 11.

	RNA + (n = 13)	RNA - (n = 12)	Healthy controls (n = 18)	Statistic	p value
Age (years ± SD)	35.59 ± 11.75	38.83 ± 7.99	48.46 ± 15.70	Kruskal-Wallis	0.06
Lobular activity (0 - 4)	2.08 ± 0.29	1.75 ± 0.62	-	Mann-Whitney U test	0.11
Portal activity (0 - 4)	2.00 ± 0.60	1.83 ± 0.58	-	"	0.49
Fibrosis (0 - 6)	1.75 ± 0.75	1.92 ± 1.24	-	"	0.69
Interface hepatitis (0 - 4)	1.50 ± 0.5	0.83 ± 0.72	-	"	**0.03**
Steatosis (0 - 3)	1.17 ± 0.94	0.58 ± 0.79	-	"	0.11

Table 11. Demographic characteristics of subjects studied by immunohistochemistry: HCV antibody and HCV-RNA positive (n = 13); HCV antibody and HCV-RNA negative (n = 12) & healthy controls (n = 18). Ages were compared with Kruskal-Wallis, while Ishak scores were compared using the Mann-Whitney U test.

Portal tracts of non-viraemic HCV subjects have a CD8+ rich infiltrate

The area of the portal tract was expanded in both groups with HCV infection when compared to healthy controls ($p < 0.05$; data not shown). There was no difference in the portal tract area between the two groups of HCV exposed patients (whether positive or negative for HCV-RNA in serum) who had been matched (intentionally) for inflammation grade.

There were no significant differences between subjects and either control group regarding the area of the portal tract that expressed CD3 (Figure 44a).

The area of the portal tract that expressed CD4 was lower in viraemic subjects with HCV when compared to healthy control subjects ($p < 0.05$, Figure 44b), but similar in both HCV exposed groups.

The portal tract area that expressed CD8 was increased significantly in both viraemic and non-viraemic HCV subjects when compared to healthy controls ($p < 0.05$ and $p < 0.0001$ respectively, Figure 44c) but similar in both HCV exposed groups. However, the number of perforin positive cells/mm^2 portal tract was similar in the three groups ($p = 0.075$, Figure 44d).

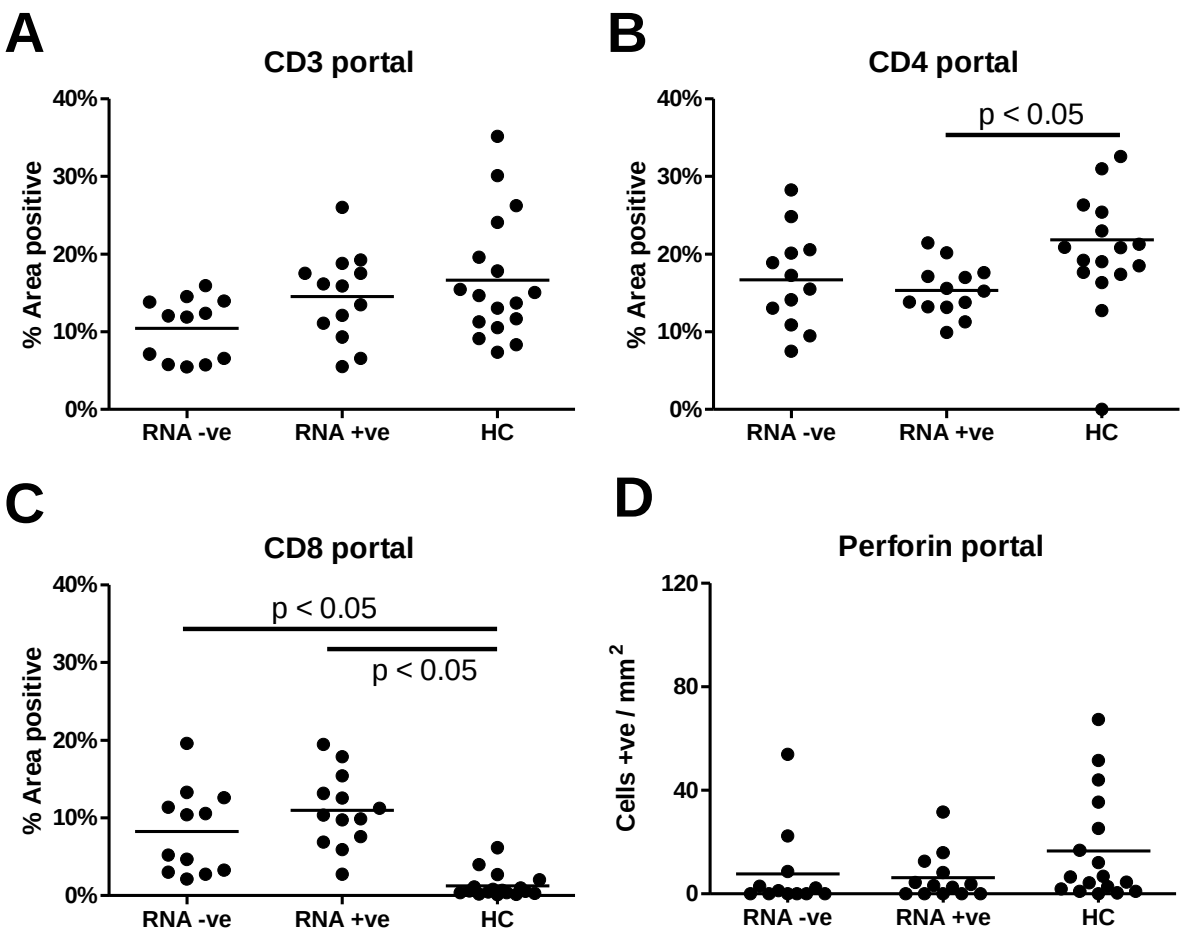

Figure 44. Characteristics of the portal tract infiltrate in subjects with non-viraemic HCV (n = 12), viraemic HCV (n = 13) and healthy controls (HC) (n = 18) stained for: A: CD3; B: CD4; C: CD8; D: perforin. Results were analysed with Kruskal-Wallis test with Dunn's multiple comparison test.

Reduced lobular CD3 and perforin expression in non-viraemic HCV subjects compared to healthy controls

The proportion of the lobular area positive for CD3 in both viraemic and non-viraemic subjects was reduced compared to healthy control subjects ($p = 0.0132$, Figure 45a), but similar in both HCV exposed groups.

There was an increase in the lobular area that expressed CD4 in viraemic subjects (median %, IQR) (2.13, 1.49 - 4.49) compared to both non-viraemic subjects (0.68%, 0.22 - 1.16, $p < 0.05$) and healthy controls (0.32%, 0.10 - 1.13, $p < 0.05$, Figure 45b). However, review of the staining pattern for CD4 in liver tissue revealed that most of the signal localised to sinusoidal lining cells, with the effect most marked in those with viraemia (Figure 46a); CD4 expression had a similar pattern but was less marked in non-viraemic cases (Figure 46b) and was rare in healthy controls. CD4 lymphocytes were detected rarely in both study groups and when identified were sinusoidal.

There were no differences between the 3 study groups in terms of the lobular area that expressed CD8 ($p = 0.477$, Figure 45c). However, perforin expression was reduced in both viraemic and non-viraemic HCV subjects compared to healthy controls, but similar in both HCV exposed groups ($p = 0.0314$, Figure 45d).

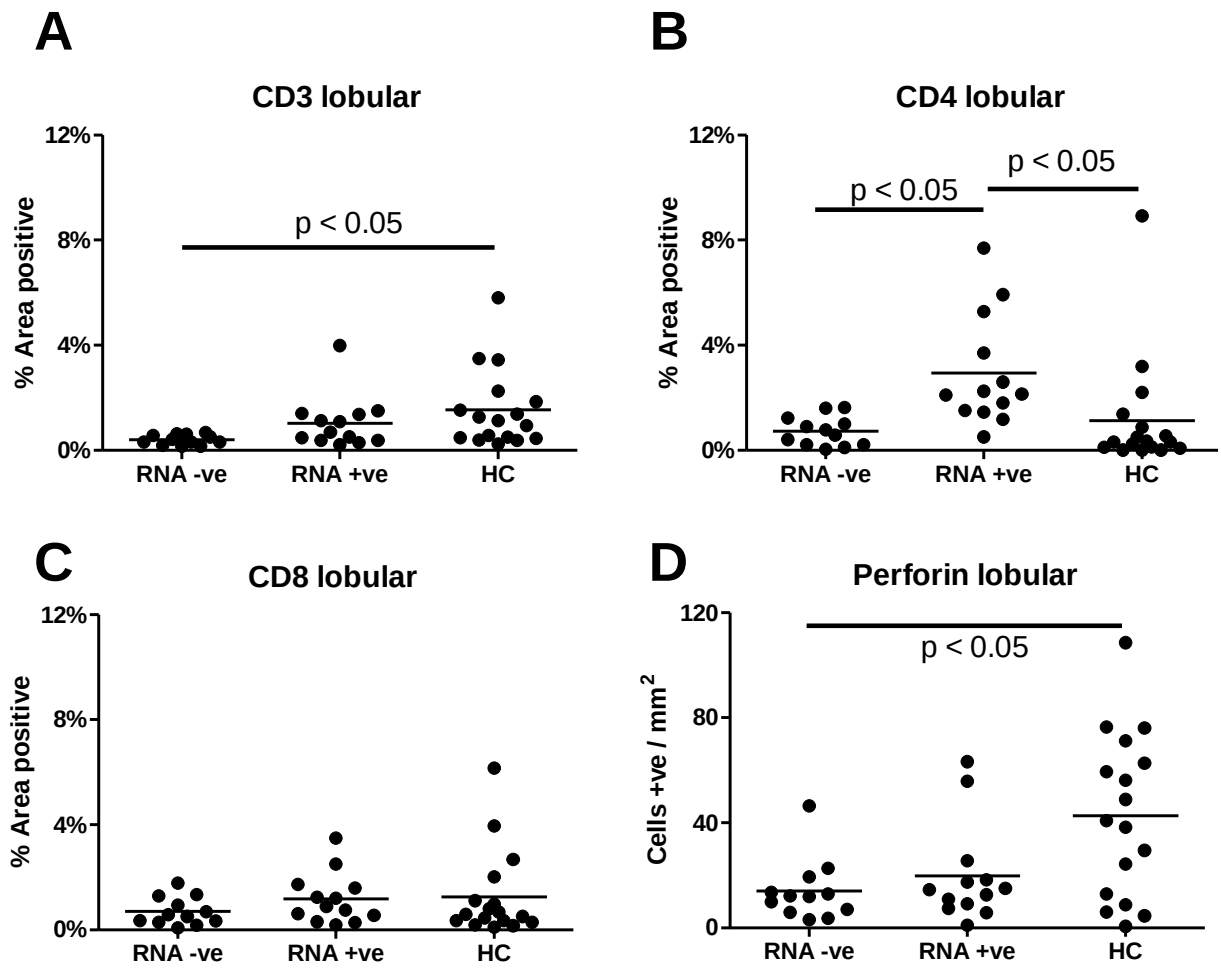

Figure 45. Characteristics of the lobular infiltrate in subjects with non-viraemic HCV (n = 12), viraemic HCV (n = 13) and healthy controls (HC) (n = 18). Biopsies were stained for: A: CD3; B: CD4; C: CD8; D: perforin. Results were analysed with Kruskal-Wallis test with Dunn's multiple comparison test.

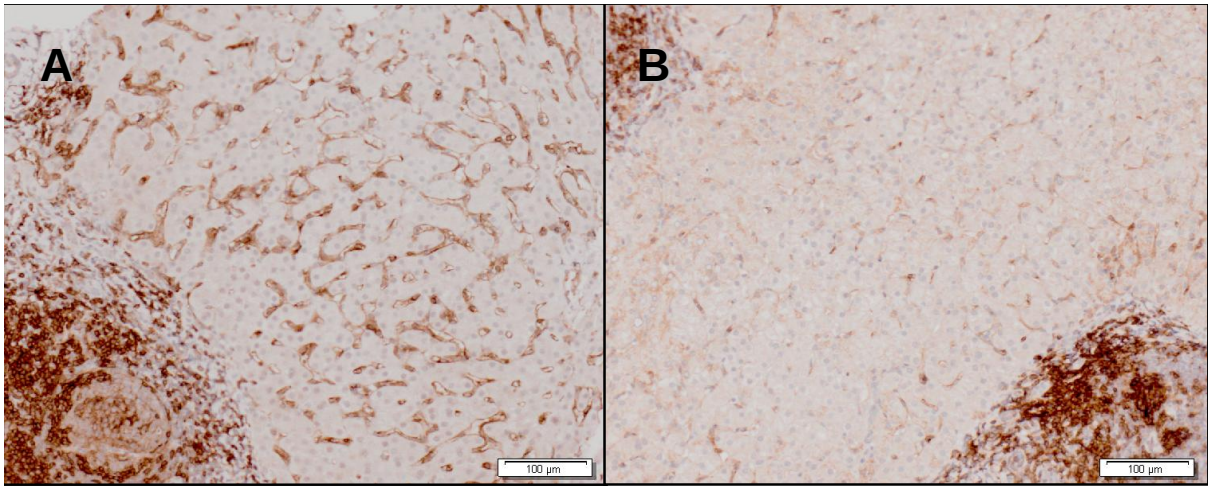

Figure 46. CD4 expression in liver. (A): a subject with viraemia and (B): a non-viraemic subject. There is strong sinusoidal lining cell CD4 expression in the viraemic patient. There is strong

lymphocytic expression in the portal tracts in both the viraemic and non-viraemic patients. Scale bars (100µm).

Portal tract lymphocytes in HCV infection are Mcm-2 negative independent of viraemia

Portal tract cells in both viraemic and non-viraemic HCV subjects had minimal expression of mcm-2 (Figure 43c); expression in both groups was reduced significantly compared to healthy controls ($p = 0.0004$, Figure 47a).

Increased lobular expression of Mcm-2 in viraemic and non-viraemic HCV subjects

Non-viraemic subjects had significantly greater expression of Mcm-2 within lobular areas as compared to healthy control subjects ($p = 0.0005$, Figure 47b). This was almost exclusively confined to hepatocytes and infiltrating inflammatory cells were always negative. There were no differences between the hepatocyte expression of Mcm-2 between viraemic and non-viraemic HCV positive subjects, as described previously [344].

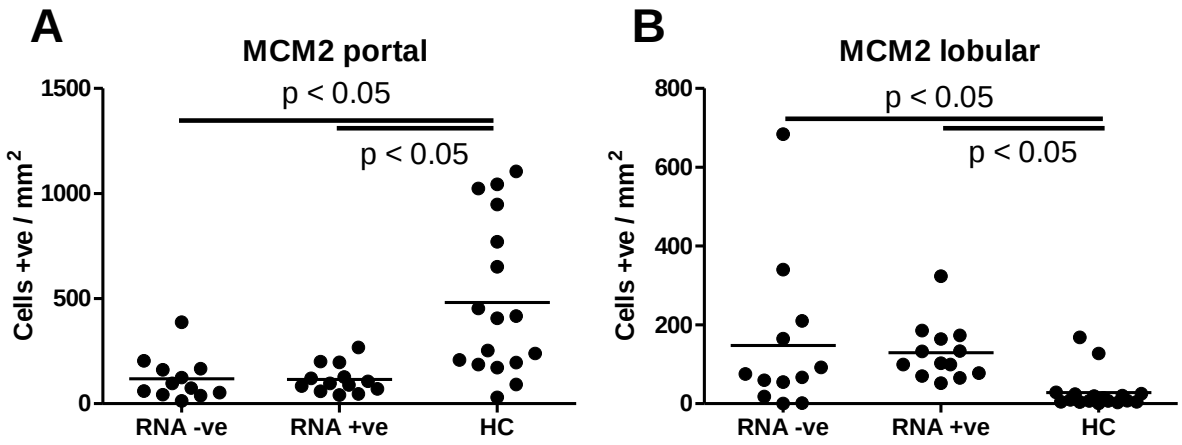

Figure 47. Proliferative activity of the inflammatory infiltrate by MCM-2 expression. (A): Mcm-2 expression within portal tracts and (B): Mcm-2 expression within hepatic lobules in non-viraemic subjects (n = 12), viraemic HCV (n = 13) and healthy controls (n = 18). Results were analysed with Kruskal-Wallis test with Dunn's multiple comparison test.

Discussion

HCV infection leads to chronic viraemia in the majority of individuals exposed to HCV; the natural history in this group, the risk factors for progressive injury and the benefits of antiviral therapy are

well established. However, the clinical status of the minority without viraemia after exposure to HCV is less clear. It is uncertain whether this group has resolved infection, with or without long term immunity and protection from further exposure to HCV or alternatively, low level viral replication, where HCV-RNA can only be detected within the liver [54, 55]. Neither the natural history nor the liver histology in this cohort has been described previously in detail.

For this study a cohort of HCV-exposed subjects without viraemia at presentation was followed for a median of 7 years, many of whom were identified at a time when there was uncertainty regarding the significance of a failure to detect HCV-RNA at first assessment. With the aid of liver biopsy in all of these patients and critically, careful subsequent exclusion of all cases with a possible alternative cause of chronic liver disease, the data presented here challenge the view that non-viraemic HCV exposed subjects have resolved infection. First, viraemia was detected eventually in 5.7% of this group, a proportion which may increase with time; second, just 7.5% cases had normal histology; third, 92% of cases had inflammation within the liver, while 82% had fibrosis, which in about a quarter would have been sufficient to prompt consideration of antiviral therapy if the patients had been viraemic; finally, when cases without viraemia were compared with viraemic patients matched for grade of inflammation and stage of fibrosis, the phenotype of the inflammatory infiltrate was similar and distinct from that in healthy controls.

These data are consistent with the hypothesis that non-viraemic patients exposed to HCV have chronic low level, probably hepatic, viral replication that is associated with a lower risk of progressive liver injury compared with viraemic patients [53]. Other possibilities to explain the histological abnormalities exist and could include as yet unknown viral infections or NAFLD without histological features of steatohepatitis.

Serum from 80 HCV-RNA negative patients was subjected to ultracentrifugation before repeating the assay for HCV-RNA; HCV-RNA was still not detected (Rolfe K and Curran MD, personal

communication). The findings are thus consistent with several studies which described the detection of HCV-RNA in liver tissue in non-viraemic HCV-exposed individuals [54, 55, 425, 437]. This view would also be consistent with a failure to date to demonstrate sterilising immunity against HCV in man or primates [438] and it is possible that HCV is a lifelong infection in many more cases than has been supposed hitherto. Perhaps the most important question to address in this cohort is why such cases have lower levels of viral replication. The long-term histology in those treated successfully with pegylated interferon-α and ribavirin will be of interest in this context, since loss of the inflammatory infiltrate would be consistent with eradication of HCV, while ongoing inflammation, as in this series, would be indicative of low level HCV replication.

Inflammation in the liver is a sensitive indication of hepatic disorder, but indirect evidence of infection. The best evidence of infection in non-viraemic HCV exposed subjects would be the demonstration of HCV genomic material and replicative intermediates in the liver of such cases. Both positive and negative strand HCV-RNA have been identified in liver tissue of non-viraemic HCV subjects with normal ALT values [55]; that study also demonstrated that HCV-RNA was present in serum after ultracentrifugation [425]. This suggests non-viraemic HCV subjects are defined by insensitive tests. In a series of patients from the same authors with HCV-RNA present in liver but without viraemia, 15% had fibrosis, including 4% with cirrhosis [52]. This contrasts with 82% with some degree of fibrosis in my series, a difference which may be explained by the longer duration of follow-up in this series compared to that of the Spanish group [55].

Immunohistochemical analysis was revealing. There were consistent differences between HCV exposed cases (irrespective of viraemic status) and healthy controls; in contrast no differences were detected between viraemic and non-viraemic HCV exposed subjects matched for inflammation for any other parameter. Thus the portal tracts were expanded with non-proliferating (Mcm-2 negative) T-cells, enriched with CD8+ T-cells and depleted of CD4+ T-cells in HCV exposed cases relative to healthy controls. However, the proportion of cells expressing perforin, a marker of cytotoxic

potential, was low and similar in all three groups [73]. The lobular infiltrate was CD3+ T-cell depleted and perforin negative in both HCV exposed groups relative to healthy controls.

Mcm-2 expression, a marker of cell cycle entry, was increased in hepatocytes in both HCV study groups. A previous study indicated that hepatocytes in HCV exposed patients had evidence of cell cycle entry without cell cycle progression - a state of cell cycle arrest - which correlated with fibrosis stage [344]. Many viruses replicate more efficiently in cell cycle arrested host cells [439] and Mcm-2 positive hepatocytes may be either HCV infected or regenerating in response to ongoing liver injury. In either case, the finding is indicative of an on-going liver insult in both viraemic and non-viraemic HCV exposed groups.

Stringent selection of non-viraemic HCV exposed subjects with HCV as the only risk factor for liver injury revealed abnormal liver histology in almost all cases. How should such cases be managed? For now it might be wise at least to continue to follow such cases to determine whether HCV-RNA will be detected eventually and to determine the natural history in this cohort. In the future, testing for HCV in serum or tissue may improve and the proportion of HCV-RNA negative subjects may fall. Intervention with antiviral therapy cannot be justified currently based on our knowledge of the natural history; however, it will be intriguing to determine the late histology in HCV-RNA positive cases treated successfully to see whether these revert to normal histology or something more akin to the findings in the non-viraemic group in this series. However, a possible role for HCV in non-viraemic subjects with a second risk factor for liver injury does need to be addressed and it is possible that the threshold for investigating such cases more thoroughly, will be reduced.

Whether this group is analogous to subjects with occult HBV infection [440] who can experience reactivation of viral replication in the face of profound immunosuppression [441] is not known. Previous studies comparing rates of HBV and HCV reactivation suggest that it is much less common with HCV and indeed may not occur; in a study of 305 patients receiving corticosteroid containing

chemotherapy for haematological malignancy, there were 9 reactivations of HBV infection but no reactivation of HCV viraemia despite a four-fold higher prevalence of non-viraemic HCV than HBV [442]. A more analogous situation may be the outcome of antiviral therapy, where a small proportion of individuals eventually become HCV-RNA positive despite sustained virological response (SVR). In one study of individuals who achieved SVR after previously failing an initial course of anti-viral therapy, the viral recurrence rate after SVR was 11.3% [443]. Further, viral RNA can also be detected in peripheral blood lymphocytes and macrophages from those individuals who have successfully achieved SVR [428].

An unexpected, but consistent, observation was that CD4 expression in the lobule was prominent in sinusoidal lining cells in HCV exposed individuals. The pattern was most consistent with endothelial expression and expression was most marked in viraemic patients. The majority of CD4 staining was sinusoidal, which caused difficulty with the semi-automated count of lymphocytes that were therefore assessed by more conventional means. The significance of sinusoidal lining cell CD4 expression will be pursued in a separate study. CD4 staining has been demonstrated in both glomerular and brain endothelial tissue in HIV-1 infection [444, 445]. In the latter study, brain endothelial cells expressed both CD4 and chemokine receptors, suggesting a permissive role in HIV infection [446].

To extend this current study, other approaches could have been adopted. Through utilisation of modern viral genome sequencing techniques on those patients who experienced viral reactivation, demonstration that the reappearing viral genotype and subtype was similar to their previous serotype, confirming reactivation rather than re-infection would be feasible. However, this was a retrospective study and many of these viral relapses occurred over 10 years ago; serum from these episodes was not stored. Further criticism could be levelled that PCR identification of positive and negative strand viral RNA from the liver tissue was not attempted. However, isolation of sufficient RNA from formalin-fixed, paraffin-embedded tissue required between eight and ten sections

(personal communication, W. Gelson) cutting. Most of the biopsy specimens had insufficient tissue to make a non-random patient selection possible.

In summary, a cohort of individuals with no risk factor for liver injury other than previous HCV exposure was identified. These subjects with non-viraemic HCV have a CD8+ rich hepatic inflammatory infiltrate and the great majority had evidence of hepatic fibrosis.

Chapter 6 - Downstream telomere signalling and response to interferon-α in CD8+ T-lymphocytes.

Abstract

Background: Age is a critical prognostic factor in the natural history and treatment outcome in hepatitis C virus (HCV) infection. Global lymphocyte telomere length declines with age. I have previously demonstrated that progressive HCV is associated with shortened CD8+ telomere length. Down-stream signalling from short telomeres occurs via the double strand break (DSB) DNA damage response (DDR) pathway. I investigated the form and function of CD8+ lymphocytes with DDR in HCV infection.

Methods: Peripheral CD8+ Lymphocytes with DSB were identified by expression of γ-H2AX (ser-139) and telomere length was determined by flow-FISH assay from healthy controls (n=27); HCV-exposed, RNA- (n=27), HCV RNA+/mild fibrosis (n=59); HCV RNA+/severe fibrosis (n=48). The in vitro response of lymphocytes with DSB to IFN-α was determined by phosho-stat1 (Y701) expression.

Results: CD8+ γ-H2AX expression was significantly higher in subjects with severe liver disease ((median; IQR) 2.8%, 1.5% - 4.6%) than healthy controls (1.8%, 0.98% - 2.4%) or subjects with mild liver disease (1.4%, 0.8% - 2.4%) ($p = 0.0023$). There was a significant inverse correlation between γ-H2AX expression and telomere length ($p = 0.006$; $Rs = -0.23$). γ-H2AX expression was significantly increased on CD27- cells compared to CD27+ (3.1% vs 0.8%; $p < 0.0001$).

Compared to the whole CD8+ population (0.4%, 0.08% - 1.2%), γ-H2AX+ lymphocytes (3.9%, 2.48% - 13.5%) had significantly higher expression of IFN-γ when unstimulated ($p = 0.0008$). Further, when stimulated with anti-CD3/CD28 γ-H2AX+ lymphocytes had higher expression of IFN-γ (20.0%, 7.8% - 26.9%) vs (9.6%, 4.9% - 18.1%) ($p = 0.02$) and perforin (17.0%, 5.8% - 28.3%) vs (6.4%, 3.9% - 9.2%) ($p = 0.05$), but lower levels of IL-2 (1.8%, 0% - 3.1%) vs (3.1%, 2% - 5.3%) ($p = 0.02$).

In response to in vitro IFN-α γ-H2AX+ lymphocytes have a significant reduction in the phosphorylation of Stat1 compared to whole CD8+ population (13.4%, 2.1% - 20.3%) vs (65.8%, 17.3% - 81.5%) ($p < 0.0001$). This is associated with a significant increase in EC50 (781 iu/ml vs 308 iu/ml).

γ-H2AX+ lymphocytes have significantly higher IFNAR-2 (17.3% (12.0% - 47.3%) vs 9.5% (4.0% - 13.8%) ($p = 0.0009$)) and IFNAR-1 expression (2.3% (1.6% - 5.5%) vs 0.3% (0.18% - 0.43%) ($p = 0.0002$)) compared to the whole CD8+ population. Further, exposure of γ-H2AX+ lymphocytes to IFN-α compared to medium alone leads to IFNAR-1 down-regulation over 24 hours ($p = 0.04$).

Conclusion: CD8+ lymphocytes with γ-H2AX expression accumulate in progressive HCV infection, have the form and function of cells with end-stage differentiation, and have an impaired ability to phosphorylate Stat1 in response to IFN-α.

Introduction

A number of factors are associated with an increased risk of progressive liver injury in the context of HCV including male sex and alcohol misuse [6]. However, one of the strongest risk factors for both clinical progression and failure to respond to anti-viral therapy is increasing age [142-144].

Increasing age is associated with reduction in telomere length within both lymphoid and non-lymphoid cells [447, 448]. Short telomeres are detected by the MRN protein complex including MRE11, NBS1 and RAD50 [276, 449] which leads to the recruitment of ATM (Ataxia telangiectasia-mutated) and ATR (Ataxia telangiectasia and Rad3 related) [282, 449] serine/threonine kinases which leads to the phosphorylation of a number of nuclear targets, including Histone 2 at serine 139 to form γ-H2AX [368, 412]. γ-H2AX is able to recruit further ATM complexes to the site of its formation in a positive feedback loop, as well as initiating the stabilisation of p53 and its down-steam target p21, ultimately leading to cell-cycle arrest [412, 450].

The cellular response to IFN-α is important for both the endogenous response to HCV infection and anti-viral therapy. IFN-α binds to the cell-surface receptor IFNAR-2, causing it to dimerise with IFNAR-1, leading to activation and intracellular signalling to modulate gene transcription.

To deliver a second messenger signal through the Jak/Stat pathway [122]. Phosphorylation of Jak1 and Tyk2 associated with the intracellular tail of IFNAR-1 leads to the phosphorylation of stat1 at tyrosine 701. It is known that the nature of the transcriptome generated in response to IFN-α, termed interferon-stimulated genes (ISG), is related to treatment outcome in patients with HCV treated with IFN-α based therapy [124-126].

HCV modulates the cellular response to endogenous IFN-α signalling through pStat1. HCV core protein prevents phosphorylation of stat1 in response to IFN-α [127] and NS5A protein prevents the translocation of pStat1 to the nucleus [130]. Further, short telomere length has been demonstrated to be associated with defects in the activation of Stat5 in mouse macrophages [451].

Data presented in chapters three and four demonstrate that progressive HCV infection is associated with shortened CD8+ and CD4+ T-lymphocyte telomeres and that short CD4+ T-lymphocytic telomeres are associated with poor clinical and treatment-related outcomes. The hypothesis to be tested is that lymphocytes with short telomeres would demonstrate increased evidence of downstream telomere signalling through γ-H2AX and that lymphocytes with short telomeres or increased γ-H2AX expression would have impaired responses to IFN-α.

Materials and methods

Subjects (Table 12)

Patients recruited at Addenbrooke's Hospital, Cambridge gave written informed consent with approved of the Local Research Ethics Committee. Patients co-infected with HIV, HBV or with other chronic liver disease identified by history, blood tests or liver biopsy were excluded. Lymphocytes from healthy controls were obtained from local volunteers; none gave a history of chronic illness or intravenous drug usage.

	Healthy Controls	HCV-RNA negative	HCV-RNA positive mild	HCV-RNA positive severe	P value†
N	27	27	59	48	
Age (years, mean ± SD)	42.0 ± 12.0	49.4 ± 7.5	48.8 ± 10.0	54.2 ± 6.2	**0.0006**
Sex (% male)	48.1%	66.6%	71.2%	79.2%	**0.046**[1]
BMI (mean ± SD)	27.6 ± 4.2	24.6 ± 3.7	25.6 ± 4.6	26.7 ± 5.5	0.102
% IgG anti-HBc positive	13.6%	52.0%	25.0%	38.3%	**0.017**[1]
% CMV antibody positive	33.3%	59.3%	50.8%	58.3%	0.16[1]
Biochemical indices					
Bilirubin (µmol/L, mean ± SD)	-	7.4 ± 2.9	10.1 ± 5.3	13.3 ± 7.4	**0.0001**
ALT (iu/L, mean ± SD)	-	27.6 ± 13.0	99.6 ± 79.4	110.3 ± 66.4	**<0.0001**
PT (seconds, mean ± SD)	-	12.6 ± 1.2	12.5 ± 0.7	13.7 ± 1.7	**<0.0001**

Table 12. Demographic characteristics of subjects in the four study groups. †Kruskal Wallis unless otherwise stated. 1Chi-squared.

Statistics

γ-H2AX expression in different study groups and cell surface phenotype of γ-H2AX+ cells were analysed by Kruskal-Wallis test. Cytokine secretion patterns and liver immunohistochemistry were analysed by Mann Whitney U test. Paired data from whole CD8+ and γ-H2AX+CD8+ lymphocytes including Stat1, phospho-Jak1, phospho-Tyk2, IFNAR1 and IFNAR2 expression were analysed by Wilcoxon signed rank test. IFNAR-1 down-regulation after IFN-α, absolute pStat1 responses and p53 expression were analysed by 2-way ANOVA. Dose-response curves and EC_{50} were calculated by Prism 5.0 for Windows (Graphpad Software, San Diego, USA).

The correlation between γ-H2AX expression and telomere length was analysed by Spearman's rank correlation. P values of < 0.05 were considered significant.

Results

Progressive HCV infection is associated with increased numbers of γ-H2AX+ CD8+ T-lymphocytes

Telomeres with critical shortening generate γ-H2AX [368, 412], a double strand DNA break signal with phosphorylation of histone-2 at serine 139. To investigate the relationship between telomere length and γ-H2AX expression in HCV, the proportion of γ-H2AX+ CD8+ and CD4+ T-lymphocytes from subjects with known telomere lengths was investigated.

Shortened CD8+CD45RO+ telomeres were associated with increased numbers of γ-H2AX+ CD8+ T-cells ($p = 0.006$; $Rs = -0.23$) but there was no evidence of a correlation between CD4+CD45RO+ telomere length and γ-H2AX+ CD4+ T-cell number ($p = 0.53$; $Rs = -0.07$) (Figure 48). Further, the proportion of γ-H2AX+ CD8+ T-lymphocytes from viraemic subjects with severe liver disease (2.8%; 1.5% - 4.6%) was higher than healthy controls (1.8%; 0.98% - 2.4%), non-viraemic HCV-exposed subjects (median 2.0%; IQR: 1.1% - 3.5%) and subjects with viraemia and mild liver disease (1.4%; 0.8% - 2.4%) (Kruskal Wallis $p = 0.0023$). There were no significant differences between study groups in terms of γ-H2AX levels in CD4+ peripheral lymphocytes ($p = 0.57$).

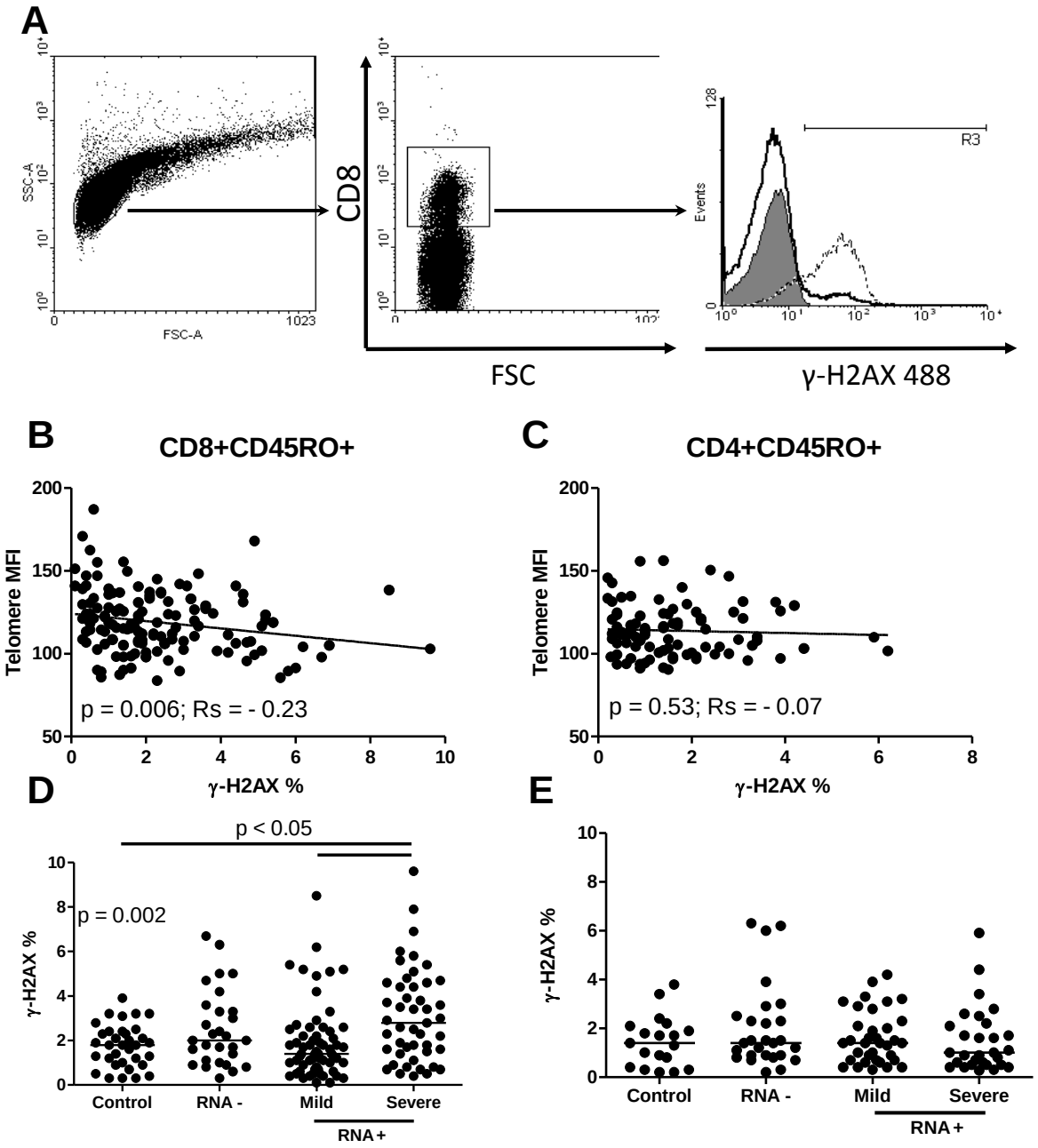

Figure 48. γ-H2AX (ser-139) expression in peripheral CD8+ (B & D) and CD4+ (C & E) lymphocytes and association with telomere length and study group allocation. Panel A. γ-H2AX was studied on cells within the live lymphocyte gate by scatter characteristics (left) and positive staining for either CD8 (centre) or CD4. Isotype control staining pattern in filled histogram; positive control staining pattern from irradiated cells in dashed histogram and experimental sample in bold histogram (right). Panel B & C. Association between telomere length in CD8+CD45RO+ and CD4+CD45RO+ subsets respectively and γ-H2AX levels. Correlation by Spearman's Rank. Panel D & E. CD8+ (27 controls, 27 non-viraemic HCV-exposed cases, 59 viraemic patients with mild fibrosis and 48 viraemic patients with severe fibrosis) and CD4+ (19 control, 27 non-viraemic HCV-exposed cases, 35 viraemic patients with mild fibrosis and 29 viraemic patients with severe fibrosis) γ-H2AX levels by study group allocation.

Therefore, shortened CD8+ T-lymphocyte telomeres were associated with increased signalling through the γ-H2AX system, but no evidence of increased signalling in CD4+ cells was demonstrated.

Attempts to demonstrate that short telomeres were associated with increased γ-H2AX expression in each cell with flow-cytometry were unsuccessful as the heat step essential for measuring telomere length led to destruction of the fluorochrome tag on the anti- γ-H2AX antibody. In a subsequent experiment lymphocytes were flow sorted into CD8+ γ-H2AX+ and CD4+ γ-H2AX- subsets and then subjected to the standard flow-FISH assay for telomere length. However, too few cells were available for meaningful analysis.

γ-H2AX expression is increased in cells with a more advanced cell-surface phenotype

To investigate the maturation status of cells expressing γ-H2AX, peripheral CD8+ or CD4+ lymphocytes were co-stained for γ-H2AX and appropriate markers of antigen-experience and maturation in 60 subjects with chronic HCV (Figure 49).

CD8+ lymphocytes were sub-divided on the basis of CD27 and CD57 expression. CD8+ lymphocytes expressing the mature CD27-CD57- (median, IQR) (3.1%, 1.4% - 5.6%) and CD27-CD57+ (3.2%, 1.7% - 6.2%) phenotypes had significantly higher expression of γ-H2AX than the less-mature CD27+CD57- subset (0.8%, 0.4% - 1.6%) (Kruskal-Wallis, $p < 0.0001$).

CD4+ lymphocytes divided by CD27 and CD45RO expression demonstrated a similar pattern of expression. The naive CD27+CD45RO- subset (0.4%, 0.2% - 0.7%) had lower expression of γ-H2AX than the central memory CD27+CD45RO+ (0.8%, 0.4% - 1.8%) and the effector memory CD27-CD45RO+ subsets (2.8%, 1.5% - 3.6%) ($p < 0.0001$).

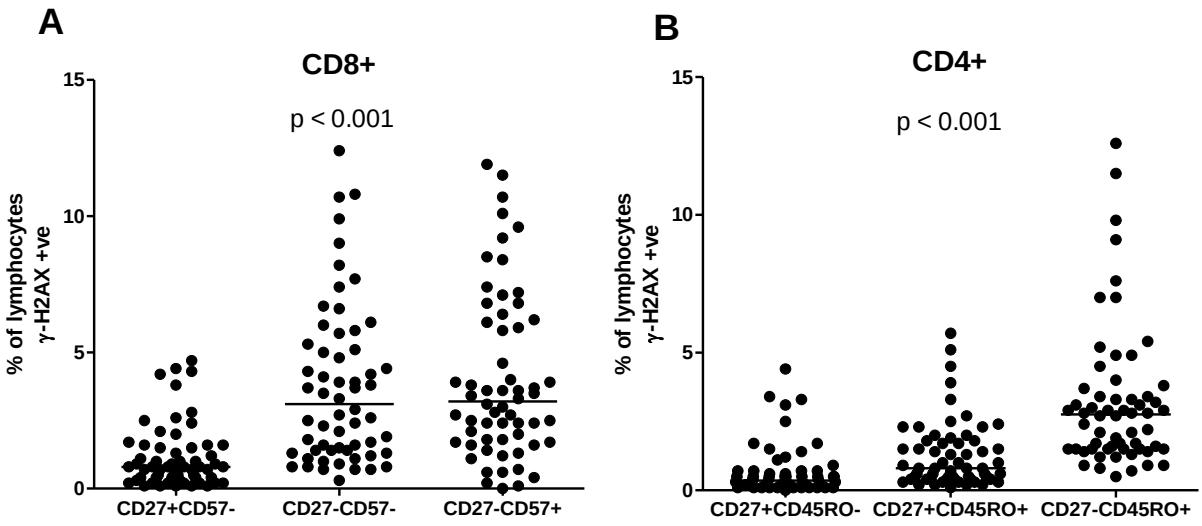

Figure 49. γ-H2AX expression patterns of different subsets of CD8+ (A) and CD4+ (B) peripheral lymphocytes from subjects (n = 60) with HCV infection. CD8+ lymphocytes were divided based on the expression of the surface markers CD27 and CD57, whereas CD4+ lymphocytes were divided based on CD27 and CD45RO expression. Statistical analysis by Kruskal-Wallis.

Intrahepatic γ-H2AX expression by immunohistochemistry

To investigate whether γ-H2AX+ cells accumulated within the portal tracts of subjects with progressive HCV infection, the proportion of portal tract area expressing γ-H2AX was analysed by immunohistochemistry in 10 HCV RNA positive patients with mild disease and HCV RNA positive patients with severe fibrosis subgroups.

There was no significant difference between the proportion of portal tract area positive for γ-H2AX staining between viraemic subjects with mild ((median, IQR) 0.17%, 0.12% - 0.34%) or severe fibrosis (0.19%, 0.14% - 0.30%) (p = 0.88) (Figure 50).

This is perhaps slightly surprising given the flow-cytometry results from peripheral lymphocytes demonstrated significant differences. This may reflect the significant liver infiltration of NK and NKT cells or may represent a failure to detect telomere-dependent γ-H2AX in the context of proliferation-related γ-H2AX [452].

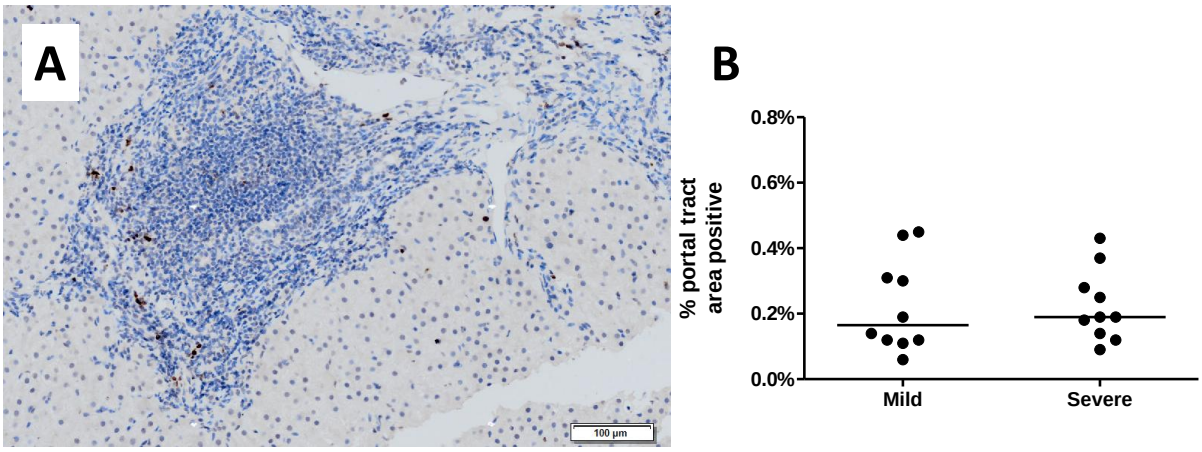

Figure 50. Intrahepatic expression of γ-H2AX on portal tract lymphocytes by immunohistochemistry. Liver biopsy sections from viraemic subjects with mild fibrosis (n = 10) or severe fibrosis (n = 10) were stained for γ-H2AX and counterstained with haematoxylin. Representative immunohistochemistry in Panel A; scale bar 100µm. Panel B. Grouped results of portal tract area positive for γ-H2AX.

Ex vivo unstimulated γ-H2AX positive T-lymphocytes secrete IFN-γ

To investigate the functional properties of γ-H2AX positive T-lymphocytes the expression patterns of IFN-γ and IL-2 in unstimulated whole CD8+ or CD4+ lymphocytes or CD8+γ-H2AX + and CD4+γ-H2AX + subsets were analysed in 10 subjects with chronic HCV infection.

IFN-γ expression was significantly higher in unstimulated γ-H2AX+ CD8+ lymphocytes (3.9%, 2.48% - 13.5%) when compared to whole CD8+ lymphocytes (0.4%, 0.08% - 1.2%) (p = 0.0008). Similarly, γ-H2AX+ CD4+ cells secreted significantly more IFN-γ (4.3%, 0.85% - 7.63%) than whole CD4+ lymphocytes (0.25%, 0% - 1.1%) (p = 0.0096) (Figure 51.

There were no significant differences in IL-2 secretion between γ-H2AX+ and whole lymphocyte subsets in either CD8+ or CD4+ populations.

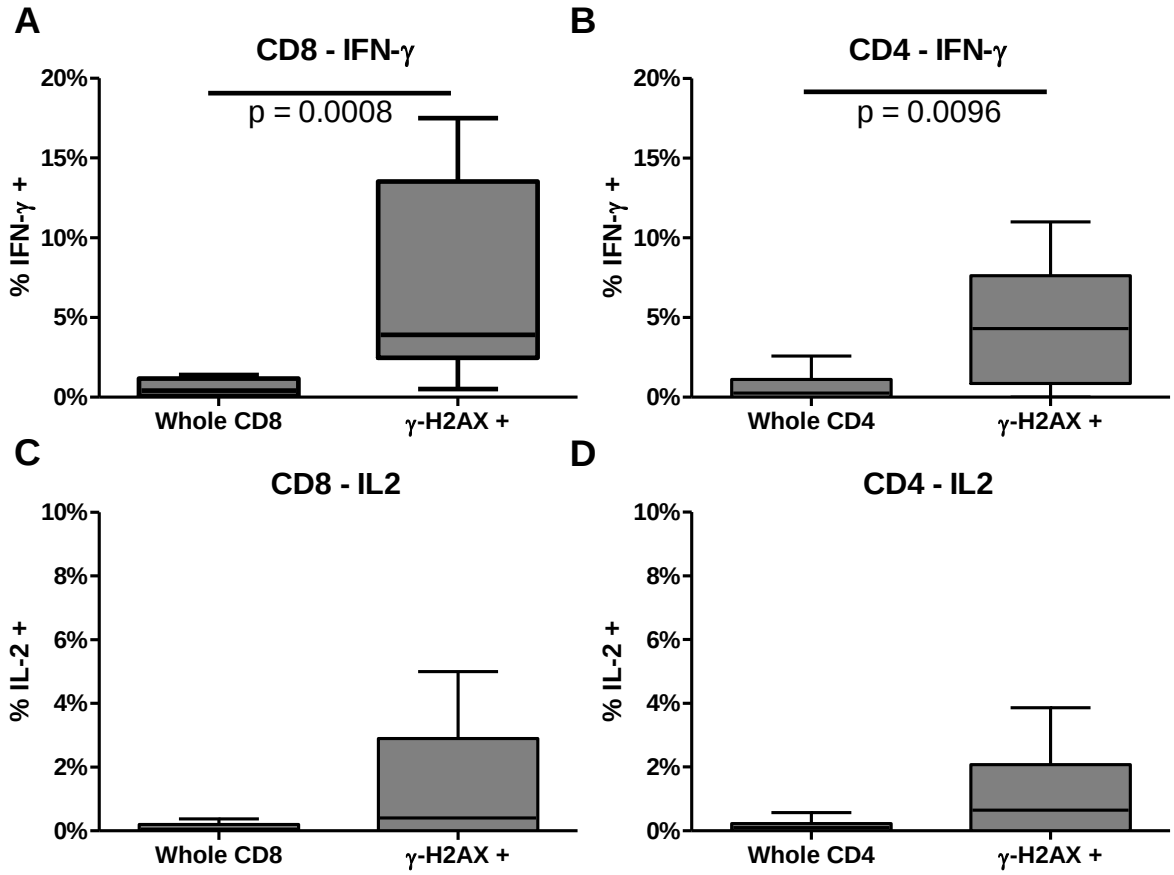

Figure 51. Cytokine expression of unstimulated peripheral CD8+ (Panel A & C) and CD4+ (B & D) T cells from 10 subjects with chronic HCV infection. Whole lymphocytes or γ-H2AX+ subsets were analysed for expression of IFN-γ (A & B) or IL-2 (C & D). Analysis by Mann-Whitney U test.

Stimulated γ-H2AX positive T-lymphocytes are pro-inflammatory

T cells were stimulated with anti-CD3 / anti-CD28 to investigate the functional properties of lymphocytes expressing γ-H2AX, measured by intracellular cytokine staining.

In comparison to the responses of the whole CD8+ population, γ-H2AX+ CD8+ lymphocytes from subjects with chronic HCV (n = 10) had higher expression of IFN-γ (median, IQR) (20.0%, 7.8% - 26.9%) vs (9.6%, 4.9% - 18.1%) (p = 0.02) and perforin (17.0%, 5.8% - 28.3%) vs (6.4%, 3.9% - 9.2%) (p = 0.05), but lower levels of IL-2 (1.8%, 0% - 3.1%) vs (3.1%, 2% - 5.3%) (p = 0.02) (Figure 52).

Similarly, γ-H2AX+CD4+ lymphocytes demonstrate similar functional properties with higher expression of IFN-γ (5.9%, 2.3% - 17.6%) vs (3.0%, 2.5% - 8.4%) (p = 0.01) and perforin (12.4%, 3.9% -

16.3%) vs (3.0%, 0.4% - 9.0%) (p = 0.002), but lower levels of IL-2 (0.6%, 0.35% - 1.4%) vs (3.9%, 2.6% - 5.2%) (p = 0.009).

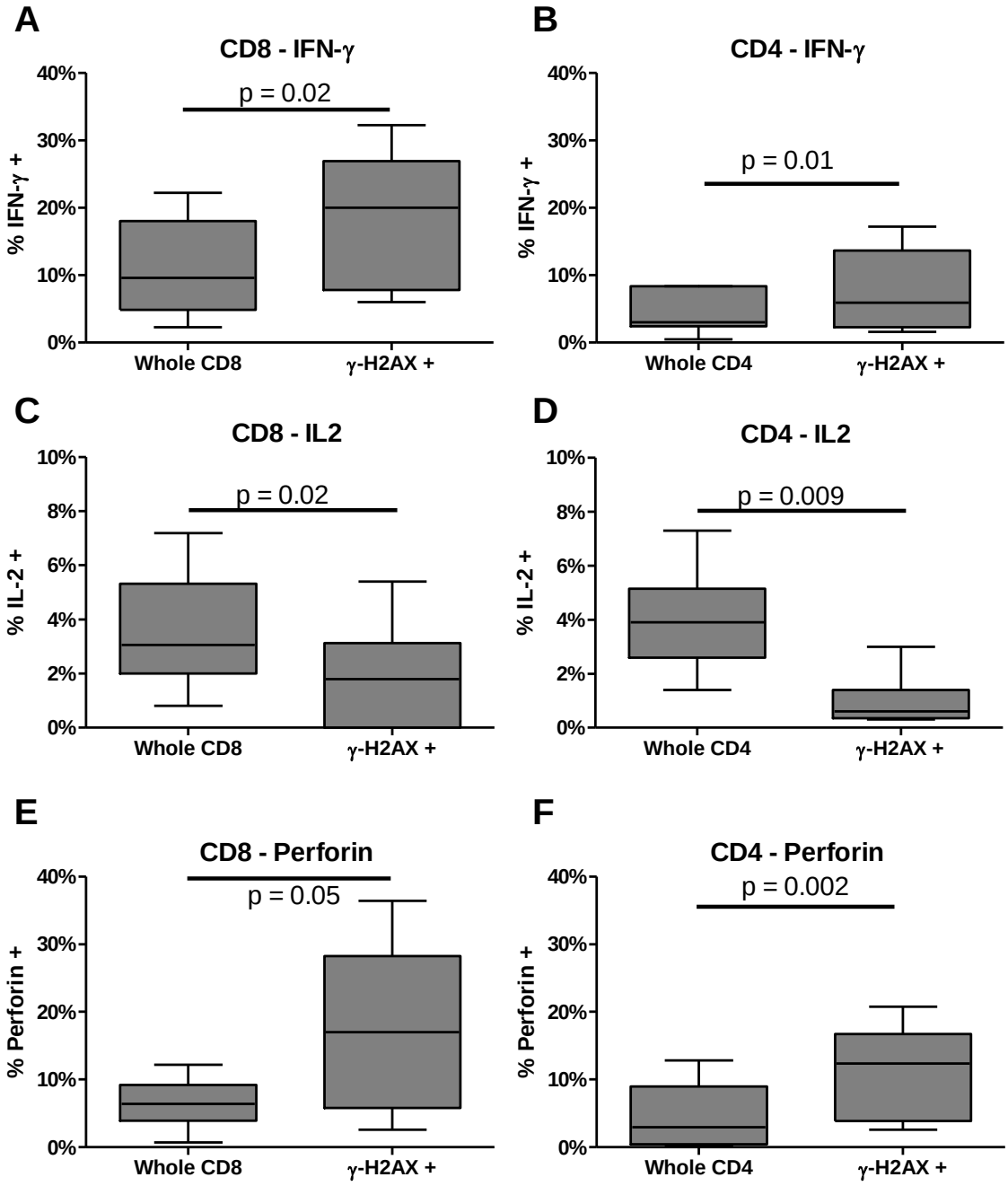

Figure 52. Cytokine expression of peripheral CD8+ (Panel A, C & E) and CD4+ (B, D & F) T-lymphocytes, in response to non-specific stimulation, in 10 subjects with chronic HCV. Cells were stimulated with anti-CD3 / anti-CD28 and then whole lymphocytes or γ-H2AX+ cells were analysed for expression of IFN-γ (A &B), IL-2 (C&D) and perforin (E&F). Analysis by Mann-Whitney U test.

CD8+ lymphocytes respond to exogenous IFN-α by phosphorylating Stat1

The intracellular signalling pathway after IFN-α binds to its surface receptor involves the phosphorylation of Stat1. To investigate the ability of lymphocytes to respond to IFN-α, a multi-colour flow-cytometric assay to analyse the phosphorylation of Stat1 was developed. Cells were cultured in supplemented RPMI-1640 and incubated with 1000iu/ml IFN-α2b and then harvested at sequential time points. Phosphorylation of Stat1 at tyrosine 701 was readily demonstrable within 30 minutes of incubation and rapidly declined thereafter (Figure 53).

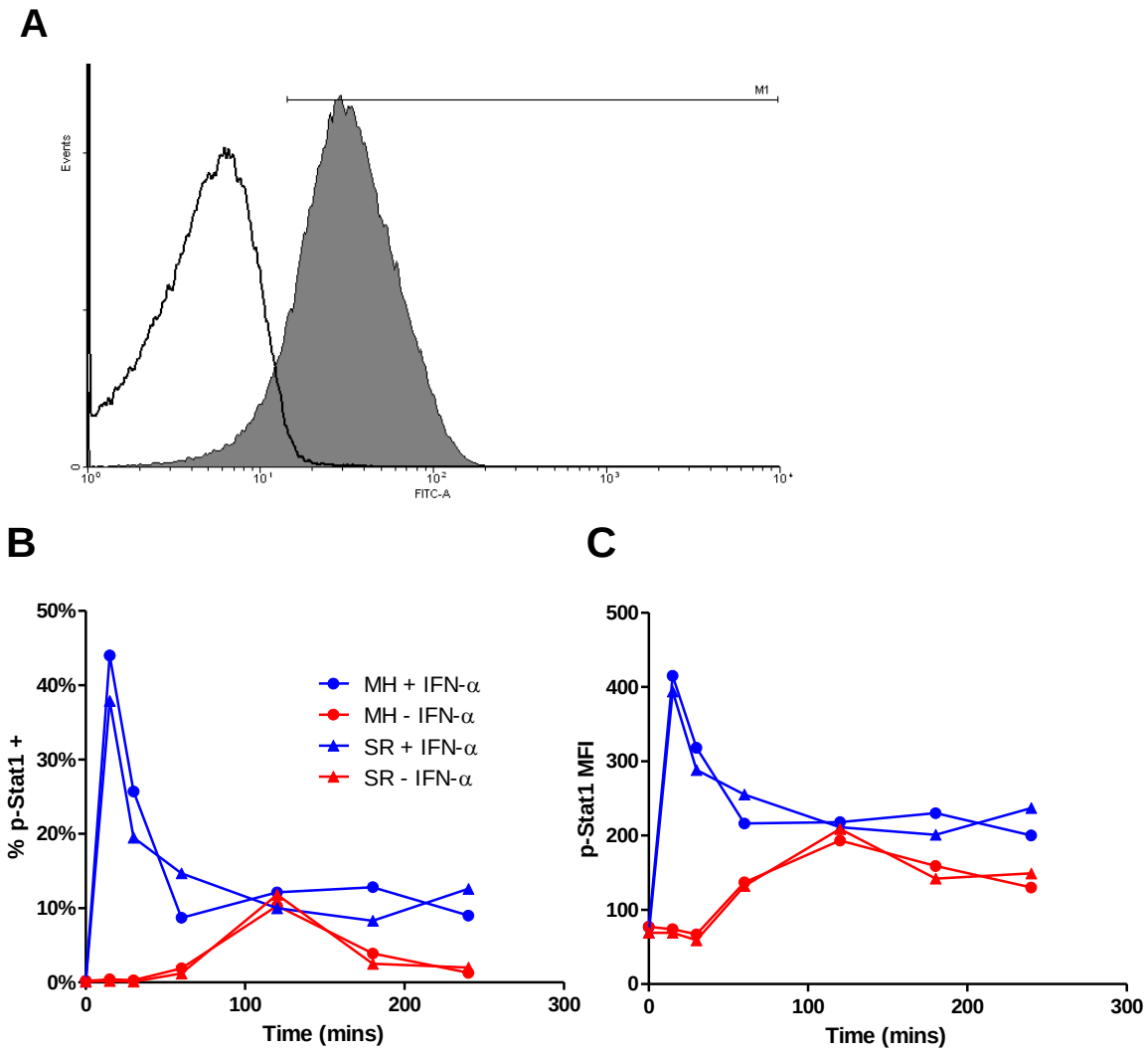

Figure 53. Phosphorylation of Stat1 at tyrosine 701 in response to exogenous IFN-α. Peripheral lymphocytes from 2 individuals (MH, SR) were cultured in the presence of 1000 iu/ml IFN-α2b and phosphorylation of Stat1 assayed at various times after start of culture. Panel A. Representative histogram of pStat1 staining after 30minutes of incubation with IFN-α, filled histogram is specific

antibody, isotype control with bold line. Time-course of pStat1 expression after incubation with interferon (blue line) or control medium (red line) in 2 individuals expressed as either pStat1 positivity (Panel B) or pStat1 mean fluorescence intensity (Panel C).

For all subsequent experiments cells were incubated with IFN-α for 30 minutes.

CD8+ γ-H2AX+ lymphocytes fail to phosphorylate Stat1 after exposure to IFN-α

To investigate the ability of γ-H2AX+ CD8+ lymphocytes to respond to IFN-α, the dose response of CD8+ lymphocytes from healthy controls (n =6) and HCV-infected subjects (n =6) was analysed. Peripheral lymphocytes were incubated with variable concentrations of IFN-α2b for 30 minutes in order to establish the dose-response curves (Figure 54).

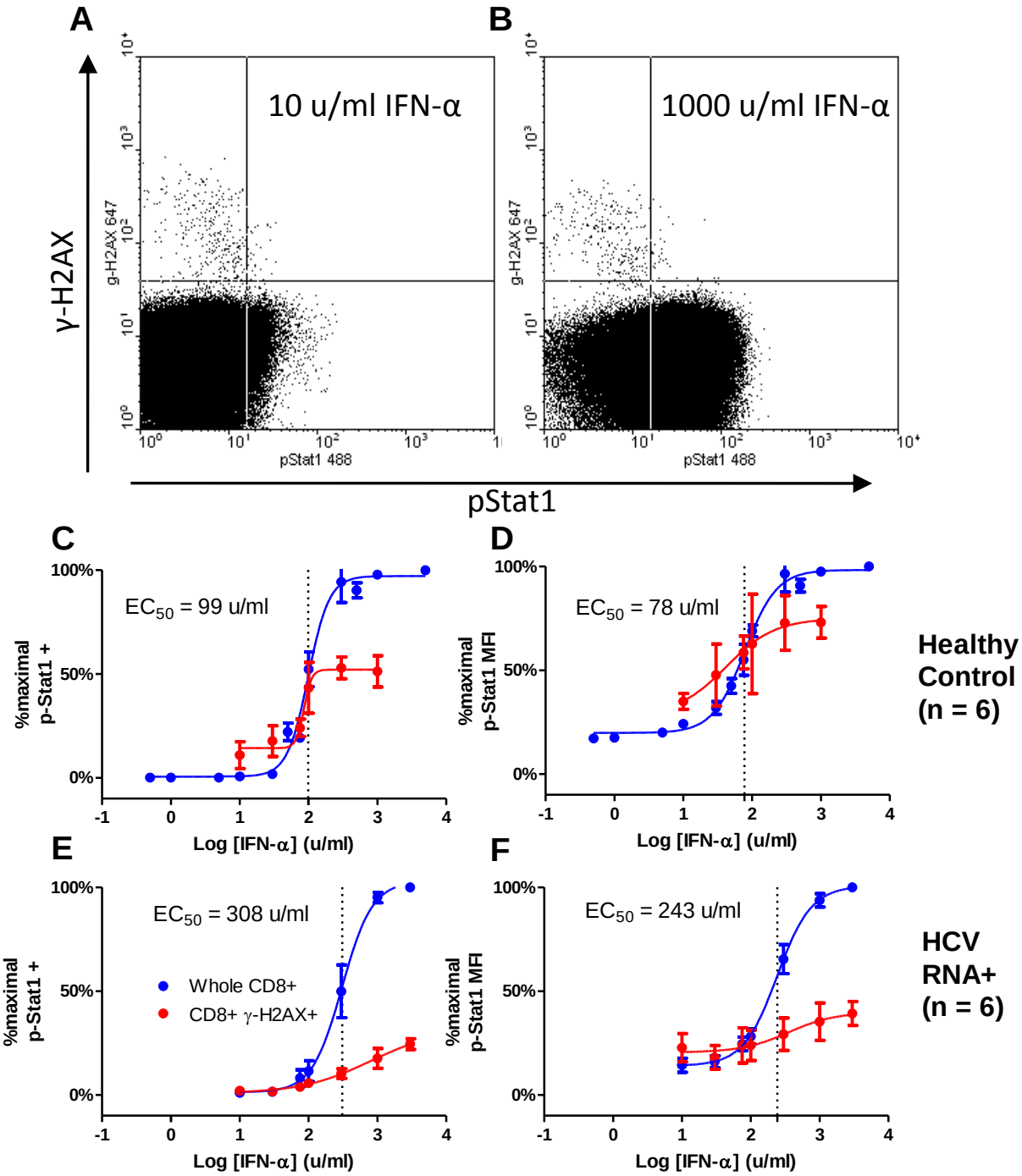

Figure 54. CD8+γ-H2AX+ lymphocytes fail to phosphorylate Stat1 after incubation with IFN-α. Peripheral lymphocytes were incubated with variable concentrations of IFN-α2b before staining for CD8, γ-H2AX and pStat1. Example dot-plots of CD8+ gated lymphocytes incubated with 10iu/ml (Panel A) or 1000iu/ml IFN-α2b (Panel B). Dose response curves of pStat1 responses by proportion of pStat1 positive cells (Panels C & E) or pStat1 MFI (Panels D & F) after incubation with IFN-α in healthy controls (n = 6) (Panels C & D) or HCV-infected subjects (n = 6) (Panels E & F).

CD8+ lymphocytes from healthy controls respond to IFN-α with an EC_{50} of 99iu/ml when analysing proportion of pStat1+ cells or 78iu/ml when analysing pStat1 MFI. γ-H2AX+ CD8+ lymphocytes have

similar EC_{50} to the whole CD8+ compartment at 85iu/ml and 42iu/ml for positivity and MFI respectively. However, γ-H2AX+ lymphocytes have a sub-maximal response when compared to the whole CD8+ compartment.

CD8+ lymphocytes from HCV-infected subjects (n = 6) had increased EC_{50} when compared to healthy controls with 308 iu/ml and 243 iu/ml for pStat1 positivity and MFI respectively, with γ-H2AX+CD8+ lymphocytes 781 iu/ml and 355 iu/ml respectively.

Whereas lymphocytes from HCV-infected individuals have an increased EC_{50} for pStat1 response to IFN-α, the absolute maximal response for global CD8+ and CD4+ cells is not different to healthy controls (Figure 55). Utilising 2-way ANOVA, γ-H2AX+ cells had a significantly reduced response when compared to the whole CD8+ compartment ($P < 0.0001$). However, HCV-infected subjects were not significantly different to healthy controls ($p = 0.29$).

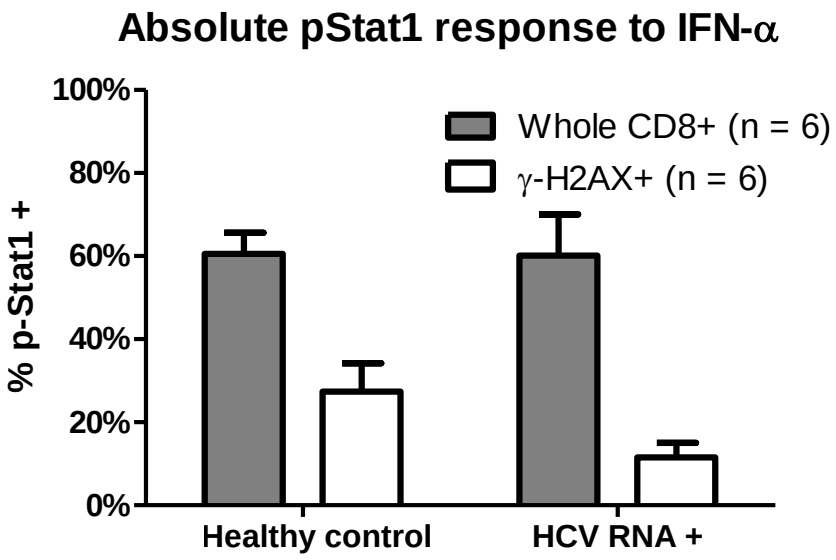

Figure 55. Maximal pStat1 response to IFN-α in healthy controls (n = 6) and HCV-infected subjects (n = 6) in different CD8+ lymphocyte subsets defined by γ-H2AX.

Therefore, whilst HCV leads to resistance to IFN-α signalling, it does not impair absolute response at high concentrations of IFN-α in T-lymphocytes.

Impaired Stat1 phosphorylation in γ-H2AX+ cells is not due to impaired Stat1 expression

To ensure that the impairment of Stat1 phosphorylation was not due to a failure to express Stat1 in γ-H2AX positive CD8+ cells, the Stat1 expression of whole CD8+ and γ-H2AX+CD8+ T-cells was compared. There was no significant difference in the proportion of Stat1 positive whole CD8+ cells (72.0% (34.4% - 85.6%)) and Stat1 positive γ-H2AX+CD8+ cells (63.5% (23.1% - 68.8%)) (Wilcoxon signed rank, p = 0.06). Nor was there a difference in the Stat1 MFI of whole CD8+ cells (4609 (2729 – 8607)) when compared to γ-H2AX+CD8+ cells (3420 (1853 – 5954)) (p = 0.06) (Figure 56).

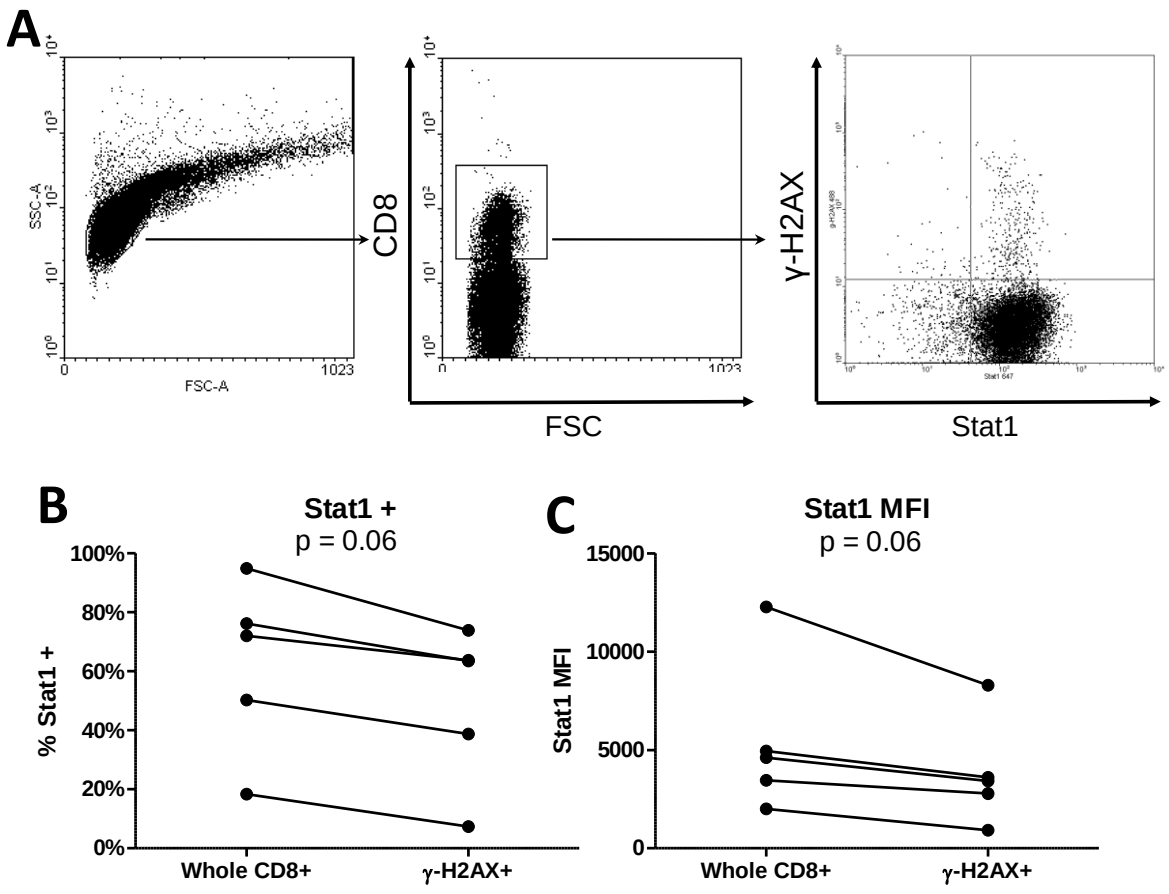

Figure 56. Stat1 expression of CD8+ and CD8+ γ-H2AX+ cells from subjects with viraemic HCV infection (n = 5). Panel A: gating strategy to demonstrate γ-H2AX+ and Stat1 expression. Left: Lymphocytes are gated by light-scatter characteristics. Middle: CD8+ cells are then gated. Right: Co-expression of Stat1 and γ-H2AX is examined. Panel B: Proportion of whole CD8+ and γ-H2AX+CD8+ cells positive for Stat1. Panel C: Stat1 MFI of whole CD8+ and γ-H2AX+CD8+ cells. Statistics by Wilcoxon signed rank analysis.

IFN-α receptor components 1 and 2 (IFNAR-1 & IFNAR-2) are up-regulated on γ-H2AX+ CD8+ lymphocytes.

The failure of the pStat1 response demonstrated in γ-H2AX positive cells may be related to a change in the expression of the surface receptor for IFN-α, which includes the components IFNAR-1 and IFNAR-2. Surface expression of these molecules on whole peripheral CD8+ lymphocytes and γ-H2AX+CD8+ lymphocytes was sought in 18 subjects with chronic HCV infection.

γ-H2AX+CD8+ lymphocytes had higher expression of IFNAR2 than whole CD8+ lymphocytes by both proportion positive (17.3% (12.0% - 47.3%) vs 9.5% (4.0% - 13.8%) (p = 0.0009)) and MFI (1420 (660 - 2826) vs 524 (317 – 777) (p = 0.0011)) (Figure 57).

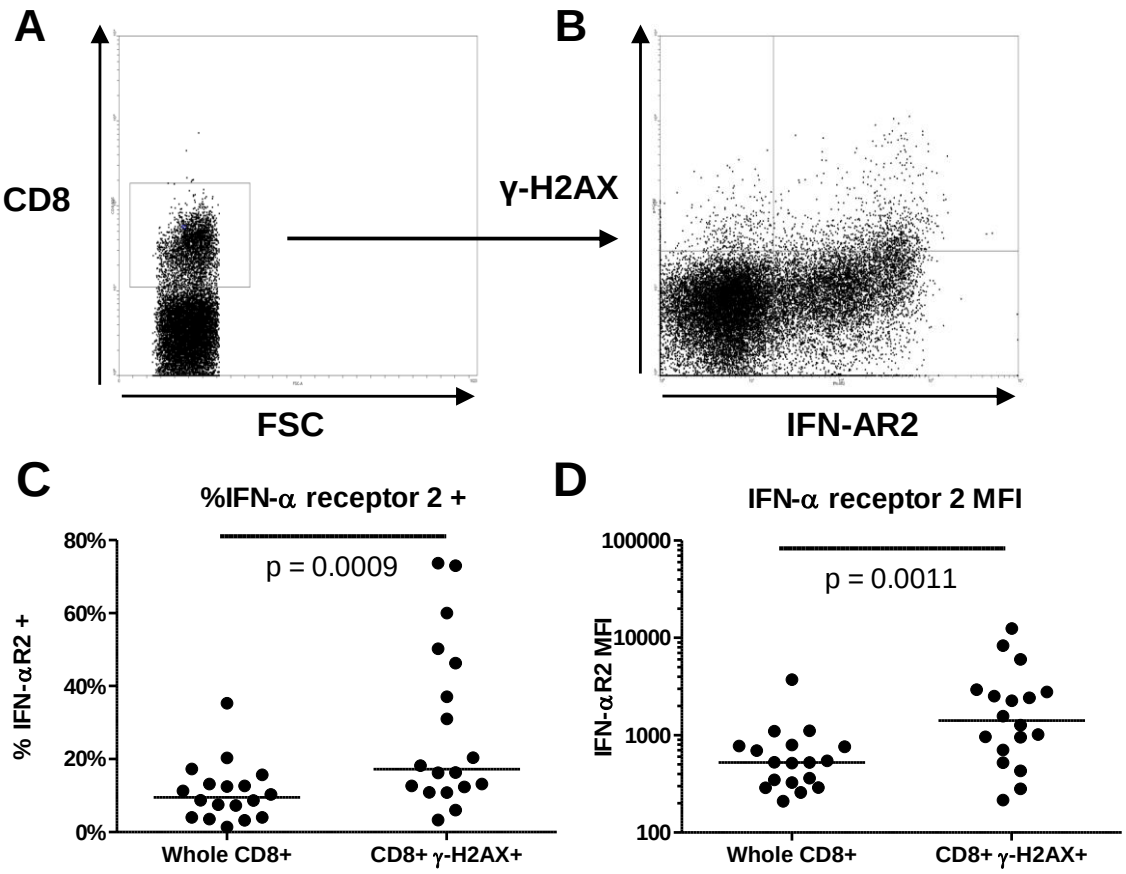

Figure 57. Expression of IFNAR-2 on whole CD8+ and γ-H2AX+CD8+ lymphocytes from HCV-infected individuals. Example cytometric data of CD8 gated PBMCs (Panel A) stained for IFNAR2 and γ-H2AX (Panel B). Proportion of IFNAR2+ (Panel C) and IFNAR2 MFI (Panel D) were

determined in ex-vivo peripheral lymphocytes from HCV-infected individuals (n = 18). Statistics by Wilcoxon matched pairs test.

γ-H2AX+CD8+ lymphocytes have higher expression of IFNAR1 than whole CD8+ lymphocytes by both proportion of IFNAR1 positive cells (2.3% (1.6% - 5.5%) vs 0.3% (0.18% - 0.43%) (p = 0.0002)) and IFNAR1 MFI (186.5 (147- 272) vs 123.5 (112.8 – 138.5) (p = 0.0003)) (Figure 58).

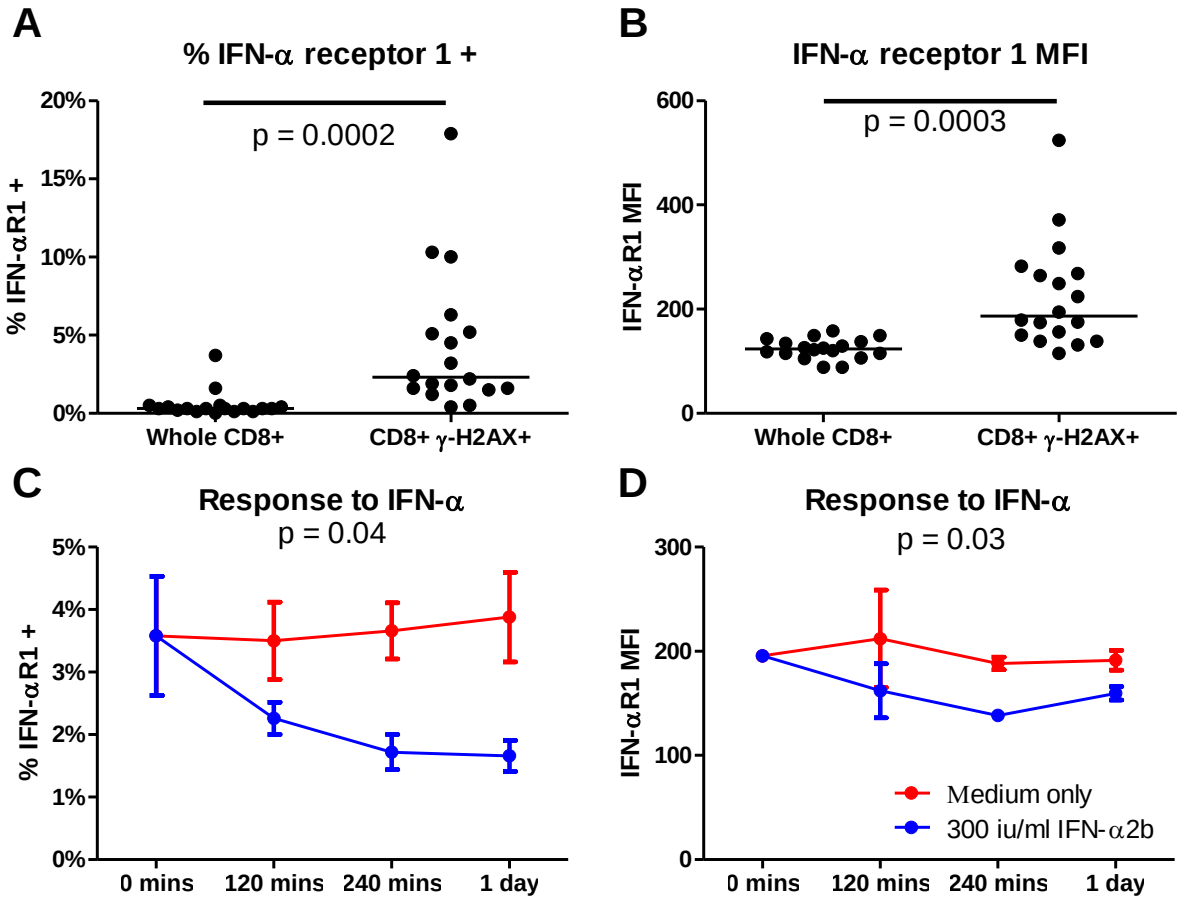

Figure 58. Expression of IFNAR-1 on whole CD8+ and γ-H2AX+CD8+ lymphocytes from HCV-infected individuals. Proportion of IFNAR1+ cells (Panels A & C) and IFNAR1 MFI (Panels B & D) were determined in ex-vivo peripheral lymphocytes from HCV-infected individuals (n = 18) (Panels A&B). Analysis by Wilcoxon matched pairs test. Receptor down-regulation following incubation with 300 iu/ml IFN-α2b (blue line) or control medium (red line) in γ-H2AX+CD8+ lymphocytes from 6 HCV-infected individuals (Panels C & D). Analysis by 2-way ANOVA.

To determine whether IFN-α was able to bind to its cell-surface receptor leading to receptor down-regulation cells were incubated with and without 300 iu/ml IFN-α2b for up to 24 hours and IFNAR-1 expression on γ-H2AX+ cells was measured at several time points.

Treatment with IFN-α led to a significant reduction in both IFNAR-1 positive cells (p = 0.04) and IFNAR-1 MFI (p = 0.03), when compared to cells incubated in medium alone. γ-H2AX+ cells are unable to phosphorylate Stat1 in response to IFN-α, but this is neither a consequence of altered receptor expression nor a failure to bind to the receptor on the cell surface.

γ-H2AX + CD8+ lymphocytes are able to phosphorylate Jak1 and Tyk2 in response to exogenous IFN-α

To further isolate the defect in the IFN-α signalling pathway, the ability of whole CD8+ and γ-H2AX+CD8+ lymphocytes to phosphorylate Jak1 and Tyk2 in response to exogenous IFN-α was investigated in subjects with viraemic HCV infection (n = 9).

Incubation with 1000 iu/ml IFN-α2b for 20 minutes led to higher numbers of phospho-Tyk2 positive cells (4.7% (3.4% - 15.6%) vs 0.4% (0.2% - 1.4%), p = 0.004) and higher phospho-Tyk2 MFI (232 (139 – 278) vs 68 (44 – 113), p = 0.004) in the γ-H2AX+ CD8+ subset than in the whole CD8+ compartment respectively (Figure 59).

Similarly, exogenous interferon led to higher numbers of phospho-Jak1 positive cells (2.0% (1.8% - 6.3%) vs 0.3% (0.2% - 0.6%), p = 0.004) and higher phospho-Jak1 MFI (173 (90 – 288) vs 53 (40 – 61), p = 0.004) in the γ-H2AX+ CD8+ subset than in the whole CD8+ compartment respectively (Figure 59).

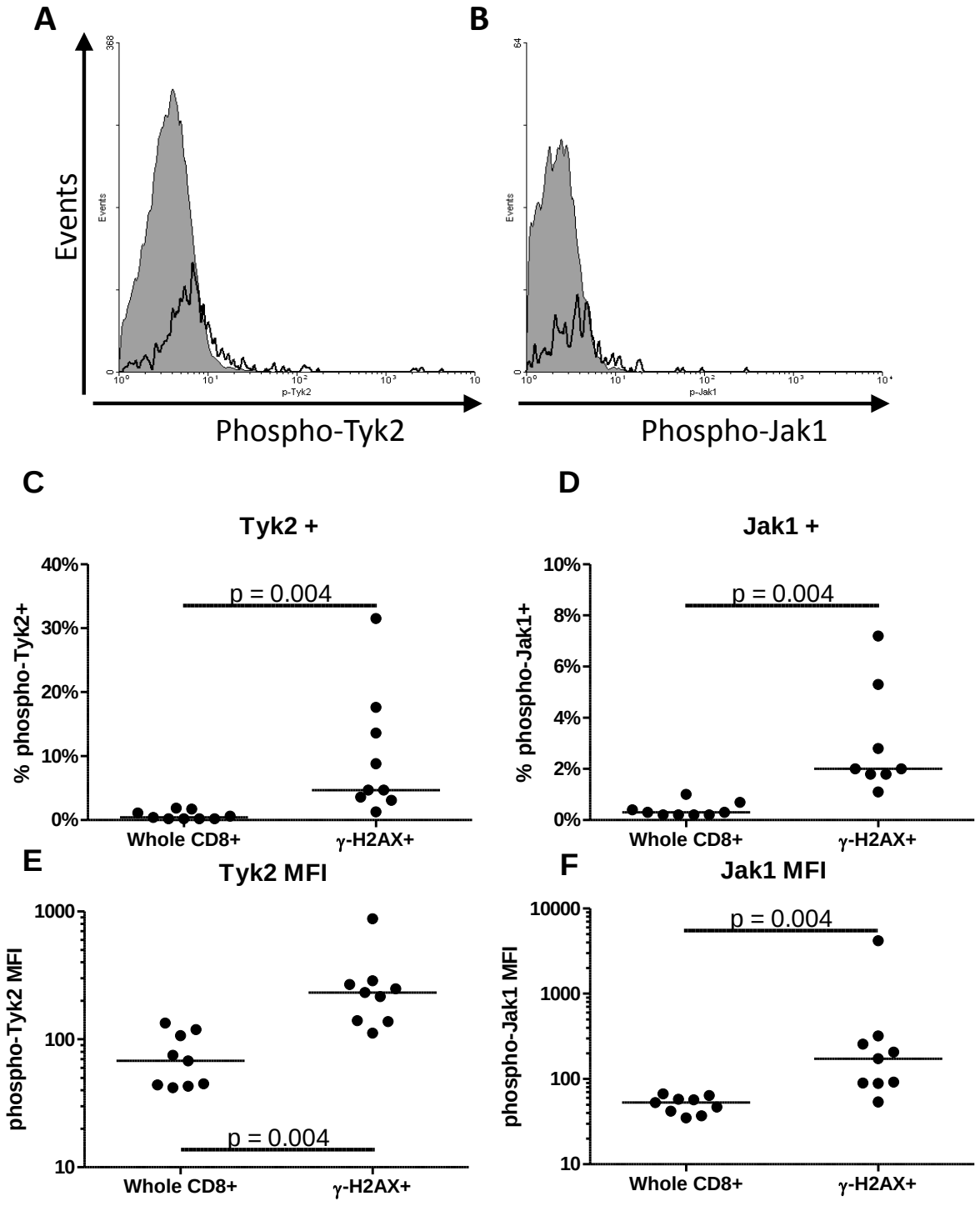

Figure 59. Interferon-induced phosphorylation of Tyk2 (Panels A, C & E) and Jak1 (Panels B, D &F) in whole CD8+ and γ-H2AX+CD8+ lymphocytes from subjects with viraemic HCV infection (n = 9). Panels A&B demonstrate cytometry plots of phospho-Tyk2 (A) and phospho-Jak1 (B) expression in whole CD8+ lymphocytes (filled histogram) and γ-H2AX+CD8+ lymphocytes (bold histogram). Panels C&D demonstrate proportion of whole CD8+ and γ-H2AX+CD8+ lymphocytes expressing phospho-Tyk2 (C) and phospho-Jak1 (D) after incubation with 1000 iu/ml IFN-α2b. Panels E&F demonstrate phospho-Tyk2 and phospho-Jak1 MFI in whole CD8+ and γ-H2AX+CD8+ lymphocytes. Stats by Wilcoxon signed rank analysis.

Increased γ-H2AX expression is associated with increased p53 in peripheral CD8+ lymphocytes

To investigate the ability of γ-H2AX to induce downstream signalling pathway components such as p53, the co-expression of γ-H2AX and p53 in peripheral CD8+ lymphocytes was analysed in four groups: healthy controls (n = 25) non-viraemic HCV-exposed (n = 25), viraemic with mild fibrosis (n = 44) and viraemic with severe fibrosis (n = 34).

P53 expression on whole peripheral CD8+ lymphocytes was similar between the 4 patient groups (Kruskal Wallis, $p = 0.72$). The expression of p53 on CD8+ γ-H2AX+ cells was much higher than on the whole CD8+ population (2-way ANOVA, $p < 0.0001$), but again the differences in p53 expression between patient groups was not significantly different ($p = 0.37$) (Figure 60).

Increased γ-H2AX expression was associated with increased p53 expression at the level of a single cell, but there was no evidence of altered p53 expression between the different study groups. The level of p53 staining was also perhaps surprising with only around 2% of γ-H2AX+ cells positive for p53. To extend these findings I would aim to repeat the experiment but study phospho-p53 expression on γ-H2AX+ cells. Phosphorylation by ATM or ATR leads to stabilisation of P53, by preventing its binding to its negative regulator MDM2 [453].

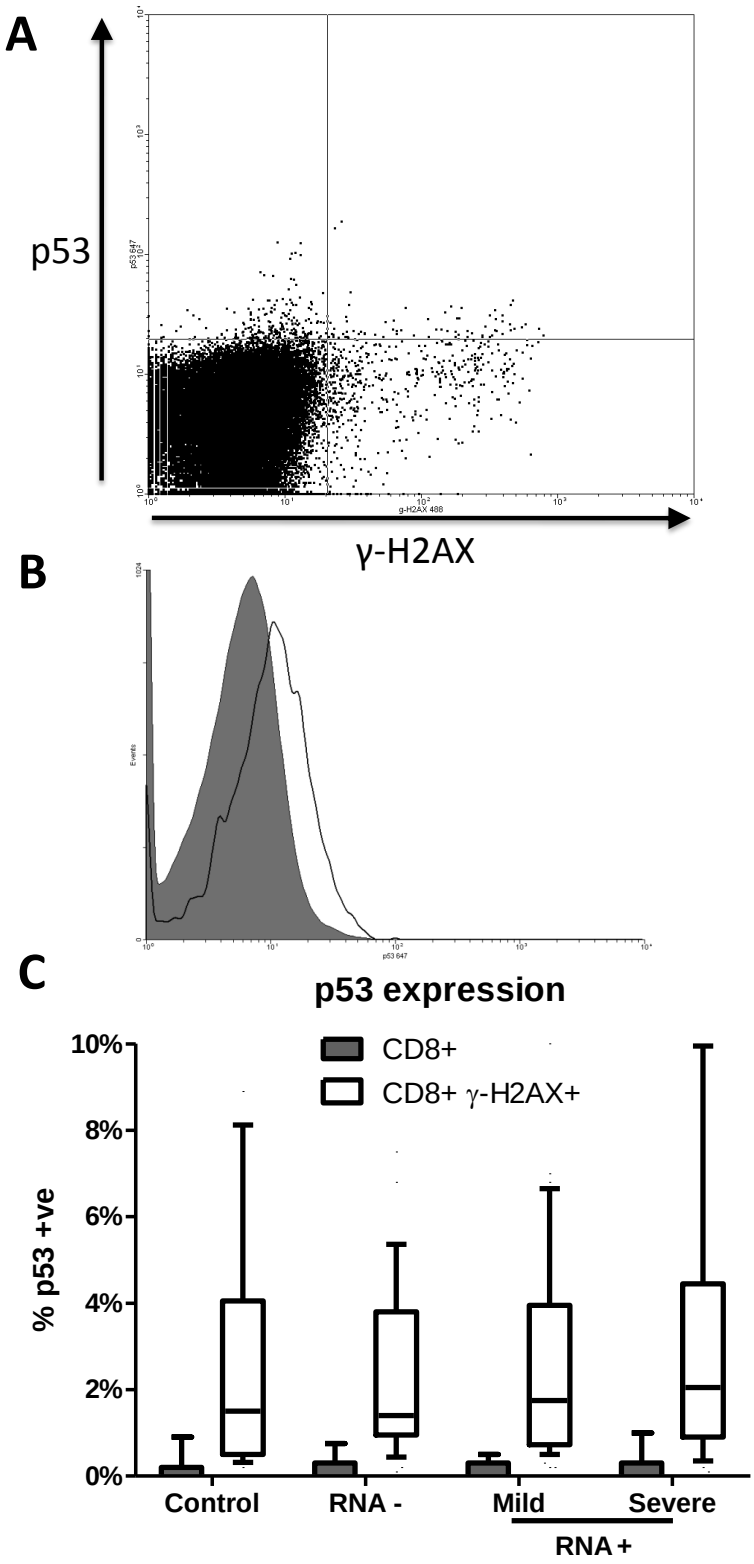

Figure 60. Co-expression of γ-H2AX and p53 on peripheral CD8+ lymphocytes. Panel A: representative dot-plot of γ-H2AX and p53 expression gated on live CD8+ lymphocytes by CD8+ and scatter characteristics. Panel B: representative histogram of p53 expression on whole CD8+ population (grey filled histogram) and CD8+ γ-H2AX+ lymphocytes (unfilled histogram. Panel C:

grouped box and whisker plots demonstrating p53 expression on whole CD8+ and CD8+ γ-H2AX+ lymphocytes across the four study groups.

P53 and p21 are not up-regulated in intrahepatic portal lymphocytes in HCV-infected patients with severe fibrosis

The portal expression of p53 and p21 was examined to determine whether the increases in γ-H2AX expression in peripheral CD8+ lymphocytes from subjects with advanced fibrosis result in increased downstream signalling. Immunohistochemistry (Figure 61) was undertaken in subgroups of viraemic patients with mild (n = 10) or severe disease (n = 11).

There were no significant differences in portal tract area positive for p53 in subjects with mild disease (median (IQR); 0.21% (0.07% – 0.44%)) compared to severe disease (0.37% (0.26% - 0.52%) (p = 0.15). Similarly, there were no significant differences in portal tract area positive for p21 in subjects with mild disease (0.58% (0.45% – 0.72%)) compared to severe disease (0.51% (0.38% - 0.72%) (p = 0.42).

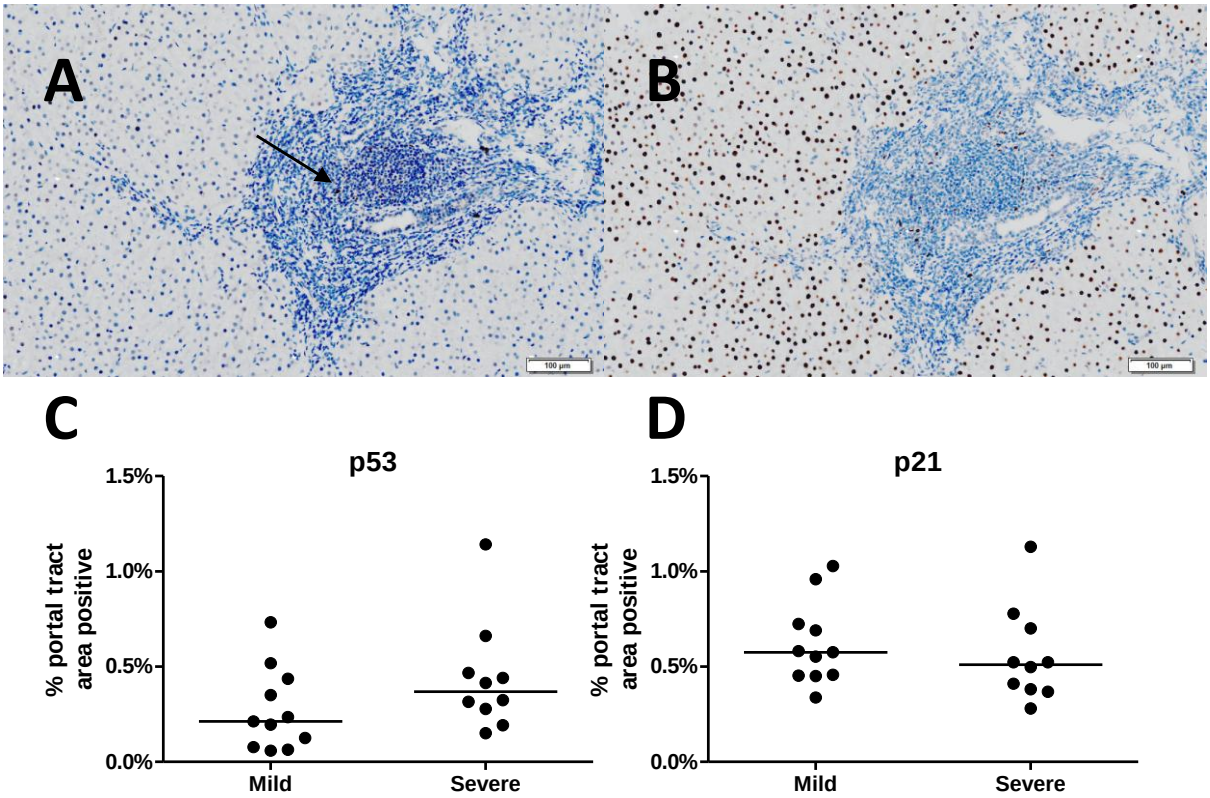

Figure 61. p53 and p21 expression by portal tract lymphocytes in HCV infection. Representative immunohistochemistry for p53 (Panel A) and p21 (Panel B) in viraemic subjects with mild disease (n = 10) and with severe disease (n = 11). Scale bar on each panel is 100 µm. Arrow in panel A demonstrates portal tract lymphocyte positive for p53; note strong hepatocyte nuclear p21 staining in HCV as described previously [344]. Panels C & D demonstrate grouped results of portal tract area positive for p53 (panel C) and p21 (panel D).

Discussion

The downstream effects of lymphocytic telomere shortening were sought. γ-H2AX expression in peripheral lymphocytes correlated with reduced telomere length in the CD8+ compartment, but unexpectedly not in the CD4+ T-lymphocytes. Further, γ-H2AX expression was localised to cells expressing an advanced cell-surface phenotype and associated with a pro-inflammatory cytokine profile i.e. IFN-γ and perforin expression.

The development of a multi-colour flow-cytometric assay to study both γ-H2AX and pStat1 simultaneously, allowed study of the ability of cells expressing γ-H2AX to respond to IFN-α. γ-H2AX positive CD8+ lymphocytes had impaired ability to phosphorylate Stat1 in response to exogenous IFN-α, which was not explained by a failure to express IFNAR-2, IFNAR-1 or down-regulate IFNAR-1.

Nor was there evidence of a failure to phosphorylate Jak1 and Tyk2, associated with the intracellular apparatus of the IFN-α receptor. Thus there was a mechanistic link between telomere shortening, downstream signalling and failure to respond to IFN-α.

Other groups have investigated the effect of DNA damage in the context of HCV previously. Grossi et al utilised the Comet assay, demonstrating DNA damage from peripheral blood lymphocytes in HBV, HCV and alcohol-related cirrhosis [454]. They found that cirrhosis in all three conditions was associated with increased levels of lymphocytic DNA damage and that the level of DNA damage correlated with the Child's Pugh score, linking DNA damage with clinical deterioration. Why circulating lymphocytes should be affected in this way in patients with cirrhosis is unclear, but may be a general effect of inflammation.

The significant correlation between γ-H2AX and telomere length was demonstrated in CD8+ lymphocytes. There was no evidence of a similar correlation in CD4+ lymphocytes. This discrepancy is unexplained. γ-H2AX expression was highest in cells with the most mature cell-surface phenotype. In chapter three I demonstrated that the same CD4+ subset also had the shortest telomere length as compared to more naive subsets. The possibility remains that correlation may exist at the single cell level but that at the less sensitive population level insufficient numbers prevented demonstration of a significant correlation. This lack of correlation between telomere length and γ-H2AX expression in CD4+ T-lymphocytes may explain why there was no difference between mild and severe fibrosis in the portal tract area positive for γ-H2AX. From chapter 5 (Figure 44) it was demonstrated that around 20% of portal tract area was positive for CD4 compared with around 10% positive for CD8.

Possible explanations for the failure of γ-H2AX positive cells to phosphorylate Stat1 in response to exogenous IFN-α include a lesion of either Jak1 or Tyk2 [455], expression of the truncated isoform of IFNAR-2 [456] or suppression of signalling through the suppressor of cytokine signalling (SOCS) family of proteins [123].

Pugnale et al demonstrated that hepatitis delta achieves suppression of IFN-α signalling through inhibition of phosphorylation of Tyk2, thereby preventing stat1 phosphorylation and activation. The results here suggest that this is not the case, and that there may be a failure of the kinase function of the activated intracellular tail of the IFN-α receptor.

Similarly, Pfeffer et al demonstrated that pre-translational processing involving mRNA splicing can result in a truncated isoform of IFNAR-2 being expressed which can suppress signalling through the IFNAR complex to intracellular second messengers [456]. Utilising a murine cell line infected with VZV they demonstrated that signalling through the isoform with the truncated cytoplasmic tail failed to suppress viral replication, in distinction to signalling through the full length IFNAR-2 isoform. Human cells normally express the truncated isoform at low levels compared to the full length biologically active form [457]. Specific antibodies do not exist to the various isoforms of the IFNAR components and therefore I have not been able to study the relative distribution in γ-H2AX+ lymphocytes.

Vlotides et al have previously demonstrated that SOCS 1 and 3 over-expression in a human hepatoma cell line could lead to a reduction of IFN-α induced Stat1 phosphorylation [458]. Therefore it is possible that γ-H2AX positive lymphocytes over-express one or more members of the SOCS family.

Chronic HCV infection is known to modulate the response to IFN-α through pStat1. Gong et al demonstrated that HCV NS5A was able to prevent phosphorylation and nuclear translocation of pStat1 [130]. Lin et al have demonstrated that HCV-transfection of Huh7 cells leads to enhanced degradation of pStat1 [459]. Further, they have shown that the N-terminal portion of HCV core is able to bind to the C-terminal portion of Stat1 blocking both homo- and hetero-dimerisation, thus preventing its intracellular actions [127]. Yao et al extended these findings to peripheral lymphocytes from subjects with HCV [129]. They demonstrated that HCV core protein was

associated with a reduction of pStat1 in peripheral T-lymphocytes but an increase of pStat1 in B-lymphocytes. These findings may explain in part the increased EC_{50} for IFN-α in HCV-infected individuals I have demonstrated here, but not the very significant pathway defects seen in cells manifesting γ-H2AX expression.

It has been assumed that γ-H2AX expression reflects the cellular response to shortened telomeres. However, γ-H2AX is generated at the site of any break in double-stranded DNA. Previous studies have demonstrated that γ-H2AX expression is related to critical telomere shortening but that is not the only explanation [368, 412, 460]. In cells exposed to conditions leading to increased levels of DNA damage, through oncogene or oxidative stress, the direct correlation between γ-H2AX expression and telomere length can be lost. Passos et al demonstrated that senescence of whatever source led to an increase in ROS production which led to increased DSB foci within the cell [330]. Other groups have demonstrated that viruses such as EBV can induce γ-H2AX in infected cells [461]. Further, in a mouse model, infection with the herpes virus γ-HV68 not only led to γ-H2AX induction, but that the presence of γ-H2AX led to increased viral replication [461]. Therefore, viruses may induce cell-cycle arrest in order to subvert cellular machinery to generate a more favourable environment for replication. A direct effect of HCV on γ-H2AX expression is feasible, but was not investigated here.

Other work has provided evidence linking IFN-α signalling pathway to cell-cycle arrest. Townsend et al demonstrated that in Stat1-deficient cells there was a failure of ATM-dependent phosphorylation of p53 and other DNA damage response proteins. However, in the same cell line the phosphorylation of H2AX in response to DNA damage was unimpaired [462].

Moiseeva et al demonstrated that chronic exposure of cells to IFN-β, but not IFN-α, led to an irreversible p53-dependent cell-cycle arrest [463]. It was demonstrated that fibroblasts exposed to

IFN-β for 6 days developed γ-H2AX expression, accumulation of mitochondrial reactive oxidative oxygen species and failure of proliferation.

Demonstrating a change in intracellular signalling pathways in senescent cells is important in the context of the recent descriptions of the change in cellular secretome in senescent cells, the SASP [343]. Kuilman et al demonstrated that cells with oncogene-induced senescence secrete large quantities of cytokines including IL-6. Further they demonstrated that IL-6, as well as IL-8, could act in both autocrine and paracrine manner to reinforce the senescent state of both the secreting and surrounding cells [343]. IL-6 signals through a number of Stat proteins including Stat1 [464]. Whether our finding of impairment in Stat1 phosphorylation extends to IL-6 signalling will be interesting to discover.

Providing a cellular pathway linking advanced fibrosis, telomere shortening, ageing and failure to respond to IFN-α therapy is crucial. However, further work is required to precisely identify the molecular lesion in this signalling pathway. There may be an alteration in the relative concentration of IFNAR-1 and IFNAR-2 on γ-H2AX+ cells or increased expression of truncated isoforms as has been demonstrated in HIV-infected children [457, 465].

Dupont el al using a Jurkat T-cell line demonstrated that IFN-β signalling through the IFNAR-1 / Stat1 pathway induced a refractory period to further signalling through the same pathway lasting around 4 days [466]. Further, Moiseeva el al demonstrated in a mouse model, that chronic exposure to IFN-β induced cell senescence associated with increased expression of γ-H2AX and stabilisation of p53 [467]. Perhaps the failure to demonstrate a stronger correlation between γ-H2AX and telomere length in patients with chronic HCV infection relates to chronic exposure of lymphocytes to IFN-α and subsequent expression of γ-H2AX. However, γ-H2AX+ cells have increased expression of IFNAR-1, which responds to IFN-α, which would suggest that these cells have not been stimulated through this pathway in vivo.

In summary, CD8+ lymphocytes with evidence of double strand DNA breaks accumulate in progressive HCV infection which have the form and function of end-stage differentiated cells and fail to respond to IFN-α due to a post-receptor defect.

Chapter 7 - Overall discussion

Age has been demonstrated to be a critically important prognostic factor in both the natural history of HCV and treatment-related outcome in HCV infection. There have been very few previous studies investigating the immunological basis for this effect. In this thesis I have sought to investigate the link between immune ageing and HCV infection.

Subjects with HCV and severe fibrosis have significantly shorter lymphocytic telomeres when compared to those with mild disease or healthy controls. Multivariate analysis demonstrated that short CD4+CD45RO+ telomere length was associated with severe fibrosis independent of other factors known to have an impact upon the prognosis of chronic HCV infection such as sex and BMI. Importantly, age in this model was not independently associated with severe fibrosis. Therefore, the previous finding of an important relationship between age and fibrosis progression [142, 143, 145], may be explained by telomere length changes.

Further, telomere length changes were associated not only with fibrosis stage but also portal inflammation and blood markers of impaired liver function. In prospective study short peripheral T-lymphocyte telomeres are associated with poor clinical outcome, independent of hepatic fibrosis or age.

There were no differences between study groups in terms of telomerase activity, peripheral or intrahepatic lymphocyte proliferation to explain the changes in telomere length. Telomerase activity was only studied in CD8+ T-lymphocytes, as these assays were performed before the results of subsequent multi-variate analyses were known. These demonstrated that whilst the telomere length changes were greater in CD8+ lymphocytes, these were not significantly associated with fibrosis and clinical outcome, independent of the length of telomeres in CD4+CD45RO+ cells.

One unexpected finding was the demonstration of short peripheral CD8+ lymphocyte telomeres in the HCV-exposed, non-viraemic cohort. There is on-going controversy about whether this group has truly developed sterilising immunity and is now non-infected or whether these individuals have occult infection with compartmentalised viral replication not detectable by current PCR techniques in serum. Despite short telomeres, no individual in this group developed any of the clinical endpoints seen in the group with severe fibrosis over a similar time-course. The possibility remains that this result is spurious; certainly increased γ-H2AX expression in CD8+ lymphocytes indicative of down-stream telomeric signalling was not detected in this group. However, CD8+ telomere length was analysed in 32 such individuals and the effect was greater than that seen in the severe fibrosis group.

As a consequence of these findings further experiments were undertaken to establish the nature of the hepatic lesion in subjects with HCV antibody without viraemia. More than 95% of these individuals had abnormal liver histology, although the changes were usually mild. Significantly, whilst the non-viraemic group had significantly shorter CD8+ telomeres than subjects with viraemic HCV and mild fibrosis, the groups had similar levels of intrahepatic lymphocyte proliferation, suggesting that chronic intrahepatic lymphocyte turnover was not the cause of the telomere length changes demonstrated.

One question regarding the telomere analysis is whether this was a feature of chronic liver disease *per se* or was specific to HCV infection. A group with chronic liver disease other than HCV was considered. However, many of the other liver diseases are restricted to particular demographics; for example patients with autoimmune liver disease or primary biliary cirrhosis are mostly middle aged females, subjects with haemochromatosis are usually forty to fifty-five year old males and those with non-alcohol related fatty liver disease carry many risk factors for telomere shortening. The second drawback of this approach is that in many other hepatic diseases liver biopsy is not performed unless there is significant concern about cirrhosis. This is not the case in HCV infection where most cases

are offered liver biopsy to stage and grade disease accurately. Therefore, subjects with a liver biopsy in the context of other liver diseases could represent a skewed cohort.

A second potential criticism of the telomere changes demonstrated in HCV infection is the lack of data with regard to telomere length changes over time. It is unclear whether those individuals who develop significant fibrosis had shorter telomeres than the population median before HCV infection and were therefore programmed from time of acute infection to develop significant fibrosis (Figure 62A). Other possibilities include a substantial fall in telomere length at the time of acute infection (Figure 62B). However, this appears unlikely as the cytokine release attendant with significant turnover of the entire peripheral lymphocyte compartment would lead to patient symptoms, whereas acute HCV is almost always asymptomatic in immunocompetent individuals.

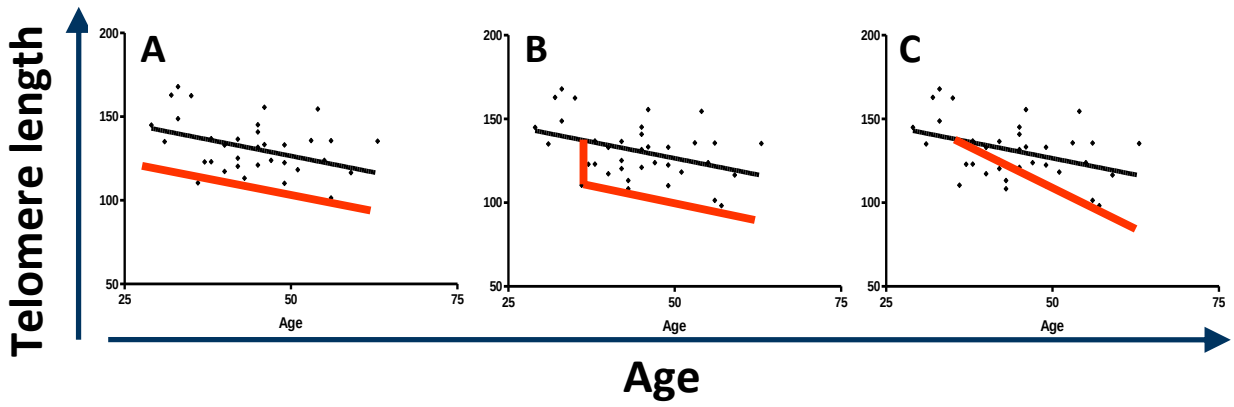

Figure 62. Possible mechanisms for telomere length changes over time in HCV infection. Black dots and black regression lines represent telomere length changes with progressive ageing in a healthy population; red lines represent possible telomere length changes explaining short telomeres seen in subjects with severe HCV-related fibrosis. Panel A: HCV subjects had short telomeres that predate HCV infection and rate of telomere length change over time is unchanged. Panel B: Acute HCV infection is associated with sudden decline in telomere length in the global T-lymphocyte population, followed by decline at a normal rate. Panel C: Patients developing significant fibrosis have an accelerated rate of telomere length loss compared to a healthy population.

The third possibility is that subjects developing significant HCV-related fibrosis have accelerated telomere loss over time when compared to a healthy control population (Figure 62C). There were no demonstrated differences in Ki67 staining in either peripheral or intrahepatic lymphocytes

between subjects with mild and severe liver disease. Certainly increased oxidative stress in the context of viraemic HCV [335] could accelerate telomeric decline in the context of similar levels of cellular proliferation between the groups. The gradient of the telomere vs age relationship was steeper in the severe cohort supporting the third model. However, as telomere length has significant variance across a population no statistical differences were demonstrated. With larger study numbers I suspect that this model would be demonstrated to be the correct one.

Duration of HCV infection was not addressed in this thesis. As between 60% and 70% of the subjects acquired HCV through intravenous drug usage and acute HCV infection is mostly asymptomatic the time point of acquisition was in most cases uncertain. A further 10% - 25% had no known risk factor for acquisition. As described in the introduction, recent data from UAPMP has found that only 22% of IDVUs acquire HCV during their first three years of intravenous drug usage. Therefore, taking the time-point of acquisition [142] as the start of injecting behaviour would have been incorrect. Whilst analysing a single time point of infection cohort such as the Dublin Anti-D cohort would allow inferences about duration of infection and immune ageing, a 'real-world' cohort as recruited herein would not.

Demonstrating telomere length changes prospectively through the flow-FISH assay proved unfeasible over the time course of a PhD thesis. The telomere changes would have been too small to detect and fibrosis progression not re-established through a second liver biopsy. This question will be further addressed in future work investigating telomere length from previously acquired liver biopsies taken five years apart in subjects who either did or did not have progressive hepatic fibrosis. I would hypothesise that subjects with progressive liver disease will have greater telomere length loss compared to subjects who did not have progressive liver disease.

It was demonstrated prospectively that lymphocyte telomere length and CD4+ telomere length in particular predicted treatment response, independent of other known predictive factors such as

viral load and viral genotype. Whilst these virus-derived variables are in routine clinical use to guide anti-viral therapy, no such factors yet exist to describe the immunological abilities or reserve of the infected host. Age is known to be important and that was confirmed here; no subject older than fifty-five years of age successfully cleared HCV on anti-viral therapy.

The data are consistent with the only other similar immunological investigation in this area. Manfras et al demonstrated that increased CD57 expression and oligoclonality of CD8+ lymphocytes, changes of immune ageing and senescence, predicted a failure to respond to anti-viral therapy [399]. These data and the work of Manfras et al should stimulate debate about whether we should be considering IFN-α therapy earlier in the course of HCV infection; a change that is already taking place in our centre. Treating patients at a younger age with lower levels of fibrosis is known to lead to better outcomes with higher rates of virological response.

Despite multiple attempts, it proved impossible to develop a reliable assay to demonstrate HCV-specific telomere length in subjects with viraemic HCV infection. The frequency of the cells in most subjects was too low to permit accurate inferences about telomere length. One avenue for future exploration is measuring telomere length in CD8+ lymphocytes that express the NK cell marker CD161. Recent work, published after my experimental work had ceased, suggested that CD8+ expression of this marker was specific for a subset of HCV-specific CD8+ lymphocytes [468]. However, choosing adequate controls for comparison could be difficult. These cells may be functionally silent; comparing with cells responding to other antigens through cytokine expression may be an erroneous comparison.

All the telomere length changes demonstrated were applicable to all T cells i.e. the global peripheral T-lymphocyte compartment. This raises significant questions about how such telomere length changes occur in the absence of HCV-antigen driven chronic cell turnover. Previous work from UCL has demonstrated that CMV can lead to telomere length changes and cell-surface phenotype

changes in both CMV-specific and non-CMV-specific CD4+ lymphocytes [356]. The unproven hypothesis is that chronic infection is associated with chronic inflammation leading to low-grade cytokine release. It has been suggested that this inflammatory cytokine release leads to either non-specific cell turnover or long-term inhibition of telomerase activity preventing its normal action of maintaining telomere length in memory cell populations. Certainly, the large Scandinavian population studies have demonstrated that past CMV infection in the healthy elderly is associated with an increased risk of dying during prospective follow-up [353, 355], leading to the suggestion that a CMV vaccination program might be justified to protect the whole immune system from the chronic effects of CMV infection [357, 410].

Similar concerns arise when discussing the findings of increased CD8+ lymphocyte γ-H2AX expression in those with significant HCV-related liver disease. As the expression occurs in the global T-lymphocyte compartment, what is the relevance for adaptive anti-HCV immunity?

Given that we have demonstrated a significant link between progressive HCV infection and shortened telomere length and other groups have demonstrated similar relationships to hepatocyte telomere length [447, 469], study of the changes in other cell types would be of interest. Certainly it is known that HCV infection is associated with B-cell non-Hodgkin's lymphoma [470], rather than T-cell neoplasms suggesting that B-cell telomeres could be significantly affected in the context of chronic HCV infection.

Criticisms could be made that the relationship between telomere length and γ-H2AX expression was not demonstrated at the level of the single cell basis. However, γ-H2AX expression could not be detected following the heat step integral to the DNA probe annealing in the telomere length assay. Further, because the cells could only be identified after fixation and permeabilisation in methanol this prevented cell sorting or separation to further investigate the nature of these cells. Further,

because the cells were fixed, Western blotting or PCR analysis to investigate intracellular second messenger pathways or telomere length by PCR was impossible.

The failure to demonstrate correlation between γ-H2AX expression and telomere length in the CD4+ population is puzzling given that subjects with severe fibrosis had the shortest CD4+ telomeres (chapter three). Further, telomeres were shorter in cells that expressed CD45RO or lacked CD27, precisely the same phenotype as CD4+ γ-H2AX+ cells. The expression of γ-H2AX in extra-telomeric DNA related to non-telomeric DDR signalling or replication-associated foci is the likely explanation [368, 412, 460].

It was demonstrated that cells expressing γ-H2AX had the form and function of cells with end-stage differentiation, with a high IFN-γ and low IL-2 expression pattern. Further, γ-H2AX+ CD8+ lymphocytes fail to respond to exogenous IFN-α by phosphorylating Stat1. On further analysis the underlying defect was found to be post-receptor; further assays demonstrated that the defect on these cells lay in the ability of the intracellular tail of the IFN-α receptor to phosphorylate Stat1.

It is unlikely that the lesion demonstrated in the IFN-α signalling pathway is isolated to one single pathway in these cells. If one were to investigate several signalling pathways, it is likely that signalling block occurs in other pathways. However, I chose to investigate the IFN-α signalling pathway due to its relevance to both the natural history and treatment outcome of chronic HCV infection. Similarly it is not likely that the failure to respond to IFN-α suddenly develops in this lymphocyte subset. Rather, that responsiveness to IFN-α may drop with progressive cellular differentiation and clonal age and that cells which express γ-H2AX+ are just one example along a spectrum of progressive pathway failure.

To further investigate the effect of cellular senescence upon IFN-α signalling would require the use of cell lines and employing more sensitive assays such as western blotting. As the γ-H2AX positive

lymphocytes proved difficult to separate from the global lymphocyte compartment, these methods were not employed in this project.

Much previous work has demonstrated the effect of different HCV proteins on the IFN-α signalling pathway; both HCV core and NS5A have been demonstrated to prevent Stat1 phosphorylation, dimerisation and nuclear translocation [127, 130]. Of great interest is the recent work of Tarakanova et al demonstrated the effect of a gamma-herpes virus upon production of γ-H2AX in macrophages [461]. They were able to demonstrate that viral encoded proteins could lead to phosphorylation of histone 2. Further, they were able to demonstrate that formation of γ-H2AX greatly enhanced viral replication. Whether HCV is able to modulate the cell cycle status or double strand DNA break pathway of hepatocytes is unknown. However, previous work from our laboratory has demonstrated increased levels of hepatocyte cell-cycle arrest in subjects with significant HCV-related hepatic fibrosis [344].

The issue of whether HCV can infect lymphocytes at all is controversial [471, 472]. There is perhaps more evidence for HCV infection of B-cells than T-cells. However, a mechanistic link between HCV-encoded proteins and the development of γ-H2AX expression in CD8+ lymphocytes has not been explored.

Future work in this area would target further characterisation of this pathway in order to establish the precise pathway location of blockage. I would also aim to study other pathways in these cells to establish the specificity of the pathway lesions. I would also aim to perform further cell phenotyping to try to establish a specific cell surface marker than correlated with γ-H2AX expression permitting Western Blotting or PCR investigation of intracellular secondary messengers; if these cells could be isolated without fixation then microarray technology would permit rapid analysis of gene expression in comparison to the global CD8+ population.

The recent data from the genome-wide association study by Ge et al demonstrating a polymorphism in the IFN-λ gene associated with anti-viral treatment success may change the context of our results [157]. It has for many years been assumed that the most important endogenous and exogenous anti-viral cytokine was IFN-α. However, in future treatment of HCV infection may involve IFN-λ; clearly the response of γ-H2AX cells to IFN-λ will become an important experiment to perform.

Of further interest from the data I have generated in this thesis is the post-transplantation course of subjects with HCV infection. Once subjects have developed cirrhosis, and as I have demonstrated significant immune changes within the global T-lymphocyte compartment, they may undergo transplantation. Is it reasonable that we expect such subjects to have a similar life expectancy to the general population when we have only addressed the nature of their liver condition? After transplantation these individuals may well have an immune system with significant impairment, related to their pre-transplant condition. In fact, subjects transplanted for HCV infection have one of the worst post-transplantation outcomes in terms of both patient and graft survival [473] when compared to subjects transplanted for other conditions, including HBV. Current work hopes to investigate the effect of transplantation upon lymphocyte telomere length. It will be interesting to see whether lymphocyte telomeres are able to elongate after transplantation. Further, if they do not, does lymphocytic telomere length predict post-transplant sepsis, graft survival and death?

Many of the problems inherent in this project have related to the study of immune ageing and senescence. Because of the complex interplay between pathogen and host over many years, even decades, this field do not lend itself for prospective interventional studies. Similarly, small animal models such as mouse and rat have such a short generation time that they do not use replicative ageing mechanisms such as telomere shortening to protect chromosomes and the organism. Similarly such organisms do not support HCV replication, therefore small animal experiments were not feasible. Similarly, whilst recent advances in the in vitro study of HCV with the replicon system

have allowed great advances in knowledge of HCV entry, translation and replication, they simply would not allow study of the long-term interplay between pathogen and host.

The only large animal model of HCV infection is the chimpanzee, which does use replicative ageing. Whilst this model would be suitable for the study of immune ageing from the time-point of acquisition the numbers of animals needed to demonstrate small changes over the chronic phase of infection make this approach uneconomic.

Therefore, whilst human studies are slow and generally descriptive rather than interventional, they currently represent the only approach to studying immune ageing in the context of HCV infection.

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