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A STUDY OF NOVEL BIOMARKERS OF HEPATITIS C-RELATED LIVER INJURY

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A thesis submitted in fulfilment of the requirements for the degree of Master of Philosophy

Faculty of Medicine
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March 2013
Preface

Unless otherwise acknowledged, the work described in this thesis was carried out by the author in the A.W. Morrow Gastroenterology and Liver Unit of Royal Prince Alfred Hospital and also at Medsaic laboratories, National Innovation Centre, Eveleigh, NSW, Australia. The work was conducted between March 2006 and March 2013.

None of this work has been submitted previously for the purpose of obtaining any other degree.

Wassim Abdul Rahman
March 2013
Acknowledgments

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Thanks are also due to Dr Alex Sharland for her keen interest and valuable thoughts. I am also very grateful to Dr Jean Yang for her very important contribution in performing complex statistical analyses.

A large part of this thesis involves the use of novel technology, the CD antibody microarray, performed at Medsaic laboratories. Special thanks go to Pauline Huang for her assistance in performing the assays, and Larissa Belov and Jeremy Chrisp for their interest in my work.

Finally, I would like to thank my parents and family for their support and encouragement, and making it possible for me to be where I am today, and to my wife for her enormous love and patience.
Abstract

Approximately 170 million people worldwide are chronically infected with the hepatitis C virus (HCV). Cirrhosis occurs in up to 20% of these people with a 30% risk of decompensation at 10 years and up to 4% annual risk of hepatocellular carcinoma (HCC). Cirrhosis related to chronic infection with HCV is the most frequent indication for orthotopic liver transplantation and it has been projected that the number of candidates referred for transplantation will double by 2020. Unfortunately HCV recurrence is universal following transplantation and many patients will experience aggressive and rapidly progressive disease recurrence with poorer overall outcomes compared to transplantation for other non-HCV indications. This thesis comprises 3 related studies that are presented as individual chapters with the main focus being an assessment of factors which may impact on the course of HCV recurrence post-transplant. The first 2 studies (Chapters 3 and 4) are published and are largely presented as they appear in press.

The first study in this thesis (Chapter 3) was a retrospective analysis of 118 consecutive HCV-positive liver transplant patients with a median duration of follow-up of 32.4 months. Both univariate and multivariate analysis of the effect of recipient, donor, surgical, and viral factors on graft and patient outcomes were undertaken. In the multivariate analysis, peak viral RNA $\geq 10^7$ in the first year post-transplant and increased donor age ($\geq 50$) were independent predictors of diminished patient survival, while azathioprine use was associated with improved outcomes. Exposure to antirejection therapy was an independent predictor of diminished graft survival. This study highlights the importance of high post-transplant viral loads in the pathogenesis and prediction of severe HCV disease recurrence and supports routine measurement in the early post-transplant period.

The second study (Chapter 4) was a cross-sectional pilot study using the recently developed CD antibody microarray to characterise human liver diseases. The assay, which uses a live cell-capture technique, enables a semi-quantitative immunophenotype
of peripheral blood mononuclear cells. The study population included patients with HCV, NASH, alcohol-related liver disease (ALD), and a normal group. Hierarchical clustering and principal components analysis demonstrated disease-specific consensus patterns of expression of CD antigens for patients with chronic liver disease and in particular, the ability to separate major stages of liver disease. This lead to the third study (Chapter 5) in which serial CD antigen expression profiles were performed in 16 consecutive patients undergoing liver transplantation for active HCV infection. Assays were performed during the Pre-transplant (day0), Early (d3–week2), Mid (w4–w10), and Late (>w12) phases. Four different definitions were used to define severe disease recurrence based on protocol liver biopsies and peak viral load within the first year post-transplant. Differential antibody expression was most significant in the pre-transplant phase, irrespective of the definition used for severe HCV recurrence, suggesting that the pre-transplant CD antigen expression profile may be the greatest determinant of recurrent HCV disease severity post-liver transplantation.

The findings in this thesis have demonstrated utility of the CD antibody microarray in the study of human liver disease and have shed light on the importance of post-transplant viral loads in determining the severity of post-transplant HCV recurrence. Further exploration may give additional insight into disease mechanisms contributing to the clinical problem of HCV recurrence.
Publications Arising from this Thesis


Abbreviations

AZA, azathioprine
ALD, alcoholic liver disease
CD, cluster of differentiation
CTL, cytotoxic T lymphocytes
CI, confidence interval
CNI, calcineurin inhibitor
CsA, cyclosporin A
HCC, hepatocellular carcinoma
HCV, hepatitis C virus
HR, hazard ratio
LSEC, liver sinusoidal endothelial cells
LT, liver transplantation
MMF, mycophenolate mofetil
MPPT, methylprednisolone
NASH, non-alcoholic steatohepatitis
NK, Natural Killer
PBMC, peripheral blood mononuclear cells
PCR, polymerase chain reaction
RNA, Ribonucleic acid
TAC, tacrolimus
TCR, T cell receptor.
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CHAPTER 1

INTRODUCTION AND
LITERATURE REVIEW
1.1 HEPATITIS C VIRUS

1.1.1 Molecular Virology

1.1.1.1 HCV Genome

HCV is a single-stranded positive-sense RNA virus that has been classified as the genus *Hepacivirus* in the Flaviviridae family. All members of this family are positive-strand RNA viruses that encode a single long polypeptide which is cleaved post-translationally [1]. The HCV genome is an uncapped, linear molecule of approximately 9600 nucleotides (nt) with a single open reading frame (ORF) that encodes a viral polypeptide precursor of approximately 3000 amino acids (Figure 1.1). The ORF is flanked at the 5′ and 3′ ends by short highly structured untranslated regions (UTRs). The polypeptide precursor is cleaved by cellular and viral proteases into structural proteins (nucleocapsid core-C, envelope proteins E1 and E2), the hydrophobic peptide p7, and the non-structural (NS) proteins (NS2, NS3, NS4a, NS4b, NS5a, and NS5b)[2]. The 5′-UTR and sections of the 3′-UTR are the most conserved parts of the HCV genome and contain signals for replication and translation. The 5′-UTR has a length of about 340 nt and contains an internal ribosomal entry site (IRES) required for translation of the HCV genome[3]. This binds the 40 S ribosomal subunit and evidence suggests that part of the IRES (domain II) induces conformational changes in the 40 S subunit that is important for assembly of the 80 S ribosome[4]. The domain II sequence overlaps with RNA signals essential for replication suggesting a possible role in the regulation of replication[2]. The 3′-UTR consists of three distinct regions - a variable region approximately 40 nts long downstream of the HCV coding sequence, a polypyrimidine tract [poly(U/UC)] of variable length, and a highly conserved 98 nt sequence designated X-tail, which has the potential to form three stem-loop (SL) structures. Studies have shown that both the X-tail and the poly(U/UC) tract are required for viral replication in cell culture and for viral infectivity of the viral genome *in vivo* [2]. The NTRs contain *cis*-acting RNA elements (CRE) essential for replication[5]. Recently a CRE has also
been identified in the 3′-terminal coding region of NS5B (designated 5BSL3.2). This CRE forms a long distance RNA-RNA interaction with SL2 in the X-tail forming a ‘pseudoknot’ structure at the 3′ end of the HCV genome that is essential for viral replication [6].

1.1.1.2 Structural and Non-Structural Proteins

Structural Proteins

The structural proteins mature by host cell signal peptidase cleavages between C/E1, E1/E2 and E2/p7. In addition signal-peptide peptidase is required to release core from the E1 signal peptide.[7]

The HCV core protein is a highly basic, RNA-binding protein thought to serve as a nucleocapsid protein. It has been reported to interact with various host cell signaling pathways and to affect such functions as gene transcription, lipid metabolism and apoptosis[8]. It has also been implicated in steatosis[9] and HCC development[10]. HCV also encodes an alternative form of core protein called ARFP (alternate reading frame protein), which is generated by ribosomal frameshifting or internal initiation of translation of an alternative reading frame within the core gene. These seem to be dispensable for RNA replication[2].

The core protein and viral RNA are encapsulated in a lipid envelope into which E1 and E2 are embedded. The envelope proteins are liberated from the viral polyprotein and undergo extensive glycosylation forming noncovalent heterodimers[11]. These E1 and E2 regions demonstrate the highest rate of mutations at both the nucleotide and amino acid levels, particularly in the hypervariable region (HVR) at the amino terminal of E2. E2 has been shown to bind specifically to CD81, a possible HCV receptor.

p7 is a small (63-amino-acid) protein that lies between the structural and nonstructural proteins. It functions as a virus encoded ion channel and seems to be critical for in vivo infectivity[12]. It is sensitive to inhibition by amantadine and may be a target for future drug development[11].
Non-Structural Proteins

The carboxy-terminal of NS2 together with the N-terminal third of NS3 form a protease (NS2/3 protease) essential for mediating cleavage between NS2 and NS3[7]. NS2 is not required for RNA replication in model culture systems but may play a role in viral assembly[11].

NS3 is a multifunctional protein, with an N-terminal serine protease domain and a C-terminal RNA helicase domain. The NS3 serine protease is responsible for the downstream cleavage of the nonstructural region. NS4A, a 54-amino acid polypeptide is required as a cofactor for the NS3 serine protease[8]. NS4A serves multiple purposes. It anchors NS3 to intracellular membranes through an N-terminal hydrophobic peptide and contributes a β-strand to the protease domain allowing its complete folding. It also stabilizes the protease against proteolytic degradation and enhances protease activity by changing the geometry of the active site residues[2]. It has recently been shown that NS3 can counteract cellular antiviral defenses by inhibition of intracellular signaling pathways, namely involving TLR-3 (Toll-like receptor-3) and RIG-1 (retinoic acid inducible gene-1)[13].

The role of the NS3 helicase is not known. It may be involved in initiation of RNA replication by unwinding stable terminal stem-loop structures and may eliminate stable RNA secondary structures within the template strand allowing processing of the replication complex. Also it may be required for dissociation of nascent RNA strands from their template during RNA synthesis and displacement of bound proteins that may interfere with RNA synthesis [2, 7].

NS4B is a highly hydrophobic protein that localizes to intracellular membrane compartments. It induces specific membrane alterations, forming structures which serve as the intracellular scaffold of the HCV replication complex. This has been termed the membranous web[2, 14].

NS5A consists of an N-terminal amphiphatic α-helix serving as a membrane anchor and three cytosolic protein domains. Domain I appears to be involved in RNA binding and possibly in the regulation of the replication complex. Domain II may have a role in modulating the IFN response[2]. Two findings suggesting the importance of NS5A in HCV replication are the high
concentration of cell culture adaptive replicon mutations within NS5A and also the impact of NS5A phosphorylation on replication efficiency[8].

NS5B is an RNA-dependent RNA polymerase (RdRp) and the catalytic centre of the replication complex. It is membrane associated through a C-terminal transmembrane domain, which is essential for RNA replication[15]. The crystal structure of the catalytic domain reveals a typical polymerase shape with finger, palm, and thumb subdomains surrounding a completely encircled active site, and creating a tunnel through which single-stranded RNA molecules are directly guided to the active site[2, 16].
Figure 1.1. **HCV genome and gene products.**

**a,** The structure of the viral genome, including the long open reading frame encoding structural and nonstructural genes, and 5′ and 3′ UTRs. The polyprotein processing scheme is shown below. Closed circles refer to signal peptidase cleavage sites; the open circle refers to the signal peptide peptidase cleavage site.

1.1.1.3 HCV Life Cycle

A schematic of the HCV life cycle is shown in Figure 1.2.

The exact mechanism of HCV cell entry has not been determined. HCV E2 binds with high affinity to the large external loop of CD81, which is likely to be involved in mediating cell entry, although not sufficient in isolation. Other candidate co-receptors including the low-density lipoprotein receptor (LDLR), scavenger receptor class-B-type-I (SR-BI), claudin-1 and occludin, and L-SIGN and DC-SIGN have also been identified as possible components of a HCV receptor complex (discussed further below)[7]. Binding of the viral envelope glycoprotein to the hepatocyte surface triggers endocytosis of the viral particle. Uncoating and penetration of the virus into the cytoplasm putatively occurs via pH dependent fusion between the viral envelope and endosomal membrane. The low pH in the endosomes induces conformational changes in the envelope glycoproteins, exposing fusion peptides, which are believed to be vital to this fusion process[17].

Upon entry of the viral genome, IRES-mediated translation takes place using the host cell’s translational machinery. Translation takes place at the rough endoplasmic reticulum, where the HCV polyprotein is subsequently cleaved by host cell and viral proteases. Formation of a membrane alteration, termed the membranous web occurs which serves as the platform for RNA replication. The assembled replication complex (including NS5B) initially generates negative strand RNA, which then serves as a template for production of excess amounts of positive RNA. These are either used for translation, further replication or packaged into new virus ready for release. The precise factors controlling these events, both host and viral, are not currently clear [2, 11].

The RNA-dependent RNA polymerase, NS5B, results in HCV replication at the enormous rate of $10^{12}$ virions per day, and through the lack of a proof-reading function, a high mutational rate that results in considerable heterogeneity throughout the genome. This has led to genetically distinct groups of HCV isolates that have arisen during the evolution of the virus called
genotypes. Nucleotide sequencing has shown variation of up to 34% between genotypes[18]. There are 6 major genotypes (designated by numbers), and more than 70 subtypes (designated by a lower case letter) within these major genotypes. The HCV genotype of an infecting HCV strain in an individual does not change over time and therefore only needs to be determined once. There is significant geographic variation with genotypes 1a and 1b most prevalent in the United States and Europe, accounting for approximately 57% and 47% of HCV infections respectively. In Egypt, genotype 4 is most prevalent accounting for more than 90% of infections[19]. In Australia 55% of the chronic HCV population are infected with genotype 1[20].

Within an infected individual, mutations that occur during viral replication can generate numerous closely related minor viral variants known as quasispecies. The development of quasispecies is one mechanism by which the virus can evade immune system and establish chronic infection. This may possibly occur due to the loss of epitopes, impaired binding of epitopes to MHC molecules, or altered recognition of variant peptide-MHC complexes. Genetic diversification in the hypervariable region of E2 during the acute phase of HCV infection is associated progression to chronic hepatitis, whereas evolutionary stasis is associated with a self-limited course of infection [18, 21].
Figure 1.2. The Hepatitis C Virus life cycle. Cell entry of HCV is followed by translation and processing of the HCV polyprotein to form the HCV replication complex. This initially generates negative strand RNA, which then serves as a template for production of excess amounts of positive RNA. These are either used for translation, further replication or packaged into new virus ready for release. See text for details. From Pawlotsky et. al. *Gastroenterology* 2007[22].
1.1.1.4 HCV Receptors

Knowledge about HCV cell binding and entry is derived mainly from *in vitro* model systems and could potentially lead to new approaches in antiviral therapy and vaccine development. These include recombinant HCV envelope glycoproteins, HCV-like particles (HCV-LPs) – generated by self-assembly of HCV structural proteins in insect cells, HCV pseudotyped particles (HCVpp) - with functional HCV envelope glycoproteins inserted onto retroviral core particles, and cell-culture-derived HCV (HCVcc)[17].

E1 and E2 are essential for host cell entry. HCVpp with both E1 and E2 demonstrated significantly greater infectivity than with either glycoprotein alone[23]. E2 appears to play a key role in HCV-host cell interactions, particularly the HVR-1 domain, the deletion of which results in decreased HCVpp infectivity in cell culture[17].

CD81 is a cell surface protein and member of the tetraspanin family that is expressed on most human cells and involved in pleiotropic activities including cell adhesion, motility, metastasis, cell activation, and signal transduction[24]. It has been identified as an E2 binding molecule, and anti-CD81 antibodies have been shown to inhibit HCVpp and HCVcc entry into Huh-7 hepatoma cells and human hepatocytes[17]. Recent studies using infectious HCVcc suggest that CD81 plays a key role in HCV entry and most likely acts at a post-attachment step[25]. However, the almost ubiquitous nature of CD81 expression, together with the specific tissue tropism of HCV (mainly hepatic, also dendritic/B cell) and the ability of CD81 to bind E2 in species resistant to HCV infection suggests that CD81 alone is not sufficient to mediate cell entry[8, 17].

SR-B1 is highly expressed in liver and steroidogenic tissues as well as dendritic cells, and is involved in bidirectional cholesterol transport across the cell membrane of both native LDL and HDL as well as modified lipoproteins such as oxidized LDL[17, 26]. SR-B1, which can bind to E2, plays an important role in HCV attachment and entry, as suggested by anti-SR-B1 antibody mediated inhibition of HCVpp infectivity [27].
Recent studies highlight the importance of the tight junction complex in the viral entry process. Claudin-1 and occludin, components of cellular tight junctions, are required for HCV cellular entry and act late in the entry process, after virus binding and interaction with CD81 [28, 29].

Other studies demonstrate that plasma lipoproteins including LDL and VLDL are associated with HCV RNA and core protein. Infectivity of these lipo-viro-particles (LVP) can be increased by up-regulation of LDLR and may be mediated by endogenous proteins rather than viral components, possibly providing a mechanism of escape from the humoral immune response[30]. LVPs are triglyceride (TG) rich and contain the VLDL components apolipoprotein B (apoB) and apoE. ApoE is involved in HCV particle morphogenesis and is essential for HCV infectivity. Apolipoprotein B (apoB) is the main protein constituent of LDL and very low-density lipoprotein (VLDL). ApoB-associated lipoproteins, including HCV-LVP are cleared post-prandialy by HCV receptors in the liver[31]. Furthermore, association of HDL with HCVpp enhances SR-B1 guided cell entry and possibly through a complex interplay also involving HVR1 could protect virions from neutralizing antibodies [32].

Another cell surface molecule proposed to mediate HCV binding is highly sulfated heparan sulfate (HS). HS are linear glycosaminoglycan chains on cell surface proteoglycans thought to have a potential role in the regulation of cell growth, differentiation and cell adhesion. They also provide primary docking sites for the initiation of various viral infections and have tissue and species specific composition[33]. Binding of specific HS configurations to viral epitopes in E2 appears to be an important factor in HCV infection and tropism. HCVpp binding to target cells is inhibited by highly sulfated HS, and HCVcc binding or proteoglycans infection is dose-dependently inhibited by heparin - a structural homolog of highly sulfated HS or by pretreatment of cells with heparinases[25]. HCV-HS binding is inhibited by antiviral antibodies derived from HCV-infected individuals, suggesting that this is a target of antiviral host immune responses[33].

The mannose binding C-type lectins DC-SIGN (CD 209) and L-SIGN (CD 209L) have been shown to bind E2 with high affinity. L-SIGN is highly expressed on liver sinusoidal endothelial cells (LSECs) but not hepatocytes, while DC-SIGN is expressed on dendritic cells. HCVpp
captured by L-SIGN+ or DC-SIGN+ can efficiently transinfest adjacent hepatocytes and thus may be an essential component of the HCV receptor complex[17, 34].

1.1.2 Immunopathogenesis of HCV infection

1.1.2.1 Innate Immunity

1.1.2.1.1 Interferon System

Interferon (IFN)-based therapy is currently the standard of care for persistent HCV infection, but can only achieve sustained response rates of 40% to 80% - depending on host and viral factors. Viral infection of mammalian cells triggers complex intracellular signaling events leading to IFN production and a cellular antiviral state. Secreted IFNs act on neighboring cells, causing them to express antiviral proteins. HCV has the ability to evade and antagonize this first line of immune defense at different levels and thus is able to establish persistent infection and resistance to IFN therapy[13].

The type 1 interferon system comprises mainly IFN-α, of which there are at least 13 subtypes, and also a single IFN-β. The importance of this powerful intracellular defense system is illustrated in knockout mice which have target deletions in the type 1 IFN receptor and despite having intact adaptive immunity, succumb to viral infections[35, 36]. Type 3 interferons (IFN-λ1-3) belong to the interleukin-10 (IL-10) superfamily and are functionally closely related to type1 IFNs[37]. Recently, four genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) near the IL28B gene (encoding IFN-λ3) to be strongly associated with spontaneous and IFN treatment-induced clearance of HCV infection[37].

On the molecular level, the induction of IFN gene expression initially involves the presentation of pathogen-associated molecular patterns (PAMPs) by the infecting virus to specific PAMP receptor factors in the host cell (see Figure 1.3). The viral RNA of HCV contains single-stranded
(ss) and double-stranded (ds) RNA PAMP signatures which can trigger a host response[13]. PAMP receptors in hepatocytes which are triggered by dsRNA (intermediates of viral transcription) include retinoic-acid-inducible gene I (RIG-I) which senses cytoplasmic dsRNA and Toll-like receptor 3 (TLR3) which senses endosomal dsRNA [13, 38, 39]. Engagement of these receptors results in activation of downstream factors including the IκB kinase (IKK)-related kinases, IKKε and TBK-1, which phosphorylate the transcription factors IRF-3 and IRF-7[13, 40]. IRF-3 and IRF-7 are members of the IFN response factor (IRF) family and play a central role in the activation of the IFN promoters. IFN-λ2/3 and IFN-α expression is induced by IRF7 and IFN-λ1 and IFN-β expression is induced byIRF3 and IRF7[41]. Phosphorylated IRF-3 and IRF-7 homo-dimerize and translocate to the cell nucleus resulting in IFN transcription and then secretion from the infected cell[35]. Nuclear factor κB(NF-κB), another transcription factor activated by this pathway, also upregulates IFN gene expression as well as chemokines and proinflammatory cytokines important in the inflammatory response to HCV[42].

Secreted IFN, through autocrine and paracrine processes, will bind to and activate different type 1 (IFN α/β) and type 3 IFN λ (Il28-Rα/IL10-R2) receptors [13, 43]. Conformational changes in the intracellular parts of these receptors activate the same JAK-STAT signaling pathway[35]. The receptor-associated Janus kinase (JAK) family members Jak1 and Tyk2 phosphorylate the signal transducer and activator of transcription (STAT) proteins. Phosphorylated STAT1 and STAT2 assemble with a third factor IRF-9 to form a heterotrimer known as IFN-stimulated gene factor 3 (ISGF-3). ISGF-3 translocates to the nucleus to bind to IFN-stimulated response elements (ISRE) within the promoter/enhancer regions of the IFN-stimulated genes (ISGs) leading to their transcription[35]. Although type 1 and type 3 IFN signaling converge in the JAK-STAT pathway, their binding to different receptors could result in different kinetics of ISG expression [44]. In addition, the tissue distribution of these receptors differ significantly in humans, with the IFN-α receptor broadly expressed while Il28-Rα/IL10-R2 expression is limited to hepatocytes, epithelial cells, and plasmacytoid dendritic cells[41].

Hundreds of ISGs are expressed in response to viral infection and their products have antiviral, antiproliferative and immunomodulatory functions which limit HCV replication. These include enzymes, transcription factors, cell surface glycoproteins, chemokines and cytokines[35].
IRF-7 is an ISG and also a transcription factor. It is activated through pathways that overlap with IRF-3 activation. IRF-7 phosphorylation and translocation to the promoter region of IFN genes result in the production of various IFN subtypes as second-wave IFNs. Through the IFN-α/β and IFN-λ receptors and the JAK-STAT pathway these signal further ISG expression, thus establishing a positive-feedback loop that amplifies IFN production and antiviral activity[13, 35].

Protein kinase R (PKR) is an ISG and a dsRNA binding protein whose RNA-dependent activation phosphorylates the alpha subunit of the eukaryotic translation initiation factor eIF2, resulting in suppressed translation of cellular and viral RNA. PKR can be activated by HCV core protein to bind RNA and thus exert its antiviral effects [13, 45].

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible) is an ISG, which is induced by type I, II, and III IFNs or after infection with a range of DNA and RNA viruses. Viperin interferes with replication of hepatitis C virus by binding to lipid droplets that are essential for hepatitis C virus replication[46].

The ISG56 product p56 binds to and inhibits the eukaryotic translation initiation factor eIF3 resulting in reduced viral RNA translation [13].

Another product of ISG expression is the 2-5 oligoadenylate synthetase (OAS)RNaseL system. Once activated, this pathway through the actions of RNaseL is able to cleave HCV RNA into non-functional products leading to viral inhibition [13, 47].

HCV can disturb the host response at multiple levels to achieve persistent infection. These include the level of IFN induction that leads to IRF-3 activation. In patients with chronic hepatitis C and early stage fibrosis, molecular profiling has demonstrated dysregulation of IFN signaling pathways, suggesting that HCV can also control or evade the host response at this level[48]. ISG effector protein products are also targeted by HCV evasion strategies[13].

At the level of IFN induction, the HCV NS3/4A protease is able to cleave adaptor proteins downstream from the RIG-1 and TLR3 signaling pathways and therefore inhibit the activation of
the IRF-3 and NF-κB transcription factors. Restoration of virus induced IFN induction can be induced by pharmacological inhibition of the NS3/4A protease, providing evidence that NS3/4A is an antagonist of the host response to infection[13].

At the level of IFN signaling, HCV protein expression in general has been associated with the induction of the protein inhibitor of activated STAT (PIAS) that inhibits STAT function. This effect is possibly mediated by the upregulation of protein phosphatase 2A within HCV-infected liver tissue[35, 49].

The suppressor of cytokine signaling (SOCS) proteins are negative regulators and inhibitors of Jak-STAT signaling. HCV core protein has been associated with increased expression of SOCS-3 which inhibits IFN signaling and attenuates ISG expression[13]. Furthermore NS5A induces IL-8, a chemokine which is elevated in chronic hepatitis C and inhibits the antiviral actions of IFN likely through attenuated ISG expression[50].

The HCV NS5A and E2 proteins both bind PKR and inhibit its catalytic activity, thus overcoming PKR-dependent inhibition of translation[51]. NS5A also interferes with 2-5 OAS/RNaseL pathway. Genotype 1 HCV sequences have fewer RNase L cleavage sites than genotypes 2 or 3, possibly suggesting a genetic basis for genotype 1 resistance to IFN therapy[52, 53].

The quasispecies nature of HCV may also be relevant to evasion of the host response. A stretch of 40 amino acids on NS5A termed the interferon sensitivity-determining region (ISDR) has been associated with responsiveness to IFN therapy. This genetically variable region influences HCV replication efficiency and thus the abundance of viral proteins, which may indirectly influence the host response [13, 54].
Fig. 1.3 IFN-α/β and IFN-λ signaling pathways. IFN-α/β and IFN-λ are both induced by IRF7/IRF3, and signal through the JAK/STAT pathway to induce a large number of widely overlapping interferon-stimulated genes (ISGs), which lead to a cellular antiviral state. See text for details. IFN, interferon; MAVS, mitochondrial anti-viral signaling protein; RIG-I, retinoic acid-inducible gene I; TRIF, Toll-IL-1 receptor domain-containing adaptor inducing IFNβ; IRF, interferon-response factor; TLR, toll-like receptor; IFNAR, IFN-α receptor complex; Jak, Janus kinase; Tyk, tyrosine kinase; STAT, signal transducers and activators of transcription; ISG, interferon-stimulated genes. From Lange et. al. J Hepatology 2011[37].
1.1.2.1.2 Cellular response

NK cells are the primary antiviral effector population of the innate immune system, eliminating virus through cytolytic and cytokine producing functions. Evidence suggests they may also play an important role in regulating the antigen-specific adaptive immune response[55]. In this regard, cross-talk between NK cells and dendritic cells (DCs) early in the immune response would impact on the activation and resolution of antigen-specific T cell responses. Many studies have demonstrated a central role of T cells in HCV viral control and clearance[56]. HCV infection may alter NK:DC interactions in such a way that impairs T cell responses leading to HCV persistence[55].

Immune regulation by NK cells is dependent on NK:DC interactions. Immature dendritic cells take up pathogens and are activated to release a variety of cytokines that act on NK cells. These include IFN-α/β which can potentiate NK cell cytotoxicity by upregulating their expression of tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL)[57], IL-12 - which enhances NK cell-mediated cytotoxicity as well as IFN-γ production[58], and IL-15 – which promotes NK cell survival and maturation. NK cell activity is further determined by the balance between the vast array of inhibitory and activatory receptors on the NK cell surface. Signals from activated NK cells, through cytokine release and cell surface contact, are essential for maturation of immature DCs into efficient APCs capable of initiating T cell responses. Thus it is through this interaction between NK and DC cells that NK cells can influence the adaptive immune response. Activated Th1 cells produce IL-2 which can stimulate NK cells to lyse immature dendritic cells, thus limiting the immune response[55].

A number of observations suggest that HCV viral persistence may be associated with HCV-induced mechanisms of NK cell inhibition. Binding of HCV E2 to CD81 on NK cells directly inhibits NK cell function[59]. MHC class I expression by hepatocytes is upregulated by HCV core protein resulting in impaired activity of NK cells[55]. HCV core protein has also been demonstrated to stabilize HLA-E expression and inhibit NK cell activity via interactions with inhibitory NKG2A receptors[60]. DC expression of the stress-inducible class I like molecules MICA/MICB is reduced in chronic HCV infection resulting in reduced NK cell activation.
through NKG2D[55]. Also despite upregulation of IL-15 in HCV-infected liver tissue, defects in availability may be a factor in NK cell inhibition[55].

HCV induced inhibition of IFN induction and signaling, as discussed above, also likely impacts negatively on NK:DC interactions. It is possible that some of the defects observed in DC function may be directly related to HCV infection as opposed to indirectly through NK:DC interactions. The downstream consequences of NK cell inhibition and loss of NK-dependent DC maturation would be an ineffective or tolerogenic adaptive response.

1.1.2.2 Adaptive Immunity

1.1.2.2.1 Humoral Immunity

HCV infection results in virus-specific antibodies against both structural and non-structural proteins approximately 7-8 weeks after infection in most immunocompetent patients. The protective role of naturally acquired antibodies is questionable as they do not prevent reinfection of immune chimpanzees or humans[61, 62], while clearance of HCV has been described in humans and chimpanzees in the absence of seroconversion[63, 64].

HCV infectivity in chimpanzees can be neutralized by in vitro treatment with hyperimmune serum against the HRV1 region of E2, which is a major target of the antibody response. This protection is strain specific and does not protect against escape mutants[65]. Similarly in humans, infection outcome can be predicted by sequence changes in the HVR1 that occur simultaneously with seroconversion[21]. Genetic diversification in the hypervariable region of E2 during the acute phase of HCV infection is associated with progression to chronic hepatitis, whereas evolutionary stasis is associated with a self-limited course of infection[21].

Recent studies using infectious lentiviral pseudotype particles indicate the existence of cross-genotype neutralisation by antibodies from chronically infected patients against genotype 1a that are able to neutralize genotype 1b, 2a, 4a and 5a. This suggests additional conserved epitopes for antibody responses, however these antibodies are rare in individuals who resolve infection[66].
1.1.2.2 Cellular Response

A number of studies highlight the variability of the cellular immune response to acute infection with HCV. Successful control of acute primary viral replication is associated with expansion of a strong, multi-specific, and sustained HCV-specific CD4 and CD8 T cell response. An analysis of the T cell response in a health care worker accidentally infected with HCV and who subsequently cleared the virus demonstrates some important points[67]. A prolonged period of hepatitis was temporally associated with the appearance of CD38+ CD8 T cells not capable of producing IFN-γ. Subsequently, a strong CD4 T cell response emerged and CD8 T cells became CD38- and capable of producing IFN-γ. This corresponded with a 5 log drop in viremia and resolution of liver disease and suggests that CD8 T cells control disease by both cytolytic and non-cytolytic mechanisms. By contrast, patients who fail to clear HCV lack significant T cell responses although a subset will demonstrate a delayed CD4 and CD8 T cell response associated with transient control of viremia and a variable rise in transaminases, but eventual contraction of the CD4 response and persistent viremia[67, 68].

Important differences exist in the characteristics of T cell responses between resolving and persistent HCV infections. Acute resolving hepatitis C is generally associated with T cell responses against multiple epitopes in structural and non-structural proteins and a high frequency of T cells against specific epitopes, sometimes exceeding 3-4%[69-71]. In contrast, T cell responses leading to persistent infection target only limited epitopes and are much less frequent, although rarely, more diverse responses have been described[68].

In patients who successfully control HCV infection, strong HCV-specific CD8 and CD4 T cells can be detected in blood for many years after infection. Subsequent re-exposure to HCV is associated with a milder course of liver disease and lower rates of persistence[72]. Reinfection in chimpanzees resulted in a shorter period of viremia - 2 weeks as compared to 12-16 weeks for first infection. This was linked with the rapid recall of liver resident virus specific CD8 T cells and expansion of memory CD4 and CD8 T cells in blood. The importance of memory CD8 T cells in protective immunity to HCV was confirmed by antibody mediated depletion of CD8 T cells before rechallenge with the same HCV strain. This resulted in prolonged viremia, which
was only terminated when HCV specific CD8 T cells recovered in the liver. In other studies antibody-mediated depletion of CD4 T cells before reinfection resulted in HCV persistence despite functional intrahepatic CD8 T cell responses and was associated with the emergence of viral escape mutations in MHC class I epitopes[73]. These findings suggest the importance of CD8 effector T cells and CD4 helper T cells in protective immunity to HCV.

In patients with chronic hepatitis C infection, HCV-specific CD8 T cells can be detected in the blood and liver for many years, albeit with impaired effector function[74]. Data regarding the relationship between viral load, which is relatively stable in chronic infection, and intrahepatic CD8 T cells is inconclusive, and thus it is unclear whether they contribute to control of viral replication in chronic disease[68]. Also data regarding the relationship between intrahepatic CD8 T cells and disease severity has been inconsistent[68].

HCV-specific CD4 T cells in chronic hepatitis C are absent or very weak when assessed using functional methods[71, 75]. One study using MHC class II tetramers to identify HCV-specific cells independent of function did not demonstrate the presence of CD4 T cells in chronic infection[76]. These findings suggest a crucial role for CD4 T helper cells in determining the outcome of HCV infection. Possible mechanisms for failure of T cell responses are discussed below.

1.1.2.2.3 Escape Mechanisms

The persistence of HCV might be promoted by the mutation of epitopes targeted by virus-specific host immune responses. The RNA-dependent RNA polymerase results in HCV replication at the enormous rate of $10^{12}$ virions per day and through the lack of a proof-reading function can generate numerous minor viral variants with the potential to evade immune recognition. This may possibly occur due to the loss of epitopes, impaired binding of epitopes to MHC molecules, or altered recognition of variant peptide-MHC complexes[68].
Many T cell epitopes are preserved and don't mutate in replicating HCV genomes despite strong focal CD8 T cell responses suggesting mechanisms other than mutational escape in evading antigen specific T cell responses[68].

Functional impairment or anergy of T cells may also play a significant role in HCV persistence. In one study, HCV persistence was associated with an impaired ability of T cells to proliferate which could be rescued in vitro by concomitant exposure to interleukin 2 and antigen[77, 78]. T-cell exhaustion and inhibition may also be additional mechanisms contributing to the failure of adequate T cell responses. The combined expression of inhibitory receptors programmed death-1 (PD-1) and T-cell immunoglobulin mucin-3 (TIM-3) on HCV-specific cytotoxic lymphocytes was associated with viral persistence[79].
1.1.3 Epidemiology of HCV Infection

1.1.3.1 Prevalence and Incidence

The worldwide prevalence of HCV infection, as determined by cross-sectional seroprevalence studies is estimated at 3%, with 170 million people chronically infected. Although HCV is endemic worldwide, there is considerable geographic and temporal variation in the incidence and prevalence of HCV infection. Countries with the highest seroprevalence are located in Asia and Africa, while lower prevalence is found in the developed nations in North America, Australia, and northern and western Europe. Relatively low prevalence rates are found in Australia (1.1%), Germany (0.6%), USA (1.8%), Japan (2.3%) and Italy (2.2%). Of the most populous developing nations, China has an estimated seroprevalence of 3.2%, India - 0.9%, Pakistan 2.4% to 6.5%, and Egypt with the highest reported rate of 22%[80, 81].

Using age-specific prevalence data, three distinct epidemiological patterns can be identified. In Australia, the United States and other developed countries, peak prevalence is found amongst people aged 30-49 years, indicating that most transmission occurred in the mid-1980s and the mid-1990s, with injection drug use being the predominant risk factor. In Japan and Italy, prevalence was highest amongst older people, suggesting the risk of infection was greatest in the distant past (>30 years). In Egypt and other developing countries, high rates of infection are observed in all age groups, indicating ongoing high risk of acquiring HCV infection. In these last two patterns of HCV transmission, unsafe injection practices and contaminated equipment in health-care related procedures are major factors in viral transmission[80].

Establishing the incidence of HCV infection is difficult because most infections are initially asymptomatic and available assays do not distinguish between acute, chronic or resolved infection. Thus mathematical models have been used to estimate incidence rates. In the USA, the incidence of acute HCV peaked in the mid-1980s but has fallen steadily through the 1990s. This decline is attributed to growing awareness of HCV, implementation of blood donor screening programs, needle exchange programs and universal precautions in medical practice[81].
In Australia it has been estimated that there were 210,000 people living with HCV antibodies at the end of 2001. HCV infection in Australia is predominantly transmitted through injecting drug use. A reduction in the heroin supply in Australia in late 2000 and early 2001 may have impacted the number of injecting drug users and the number of new hepatitis C infections. Mathematical models suggest that HCV incidence decreased from a peak of 14,000 infections in 1999 to 9700 infections in 2005, largely attributable to a reduction in injecting drug use. It was estimated that 6500 people were living with HCV-related cirrhosis in 2001, and that without effective therapeutic intervention this as well as the incidence of HCV related liver failure and HCC is projected to triple by 2020[20, 82, 83].

1.1.3.2 Transmission

Modes of transmission of HCV are divided into percutaneous, including injection drug use and blood transfusion, and nonpercutaneous, including sexual contact and perinatal transmission.

Injection drug use is the main mode of transmission of HCV in the developed world and accounts for 68% and 80% of current infections in the USA and Australia, respectively[81]. Amongst current injecting drug users in Australia, HCV infection remains very high, with incidence and prevalence estimates at 10-20/100 person-years and 50-55%, respectively[20]. In contrast, the fall in estimated incidence of HCV in the USA is significantly due to a reduction in injection drug use related HCV[84]. Factors associated with HCV acquisition in injection drug users include older age, longer duration of injecting, incarceration, and frequent sharing of injecting equipment[85].

Blood transfusion was a significant mode of HCV infection in previous decades in developed nations. Numerous measures have led to the progressive reduction in transfusion-transmitted disease including an all-volunteer donor system, screening donors with regard to HIV risk factors, and HCV serologic and nucleic acid testing which is estimated to reduce the risk to one case per million.. The risk was greatest for haemophiliacs receiving blood products from multiple donors, with a prevalence of 75%. In developing nations, ongoing transfusion-
transmitted HCV is much more significant as most blood donations are not from voluntary, non-
remunerated donors and because of inadequate HCV screening[81].

Other important percutaneous modes of transmission include haemodialysis, with prevalence
rates between 10 and 20%, although this is declining with strict adherence to “universal
precautions”. The risk of anti-HCV seroconversion in health care workers who have sustained
needlestick injuries from anti-HCV positive sources are variable, but recent estimates of 0.3%
have been reported[86, 87].

Transmission of HCV by nonpercutaneous routes occurs much less efficiently than through
repeated or large percutaneous exposures. Sexual transmission of HCV is far less efficient than
with other sexually transmitted viruses, being extremely low in people in long term monogamous
relationships[81]. Anti-HCV is detected in no more than 3% of partners of haemophiliacs, the
majority of who are infected with HCV. The risk of transmission is increased among
promiscuous heterosexuals, homosexuals, and coinfection with HIV. “Safe sex” practices
including barrier precautions are recommended in high risk and non-monogamous
relationships[80, 87].

The risk of perinatal transmission of HCV is estimated at 2.7-8.4% for infants born to HCV
infected mothers, and higher in those born to women coinfected with HIV, although this can be
reduced with the use of highly active anti-retroviral therapy (HAART)[81]. The level of HCV
viremia also likely influences the risk of transmission. There is inconsistent data comparing
vaginal and cesarean delivery and transmission risk, while the risk of breastfeeding is negligible
[87, 88].
1.1.4 HCV infection in Immunocompetent Host

1.1.4.1 Clinical Features

Hepatitis C accounts for 20% of cases of acute hepatitis. The vast majority of these patients are asymptomatic and rarely seen in clinical practice. Jaundice is present in fewer than 25% of patients and additional symptoms similar to those present in other forms of viral hepatitis occur in about 20%, lasting 2-12 weeks and include malaise, nausea, and right upper quadrant pain. The mean incubation period is 6-12 weeks. Fulminant hepatic failure is very rare. HCV RNA is detectable in blood within 2 to 3 weeks of exposure and seroconversion typically occurs a few weeks later, with HCV antibodies appearing after 3 to 6 weeks. Serum aminotransferases become elevated approximately 6 to 12 weeks after exposure and follow a fluctuating pattern for the first few months[89, 90]. In those infection is self-limited, loss of HCV RNA and normalisation of ALT usually occurs within 3 to 4 months of clinical disease. The rate of spontaneous clearance of HCV infection varies from 10 to 50% and is affected by a number of factors. Long term follow-up of cohorts of intravenous drug users (most common mode in West) with HCV suggest spontaneous clearance of approximately 15% [91]. Factors associated with increased clearance rates include, younger age, female sex, mode of transmission (injection drug use > blood transfusion), the development of symptomatic infection with jaundice, persistence of a specific CD4 T cell response, specific HLA-DRB1 and DQB1 alleles, IL28B genotype, and low peak levels of HCV viremia during acute infection. Those associated with reduced clearance rates include immunodeficiency states (HIV and agammaglobulinaemia) and black race [73, 90-92].

Chronic hepatitis C is marked by the presence of HCV RNA for at least 6 months after the onset of infection. During the evolution of acute to chronic disease at least 25% of patients will have a fluctuating or 'stuttering' pattern in their HCV RNA and ALT levels and some may even have periods when HCV RNA is undetectable and ALT levels normal, so that a single negative result during convalescence does not prove resolution of infection and continued follow-up for 6 to 12 months after onset is required. In up to one third, ALT levels remain persistently normal despite evidence of continued liver injury in some. Most patients with chronic hepatitis C are
asymptomatic or only have mild symptoms. The most frequent complaint is fatigue, while less frequent complaints include anorexia, weakness, myalgia, arthralgia and weight loss. Symptom severity is not closely related to disease activity or severity. The sequelae of chronic hepatitis C are progressive fibrosis, cirrhosis and the risk of developing hepatocellular carcinoma (HCC) and complications of portal hypertension including gastrointestinal bleeding, ascites and encephalopathy [89].

1.1.4.2 Natural History

The natural history of chronic hepatitis C has been difficult to define due to the long and variable course of disease and the many factors associated with disease progression. Studies used to assess natural history include cross-sectional, retrospective, long-term cohort and prospective studies. The most accurate assessment is likely to be derived from prospective studies, possibly due to a reduction in referral bias and which also generally paint a more favourable outcome.

A series of 131 patients with chronic post-transfusion hepatitis C seen in a referral center in the United States demonstrated an aggressive course after an average duration of infection of 22 years. The rates of chronic active hepatitis and of cirrhosis were 23% and 51% respectively[93]

Iatrogenic outbreaks of HCV have also provided some insight into the natural history of HCV infection. One study examined 1980 (70% of whole cohort) women who received HCV contaminated anti-D immunoglobulin in East Germany. After 25 years of follow up 86% and 46% of women tested positive for HCV antibodies and HCV RNA (chronic infection), respectively. Cirrhosis and advanced fibrosis developed in only 9 (0.5%) and 30 (1.5%) patients respectively. This corresponds to a low risk of progression from chronic hepatitis C to advanced fibrosis and cirrhosis (4-5%) over 25 years[94].
In another study with the longest follow-up to date, frozen stored blood from 8568 young healthy military recruits was examined for HCV antibodies and HCV RNA, which were present in 17 (0.2%) and 11 patients respectively. After 45 years of follow-up, liver disease occurred in only 2 of the 17 patients (12%) who were antibody positive, suggesting a low risk of progressive liver disease in healthy HCV positive people[95].

Studies performed in children infected early in life also show a benign clinical course for HCV infection. In one such study, 31 adults who received HCV infected mini blood transfusions at birth were examined 35 years after the initial exposure. Sixteen tested positive for HCV RNA (50%), and of these 11 consented for liver biopsy, in which 2 demonstrated advanced fibrosis or cirrhosis [96].

The majority of those who develop cirrhosis will remain well and compensated for most of their lives. In the absence of decompensation, actuarial survival rates are as high as 80% at 10 years. The probability of decompensation is 4-5% at one year and increases to 30% at 10 years from the development of cirrhosis, and is manifest by ascites, encephalopathy, variceal bleeding or marked synthetic dysfunction. The development of decompensation confers a poor prognosis with only 50% 5-year survival and is therefore an important indicator for transplantation[90, 97, 98].

HCC in patients with chronic hepatitis C occurs almost exclusively in the setting of cirrhosis. Once cirrhosis has developed, the risk of HCC is 1% to 4% per year[90].

Thus, among different cohorts there is significant variation in the natural history of hepatitis C infection, both in terms of chronicity rates and progression of chronic infection. Many factors are known to influence the progression of chronic hepatitis C, including host and possibly viral factors.
1.1.4.3 Factors Associated with Disease Progression

Age at infection appears to one of the strongest predictors of developing cirrhosis. Progression is much faster in those with a later onset of infection. In one study, within 20 years of infection, cirrhosis developed in 2% of those infected before the age of 21, in contrast to 63% of those infected after the age of 50 [99]. This may be related to ageing associated reduction in hepatic immune function. The duration of infection was also an important predictor of the rate of cirrhosis development in all age groups, increasing progressively with longer follow-up.

Male gender is associated with a higher risk of progression to cirrhosis, as is race, with a worse outcome of chronic hepatitis C seen in African Americans compared to white patients[90, 99].

Host genetic factors influencing fibrogenesis may also affect the progression to cirrhosis. A positive relationship has been demonstrated between inheritance of high transforming growth factor beta1 (TGF-β1) and high angiotensin producing genotypes and the development of progressive hepatic fibrosis in chronic hepatitis C, suggesting that genetic polymorphism in these genes may influence rate of fibrosis progression [100].

Genome wide association studies have identified single nucleotide polymorphisms (SNPs) near the IL28B gene to be strongly associated with spontaneous clearance of HCV. Thomas et al. found that IL28B rs12979860 is strongly associated with spontaneous HCV clearance in populations of African or European ancestry with a threefold higher clearance rate in the protective C/C genotype versus C/T and T/T [101].

Alcohol enhances HCV replication, possibly through activation of the nuclear factor kappaB (NF-κB) promoter[102] and is known to reduce responsiveness to interferon therapy. Alcohol consumption leads to increased viraemia and accelerated hepatic fibrosis, even with relatively low intake[103, 104]. Daily use of marijuana has also been associated with more rapid fibrosis progression, possibly through their actions on hepatic cannabinoid receptors[105].
Metabolic abnormalities including type 2 diabetes, obesity, hepatic steatosis, and increased iron stores have also been associated with accelerated progression of fibrosis and decreased response to interferon therapy in some but not all studies[106, 107].

The degree of inflammation and fibrosis on liver biopsy is also an important predictor of progressive liver injury, in particular the presence of high grade necroinflammation[108].

Although the level of HCV RNA and genotype have an established impact on antiviral therapy, they do not appear to influence disease progression in the immunocompetent host[99]. Low quasispecies heterogeneity in the HVR region in chronic hepatitis C has been associated with normal ALT and mild histological lesions although this association is not established[109].

1.1.4.4 Treatment

The standard of care for HCV infection has been combination therapy with pegylated interferon alpha and ribavirin, which achieves sustained virological response (SVR) of 40-50% in patients infected with the most common genotype 1. SVR is associated with improved long-term outcome, but there remains the risk for HCC development in those with cirrhosis. The NS3/4A protease inhibitors, boceprevir and telaprevir have recently been approved for treatment of chronic hepatitis C in combination with PegIFN and ribavirin with improvement in SVR up to 80% in genotype 1 infection [110].

1.1.5 HCV in the Setting of Liver Transplantation

HCV-related end-stage liver disease is the most frequent indication for orthotopic liver transplantation in western countries and despite anticipated improvements in the efficacy of antiviral therapy, it has been projected that the number of candidates referred for transplantation will double by 2020[111]. Unfortunately HCV reinfection of the allograft is universal leading to recurrent disease of variable outcome. In comparison to primary infection in immunocompetent
individuals, the median duration from transplantation to cirrhosis is 9 to 12 years, followed by a 42% risk for clinical decompensation at 1 year[112].

1.1.5.1 Post-Transplant Viral Kinetics

Studying viral kinetics posttransplantation may be helpful in understanding the pathogenesis of recurrent HCV and also in designing antiviral therapy. Negative-strand HCV RNA, the best indicator of HCV replication has been detected in serum as early as 48 hours following liver transplantation, while hepatocyte expression of HCV antigens has been detected as early as 10 days after transplantation[113, 114]. Garcia-Retortillo performed a detailed study of HCV viral kinetics during and immediately after liver transplantation in 20 patients with HCV-related cirrhosis[115]. HCV viral load decreased significantly during the anhepatic phase, presumably due to a loss of viral synthesis after hepatectomy. Following transplantation and reperfusion, viral load decreased exponentially for 8 to 24 hours, suggesting that binding and uptake of HCV by hepatocytes is the likely mechanism, and that allograft infection occurs immediately after transplantation. In the following days, HCV RNA levels varied. Viral load increased exponentially in approximately half of the patients, reaching pre-transplant levels by the fourth postoperative day. In a third, viral load continued to decrease during the first postoperative week and of particular note is that most of these patients were on a steroid-free immunosuppression protocol, suggesting a role for steroids in early posttransplant viral kinetics[115, 116].

Other studies have examined medium and late posttransplantation kinetics[113]. These have demonstrated that viral load increases rapidly from about 2 weeks posttransplantation and peaks between the first and fourth post-operative months. By the end of the first postoperative year, viral load is on average, 10 to 20 times greater than pretransplantation levels[116].

Corticosteroid treatment of acute cellular rejection is associated with marked increases in HCV viral load. Transient 4- to 100- fold increases in serum HCV RNA are noted after pulsed methylprednisone therapy. The effect of calcineurin inhibitors on viral load in posttransplant patients is unclear. Cyclosporine inhibits HCV replication in vitro although in vivo activity is
controversial. Tacrolimus does not have any anti-HCV activity[117]. No changes are observed in viral load with calcineurin inhibitors in immunocompetent patients[116, 118].

1.1.5.2 Histological Patterns of Disease Recurrence

While reinfection is defined by the reappearance of HCV RNA in the serum, the diagnosis of recurrent HCV disease is based on histological findings. Elevated serum transaminases lack sensitivity and specificity in the post-transplant setting and may underestimate the presence of liver damage. About 20% to 30% of patients with histological evidence of chronic recurrent HCV have persistently normal serum ALT levels and do not develop biochemical or clinical hepatitis posttransplantation [114, 119]. Thus the natural history of recurrent hepatitis C is best determined through prospective, longitudinal studies with serial protocol liver biopsies.

Acute hepatitis typically develops between the first and third month posttransplantation and is characterized by histologic features of hepatocyte swelling, macrovesicular fatty change, mild lobular inflammation with focal hepatocyte necrosis, and acidophil bodies. In longitudinal studies, acute hepatitis C has been diagnosed in 70% of patients within the first 6 months posttransplantation, and was associated with flares in serum ALT in less than half of these. It is always associated with a steep increase in HCV viral load[120].

An important issue in clinical practice is to distinguish recurrent HCV hepatitis from acute cellular rejection (ACR). ACR does not occur more frequently in patients with hepatitis C, but can be difficult to differentiate from recurrent HCV beyond 2 months posttransplant. This has importance in view of the prognostic and therapeutic implications. While HCV-negative recipients have reduced mortality if they experience an episode of ACR, HCV-positive recipients have a threefold increase in morality if they are treated with steroids for a rejection episode[121]. Increased immunosuppression in the absence of ACR would also negatively impact on HCV recurrence. In the setting of recurrent HCV, distinguishing histological features of ACR include the presence of endothelitis, severe bile duct damage and a mixed portal tract infiltrate (versus mononuclear in chronic HCV)[114]. Recently, some investigators have examined differential expression of certain genes as a way to differentiate between ACR and HCV recurrence[122].
Fibrosing cholestatic hepatitis (FCH), an accelerated course of liver injury, develops in a small proportion of patients (5%). It usually begins 1 month posttransplant and progresses over a 3- to 6-month period to liver failure. It is characterized by biochemical cholestasis (in the absence of biliary/vascular complications) with elevated bilirubin (>100μmol/L), alkaline phosphatase (>500U/L), and γ-glutamyltransferase (>1000U/L)\[114\]. Also viral loads are characteristically much higher than seen in other forms of recurrent HCV (>2x10^6IU/mL). Histologically, severe hepatocyte ballooning, intrahepatic cholestasis, pericellular and portal fibrosis, ductular proliferation, and paucity of inflammation characterize FCH. This often occurs in the context of high levels of immunosuppression, and has also been reported in HIV/AIDS patients and other solid organ transplant recipients with HCV infection\[120\].

Histologic features of chronic hepatitis C are present in 70% to 90% of transplant recipients after 1 year and in 90% to 95% after 5 years. These are similar to findings in immunocompetent hosts and include mixed portal infiltrate with lymphoid aggregates, periportal inflammation and fibrosis, variable lobular inflammation, and macrovesicular steatosis.

1.1.5.3 Natural History and Progression of Disease

The progression of chronic hepatitis C to cirrhosis in transplant recipients is markedly accelerated, with 20% to 40% progressing within 5 years of transplantation, in comparison to 5% of non-transplant individuals with chronic HCV infection\[120\]. The median rate of fibrosis progression in the posttransplant setting (based on Desmet fibrosis score of 0 to 4) is estimated to be between 0.3 to 0.6 stages per year compared to 0.1 to 0.2 stages per year in immunocompetent HCV-infected patients\[114, 120\].

After the development of cirrhosis, the rate of decompensation is also greatly accelerated in the transplant setting. The rate is > 40% at 1 year and >60% at 3 years in transplant recipients with cirrhosis compared to <5% at one year and <20% at 5 years in immunocompetent patients\[120\].
Survival after hepatic decompensation is also much worse in the transplant setting, with 3-year survival <10%, compared with >50% in immunocompetent patients[112].

Compared to transplantation for other causes of liver disease, recurrent hepatitis C is associated with a significant reduction in both graft and patient survival. In one study, the 5-year graft and patient survival of 57% and 70% in HCV +ve patients compared with graft and patient survival of 68% and 77% in HCV –ve patients[123].

1.1.5.4 Factors Associated With Disease Progression

High pretransplant HCV RNA is associated with increased graft loss and mortality. Charlton et al reported that the relative risk of graft loss was 3.6-fold higher in patients with a pretransplant viral load greater than $1 \times 10^6$ viral Eq/mL, than in those with lower viral loads, with 5-year patient survival rates of 57% and 87%, respectively[124]. Sreekumar et al have reported that HCV RNA at 4 months posttransplant is a sensitive and specific predictor of subsequent necroinflammatory activity and fibrosis stage[125].

While some studies have demonstrated a relationship between the severity of liver disease posttransplant and HCV genotype, in particular genotype 1b[126], results from other studies have been inconsistent[124].

Greater rates of HCV quasispecies diversification in the posttransplant setting have been associated with mild or moderate liver disease activity compared with less genetic diversity in severe disease patterns, particularly in the viral envelope region[127]. It is likely that this association is linked with the level of immunosuppression, the strength of which relates to the degree of immune selective pressure and quasispecies emergence. It has been shown that the rate of change of genetic diversity is greater early after transplantation and becomes more stable after 36 months [128, 129].

Recipient age, female gender, non-white race and high Child Pugh score (>10) have been identified as significant risk factors for reduced patient survival. Being non-Caucasian is also
associated with increased severity of recurrent HCV. Cytomegalovirus (CMV) infection has also been identified as an established risk factor for the severity of HCV recurrence. Data on HBV co-infection and the presence of HCC as risk factors has been inconsistent [130].

The most consistent donor factor to demonstrate an impact on patient survival and severity of HCV recurrence is advanced donor age. In one series, only 14% of the recipients who received an organ from a donor younger than age 30 developed recurrent HCV-related cirrhosis. In contrast, 45% and 52% of those receiving an organ from donors aged 31 to 59 or older than 59, respectively, developed graft cirrhosis[131]. This correlates with data from immunocompetent patients, in which age at time of infection is a significant factor for increased disease severity. The reasons for this association are unclear but may be due to age-related changes in the liver response to infection or possibly increased arteriosclerotic complications in older grafts[132]. Other possible explanations include higher degrees of steatosis found in older grafts, although the true significance of this is controversial[133].

Hepatic graft iron content, which increases with donor age, may play an important role in accelerated fibrogenesis post-transplant, although this is not established on current data. One study has demonstrated an association between increased graft iron concentration and early fibrosis progression in female patients with recurrent hepatitis C[134].

In liver transplant patients, IL28B variations in the recipient, but not donor have been associated with histologic recurrence in the liver graft and the progression of fibrosis. In contrast, both recipient and donor IL28B variations are independent predictors of post-transplant peginterferon treatment response [37, 135].

Living donor transplantation has been associated with increased severity of HCV recurrence in small studies, although a recent analysis has demonstrated 2-year patient and graft survival similar to deceased-donor transplantation. In this analysis, living donor recipients were less ill at the time of transplantation, more likely to be female and received grafts from younger donors[136].
In view of the shortage of cadaveric liver donation and recent practice of living-donor transplantation, the impact of donor-recipient HLA matching has been examined in a number of studies. While some studies have reported an association between HLA-DRB1 mismatch and increased disease severity, others have shown that sharing of HLA-DRB1 alleles resulted in more rapid disease recurrence[137, 138]. Thus at present donor-recipient HLA-matching is considered a controversial risk factor.

Some centers have reported that more recent year of transplantation is associated with poorer transplant outcomes. An analysis from Berenguer et al included 283 HCV infected transplant patients between 1991 and 2000. At one year post-transplant, graft survival was 58% and 81% for patients who received transplants between 1999-2000 and 1991-1992, respectively. Increased donor age and stronger immunosuppression were identified as important contributing factors[131].

Increased severity of histological findings observed on initial biopsy is predictive of more severe progression of liver disease. Specific histologic features predictive of worse outcome include, cholestasis, ballooning degeneration, and confluent necrosis[133]. A Hepatitis Activity Index (HAI) of ≥ 3 at 4 months was associated with high fibrosis score at 1 and 3 years post-transplant[125]. Also early recurrence is associated with worse graft and patient survival [139, 140].

Prolonged ischaemic rewarming time prior to implantation may also contribute to more severe recurrence. Baron et al reported the risk of severe HCV recurrence at one year was 19% and 65% for rewarming times of 30 and 90 minutes, respectively[141].

The treatment of acute rejection episodes with corticosteroid (CS) boluses is associated with an increased severity of recurrent HCV, progression to cirrhosis and patient mortality. Charlton et al demonstrated that such treatment of ACR resulted in increased mortality in HCV infected patients (relative risk 2.9) compared to a protective effect observed in HCV-negative patients (relative risk 0.6)[121]. Treatment of multiple rejection episodes also correlates with the
incidence of cirrhosis, with Prieto et al observing a 5 year actuarial rate of cirrhosis of 50% and 5% in patients with 2 episodes and no episodes of rejection, respectively.

The cumulative dose of steroids has also been shown to impact on transplant outcome[140]. Charlton et al reported increased mortality in patients with a mean daily CS dose of 100mg in the first 42 days compared with those administered 50mg or less (RR 2.7)[124].

Corticosteroid treatment is also associated with increased viral load. Pulsed steroids were associated with a 4-100 fold increase in HCV RNA levels compared to pre-transplant[113], while at 1 year post-transplant, HCV RNA levels were correlated with the duration of CS treatment[142]. Increased early post-transplant viral load is also associated with more aggressive disease recurrence.

In view of these findings, various centers have used different approaches to limit the potential deleterious effects of CS therapy. Belli et al reported no difference in recurrent HCV, advanced fibrosis, acute and chronic rejection, and death after 5-years follow-up in patients who were randomized to steroid withdrawal 3 months after transplantation[143]. Other studies have reported that patient and graft survival are not adversely affected by very early steroid withdrawal (14 days)[144]. Some have questioned the safety of early steroid withdrawal, arguing that this may lead to rapid immune reconstitution and immune mediated liver injury in the setting of inadequate viral clearance due to strong initial immunosuppression[133]. In this regard, Brillanti et al observed reduced disease progression in patients with a slower taper off steroids, with a higher median daily dose of prednisone, at 6 months after transplantation, being lower with moderate/severe chronic hepatitis (6.3mg) than in those who had milder histologic changes (9.6mg)[145].

The use of antithymocyte antibodies, mainly OKT3 for acute rejection has also been associated with more aggressive HCV recurrence. Rosen et al compared the outcome of 19 HCV-positive transplant recipients administered OKT3 for steroid-resistant rejection with 33 well matched patients who received at least one steroid dose, but no OKT3. Rates of recurrence of HCV were
greater (84% vs 52%), time to recurrence was shorter, and risk for cirrhosis greater in OKT3 treated patients[146].

Data on calcineurin inhibitors, have not shown any consistent difference between cyclosporine and tacrolimus with regards to patient and graft outcome[130, 133]. A meta-analysis of 16 randomised controlled trials demonstrated improved patient and graft survival in tacrolimus versus cyclosporin[147].

The current major use of azathioprine is as a steroid sparing or calcineurin sparing agent. It has been associated with variable effects on recurrent HCV. Mycophenolate has similarly been associated with contrasting effects on disease recurrence[133, 148]. It is an inhibitor of inosine monophosphate, like ribavirin, and has been demonstrated to have some antiviral properties. Platz et al demonstrated reduced HCV viral load 3 weeks after mycophenolate based therapy for ACR[149]. One study has demonstrated that patients who received high doses of MMF experienced reduced HCV RNA at 3 months and decreased fibrosis at 1 year compared to patients who received lower doses of MMF, although fibrosis was similar in both groups at 2 years[133, 150]. In another study HCV RNA was found to double in stable liver transplant patients 3 months after switching from azathioprine to MMF[151]. Studies comparing azathioprine and mycophenolate have also had conflicting results but suggest that maintenance with azathioprine is associated with less fibrosis progression, particularly if given for 6 months or longer[117].

1.2 SYSTEMS BIOLOGY IN HEPATITIS C LIVER DISEASE

Systems Biology is an approach using holistic computational methods to analyse complex interactions in biological systems and disease. Numerous research studies utilising high throughput genomic and proteomic methods are examples of systems biology approaches which have aided our understanding of HCV-related liver disease[152].
1.2.1 Genomics in Hepatitis C

Acute and chronic HCV infection has been studied using functional genomics techniques. These experiments have examined acute HCV infection in primates, as well as the sequelae of chronic infection. The results from microarray studies of acute HCV infection in the chimpanzee are intriguing. Acute HCV infection is characterised by a rapid (within 2 weeks) as well as a delayed induction (up to 6 weeks) of genes involved with the innate immune response[153]. Most of these genes are associated with interferon gene expression and are known as interferon response genes (IRG’s; including ISG15, ISG16, CXCL9, CXCL10, Mx-1, stat-1, 2’5’-oligoadenylate synthetase and p27). Viral clearance appears to be associated with rapid induction of these interferon stimulated genes (ISG). Overall HCV persistence appears to be associated with comparatively less induction of ISG’s compared to viral clearance. Further, chronic HCV related liver injury appears to be characterised by an ISG associated chronic Th1 immune response, which is insufficient to clear the virus, but is chronic and responsible for ongoing liver injury. The situation with interferon treatment of HCV infected individuals is similar to acute infection as an immune response characterised by a significant increase in ISG expression following treatment is associated with a greater likelihood of a sustained long-term therapy response [154].

The recent identification of single nucleotide polymorphisms in the IL-28B gene that predicts the likelihood of response to HCV treatment with interferon and spontaneous clearance of the virus has highlighted the importance of the ISG’s in determining HCV outcomes. Clearly, the immune response to HCV drives fibrogenesis as interferon administration is associated with a reduction in intrahepatic inflammation and fibrosis even in the absence of a long-term virological clearance following treatment. Importantly, in HCV the newly identified IL-28B polymorphisms are the first specific example of a genomic medicine approach, which will lead to individualised treatment approaches in liver disease.

A recent study in liver transplantation HCV recurrence has employed a novel computational method known as Singular Value Decomposition initialized MultiDimensional Scaling (SVD-MDS) to eliminate the biological noise that arises with multidimensional data analysis [155, 156]. This study of 111 liver biopsy specimens from 57 individuals identified a molecular signature of 400 genes associated with the risk of rapid fibrosis [156]. These results suggest that
the transcriptional events observed early during the disease, in the absence of classical morphological changes of advanced fibrosis at the time of biopsy, predict subsequent rapid fibrosis progression [156].

1.2.2 Proteomics in Hepatitis C

Proteomic methodologies have been applied to a number of aspects of HCV related liver injury. Proteome analysis of HCV has identified fourteen cellular proteins binding to the core protein [157]. These proteins include DEAD-box polypeptide 5 (DDX5) and intermediate microfilament proteins, including cytokeratins (cytokeratin 8, cytokeratin 19 and cytokeratin 18) and vimentin. Interestingly, DDX5 gene polymorphisms are associated with accelerated fibrosis development in HCV infected individuals [158].

In HCV infection IFN treatment responses has been studied using proteomics. The response of hepatocyte cell lines to IFN gamma treatment has uncovered over 54 IFN response genes including many novel targets, an approach that may pave the way for novel therapies. Examination of protein extracts that bind to the HCV IRES has identified a number of novel protein targets such as Ewing Sarcoma breakpoint 1 region protein EWS and TRAF-3. The final aspect of HCV liver injury receiving attention is the study of potential biomarkers such as heat shock protein HSP-70 associated with HCV infection progression to HCC [159].

The search for fibrosis Biomarkers in an area in which Systems Biology Approaches are being widely adopted. In a study of fibrosis progression to cirrhosis in the HALT-C HCV cohort, Qin et al identified that the combination of protein C and retinol-binding protein 4 in serum distinguish patients at different stages of fibrosis [160]. Further, they identified that the expression of alpha-1B glycoprotein, complement factor H and IGFBP acid labile subunit distinguished HCV infected patients from normal controls [160].

In a recent study of post-transplant HCV recurrence, proteome profiling of 24 liver biopsy specimens taken at 6 and 12 months post-transplant identified protein expression associated with
early disease progression. This was correlated with serum sample proteomic analysis of an independent cohort of 60 HCV post-transplant samples [155]. The researchers identified a group of 250 proteins in patients with rapid fibrosis progression and an impairment of oxidative stress [155]. They suggest that the data supports a role for oxidative stress in the propensity to develop rapid fibrosis post HCV recurrence.

1.3 CD ANTIBODY MICROARRAYS

1.3.1 Overview of CD Antigens

The human CD antigens are a family of heterogeneous proteins, glycoproteins, and glycolipids expressed on the cell membrane of leucocytes and also other tissues including endothelium which perform important immune system functions[161]. In the early 1980s immunologists generated multiple monoclonal antibodies reactive with leucocyte cell-surface molecules but it was not clear if the same molecule was being targeted by more than one antibody. The Human Leucocyte Differentiation Antigen (HLDA) workshops were formed, whose purpose was to identify all the antigens expressed on the cell-surface of leucocytes. Antibodies submitted to the workshop were coded and blindly tested against multiple cell types. The data would then be analysed by heirarchial cluster analysis to identify “clusters” of antibodies with similar patterns of binding to leucocytes at various stages of differentiation, hence the “cluster of differentiation” (CD) nomenclature[162]. Various biochemical techniques were then used to identify the new molecules defined by workshop antibodies. However, with the development of molecular genomic and proteomic techniques, monoclonal antibodies are no longer the primary tool for new protein discovery[162]. The full list of CD antigens is accessible through the HLDA workshop website (www.hcdm.org).

1.3.2 CD Antibody Microarray Studies in Non-hepatic Diseases

A CD antigen monoclonal antibody microarray has been developed consisting of a large number of monoclonal antibodies against different CD antigens immobilized as dots on a microarray
platform. Live peripheral blood mononuclear cells (PBMC) are incubated with the microarray and those cells expressing corresponding antigens on their surface are captured by the antibodies[163]. The resulting pattern of cell binding densities obtained by optical or fluorescent scanning of cells bound to immobilized antibodies represents the CD antigen expression signature of the leucocyte population. The number of cells captured by an antibody dot reflects the proportion of cells expressing each CD antigen, the number of antigen molecules on the cell surface, and the affinity of the antibody-antigen interaction. Thus the overall CD antigen expression signature is a semi-quantitative immunophenotype for the leucocyte population assayed[164]. The results generally correlate well with results from flow cytometric analysis [165].

The CD antibody microarray has been used to characterise a comprehensive range of human leukemias and lymphomas, and prediction analysis using the expression profiles has shown excellent consensus with diagnoses obtained using conventional clinical and laboratory methods [166]. The CD antibody microarray has also been used to define a robust HIV-induced CD antigen signature in patients with HIV-1 infection at different clinical stages[167], and to monitor changes in antigen expression patterns in a longitudinal retrospective study of patients undergoing antiretroviral therapy [168].
1.4 HYPOTHESES AND AIMS

1.4.1 Hypotheses

1. The severity of HCV disease recurrence post-liver transplantation is predicted by post-transplant HCV viral load.

2. The liver is a complex immunological organ with dynamic inflow and outflow of lymphocytes. Peripheral blood mononuclear cells and their immunophenotypes are reflective of intrahepatic disease states.

1.4.2 Aims

1. To examine factors which impact on the course of HCV disease recurrence post-liver transplant. In particular, to assess the importance of hepatitis C viral load in patients within the first year post-liver transplant in determining post-transplant survival.

2. To utilise a CD antibody microarray to determine CD antigen expression profiles for patients with various liver diseases, looking for preserved disease-specific signatures.

3. To use a CD antibody microarray to determine serial CD antigen profiles for patients undergoing liver transplantation for HCV, looking for preserved disease-specific signatures and associations with disease outcomes.
CHAPTER 2

GENERAL METHODS AND MATERIALS
CHAPTER 2  GENERAL METHODS AND MATERIALS

This chapter details the general techniques used in this research thesis. For each individual study, the experimental design, definitions, and study populations are outlined in the corresponding chapters.

2.1 Quantitative HCV RNA Determination

HCV viral load determinations were performed according to the clinical progression of individual patients. Quantitative HCV RNA detection was performed with the Amplicor HCV assay (Roche Diagnostics, New Jersey) according to the manufacturer’s instructions. In brief, 0.1 mL of serum was added to 0.4 mL of Lysis reagent and incubated at 60°C for 10 minutes. The RNA was precipitated by the addition of 0.5 mL of isopropyl alcohol and then pelleted by centrifugation at 13,000 g for 15 minutes. The pellet was washed with 70% ethanol and then resuspended in 1 mL of specimen diluent. A total of 0.05 mL of this suspension was added to 0.05 mL of PCR Master mix. Reverse transcription and PCR were performed on the Roche Amplicor instrument. High-level samples (> 800,000 IU/mL) that fell outside the linear range of the assay were diluted with normal human serum to produce an accurate concentration within the assay’s linear range.

2.2 HCV Genotyping

HCV genotypes were identified with the Versant HCV (LiPA II) line probe assay (Bayer Corp., New York). In brief, 20 μL of amplified Amplicor PCR product was added to 1 mL of hybridization buffer and incubated with genotyping strips (with type-specific oligonucleotides attached) for 60 minutes. The strips were then stringently washed, and conjugate solution was added before the addition of substrate solution. The strips were analyzed and genotypes were assigned according to the reactivity pattern interpretation table.
2.3 CD Array Construction and Assay Technique

CD antibody microarrays were constructed by Medsaic Pty Ltd and the assays were performed at Medsaic laboratories. Each microarray consisted of 90 different 10nl antibody dots (including isotype controls) applied in duplicate to a film of nitrocellulose on a glass microscope slide, then blocked with 5% skim milk, air dried and stored with desiccant at 4°C.

After obtaining informed consent, blood (5ml) was collected from patients by venipuncture and placed into tubes containing EDTA. These were kept at room temperature and assayed within 24hrs of collection. Leucocytes were isolated from peripheral blood using Histopaque density gradient centrifugation, washed in phosphate buffered solution (PBS) and adjusted to a density of $1.3 \times 10^7$ cells/mL in PBS containing 1µM EDTA. Leucocyte suspensions were then incubated with the antibody microarrays (300µL of suspension/slide) for 30min at room temperature. Unbound cells were gently washed off with PBS and the microarrays were fixed for at least 20min in PBS containing 3.7%(w/v) formaldehyde and then washed in PBS.

2.4 CD Antibody Microarray Image Analysis

Scanning and image analysis of cell binding intensities were performed using a DotScan™ array reader and software (Medsaic). Dot intensities for each antibody were quantified in duplicate on an 8-bit pixel greyness scale from 0 to 255. The dot intensity reflects the number of cells captured by a particular immobilised antibody. Dot intensity is proportional to the density of the complementary surface antigen in the cell suspension and generally correlates well with results from flow cytometry [164].
2.5 Statistical Analyses

**Statistical Analysis for HCV Viral Load and Post-Transplant Outcomes Study**

The cohort was described with estimates of central tendency (means and medians) and spread (standard deviation and range) for continuous variables and frequencies and percentages for categorical variables. Patient and graft survival rates were determined with Kaplan-Meier analysis.

Univariate data comparison was performed by Kaplan-Meier estimation using the log-rank test to assess statistical significance. Variables with $P$ values of $<0.20$ in the univariate analysis or those thought to be clinically relevant were included in the final multivariate model. Multivariate analysis was performed with the Cox proportional hazard regression model.

Because data were retrospectively collected, the potential influence of missing data on the analysis was addressed using the complete case analysis or listwise deletion approach in which those cases with missing data were simply omitted. All statistical analyses were performed with SPSS version 14 software. All reported $P$ values were 2-tailed, and all confidence intervals were 95%.

**Statistical Analysis for CD Antibody Microarray Study in Human Liver Diseases Study**

Several normalization (pre-processing) approaches for protein microarray data were investigated. The data indicate that two housekeeping antibodies (CD29 and CD44) are very stable across all samples and thus no further adjustment is required. The behaviour of non-control spots are very different to the control or housekeeping spots.

Various statistical analyses were performed on the expression data to assess structure within and relationships between different disease groups and to identify antigen-binding patterns, which differed between these groups. Further the usefulness of the expression data in disease prediction
was examined. The analyses were performed using the TMEV 4.3.0 package (www.TM4.org) unless otherwise indicated.

The CD antigen expression data were analysed without *a priori* knowledge of the samples. Such data is highly dimensional and difficult to visualise. Principal component analysis (PCA) enables projection from a high-dimensional space to a low dimensional space while capturing much of the variability in the data and thus aids in the visualisation of relationships and differences between different data samples [169]. The first principal component captures the greatest variance in the data and subsequent components progressively less. The array expression data were visualised by projection onto the first 3 principal components in three-dimensional space and conclusions were then made concerning clustering of samples from various disease categories.

Analyses for differential antibody binding between groups may identify cell surface proteins with biological significance. The expression data were analysed using significance analysis of microarrays (SAM) and the R statistical language and environment contributed library “LIMMA” [170, 171]. The results for the top ranking antibodies were comparable with both these methods. LIMMA uses empirical Bayes methods and fits a linear model to the microarray expression data for each antibody. Adjusted p-values (for multiple testing) and B-statistics (log-odds of differential expression) were used to determine significant differential antibody expression[171].

To assess the accuracy with which patient groups may be predicted based on their expression profiles, K nearest neighbours classification was performed and prediction success was evaluated using leave one out cross-validation[172]. Receiver operating characteristic (ROC) analysis was also performed for various pairwise comparisons between different disease groups.
Statistical Analysis for CD Antibody Microarray in Post-Liver Transplant HCV Recurrence Study

For each phase of transplantation and recurrence definition, a moderated t-test (based on LIMMA) was performed to identify differential expressed antibodies. An antibody was considered of potential interest if it has greater than 2-fold change[171]. Hierarchical clustering was performed in the pre-transplant phase, based on Euclidean distance and ward agglomeration method. The four different outcome measures are displayed as horizontal color vectors.
CHAPTER 3

A STUDY OF HCV VIRAL LOAD AND POST-LIVER TRANSPLANT OUTCOME
CHAPTER 3  A STUDY OF HCV VIRAL LOAD AND POST-LIVER TRANSPLANT OUTCOMES

3.1 Introduction

Cirrhosis related to chronic infection with HCV has emerged as the most frequent indication for orthotopic liver transplantation. Presently, approximately 50% of transplants performed in the United States and Europe are for patients infected with HCV. The health burden due to HCV is expected to increase to such a degree that by the year 2020, the proportion of untreated HCV patients developing cirrhosis will have increased by 30%. Furthermore, it is projected that the number of HCV patients with cirrhosis will double and the number of HCV patients with cirrhosis developing hepatocellular carcinoma (HCC) will increase by 80%[111].

HCV recurrence in the liver allograft is universal. The clinical course is characterized by more rapid disease progression after transplantation in comparison with the nontransplant population. Previously, it has been reported that the median duration from transplantation to HCV-related graft cirrhosis is approximately 9 to 12 years with a 42% risk for clinical decompensation at 1 year after the development of cirrhosis[120]. A recent analysis of the United Network for Organ Sharing database demonstrated significantly diminished survival at 5 years after primary orthotopic liver transplantation among HCV-positive patients (65.6% of HCV-negative recipients versus 56.7% of HCV-positive recipients)[173]. Moreover, there is a death rate on the transplant waiting list of 20%, highlighting the shortage of liver allografts. Therefore, there is clearly a need to optimize the outcomes of liver transplantation among HCV-infected patients. This can be achieved only with a better understanding of the host, viral, and external factors influencing patient and graft survival.

Factors influencing HCV-related disease progression in the posttransplant setting include high pretransplant HCV RNA levels[124], early acute hepatitis[174], an increased number of acute rejection episodes and methylprednisolone (MPPT) boluses, OKT3 use[146], and utilization of older donors[175]. Viral factors, including a persistently high viral burden posttransplant have also been shown to be associated with allograft damage [125], while some studies have demonstrated a relationship between the severity of disease recurrence and HCV genotype, in particular genotype 1b[126], results from other studies have been inconsistent[124]. However, it
is unclear if high serial viral load levels are an important independent factor in predicting outcome after liver transplantation.

In this study, the impact of serial serum HCV viral load levels within the first year of transplantation on posttransplant disease outcomes has been examined. A multivariate analysis incorporating recipient, donor, surgical, and other viral factors on patient and graft survival has been undertaken.

### 3.2 Methods

#### Study Population

Clinical and laboratory data were collected retrospectively from a total of 118 consecutive patients who underwent liver transplantation for HCV-related cirrhosis with or without HCC at the Australian National Liver Transplant Unit from January 1997 to September 2005. The following data were recorded:

1. Demographics factors, including the age at transplantation, gender, and year of transplantation.
2. Presence of intercurrent pathology at the time of transplantation, including HCC, hepatitis B virus coinfection, and a pretransplant history of excessive alcohol intake (more than 80 g/day for > 10 years).
3. HCV genotype.
4. Serial viral load levels post-transplant.
5. Episodes and treatment of acute rejection (steroid pulse and/or Orthoclone OKT3).
7. Type of organ transplantation (whole or split).
8. Immunosuppressive therapy [calcineurin inhibitor (CNI) immunosuppression: tacrolimus (TAC) or cyclosporine A (CsA); azathioprine (AZA) or mycophenolate mofetil (MMF) use].
Recurrent HCV infection was confirmed by the presence of HCV RNA in serum as detected by reverse transcription polymerase chain reaction (PCR). Human immunodeficiency virus–coinfected individuals were excluded from this study.

**Definitions**

Primary endpoints were patient death and graft failure. Patient survival was defined as the time from the initial transplant until the time of death or last known follow-up. Graft survival was defined as the time from transplant until retransplantation or death, whichever came first. Patients lost to follow-up were censored at the date on which they were last known to be alive for analyses of both patient and graft survival. Hepatitis C–related death was defined as death resulting from graft failure secondary to either severe recurrent hepatitis C or fibrosing cholestatic hepatitis. Non–hepatitis C–related deaths were those deaths related to other causes such as infection, perioperative complications, recurrence of HCC, and biliary complications. Use of AZA or MMF was defined as the use of either agent for at least 3 months.

**Immunosuppression**

According to institutional protocol, patients received 500 mg of MPPT intravenously on the day of transplantation (intraoperatively) and on day 1 after transplantation, followed by a taper to 20 mg/day over the next 7 to 10 days. Oral prednisone was subsequently begun at 20 mg/day and tapered progressively as tolerated. In cases of acute rejection, therapy consisted of 3 daily doses of 1 g of intravenous MPPT followed by a taper to 100 mg over the next 4 days. On day 8, oral prednisone was started and reduced by 10 mg daily until a dose of 20 mg was reached, which was then tapered progressively as tolerated. Maintenance immunosuppression included combination therapy with either TAC (Prograf) or CsA (Neoral) and prednisone with or without AZA or MMF. TAC and CsA doses were adjusted on the basis of target trough levels. On occasion, the CsA dose was adjusted on the basis of C2 levels. Steroid-resistant acute rejections were treated with OKT3 for a course of 7 or 10 days.
3.3 Results

3.3.1 Patient Demographics and Baseline Characteristics

Demographic and baseline characteristics of the patients are shown in Table 3.1. The study comprised 118 patients, of whom 99 (83.9%) were male, with a median age at transplant of 49 years (range, 29-69 years). The median duration of follow-up was 32.4 months (range, 0-109.5 months). A history of intercurrent alcohol abuse was present in 28 (23.7%). Six patients (5.1%) were coinfected with hepatitis B virus. HCC, known or incidental at the time of transplantation was present in 44 patients (37.3%). A single human immunodeficiency virus and HCV–coinfected patient was excluded from this study. The donor organ cold ischemic time was 8 to 10 hours, and the warm ischemic time was 30 to 60 minutes.

3.3.2 Patient and Graft Survival

Thirty-one patients died at a median time of 28.1 months (range, 0-109.5 months) after transplantation. Overall patient survival was 87.8%, 79.9%, and 70.1% at 1, 3, and 5 years, respectively (Fig. 1A). Twelve of 31 (38.7%) deaths were hepatitis C–related. Causes of non–HCV related deaths included sepsis (n = 6), multiorgan failure (n = 3), cardiovascular events (n = 1), malignancy (n = 3), surgical complications (n = 3), chronic rejection (n = 1), and other multifactorial nonviral causes (n = 2). Overall, 1-, 3-, and 5-year graft survival was 87.0%, 79.2%, and 68.2%, respectively (Fig. 1A). Five patients (4.2%) underwent retransplantation at a median time of 1.4 months (range, 3 days to 21.7 months) after first transplant. The indications for retransplantation were hepatic artery thrombosis (3 patients, 60.0%), primary nonfunction (1 patient, 20.0%), and HCV-related cholestatic hepatitis (1 patient, 20%).
<table>
<thead>
<tr>
<th>Recipient Demographics</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at transplant (mean +/- SD)</td>
<td>50.0 (+/- 7.8)</td>
</tr>
<tr>
<td>Male</td>
<td>99 (83.9%)</td>
</tr>
<tr>
<td>Female</td>
<td>19 (16.1%)</td>
</tr>
<tr>
<td>Intercurrent Hepatocellular carcinoma</td>
<td>44 (37.3%)</td>
</tr>
<tr>
<td>Intercurrent alcohol abuse</td>
<td>28 (23.7%)</td>
</tr>
<tr>
<td>Hepatitis B virus coinfection</td>
<td>6 (5.1%)</td>
</tr>
<tr>
<td>Year of Transplant</td>
<td></td>
</tr>
<tr>
<td>1997-1999</td>
<td>28 (23.7%)</td>
</tr>
<tr>
<td>2000-2005</td>
<td>90 (76.3%)</td>
</tr>
<tr>
<td>Steroids at 1 year</td>
<td>77 (81.1%)</td>
</tr>
<tr>
<td>Primary calcineurin immunosuppression</td>
<td></td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>13 (11.0%)</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>105 (88.9%)</td>
</tr>
<tr>
<td>Other immunosuppression</td>
<td></td>
</tr>
<tr>
<td>Azathioprine</td>
<td>70 (59.3%)</td>
</tr>
<tr>
<td>Mycophenolate</td>
<td>13 (11.0%)</td>
</tr>
<tr>
<td>None</td>
<td>35 (29.7%)</td>
</tr>
<tr>
<td>Duration of follow-up (median, range)</td>
<td>32.4 months (0-109.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Donor Demographics</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age (mean +/- SD)</td>
<td>42.5 (+/- 15.1)</td>
</tr>
<tr>
<td>Male</td>
<td>75 (63.6%)</td>
</tr>
<tr>
<td>Female</td>
<td>43 (36.4%)</td>
</tr>
<tr>
<td>Donor recipient sex mismatch</td>
<td>37 (31.4%)</td>
</tr>
<tr>
<td>Whole organ/Split</td>
<td>108/10 (91.5%/8.5%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Viral Characteristics</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV Genotype'</td>
<td></td>
</tr>
<tr>
<td>Genotype 1</td>
<td>60 (52.6%)</td>
</tr>
<tr>
<td>Genotype 2</td>
<td>4 (3.5%)</td>
</tr>
<tr>
<td>Genotype 3</td>
<td>25 (21.9%)</td>
</tr>
<tr>
<td>Genotype 4</td>
<td>15 (13.2%)</td>
</tr>
<tr>
<td>Mean pretransplant viral load (IU/mL)'</td>
<td>$9.0 \times 10^5$</td>
</tr>
<tr>
<td>Mean peak viral load post-LT (IU/mL)</td>
<td>$2.0 \times 10^7$</td>
</tr>
<tr>
<td>Peak viral load</td>
<td></td>
</tr>
<tr>
<td>Viral load &gt; $10^8$</td>
<td>4 (3.7%)</td>
</tr>
<tr>
<td>Viral load $\geq 10^7$ to $&lt; 10^8$</td>
<td>11 (10.1%)</td>
</tr>
<tr>
<td>Viral load $&lt; 10^7$</td>
<td>94 (86.2%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alive</td>
<td>87 (74.6%)</td>
</tr>
<tr>
<td>Dead</td>
<td>31 (26.3%)</td>
</tr>
<tr>
<td>Causes of death</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C related</td>
<td>12 (38.7%)</td>
</tr>
<tr>
<td>Non-hepatitis C related</td>
<td>19 (61.3%)</td>
</tr>
<tr>
<td>Graft Failure</td>
<td>34 (28.8%)</td>
</tr>
<tr>
<td>Retransplantation</td>
<td>5 (4.2%)</td>
</tr>
<tr>
<td>Hepatic artery thrombosis</td>
<td>3</td>
</tr>
<tr>
<td>Primary nonfunction</td>
<td>1</td>
</tr>
<tr>
<td>Cholestatic hepatitis</td>
<td>1</td>
</tr>
<tr>
<td>Episodes of acute rejection</td>
<td>40 (34.8%)</td>
</tr>
<tr>
<td>Antirejection therapy</td>
<td>16 (13.6%)</td>
</tr>
<tr>
<td>Steroid pulse</td>
<td>13 (11%)</td>
</tr>
<tr>
<td>OKT3</td>
<td>6 (5.1%)</td>
</tr>
</tbody>
</table>

Table 3.1. Demographic and Clinical characteristics of the cohort of consecutive patients transplanted for HCV-related cirrhosis with or without HCC. LT, liver transplantation.

' Data were available for 114 patients only. * Data available for 75 patients only.
3.3.3 Effect of Posttransplant Peak Viral Load on Survival

There were a total of 620 viral load estimations in the first year after transplant (Fig. 3.2). An examination of the mean viral load in each week following transplantation demonstrated that in week 11, the viral load peaked at $1.0 \times 10^7$ IU/mL.

The relationship between the peak viral load within 1 year post-transplant and patient and graft survival was then analysed by the division of the cohort into 2 subgroups: patients with a posttransplant peak viral load of $\geq 10^7$ IU/mL and those with a peak viral load of $<10^7$ IU/mL.

We observed that patients with a posttransplant peak viral load $\geq 10^7$ IU/mL had reduced overall patient survival (52.8% versus 89.1%, $P = 0.003$) and reduced overall graft survival (52.8% versus 85.9%, $P = 0.010$) in comparison with those with a peak viral load $< 10^7$ (Fig. 3.1C). The mean survival times for patients with peak viral load levels of $>10^8$, $<10^8$ to $\geq 10^7$, and $<10^7$ IU/mL were 11.8, 70.6, and 89.1 months, respectively ($P < 0.05$; Fig. 3.2).

Although it was clear that peak viral load levels were associated with differing outcomes, other factors thought to be potentially important were examined.

3.3.4 Effect of Donor Age on Survival

There was a decrease in both patient and graft survival with increasing donor age. In recipients who received deceased donor organs from donors < 50 years and $\geq$ 50 years old, 1-, 3-, and 5-year patient survival was 89.8%, 82.4%, and 79.3% versus 83.5%, 73.7%, and 45.2%, respectively ($P = 0.02$), whereas graft survival was 88.6%, 81.3%, and 76.3% versus 83.5%, 73.7%, and 45.2%, respectively ($P = 0.05$; Fig. 3.1B).
3.3.5 Effect of Immunosuppression on Survival

All patients were maintained on prednisone therapy for a minimum of 6 months, with 83.4% being on low-dose steroids (1-10 mg; median, 5 mg) at 1 year. Importantly, all the peak viral load determinations within the first year occurred while the patients were on prednisone therapy. Overall, 75% of the patients received TAC-associated immunosuppression, with the remaining 25% receiving CsA. In patients receiving TAC, the median duration of therapy was 32 months (mean, 38.1), whereas for CsA, the median was 39 months (mean, 53.8). There was diminished patient survival for recipients whose initial immunosuppression regimen included CsA. In recipients who received CsA, the 1-, 3-, and 5-year patient survival was 76.9%, 53.8%, and 46.2% versus 89.1%, 84.1%, and 73.5% for those on TAC ($P = 0.05$). However, graft survival was not significantly different between those on CsA and those on TAC. The graft survival was 76.9%, 53.8%, and 46.2% versus 88.3%, 83.3%, and 71.2%, respectively ($P = 0.10$; Fig. 3.3B). Furthermore, there were 34% more rejection episodes in patients treated with CsA versus TAC ($P = 0.11$).

Additionally, there was diminished patient survival for recipients who had treated episodes of acute rejection. There were a total of 13 episodes of treated acute rejection with corticosteroids and 6 cases treated with OKT3 in the first year after transplantation (median of 33.5 months with a mean of 41.5 months post-transplantation). The mean survival of individuals treated for acute rejection was 65.7 ± 9.6 months versus 80.6 ± 4.2 months for individuals who did not have treated acute rejection ($P < 0.04$).

Patients who received AZA had a median follow-up of 37 months (mean, 42.6) versus 45 months (mean, 42.6) for those individuals who did not receive AZA. There was better patient and graft survival for those recipients who had AZA as part of their initial immunosuppression regimen. The 1-, 3-, and 5-year patient survival for those on AZA and those not on AZA was 96.9%, 91.3%, and 91.3% versus 74.3%, 63.9%, and 45.4%, respectively ($P < 0.001$), whereas graft survival was 96.9%, 91.3%, and 88.3% versus 72.5%, 62.3%, and 44.3%, respectively ($P < 0.001$; Fig. 3.3A).
3.3.6 Effect of Transplantation Year on Survival

Patient and graft survival did not differ among recipients who were transplanted before 1999 and those who were transplanted between 1999 and 2005. In recipients who were transplanted before 1999 and between 1999 and 2005, the 1-, 3-, and 5-year patient survival was 82.1%, 71.4%, and 60.7% versus 89.7%, 83.9%, and 76.0%, respectively ($P = 0.13$), whereas graft survival was 82.1%, 71.4%, and 60.7% versus 88.6%, 82.9, and 72.9%, respectively ($P = 0.36$). Furthermore, a survival analysis was undertaken according to the year of transplantation, and there was no significant difference in either patient or graft survival.
Figure 3.1. Graft Survival Post Liver Transplantation for Hepatitis C Infection.

Graft survival post liver transplantation for hepatitis C virus infection. Panel A graft survival and patient survival. Panel B graft survival stratified for donor age > or <= 50 years (p=0.05). Panel C graft survival stratified for HCV viral loads > or <= 10^7 IU/ml (p=0.01).
Figure 3.2 Hepatitis C Viral Load Determinations in the First Year Post Liver Transplantation.

The viral load determination in the first year post liver transplantation; all viral load determinations (Panel A), viral loads from patients with a peak viral load of >10^8 IU/ml (Panel B), peak viral load of >10^7 and <10^8 IU/ml (Panel C) and a peak viral load of >10^7 and <10^8 IU/ml (Panel D).
Figure 3.3. The Effect of Immunosuppression on Graft Survival Post Liver Transplantation for Hepatitis C Infection.

The effect of immunosuppression on graft survival post liver transplantation for hepatitis C virus infection. Panel A graft survival associated with azathioprine (AZA) use post liver transplantation (p<0.001). Panel B graft survival stratified for Tacrolimus or Cyclosporine calcineurin immunosuppression use post liver transplantation (p=0.13).
3.3.7 Independent Predictors of Patient Survival

In the univariate analysis, 10 variables were significantly associated with an increased risk of patient death after transplantation (P < 0.20). (Table 3.2):

1. Recipient age greater than 50 years.
2. A significant history of alcohol intake before transplantation.
3. Posttransplant peak RNA greater than $10^7$ IU/mL.
4. Donor age greater than 50 years.
5. Split organ transplantation.
6. CsA immunosuppression.
7. Use of AZA.
8. Use of antirejection therapy.
9. OKT3 use.

Of these, only a posttransplant peak viral load of $\geq 10^7$ IU/mL [hazard ratio (HR), 3.71; $P = 0.02$] and use of AZA (HR, 0.35; $P = 0.05$) were shown to be independent predictors of patient survival in the Cox regression analysis (Table 3.2).

3.3.8 Independent Predictors of Graft Survival

In the univariate analysis, the same variables were associated with an increased risk of graft failure, except for the presence of intercurrent HCC and split organ transplantation. Of these, only a posttransplant peak viral load of $\geq 10^7$ IU/mL (HR, 3.70; $P = 0.02$), use of AZA (HR, 0.30; $P = 0.02$), and treated acute rejection (HR, 3.16; $P = 0.03$) were shown to be independent predictors of graft survival (Table 3.2).
3.3.9 Independent Predictors of HCV Related Graft Failure.

In the univariate analysis, variables associated with hepatitis C–related graft failure included recipient age $\geq 50$ years, female recipient, intercurrent alcohol abuse, a posttransplant peak viral load of $\geq 10^7$ IU/mL, CsA immunosuppression, nonuse of AZA, treated acute rejection, and exposure to OKT3. Of these, only a posttransplant peak viral load of $\geq 10^7$ IU/mL (HR, 8.68; $P = 0.004$) and use of AZA (HR, 0.25; $P = 0.04$) were independently associated with hepatitis C–related graft loss (Table 3.2).
<table>
<thead>
<tr>
<th>Predictor</th>
<th>Univariate Analysis</th>
<th>Multivariate Hazard ratio</th>
<th>Univariate Analysis</th>
<th>Multivariate Hazard ratio</th>
<th>Univariate Analysis</th>
<th>Multivariate Hazard ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>(95% CI)</td>
<td>p-value</td>
<td>(95% CI)</td>
<td>p-value</td>
<td>(95% CI)</td>
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<tr>
<td>Recipient age</td>
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<td>Recipient gender</td>
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<td>0.66</td>
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<td>0.20</td>
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<tr>
<td>Intercurrent HCC</td>
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<td>0.28</td>
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<td>0.74</td>
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<tr>
<td>Intercurrent alcohol</td>
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<td>0.03</td>
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<tr>
<td>Genotype (1 vs non 1)</td>
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<td></td>
<td>0.64</td>
<td></td>
<td>0.69</td>
<td></td>
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<tr>
<td>Peak RNA post LT(IU/ml) (≥10⁷ vs &lt;10⁷)</td>
<td>0.003</td>
<td>4.03</td>
<td>0.01</td>
<td>3.04</td>
<td>0.004</td>
<td>8.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.45-11.2)</td>
<td>p= 0.0007</td>
<td>(1.17-7.90)</td>
<td>p=0.02</td>
<td>(2.04-37.02)</td>
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<td></td>
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<td>(0.92-6.35)</td>
<td>p=0.05</td>
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<td>0.40</td>
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<td>0.87</td>
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<td>Whole organ vs Split</td>
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<td>0.46</td>
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<td>0.13</td>
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<td>&lt;0.001</td>
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<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.10-0.64)</td>
<td>P=0.004</td>
<td>(0.10-0.56)</td>
<td>P=0.05</td>
<td>(0.07-0.91)</td>
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<td>2.26</td>
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<td>(1.0-5.38)</td>
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<td>0.32</td>
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<td>0.36</td>
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<td></td>
</tr>
<tr>
<td>OKT3 use</td>
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<td></td>
<td>0.05</td>
<td></td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Year of Transplant (after 1999 vs on or before 1999)</td>
<td>0.13</td>
<td></td>
<td>0.36</td>
<td></td>
<td>0.89</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Predictors of Patient Death, Graft Failure, and Hepatitis C-Related Graft Failure

Abbreviations: AZA, azathioprine; CI, confidence interval; CNI, calcineurin inhibitor; CYA, cyclosporine A; HCC, hepatocellular carcinoma; LT, liver transplantation; MMF, mycophenolate mofetil. *Only significant variables are shown in the multivariate analysis.
3.4 Discussion

This study investigated the relative importance of early peak viral load levels in predicting overall outcome for patients with HCV infection undergoing liver transplantation. Transplantation of 118 HCV-positive patients showed overall patient survival rates of 87.8%, 79.9%, and 70.1% and graft survival rates of 87.0%, 79.2%, and 68.2% at 1, 3, and 5 years, respectively. These observations are in keeping with previous studies [126]. Following multivariate analysis, a peak viral RNA of ≥10^7 IU/mL within the first year of transplantation and exposure to acute antirejection therapy were associated with worse graft outcomes, while use of AZA as part of the initial immunosuppressive regimen was associated with improved graft outcomes. This study is by far the largest sampling of viral load estimations following liver transplantation and is the first to show that early high peak viral load levels are independently associated with worse clinical outcomes.

In the nontransplant setting, HCV RNA levels do not correlate with the severity of liver disease[176]. However, pretransplant viremia levels have been associated with more severe recurrent HCV[124]. The effect of viral load in the posttransplant setting has not previously been incorporated into a multivariate analysis. The levels of viremia start to rise within the first week of surgery and progress in the following weeks [115, 116]. Posttransplantation viremia has been found to be a mean of 1 order of magnitude greater than pretransplantation viremia levels. These elevated viremia values have been reported during short-term follow-up (1-2 years posttransplantation) and could correspond to the invasive phase of recurrence and lobular hepatitis in most patients [177]. However, the association of posttransplant HCV RNA levels with the severity of HCV recurrence is unclear. Papatheodoridis et al. found a correlation between levels of fibrosis and HCV RNA levels at 12 months post- transplantation[142]. Moreover, Gane et al. also reported an association between the level of viremia and histological severity of early HCV recurrence[119].

In this study, the greatest mean peak viral load was seen at week 11, and this suggests that early viral load estimation within the first 3 months after transplantation may be important and raises the question of whether or not prospective serial measurement of the viral load in the early posttransplant period could predict disease outcomes.
Among recipients with a posttransplant peak viral load of $\geq 10^7$ IU/mL, 4 patients who had a peak viral load of $>10^8$ IU/mL were identified. All of these patients died within the first 2 years after transplant (mean, 11.8 months). Doughty et al reported similar observations. They observed that recipients with more severe graft injury, progressive cholestasis, and jaundice with marked central zonal ballooning on liver biopsy had the highest viral loads, which repeatedly were measured in excess of $10^7$ copies/mL[178].

Many studies have shown that advanced donor age is associated with lower graft survival[179]. However, it should be recognized that these studies did not include extensive analysis of posttransplant viral load as was done here.

Immunosuppression is a major factor that accounts for the accelerated history of HCV infection post-transplant. Worse outcomes have been reported in recent years that parallel changes in immunosuppression with the introduction of newer and more potent immunosuppressive regimens [124, 140]. In particular, there has been a shift to the greater use of TAC and less use of CsA. This has led to the speculation that the choice of CNI may affect the severity of HCV recurrence.

CNIs are the cornerstone of current immunosuppressive regimens in liver transplantation. Importantly, the CNI choice (TAC or CsA) was not an independent predictor of patient and allograft outcomes in our study. Interestingly, all patients with a peak viral load of $>10^7$ IU/mL were on TAC immunosuppression. Although this may imply a possible deleterious effect of TAC, previous studies have shown no significant difference in the HCV RNA levels in CsA versus TAC-treated patients[180]. Although in the present cohort there was worse patient survival with CsA ($P = 0.05$), this observation was not explained by an increased number of rejection episodes (and therefore a greater likelihood of exposure to steroid pulses and OKT3) because the number of rejection episodes was not significantly different between the 2 CNIs ($P = 0.11$), although there was a non-significant trend towards increased rejections for CsA. However, after adjusting for other clinically significant factors in the multivariate analysis, it was shown that the type of CNI is not an independent predictor of worse outcomes (both patient and graft
survival). Therefore, these findings suggest that the choice of initial CNI does not per se adversely affect outcomes of patients undergoing liver transplantation for HCV but rather, that viral load may be a surrogate measure of the overall level of immunosuppression.

AZA is primarily used in maintenance immunosuppressive regimens as a steroid-sparing agent. Data on the effect of AZA use on the posttransplant course are few but suggest a beneficial effect of maintenance therapy given for 6 months or longer [117]. In our cohort, AZA use was associated with better patient and graft survival. Long-term analysis by Hunt et al. [181] previously showed that patients treated with AZA-containing regimens experienced significantly less recurrence and progression than those without AZA as part of their immunosuppression regimen. Furthermore, AZA has a direct antiviral effect on HCV [182]. The exact mechanism by which AZA confers a survival advantage is likely to be multifactorial, including patient selection, avoidance of acute rejection episodes, and a direct antiviral effect of AZA.

Multiple studies have shown a strong association between treatment of rejection and severe recurrence of hepatitis C [183, 184]. Patients with multiple rejection episodes, exposure to steroid pulses, and greater daily exposure to steroids have a greater incidence and severity of recurrent hepatitis C[117]. In the National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplant Database study, treatment of acute cellular rejection increased mortality in HCV-positive patients (relative risk, 2.9) in contrast to a seemingly protective effect of treatment of 1 rejection episode in non–HCV-positive patients (relative risk, 0.6) [121]. Treatment of rejection episodes as an important determinant of worse outcomes is likewise confirmed in the present study. These findings confirm the importance of preventing acute rejection with adequate immunosuppression, although this should be balanced against the risks of excessive immunosuppression, which likewise negatively affect outcomes of HCV infection after transplantation.

In conclusion, this study shows that posttransplant peak HCV viral load levels of $>10^7$ IU/mL within the first year are independently associated with worse patient and graft outcomes. This study is unique in presenting HCV transplantation outcomes from the largest documented cohort of individuals with serial HCV viral loads. Previously, viral load estimations have not been
incorporated into predictor models of HCV-related outcomes post–liver transplantation. However, these results clearly show that viral load is a very important predictor of patient death and allograft failure. HCV viral load may be an alternate and superior surrogate marker of immunosuppression post-transplantation. Therefore, frequent early HCV viral load monitoring after transplantation should be incorporated into clinical practice.
CHAPTER 4

CD ANTIBODY MICROARRAY ANALYSIS OF HUMAN LIVER DISEASES
CHAPTER 4   CD ANTIBODY MICROARRAY ANALYSIS OF HUMAN LIVER DISEASES

4.1 Introduction

Advances in high-throughput technologies in genomics and proteomics have been embraced by researchers studying liver disease and applied to a broad range of disease states [185-187]. This has given insights into pathogenesis and opened avenues for drug discovery. Further, there are a growing number of studies applying this technology to diagnosis, prognostication, and treatment prediction in liver disease [185].

Gene expression profiling of hepatocellular carcinoma (HCC) has identified dysregulation of numerous molecular pathways including those associated with cell cycle control, cell proliferation, angiogenesis, apoptosis, transcriptional regulation and immune response. Patterns of gene expression have also been correlated with differential prognosis in patients diagnosed with HCC [188]. A number of studies have demonstrated that intrahepatic gene expression profiling may have predictive value in the treatment of chronic hepatitis C with interferon-based therapy and studies of gene expression in non-alcoholic fatty liver disease have highlighted a number of possible molecular processes involved in disease pathogenesis [189, 190].

The CD antibody microarray is a recent development consisting of a large number of CD antibodies immobilized on a microarray platform. Peripheral blood mononuclear cells (PBMC) are incubated with the array and those cells expressing corresponding antigens on their cell surface are captured by the antibodies [164]. The resulting pattern of cell binding densities is thus a semi-quantitative immunophenotype for that particular sample. The CD antibody microarray has been used to determine extensive immunophenotypes of human leukaemias and lymphomas and has shown high levels of consistency with diagnoses obtained using conventional methods. Also a robust CD antigen signature in retroviral disease has been demonstrated[163].
The liver can be considered a lymphoid organ with a rich and unique composition of resident lymphocytes determined by a dynamic process of recruitment of specific subsets of lymphocytes from the periphery [191, 192]. It is not clear whether the characteristics of peripheral circulating lymphocytes are reflective of the complex intrahepatic immune environment in disease processes leading to liver injury. However, there is now increasing data that PBMC gene expression can predict treatment outcomes in HCV liver disease [154, 193, 194].

The present study represents the first use of the CD antibody microarray in human liver disease. The aim was to determine CD antigen expression profiles for patients with various causes of liver disease and to look for preserved disease-specific signatures. The data were also used to generate disease prediction algorithms and to identify differential antibody expression.

4.2 Methods

Patients were recruited from liver clinics at Royal Prince Alfred and Westmead hospitals. Project approval was obtained from the Ethics Review Committee at Royal Prince Alfred Hospital and the University of Sydney (Ethics Protocol number 4292). The underlying cause of liver disease and the stage of disease as determined by the patient’s regular physician were based on clinical, radiological, and histological findings. The diagnosis of NASH was made histologically in all patients. Patients were excluded if there were any clinical or laboratory features to suggest active sepsis as this would likely affect the CD antigen expression profiles. The patients selected for this study were representative of patients commonly encountered in the liver clinics. Consequently, there was a broad range of disease severity including patients awaiting liver transplantation. Normal controls had no apparent liver disease.

4.3 Results

4.3.1 Patient Characteristics
Patients from three liver disease groups including HCV, NASH, and alcohol-related liver disease (ALD) were compared to a normal group. Table 4.1 lists the patient groups with average values for a number of laboratory markers of liver function and clinical characteristics. Almost all patients with NASH-related cirrhosis had compensated disease with a mean MELD (Model for End Stage Liver Disease) score of 10, whereas most of those with alcohol and HCV-related cirrhosis had more advanced cirrhosis with mean MELD scores of 18.4 and 15.2 respectively. Also notable was a male predominance in patients with alcohol and HCV-related cirrhosis compared to a more even sex distribution in patients with NASH. Amongst the patients with cirrhosis, 5 patients with HCV and 4 with alcohol-related disease also had hepatocellular carcinoma (HCC). The characteristics of these subgroups did not differ significantly from the parent groups. A normal group was also included in the analysis and consisted of 23 healthy individuals without any significant medical history or liver disease. This group had a mean age of 69 years (54-75), and had laboratory values in the normal range for age.

4.3.2 Dot Patterns and Expression Profiles

A representative CD array with cell binding for a patient with liver disease is shown in Figure 4.1. Cell binding was reproducible on the duplicate arrays with good correlation (mean $R^2 > 0.90$). Expression profiles displaying the average cell binding densities for the different groups of liver disease and normal patients are shown in Figure 4.2. It is clear from inspection that the pattern of cell binding densities is very similar between all groups, including the normal group, and that only subtle differences in cell binding differentiate the groups. This is not surprising as in most disease processes only a very small fraction of genes or protein products are affected.

4.3.3 Differential Groups

Plots of the first three principal components are shown in Figure 4.3. The most striking feature on these plots is the excellent statistical clustering of the normal group which is distinct from most other samples. When considering all patients according to the underlying cause of liver disease (Figure 4.3a), there is no apparent clustering or separation of groups. When all the samples are categorised as either cirrhotic or non-cirrhotic (Figure 4.3b), there was loose
clustering and significant overlap between the two groups. This improved when patients with advanced cirrhosis (i.e. with portal hypertension) were considered (Figure 4.3c). Patients with HCV related cirrhosis and portal hypertension clustered closely with patients with alcohol-related cirrhosis and portal hypertension, while patients with non-cirrhotic HCV clustered as a separate distinct group. When examining the HCV group alone (Figure 4.3d), it is clear that samples from non-cirrhotics cluster separately to those from patients with cirrhosis, and also demonstrate tighter clustering. When only NASH samples were considered, there was no clustering or separation of cirrhotics and non-cirrhotics. There was also no apparent clustering or separation of the subgroup of samples with HCC.

Table 4.1

Patient Characteristics#

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<tr>
<th></th>
<th>HCV cirrhotic</th>
<th>HCV noncirrhotic</th>
<th>NASH cirrhotic</th>
<th>NASH noncirrhotic</th>
<th>ETOH cirrhotic</th>
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<tbody>
<tr>
<td>N</td>
<td>20</td>
<td>15</td>
<td>6</td>
<td>15</td>
<td>14</td>
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<tr>
<td>Age</td>
<td>51# (36-66)</td>
<td>46 (31-62)</td>
<td>70 (56-81)</td>
<td>53 (36-66)</td>
<td>60# (49-78)</td>
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<tr>
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<td>85 (16-718)</td>
<td>40 (4-22)</td>
<td>50 (9-19)</td>
<td>60 (4-19)</td>
<td>93 (11-316)</td>
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<td>Bili</td>
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<td>45 (41-48)</td>
<td>32 (23-39)</td>
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<tr>
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<td>1.0 (1-2.3)</td>
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<td>30.4 (26-36)</td>
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<td>112 (20-470)</td>
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<td>ALT</td>
<td>14.9* (9-29)</td>
<td>n/a (6-11)</td>
<td>9 (6-11)</td>
<td>n/a (6-11)</td>
<td>17.4* (6-27)</td>
</tr>
</tbody>
</table>

# Mean values and (ranges) for various clinical and laboratory measurements.
*p < 0.015 compared to NASH,
*#p<0.05 compared to NASH.

Bili – bilirubin (umol/L); ALT – alanine aminotransferase (U/L); Alb – albumin (g/L); INR – International Normalised Ratio; BMI – body mass index; MELD – Model for End Stage Liver Disease, MELD = 3.8[Log_e 17.1*bilirubin/17.1 (umol/L)] + 11.2[Log_e INR] + 9.6[Log_e creatinine/88.4 (umol/L)] + 6.4; n/a – not applicable.
Figure 4.1. Representative CD array for a patient with HCV-related cirrhosis. (A) Image of CD array slide with cell binding to antibody dots; (B) Key to CD antibodies: The numbers represent the different CD antibodies. TCRα/β and TCRγ/δ are T-cell receptors α/β and γ/δ, HLA-DR is a Class II histocompatibility leukocyte antigen, FMC7 detects a conformation epitope on CD20, kappa and lambda are immunoglobulin light chains, and sIg is surface immunoglobulin. Murine isotype controls, mIgG1, mIgG2a, mIgG2b, and mIgM did not demonstrate significant cell binding.
Figure 4.2. Expression profiles for different liver disease groups and normals. These bar charts are average cell binding densities for all patients in the respective disease groups. The horizontal axis represents the antibodies on the array and the vertical axis the average density of cell binding. The overall pattern of cell binding is similar in all groups and only a few antibodies are differentially expressed between groups. As an example, the binding of CD11b and CD11c (*) were significantly increased in all disease groups compared to normal.
4.3.4 Differential Antibody Binding

A number of pairwise comparisons were made between different disease categories (Table 4.2). Comparison of individual disease groups with the normal group demonstrated differential expression of multiple antibodies. Interestingly, the relative expression compared to normal of 6 antigens, CD45RO, CD11b, CD11c, CD36, CD16 and CD31 was increased in all the liver disease groups and featured amongst the antigens with the most significant differential expression. CD45RO has an important role in T-cell and B-cell receptor mediated activation, CD11b and CD11c in neutrophil adhesion and also phagocytosis of complement coated particles, CD36 in platelet adhesion and recognition and phagocytosis of apoptotic cells; CD16 is the low affinity Fc receptor and plays an important role in phagocytosis, and CD31 functions as an adhesion molecule. No significant differential expression was seen between HCC and the parent cirrhosis group without HCC. A number of T cell markers were underrepresented in leukocytes amongst patients with cirrhosis compared with non-cirrhotic patients. These include TCRαβ, its signalling partner CD3, the CD8 coreceptor, and the CD28 costimulatory receptor. The ratio of CD45RO to CD45RA was increased in cirrhotic patients suggesting an increased frequency of antigen-experienced T cells in the cirrhotic patients.
Figure 4.3. Principal component analysis (PCA) for various disease categories. (A) All disease groups according to underlying cause. (B) Cirrhosis and non-cirrhosis (all samples); (C) Advanced cirrhosis with portal hypertension (HCV and Alcoholic-ALD) and HCV noncirrhosis; (D) HCV cirrhosis and non-cirrhosis; These 3-dimensional plots of the first 3 principal components (1,2,3) were rotated to demonstrate the maximum separation between disease categories. Each sphere represents one patient.
4.3.5 Disease Prediction

Predicted disease association based on expression profiles was compared to known disease categories. When samples where categorised as cirrhotic and non-cirrhotic, the disease prediction algorithm (using leave one out cross-validation) had a sensitivity of 80% and a specificity of 60% for the diagnosis of cirrhosis (overall success 71%). When only HCV samples were used, the prediction algorithm had a sensitivity of 80% and a specificity of 73% for the diagnosis of cirrhosis (overall success 77%). The calculated prediction algorithm incorrectly classified most of the samples with HCC, suggesting a relatively weak disease signature for HCC.

The CD expression data was also used to make pairwise comparisons between different disease groups. These results expressed as receiver operator characteristics (ROC) area under curves (AUC) are represented in Table 4.3. Kernel smoothed ROC AUC (upper triangle, shown in white) and raw ROC AUC (lower triangle, shown in grey) are presented in Table 4.3. Performance of the ROC AUC where assessed as >0.8 being good and >0.85 being excellent. NASH was most readily distinguished from other types of liver disease with ROC AUC ranging from 0.76 – 0.95. Conversely alcoholic cirrhosis was poorly distinguished from HCV cirrhosis (ROC AUC 0.64) and HCV non-cirrhosis (ROC AUC 0.68/0.72).

In the sub-group of patients with HCV, the ability of the CD array to predict cirrhosis was compared with that of the APRI - aspartate aminotransferase-to-platelet ratio index [calculated as aspartate aminotransferase (AST) (U/L)/upper normal limit x100/platelet count (10⁹/L)]. The APRI is easy to calculate and has been extensively studied. Using an APRI threshold of 1.0, a recent systematic review showed an overall sensitivity and specificity of 76% and 71% respectively for the identification of cirrhosis in patients with HCV [195]. Using the same threshold in our patient group yielded a sensitivity and specificity of 90% and 60% respectively. This is comparable to the results using the CD array (sensitivity 80%, specificity 73%).
Table 4.2

Significant Differential Antibody Expression

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Differential antibody expression</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD11b</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>CD11c</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>HCV</td>
<td>CD16</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>ALD vs. *Normal</td>
<td>CD36</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>NASH</td>
<td>CD16</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>CD45RO</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>CD31</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>*Cirrhosis vs. Non-cirrhosis</td>
<td>CD45RA</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>CD28</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>TCR a/b</td>
<td>0.034</td>
</tr>
<tr>
<td>*HCV cirrhosis vs. HCV noncirrhosis</td>
<td>CD45RA</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>CD3</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>TCR a/b</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td>CD7</td>
<td>0.046</td>
</tr>
</tbody>
</table>

(*) Expression of antigen significantly lower in normal, cirrhosis (all causes), and HCV cirrhosis.
Table 3.

Receiver Operating Characteristics (ROC) Analysis

<table>
<thead>
<tr>
<th>ROC</th>
<th>Alcohol Cirrhosis</th>
<th>HCV Cirrhosis</th>
<th>NASH</th>
<th>HCV Non-cirr.</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol Cirrhosis</td>
<td></td>
<td>0.64</td>
<td>0.90</td>
<td>0.68</td>
<td>0.91</td>
</tr>
<tr>
<td>HCV Cirrhosis</td>
<td>0.64</td>
<td></td>
<td>0.90</td>
<td>0.67</td>
<td>0.82</td>
</tr>
<tr>
<td>NASH</td>
<td>0.94</td>
<td>0.95</td>
<td></td>
<td>0.76</td>
<td>0.94</td>
</tr>
<tr>
<td>HCV Non-cirr.</td>
<td>0.72</td>
<td>0.70</td>
<td>0.82</td>
<td></td>
<td>0.83</td>
</tr>
<tr>
<td>Normal</td>
<td>0.92</td>
<td>0.83</td>
<td>0.92</td>
<td>0.84</td>
<td></td>
</tr>
</tbody>
</table>

ROC Area Under Curve (AUC) for various pairwise comparisons. These were assessed as >0.8 being good and >0.85 being excellent.
4.4 Discussion

The liver has a rich and unique composition of lymphocytes, which is shaped by a dynamic equilibrium between recruitment, retention, apoptosis, and emigration. The molecular mechanisms by which lymphocytes are recruited to the liver and in which they perform effector functions crucial in the immunopathogenesis of liver disease are areas of active research [196, 197]. The majority of liver diseases are associated with significant immune processes. Hepatitis C associated liver disease is a good example and many studies have demonstrated a central role of T cells and natural killer (NK) cells in HCV viral control and immunopathogenesis [56, 198]. Thus it may be expected that the leukocyte cell surface proteome would reflect to an extent the state of the liver disease and that this ‘immunophenotype’ may have research and diagnostic utility.

There are some clear advantages in using a CD array compared to other proteomic or genomic arrays. The assay itself is much easier to perform and does not require multiple complex steps to purify the samples. Also PBMCs are much more accessible than the liver tissue used in most genomic studies. There is also the possibility that the cell surface proteome provides a more accurate reflection of function than the expression of mRNA.

The results presented here show that the CD antibody array is able to distinguish major stages of human liver disease. The lack of any significant clustering according to the underlying cause of liver disease may reflect a more dominant signature for advanced stages of liver disease than for the underlying causes. Compared to normal samples, samples from liver disease groups showed increased expression of CD45RO which is a marker of a primed or memory T cell phenotype. Further, there was increased expression of a number of adhesion molecules including CD11b and CD31 which may play a role in immune mediated liver injury.

The results obtained when samples were categorised as advanced cirrhosis and non-cirrhosis are interesting. Although there were outliers and some overlap there was definite clustering and separation between these groups. This was also true when the subgroup of patients with HCV was considered. Cytotoxic T lymphocytes (CTL) play an important role in the pathogenesis of
liver disease, particularly HCV related disease [198]. The reduced expression of the CTL marker CD8 in patients with cirrhosis suggests a depletion of this sub-population of T-cells. In addition, reduced CD45RA expression indicates an overall depletion of naïve T-cells. These changes, together with the reduction of T-cell markers (CD3, TCRα/β) and the T-cell activation marker CD28, suggest a less effective or an exhausted (anergic) response in patients with more advanced disease.

Samples from patients with HCC did not show a distinct disease signature, nor did they cluster with the parent liver disease group. This does not necessarily imply that the development of HCC does not cause changes in the peripheral lymphocyte surface proteome but may suggest a stronger overriding signature due to the stage of liver disease and possibly a lack of relevant antibodies on the microarray used.

The inability to discriminate between lower stages of fibrosis was disappointing but may improve with further refinement. On the other hand the ability to discriminate advanced cirrhosis from earlier stages of liver disease is more promising and may reflect a signature for portal hypertension. This may have potential clinical utility as the current methods available to measure portal pressure are invasive and there is ample data suggesting usefulness of portal pressure estimation in predicting outcomes in cirrhosis such as HCC, bleeding, and clinical decompensation [199, 200].

Future clinical applications of this technology would likely focus on more homogenous patient groups and this would be expected to improve performance. Additionally, the array used in this study was initially designed to study and diagnose leukaemia and lymphoma, so the antibodies were selected for this purpose. Further improvements in performance may be obtained by designing an array with antibodies against cell surface molecules which have been shown to be important in liver disease.

This study represents the first use of the CD antibody microarray in human liver disease and serves as a ‘proof of concept’ that this technology may have clinical and research utility.
CHAPTER 5

CD MICROARRAY ANALYSIS OF POST-LIVER TRANSPLANT HCV RECURRENCE
5.1 Introduction

HCV-related end-stage liver disease is the most frequent indication for orthotopic liver transplantation in western countries and despite anticipated improvements in the efficacy of antiviral therapy, it has been projected that the number of candidates referred for transplantation will double by 2020[111].

Following transplantation, reinfection of the liver allograft is universal leading to recurrent disease of variable outcome. While recurrent infection is evidenced by the presence of HCV RNA in serum, recurrent disease requires histological confirmation. The commonest pattern of recurrence is the development of chronic hepatitis with accelerated fibrosis progression compared to non-transplant patients with HCV. Fibrosis can progress rapidly during the first few years or present a delayed onset of rapid progression. The median duration from transplantation to cirrhosis occurs after 9 to 12 years, followed by a 42% risk for clinical decompensation at 1 year[112]. A less common but more severe pattern of disease recurrence is fibrosing cholestatic hepatitis, which usually begins a few months after transplantation and rapidly progresses to graft failure within 3-6 months of onset. This typically occurs in the context of viral loads much higher than that seen in other patterns of HCV recurrence[120]. Other patients have absent or very little disease progression for at least the first decade after transplantation[111].

Patients transplanted for HCV have poorer outcomes compared to transplantation for other indications, with up to 10% difference in survival after 10 years. In addition, while survival rates in non-HCV recipients have improved over time, survival of HCV+ve recipients has decreased over the same time, a finding likely due to changes in immunosuppression and increasing donor age[123].

Successful antiviral therapy with PegIFN is associated with improved outcomes but suffers from poor tolerability and efficacy, particularly in advanced stages of disease. Monitoring with protocol liver biopsies is important to identify and offer treatment to those patients with rapid
progression. Non-invasive means of predicting severe recurrence are required. About 20% to 30% of patients with histological evidence of chronic recurrent HCV have persistently normal serum ALT levels[114, 119]. Fibroscan, a combination of elastography and serum fibrosis markers may be useful in identifying rapid fibrosers[201].

In Chapter 4, the CD antibody microarray was used to demonstrate disease specific consensus patterns of expression of CD antigens for patients with chronic liver disease including HCV infection.

The aim of this study was to determine serial CD antigen expression profiles for patients undergoing liver transplantation for active HCV infection looking for preserved disease-specific signatures predictive of outcomes.

5.2 Methods

Patients recruited for this study were attendees of the liver clinics or inpatients with end-stage HCV with or without hepatocellular carcinoma (HCC) and on the liver transplant waiting list. Project approval was obtained from the Ethics Review Committee at Royal Prince Alfred Hospital and the University of Sydney (Ethics Protocol number 4292). After obtaining informed consent, blood (5 ml) was collected from patients by venepuncture and placed into tubes containing EDTA. These were kept at room temperature and assayed within 24 h of collection. In each patient, CD antibody microarray assays were performed during the Pre-transplant (day0), Early (d3–week2), Mid (w4-w10), and Late (>w12) phases. Assays were not performed if there were any clinical or laboratory features to suggest active sepsis as this would likely affect the CD antigen expression profiles. The assay technique is described in detail in Chapter 2.

Concomitant clinical data was also collected during each phase. This included clinical progress, haematological and biochemical parameters, and also HCV RNA viral load. Protocol liver biopsies were performed at 1 and 2 years post-transplant, and also when clinically indicated.
Although it is agreed that protocol histological assessment is required, there is no consensus on the definition of severe HCV recurrence. This relates to the cut-off value for fibrosis stage and also the timeframe within which this is deemed to be severe or indicative of rapid disease progression. This is likely a reflection of the variable nature of disease recurrence. While studies consistently demonstrate poor outcome with higher stages of fibrosis (F=3-4), the finding of low or intermediate stages of fibrosis in the first few years post-transplant is associated with variable outcomes. In one study, 35% of patients with no or mild fibrosis during the first 3 years post-transplant progressed on to more aggressive liver injury, with F3 or F4 fibrosis after 4-7 years of follow-up, which was referred to as late-onset severe recurrence [184, 202]. For this reason, in this study we used four definitions to classify the severity of disease recurrence (Table 5.2). In the first classification, severe recurrence was defined as F3-4 fibrosis stage with the first 2 years of follow-up. In classification 2, severe recurrence was F2-4 fibrosis, while in classification 3 severe recurrence was defined as F3-4 and mild recurrence as F<2, leaving those with F2 fibrosis unclassified. With these definitions, the uncertain predictive value of F2 fibrosis could be assessed. In Chapter 3 it was demonstrated that a peak viral load $\geq 10^7$ IU/mL in the first year post-transplant was an independent predictor of patient and graft survival. Therefore a fourth classification with this viral load cut-off within the first year post-transplantation was included.

5.3 Results

5.3.1 Patient Characteristics

Sixteen patients from the liver transplant waiting list with HCV related liver disease with or without hepatocellular carcinoma were recruited consecutively for inclusion in this study (Table 5.1). A total number of 98 assays were performed. The average age of patients was 52 with only one female recruited. For more than half of the patients (9/16), HCC was a contributing indication for transplantation. The average MELD for the whole group was 16, while the MELD scores for those with and without HCC were 19 and 13 respectively. HCV genotypes 1 and 3 were present in 31% and 56% of patients respectively. Clinical outcome was followed up for 3-5 years after transplantation. Three patients underwent antiviral therapy
(combination peg Interferon/Ribavirin) during the first 2 years post-transplantation, two achieving a sustained virological response, while treatment in the third had to be ceased due to rejection. This patient required re-transplantation at three years.

5.3.2 Recurrence Severity versus Classification Definition

Patients were classified as having severe or mild HCV recurrence according to four definitions as outlined in Table 5.2. The rationale for the selection of these criteria was based on the presence or absence of advanced fibrosis or viral load. Liver injury was classified on the basis of fibrosis stage and peak viral load post-transplant. Development of rapid fibrosis is a strong predictor of allograft loss due to HCV recurrence. However, F2 fibrosis in particular is difficult to classify – whilst not normal, it may not carry the same importance in determining allograft loss due to HCV recurrence as F3 or F4 fibrosis. Similarly F2 fibrosis is unlikely to be a negative discriminator. Therefore in our outcome classification we attempted to account for this variability by grouping F2 fibrosis with both higher and lower fibrosis stages, as well as excluding it in one of the definitions. Comparing the first two definitions of recurrence severity, the number of patients classified as severe increased from 4 to 10 by including F2 fibrosis in the definition of severe recurrence within 2 years. Recurrence severity did not vary with classification definition for seven patients (patient numbers 1, 2, 6, 10, 11, 12, 13). Using the third definition, which excludes fibrosis stage F2 in the definition during the first 2 years, 6 patients were unclassified.

5.3.3 Distribution of Binding Intensities

Duplicate arrays demonstrated reproducible binding with correlation $R^2 > 0.90$. Figure 5.1 demonstrates the mean binding intensity of all the antibodies on each assay and control antibodies CD44 and CD29. The variance in binding versus the mean binding intensity for each antibody on the microarray is shown in Figure 5.2. Control antibodies CD44 and CD29 are saturated by binding and show little variance in comparison to most other antibodies.
Table 5.1

Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>MELD</th>
<th>Post-Transplant Clinical Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>M</td>
<td>HCV 3a/HCC</td>
<td>20</td>
<td>Required retransplant (cirrhosis at 2 years)</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>M</td>
<td>HCV 3a</td>
<td>17</td>
<td>Successful AVT at 1 year</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>M</td>
<td>HCV 3a/ HCC</td>
<td>18</td>
<td>Pancreatitis sepsis - died 1yr 8mth</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
<td>M</td>
<td>HCV 3a/ HCC</td>
<td>13</td>
<td>Successful AVT at 2yr 6mth</td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>F</td>
<td>HCV 1b/ HCC</td>
<td>16</td>
<td>Recurrent HCC with metastases</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>M</td>
<td>HCV indet#</td>
<td>26</td>
<td>Cholestatic hepatitis</td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>M</td>
<td>HCV 3a</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>47</td>
<td>M</td>
<td>HCV 3a/HCC</td>
<td>9</td>
<td>AVT SVR at 2yr 4mth</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>M</td>
<td>HCV 3a</td>
<td>14</td>
<td>Failed AVT at 3 years</td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>M</td>
<td>HCV 1b/ HCC</td>
<td>12</td>
<td>Successful AVT at 3 years</td>
</tr>
<tr>
<td>11</td>
<td>56</td>
<td>M</td>
<td>HCV/HCC 1a</td>
<td>6</td>
<td>ACR</td>
</tr>
<tr>
<td>12</td>
<td>56</td>
<td>M</td>
<td>HCV 1a</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>42</td>
<td>M</td>
<td>HCV indet#</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>53</td>
<td>M</td>
<td>HCV 3a/HCC</td>
<td>6</td>
<td>Successful AVT 2yr 10mth</td>
</tr>
<tr>
<td>15</td>
<td>48</td>
<td>M</td>
<td>HCV 3a/HCC</td>
<td>21</td>
<td>Successful AVT 2yr 9mth</td>
</tr>
<tr>
<td>16</td>
<td>49</td>
<td>M</td>
<td>HCV 1a</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>52</td>
<td></td>
<td>Genotype 3 - 56%</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Genotype 1 - 31%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#HCV genotype was indeterminate in 2 patients

AVT, antiviral therapy; SVR, sustained virological response; ACR, acute cellular rejection
Table 5.2
Classification Definition versus HCV Recurrence Severity

<table>
<thead>
<tr>
<th>Classification Definition</th>
<th>HCV Recurrence Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild (patient identifier)</td>
</tr>
<tr>
<td>1. <strong>Severe</strong> - F3-4 within 2 years, <strong>Mild</strong> - F≤2 within 2 years</td>
<td>12(2,5,7,9,10,12-16)</td>
</tr>
<tr>
<td>2. <strong>Severe</strong> - F2-4 within 2 years, <strong>Mild</strong> - F&lt;2 within 2 years</td>
<td>6(2,3,5,10,12,13)</td>
</tr>
<tr>
<td>3. <strong>Severe</strong> - F3-4 within 2 years, <strong>Mild</strong> - F&lt;2 within 2 years (this excludes F2 fibrosis)</td>
<td>6(2,3,5,10,12,13)</td>
</tr>
<tr>
<td></td>
<td>6(4,7,9,14-16)</td>
</tr>
<tr>
<td>4. <strong>Severe</strong> - Peak VL &gt;10^7, <strong>Mild</strong> - Peak VL ≤10^7 within 1st year</td>
<td>11(2,4,7-10,12-16)</td>
</tr>
</tbody>
</table>

The right columns show the numbers of patients classified as mild or severe HCV recurrence according to classification definitions. The patient identifiers correspond to individual patients listed in Table 5.1.

Classification definition fibrosis scores are based on Scheuer scoring system.

VL, viral load (in IU/ml)
Figure 5.1. Mean Binding Intensities of all Antibody Dots

This demonstrates the mean binding intensity of all antibody dots per assay (black dots). Shown in red and green are the binding intensities of the control antibodies CD29 and CD44 for each assay which are clearly saturated.
Figure 5.2 Variance versus Mean Binding Intensity For Each Antibody.
This demonstrates the variance versus the mean binding for each antibody across all assays performed. CD29 and CD44 are the control antibodies. The blue antibodies represent the most significant differentially expressed antibodies (Section 5.3.4)
5.3.4 Differential Expression Analysis

Differential antibody expression analysis was performed for each transplant phase and across the 4 different recurrence severity definitions. Most interesting is that the greatest differential antibody expression was seen in the pre-transplant phase, irrespective of the definition used for severe HCV recurrence (Figure 5.3). This suggests that the pre-transplant CD antigen expression profile may be the greatest determinant of recurrent HCV disease severity post liver transplantation.

Table 5.3 lists the differentially expressed antibodies which were common across the 4 recurrence severity definitions. In the pre-transplant phase these include reduced expression of the T-cell activation molecule CD27 and CD182, and increased expression of CD41, CD260, and CD34 in those who later developed severe recurrence. In the Late phase (>w12) of sampling, expression of a single antigen, CD152, which suppresses cytotoxic lymphocyte activity was reduced in those with severe recurrence.

Table 5.4 lists the antibodies which were significantly expressed in 3 of 4 definitions. What is clear from this is that there are a greater number of antibodies in the pre-transplant phase compared to post-transplant phases. The definitions of recurrence in which severe disease requires more advanced stages of fibrosis (ie F3-4, classifications 1 and 3) contained a greater number of significant antibodies compared to definitions in which intermediate stages of fibrosis were included in the definition of severe (F2-4, classification 2). Also clear is the significant discordance between classification 2, in which F2 was included in the definition of severe recurrence and classification 4 in which severity was defined by peak viral load. This was not seen with classifications 1 and 3 where F3-4 defined severe recurrence. Significant antibodies with reduced expression in patients with severe recurrence include CD81, which is known to mediate HCV cellular entry, the costimulatory receptor CD28, TCRαβ, the death receptor CD95, and CD127 which blocks apoptosis of activated T cells[203].
5.3.5 Heirarchial Clustering of CD Antibody Expression with Outcome

Heirarchial clustering was performed for assays from the Pre-Transplant period and using the antibodies which were significantly expressed in 3 out of 4 classification definitions as shown in Figure 5.4. It is clear that there is considerable heterogeneity in individual CD antibody expression. However the combined signature does predict outcomes.

![Number of Differential Antibodies vs Transplant Phase](image)

**Figure 5.3. Number of Differentially Expressed Antibodies versus Transplant Phase**

The number of differentially expressed antibodies between patients who develop severe and mild disease for all 4 classification definitions is represented by the collection of dots at each transplant phase. It is clear from this that the greatest number of differentially expressed antibodies irrespective of classification definition were seen in the Pre-Transplant phase.
Table 5.3
Differentially Expressed Antibodies Common Across the 4 Recurrence Severity Definitions.

<table>
<thead>
<tr>
<th>CD Antibody</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Classification 1</td>
</tr>
<tr>
<td><strong>Pretransplant Phase</strong></td>
<td></td>
</tr>
<tr>
<td>CD27 PH</td>
<td>-5.24</td>
</tr>
<tr>
<td>CD182</td>
<td>-2.38</td>
</tr>
<tr>
<td>CD260</td>
<td>6.06</td>
</tr>
<tr>
<td>CD41</td>
<td>6.41</td>
</tr>
<tr>
<td>CD34</td>
<td>18.13</td>
</tr>
<tr>
<td><strong>Late Phase</strong></td>
<td></td>
</tr>
<tr>
<td>CD152</td>
<td>-4.20</td>
</tr>
</tbody>
</table>

Fold changes are absolute. Positive fold changes reflect increased expression in patients developing severe recurrence. Classification definitions are shown in Table 5.2.
Table 5.4
Differentially Expressed Antibodies Across 3 of 4 Recurrence Severity Definitions

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Figure 5.4. Hierarchical Clustering of CD Antibody Expression with Outcome.

The vertical axis represents the differentially expressed antibodies which were common in 3 out of 4 classification definitions. The horizontal bars at the bottom show the severity outcomes for the 4 different classification definitions.
5.4 Discussion

There are a number of clearly established factors associated with severe post-transplant HCV recurrence. These include donor age, particularly greater than 65 years, prolonged ischaemia time, steroid boluses or OKT3 for rejection, and post-transplant metabolic syndrome. We have previously shown peak post-transplant HCV RNA levels to be an independent predictor of severe recurrence, while the impact of pre-transplant HCV RNA is uncertain [111, 204, 205]. In addition, necroinflammatory activity at 1 year post-transplant has been shown to be an excellent predictor of the risk of cirrhosis at 5 years [206].

The only strategy shown to modify disease outcome is antiviral therapy. Effective means of predicting which patients will develop severe recurrence are required so that antiviral therapy can be offered in a timely fashion. Overall, sustained virological response is achieved in approximately 35% of patients given peginterferon and ribavirin, with worse results seen in those with more advanced stages of disease recurrence. Side effects, particularly anaemia, are very common, and require frequent dose reduction [207].

Intriguingly, while common differentially-expressed CD antigens were detected through multiple classifications, when considering common antibodies across 3 of 4 classification definitions, there was clearly identifiable discordance between Classification 2, in which F2 was included in the definition of severe disease progression, and Classification 4, in which severe disease progression was defined by high post-transplant HCV viral load. This suggests that, although there are common immune factors that affect progression of fibrosis and HCV replication evident from the differentially-expressed CD antigens common in all 4 classifications, there are also clear differences. This was not seen when F3-4 defined severe recurrence. Therefore, if detected clinically, F2 fibrosis may indicate a time of urgent intervention to ensure mild outcome. The immunological basis of this is not clear but we can speculate that immune responses at this point can be manipulated to control HCV replication. While this hypothesis is supported by our data, it needs to be tested in future studies.
The strength of this study is not in the number of individuals, but rather the number of serial assays performed, a total of 98, the majority of which were post-transplant samples. This experimental approach was adopted to understand if samples in the post-transplant setting were predictive of disease recurrence.

Importantly, these results show in fact, that pre-transplant factors are the greatest determinant of severe disease recurrence and did not change with different definitions for recurrence severity. This suggests that pre-transplant factors, possibly related to the primary course of disease (i.e. infection in the immunocompetent state) and contributing to an individual’s inability to clear primary infection still exist and shape the outcome HCV reinfection. These may include previously acquired viral adaptations, ineffective T cell responses, and genetic factors.

The transplantation process acts as a bottleneck, with emergence of a more homogenous quasispecies population which are closely related to viral variants from the explanted liver and likely represent evolutionarily superior forms[208]. Longitudinal analysis of T-cell epitope-coding regions of HCV has demonstrated that they remain largely conserved after transplantation[209]. In this regard, a stable quasispecies pattern over time has been associated with more severe recurrence, while increased viral genetic diversity is predictive of milder disease recurrence[210-212].

Intrahepatic T-cell responses occur in the context of a complex hepatic microenvironment, and although our study relies only on peripheral mononuclear cells, there is evidence of significant overlap in the intrahepatic and peripheral T-cell responses in chronic HCV[213]. Failure or exhaustion of intrahepatic T-cell responses is well documented in chronic HCV and likely reflects mutational escape and possibly other inhibitory factors [79, 214]. It is likely many of these factors persist in the post-transplant setting and contribute to ineffective viral clearance. These would be further accentuated by immunosuppression, including corticosteroids, with resultant increase in HCV replication, and calcineurin inhibitors which dampen T-cell responses and antigen presentation [113, 215].
Recent genome-wide association studies have identified single nucleotide polymorphisms near the IL28B gene to be strongly associated with spontaneous and treatment induced clearance of HCV. In liver transplant patients, IL28B variations in the recipient, but not donor have been associated with histologic recurrence in the liver graft and the progression of fibrosis. In contrast, both recipient and donor IL28B variations are independent predictors of post-transplant peginterferon treatment response [37, 135]. Recent studies suggest a greater role for innate antiviral immunity, in particular NK cells, in control of HCV recurrence, so this may be one strategy to reduce the burden of recurrent disease[216].

These findings argue for further investigation of pre-transplant factors, both for better prediction and understanding of HCV disease recurrence.
CHAPTER 6

DISCUSSION AND CONCLUSIONS
CHAPTER 6 DISCUSSION AND CONCLUSIONS

The first study in this thesis (Chapter 3) was a retrospective analysis of 118 consecutive HCV-positive liver transplant patients with a median duration of follow-up of 32.4 months. Both univariate and multivariate analysis of the effect of recipient, donor, surgical, and viral factors on graft and patient outcomes were undertaken. A total of 620 viral load measurements were undertaken in the first 12 months following transplantation, making this the largest study to assess if viral load post-transplant is predictive of patient survival or allograft loss. Overall patient survival rates of 87.8%, 79.9%, and 70.1% and graft survival rates of 87.0%, 79.2%, and 68.2% at 1, 3, and 5 years, respectively are in keeping with other studies. In the multivariate analysis, peak viral RNA $\geq 10^7$ in the first year post-transplant and increased donor age ($\geq 50$) were independent predictors of diminished patient survival, while azathioprine use was associated with improved outcomes. Exposure to antirejection therapy was an independent predictor of diminished graft survival.

Studies of post-transplant viral kinetics demonstrate that viral load increases rapidly from about 2 weeks post-transplantation and peaks between the first and fourth post-operative months. By the end of the first postoperative year, viral load is on average, 10 to 20 times greater than pretransplantation levels[116]. A number of factors likely influence this increased post-transplant viraemia.

The introduction of an uninfected liver may provide an environment more suited to viral replication[217]. This could be due to differences in hepatocyte metabolic state, the degree of inflammation in the allograft, and properties of other non-parenchymal cells which are involved in immunity and fibrosis development. The new allograft may also present a more favourable set of target cell receptors for viral binding and entry. In this regard, in a recent study it was shown that the levels of HCV receptors claudin-1 and occludin significantly increased after liver transplantation for HCV and were positively correlated with early post-transplant HCV kinetics[218]. Further it is now apparent that the interaction between donor and recipient
genetics, in particular IL-28B genotype also influence outcomes associated with HCV recurrence in the allograft[37].

The transplantation “bottleneck” may also impact on post-transplant viraemia. This describes emergence of a more homogenous quasispecies population which are closely related to viral variants from the explanted liver and likely represent evolutionarily superior forms[208]. A stable quasispecies pattern over time has been associated with more severe recurrence, while increased viral genetic diversity is predictive of milder disease recurrence[210-212]. A recent study by Dragun et al examined early viral kinetics and viral genetic evolution after HCV liver transplantation. They found that conserved sequences in hypervariable region 1 (HVR1) were associated with increasing viral loads while adaptive mutations in HVR1 were associated with a plateau early HCV kinetic, suggesting that HCV genetic evolution may account for early viral kinetics post-liver transplantation [219].

Immunosuppression is an important and well documented factor in post-transplant viraemia. Pulsed steroids for acute rejection were associated with a 4-100 fold increase in HCV RNA compared to pre-transplant levels in one study[113], while in another study, HCV RNA levels at one year post-transplant were correlated with the duration of corticosteroid treatment[142].

The effect of calcineurin inhibitors on viral load in posttransplant patients is unclear. Interestingly in the present study, all patients with a peak HCV RNA ≥ 10^7 were on tacrolimus-based immunosuppression which may suggest a deleterious effect of tacrolimus or alternatively, antiviral properties of cyclosporine. Cyclosporine inhibits HCV replication in vitro but in vivo activity is controversial while tacrolimus does not appear to have any anti-HCV activity[117]. In the current study it was also shown that cyclosporine was associated with reduced patient survival compared to tacrolimus, but this was not borne out in the multivariate analysis. A recent meta-analysis of 16 randomised controlled trials demonstrated improved patient and graft survival in tacrolimus versus cyclosporine-treated patients which is reflected in the current trend which favours tacrolimus-based immunosuppression[147]. It is plausible but not shown that tacrolimus avoids more aggressive anti-rejection regimes and this may be the over-riding difference, nullifying any antiviral benefit of cyclosporin.
In this present study azathioprine use was independently associated with improved patient and graft survival. Maintenance azathioprine is used as a steroid-sparing agent and likely has a beneficial effect when given for 6 months or longer. Azathioprine has a direct antiviral effect on HCV. The exact mechanism by which azathioprine confers a survival advantage is likely to be multifactorial, including patient selection, avoidance of acute rejection episodes, and a direct antiviral effect [182].

The results from this first study show that high viral loads in the early post-transplant period are predictive of poor overall outcomes and argue for routine monitoring of early post-transplant viraemia. Increased immunosuppressive therapy impairs host viral responses, while inadequate immunosuppression increases the risk of cellular rejection and graft loss. As no satisfactory measure of overall immunosuppression is available, post-transplant HCV RNA as a surrogate marker may fulfill this role.

The second study in this thesis (Chapter 4) was a cross-sectional pilot study using the recently developed CD antibody microarray to characterise human liver diseases. The study population included patients with HCV, NASH, alcohol-related liver disease (ALD), and a normal group. This has demonstrated disease-specific consensus patterns of CD antigen expression for patients with chronic liver disease. There was clear separation of liver disease groups from the normal group but no overall separation of liver disease groups when considered as a whole. A prominent finding was the ability to separate major stages of liver disease. Patients with advanced cirrhosis and portal hypertension (with mean MELD >15) clustered separately to those without cirrhosis. In addition, when only patients with HCV were considered, the separation between advanced and early stage disease persisted, suggesting a stronger signature for disease stage than disease aetiology.

Disease prediction was also undertaken using leave one out cross-validation. Predicted disease association based on expression profiles was compared with known disease categories. When only HCV patients were considered, the prediction algorithm had an overall success of 77%. Differential expression analysis demonstrated reduced expression of the CTL marker CD8 in patients with cirrhosis. In addition, reduced CD45RA expression indicates an overall depletion of
naïve T-cells. These changes, together with the reduction of T-cell markers (CD3, TCRα/β) and the T-cell activation marker CD28, suggest a less effective or an exhausted (anergic) response in patients with more advanced disease. This is consistent with the finding of impaired or inhibited T-cell function in various liver diseases [79, 213, 214, 220].

The liver is a complex immune organ with a rich and unique composition of lymphocytes which is shaped by a dynamic equilibrium between recruitment, retention, apoptosis, and emigration. [196, 197]. There appears to be significant overlap in intrahepatic and peripheral T-cell responses[213] and therefore the peripheral CD antigen profile may, to an extent, reflect intrahepatic immune processes. The obvious advantage of using the CD antibody microarray is the easy access to peripheral blood and uncomplicated assay technique. Refinement of the microarray with more relevant antibodies to the disease under study would also likely improve performance. This was the first study to use the CD antibody microarray in human liver disease and demonstrates disease-specific expression patterns of CD antigens. Based on these promising results, the next study in this thesis (Chapter 5) also utilised the CD antibody microarray, but in a different population group, HCV positive liver transplant patients.

In chapter 5, serial CD antigen expression profiles were performed in 16 consecutive patients undergoing liver transplantation for active HCV infection. Assays were performed during the Pre-transplant (day0), Early (d3–week2), Mid (w4-w10), and Late (>w12) phases. Protocol liver biopsies were performed at 1 and 2 years post-transplant, and clinical follow-up occurred for at least 3 years. Due to the variable nature of disease recurrence, four different definitions were used to classify disease recurrence severity. F2 fibrosis within the first 2 years was grouped with both higher and lower fibrosis stages, as well as excluding it in one of the definitions of severe recurrence. The rationale for this is evidence that suggests that intermediate stages of fibrosis in the first few years post-transplant may not be good indicators of long-term outcome[202]. Based on the results of Chapter 3, a fourth classification of disease severity with peak viral load ≥ 10^7 IU/mL in the first year post-transplant was also included.

The most significant finding was that the greatest differential antibody expression was seen in the pre-transplant phase, irrespective of the definition used for severe HCV recurrence,
suggesting that the pre-transplant CD antigen expression profile may be the greatest determinant of recurrent HCV disease severity post-liver transplantation. This suggests that factors present prior to transplantation and contributing to failure to clear primary disease are inherited and shape the subsequent disease course. These may include host genetic factors, previously acquired viral adaptations, and impaired or ineffective immune responses.

IL28B variations in the recipient, but not donor have been associated with histologic recurrence in the liver graft and the progression of fibrosis, while both recipient and donor genotype are important in the outcome of antiviral therapy [37, 135]. As already discussed, a stable quasispecies pattern over time has been associated with more severe recurrence.

When antibodies which were significantly expressed in 3 of 4 definitions were considered, it was clear that relatively less antibodies were seen in the definition of severe recurrence which required intermediate stages of fibrosis (F=2). In addition, there was clearly identifiable discordance between Classification 2, in which F2 was included in the definition of severe disease progression, and Classification 4, in which severe disease progression was defined by peak post-transplant HCV viral load. This was not seen when F3-4 defined severe recurrence. This suggests that, although there are common immune factors that affect progression of fibrosis and HCV replication as seen by the common CD antigens across all 4 classifications, there are also clear differences. These findings would be consistent with F2 being a point at which a patient could progress to severe disease recurrence, possibly through immune factors which alter HCV replication. Therefore, if detected clinically, F2 fibrosis could indicate a time of urgent intervention to ensure mild outcome.

Overall, our findings argue for further investigation of pre-transplant factors, both for better prediction and understanding of HCV disease recurrence.

One strategy to improve disease outcomes is to intensify innate antiviral immunity. High pre-transplant NK cell populations in peripheral blood have been associated with reduced severity of HCV recurrence [216, 221]. Adoptive immunotherapy 3 days after transplantation with in vitro-
activated allograft-derived lymphocytes which includes an abundance of NK and NKT cells has been shown to markedly reduce HCV RNA in the first month post-transplant[222].

Direct-acting antivirals (DAA) such as protease inhibitors, non-structural protein inhibitors, and polymerase inhibitors represent a new paradigm in treatment of HCV-associated liver disease. The NS3/4A protease inhibitors, boceprevir and telaprevir have been approved for treatment of chronic hepatitis C in combination with PegIFN and ribavirin with improvement in SVR up to 80% in genotype 1 infection [110]. Preliminary results of this triple therapy regimen in liver transplant patients suggest limitations due to safety and tolerance, in particular interactions with calcineurin inhibitors[223].

In conclusion, this thesis has demonstrated utility of the CD antibody microarray in the study of human liver disease and has shed light on the importance of post-transplant viral loads and other pre-transplant factors in determining the severity of post-transplant HCV recurrence.

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