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Association between T Cells-Related Gene Expression and Fibrosis Progression in HCV Recurrence disease.

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Association between T Cells-Related Gene Expression and Fibrosis Progression in Recurrent HCV Disease

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

by

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Richmond, Virginia
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<td>AIDs</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<td>ALT</td>
<td>Alanine Aminotransferase</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>AST</td>
<td>Aspartate Aminotransferase</td>
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<tr>
<td>B2M</td>
<td>Beta-2-Microglobulin</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNI</td>
<td>Calcineurin Inhibitor</td>
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<tr>
<td>CDNA</td>
<td>Complimentary DNA</td>
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<tr>
<td>CT</td>
<td>Critical Threshold</td>
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<td>CTLA4</td>
<td>Cytotoxic T-Lymphocyte Antigen 4</td>
</tr>
<tr>
<td>CTP</td>
<td>Child-Turcotte-Pugh</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
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<td>E</td>
<td>Envelope Protein</td>
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<td>EBV</td>
<td>Epstein–Barr Virus</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-Fixed Paraffin-Embedded</td>
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<td>HCV</td>
<td>Hepatitis C Virus</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis Virus</td>
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<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LRRC32</td>
<td>Leucine-rich Repeat-Containing Protein 32</td>
</tr>
<tr>
<td>LT</td>
<td>Liver Transplant</td>
</tr>
<tr>
<td>MELD</td>
<td>Model for End Stage Liver Disease</td>
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<tr>
<td>MFB</td>
<td>Myofibroblasts</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
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<td>NK</td>
<td>Natural Killer Cell</td>
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<td>NS</td>
<td>Nonstructural Protein</td>
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<tr>
<td>OLT</td>
<td>Orthotopic Liver Transplant</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>QPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RIN</td>
<td>RNA Integrity Number</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>SSRNA</td>
<td>Single Stranded RNA</td>
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<tr>
<td>TGF-Beta</td>
<td>Transforming Growth Factor Beta</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>TNFRS18</td>
<td>Tumor Necrosis Factor Receptor Superfamily Member 18</td>
</tr>
<tr>
<td>UNOS</td>
<td>United Network for Organ Sharing</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
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<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
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THE ASSOCIATION BETWEEN T CELL-RELATED GENE EXPRESSION AND FIBROSIS PROGRESSION IN RECURRENT HCV DISEASE

By Alexander A. Philip, M.P.H.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Thesis Director: Valeria Mas, Ph. D., Associate Professor of Surgery and Pathology, Division of Transplant in the Department of Surgery

Hepatitis C virus (HCV) is the major cause of chronic hepatitis worldwide and a leading cause for liver transplant. Unfortunately, graft HCV infection is a universal phenomenon despite of pre-transplant prophylactic strategies. Acute HCV infection and innate immune responses elicit an inflammatory scenario that triggers the recruitment of adaptive immune response cells. Of those chronically infected, 30% experience accelerated fibrosis with concomitant cirrhosis development within 5 years post-LT and require re-transplant. With many patients responding unfavorably to antivirals and ineffective vaccines, much attention is now placed on T cell immunity in controlling HCV infection.

This study represents a retrospective analysis that examined the association of T cells with respect to liver fibrosis severity progression in a prospective cohort of biopsy samples taken from 27 patients at the time of HCV recurrence disease diagnosis post-LT. For those patients, the fibrosis progression was scored 36 months post-LT by Metavir scoring system. Liver biopsies were classified based on fibrosis severity as Mild (G1; n = 12), Moderate
(G2; n = 6), and Severe (G3; n = 9). Additionally, an independent set of liver biopsy samples, taken according to fibrosis severity progression, was classified (G1; n = 3, G3; n = 4) and used as a validation set for CD4 gene expression.

Real time PCR was performed to study the expression of immune-related genes using the Taqman® probe system. From the results analysis, the CD4 T cell marker encoding gene was down-regulated (2.9-fold) in G3 with respect to G1; although, only borderline significant (p = 0.052). This suggests an inverse relationship of CD4+T cell related-genes expression with respect to worse fibrosis progression in HCV recurrence diagnosed recipients. The validation samples showed a similar trend (1.8 fold decrease in G3 with respect to G1), although not significant. This may be due to impaired T cell function resulting from T cell exhaustion, poor dendritic cell priming and activation, or the use of immunosuppressant drugs. To conclude, CD4 could be a potential biomarker to help in identifying HCV recurrent patients with a high risk of fibrosis development soon after LT.
Introduction

Hepatitis C is an infectious liver disease that affects more than 170 million people worldwide (1). Within etiological agents, Hepatitis C virus (HCV) represents the major cause of the disease in US and Europe (2,3). Hepatitis is a medical condition that is defined by inflammation and swelling of the liver, and characterized by the presence inflammatory cells within the organ. The length of HCV infection varies. Few individuals with acute HCV infection (infection within the first 6 months) will clear the virus spontaneously, whereas the majority will develop long-term chronic HCV infection. This can progress to scarring of the liver (fibrosis) and may lead to liver failure. Hepatic fibrosis development is the main complication of HCV infection and results from an inflammatory scenario that triggers the production of cytokines and the induction of fibrogenesis. This can further progress to disease state known as cirrhosis and also hepatocellular carcinoma (4-7).

Orthotopic liver transplantation (OLT) represents the optimal treatment for HCV-chronically infected patients diagnosed with end-stage liver disease due to cirrhosis in the USA, Europe, and Japan (7). Unfortunately, HCV recurrence disease due to virus re-infection of the graft constitutes a nearly universal phenomenon. The disease recurrence is associated with graft damage, but interestingly the disease progression has been documented as variable among recipients. In this sense, 30% of patients experience accelerated fibrosis progression and cirrhosis development 5-years post-LT with the concomitant needs of liver re-transplantation (7).
Organ availability constitutes an added important issue. Because there is an already limited supply of organs available, it is a nearly impossible task to keep supplying new livers to patients in need (7). Furthermore, there is no vaccine for HCV, and only about 50% of chronically infected patients respond favorably to antiviral treatment (combination interferon and ribavirin) (7). With all these factors taken into account, it is expected that the death rate from HCV infection will surpass that of AIDS by the turn of the 21st century (2,4-6). Therefore, since fibrosis development is the main complication of chronic HCV infection, it is of critical importance to understand disease progression and to find new ways to predict patients at increased risk for fibrosis development.
Hepatitis C Virus

Hepatitis C virus (HCV) is an enveloped, single-stranded, positive sense RNA virus that belongs to the genus *Hepacivirus* within the family *Flaviviridae* (7-10). Because HCV is an RNA virus, it lacks efficient proofreading mechanisms during replication. This allows for the virus to be highly mutable and has led to the rise of various virus quasispecies (4). Phylogenetic analysis has identified multiple HCV genotypes that have been classified into six groups or clades, with more than 100 subgroups or subclades within each group (2, 6, 10). This constant and rapid mutation of the viral genome (specifically the hypervariable region coding for the envelope proteins) is believed to allow the virus to escape immunological detection and elimination by the host (4).

The only known natural host of the HCV virus is humans, with over 170 million affected worldwide (6). This makes the HCV a major global public health problem. It is a blood borne pathogen that primarily replicates within the hepatocytes of the liver (4). The virus itself is comprised of a 9.6 kb, single-stranded RNA (ssRNA) molecule which consists of a single open reading frame. This codes for a large poly-protein, which is later cleaved into individual proteins. The ssRNA molecule is surrounded by an icosahedral protective core (30-35nm) that is further encapsulated in a glycoprotein rich envelope (55-65nm) (11,12).
HCV and the Liver

The primary site of HCV replication and disease progression is the liver. It is the largest organ in the body and performs over 500 vital processes, which include excretory functions, detoxification, and various metabolic functions. These functions are performed by the working cells of liver, called hepatocytes. Hepatocytes have the unique capacity to reproduce in response to liver injury. However, despite the liver’s remarkable ability to repair and recover after injury, repetitive insults can lead to liver failure, such as seen in HCV infection (6, 13).

In addition to its various functions, the liver is also considered an intrinsic lymphoid organ (14). It contains a unique microenvironment that allows for the development and differentiation of immune cells distinct from those in the periphery. Studies have indicated that naïve T cells in the liver undergoing primary activation exhibited defective cytotoxic function, shortened half-life, and were not able to mediate hepatocellular injury; whereas, naïve T cells activated within the lymph nodes were capable of mediating HCV (15). It is unclear why the liver demonstrates intrahepatic tolerance, but it is believed that the constant presence of non-self antigens from incoming blood, directed from the intestines, has resulted in a tolerant environment (16). However, this constraint on liver immunity does not prevent the immune system from mounting a vigorous response against liver specific pathogens such as HCV (17). Of those acutely infected with HCV, 20% will resolve the virus spontaneously, while the majority will develop chronic hepatitis that may lead to end-stage liver disease (3,18).
The Innate Immune Response to HCV

HCV progresses to chronic hepatitis in 50-80% of cases; however, a small fraction of those infected (20-50%) will spontaneously clear the virus (3,7). This indicates the immune system’s ability to control and clear HCV. Similar results were seen when study performed on persistent intravenous drug users showed that users who resolved previous HCV infections were 12 times less likely to be re-infected and to develop chronic infection. Those who were re-infected demonstrated HCV RNA levels two logs lower than people who were infected for the first time and developed chronic infection. These findings suggest that protective immunity does exist against HCV (18). The mechanisms underlying the different outcomes of HCV clearance is not clearly understood; however, it is clear that the innate immune response plays a key role in determining the outcome of viral infection and the extent of hepatic injury due to HCV by regulating adaptive immune response T cells (19).

Two specific cell types of the innate immune system – dendritic cells and natural killer cells – are known to aid in the control and clearance of HCV by aiding in the development and differentiation of T cells into functional memory and effector cells. Studies indicate that HCV may affect both types of cells, which in turn, could affect T cell activation (Figure 1).

T cell development is modulated by a group of antigen presenting cells (APC) known as dendritic cells (DC). DCs play a crucial role in the processing and presenting of antigens to initiate B and T cell responses, and also in
tolerizing T cells to self-antigens. Both these roles are related to the maturity of DCs and their cytokine production patterns. Hence, DC function is precisely controlled to activate or tolerize T cells, and any perturbation to this control can lead to impaired immunity (18, 20-22).

In addition to the activation of the adaptive immune system, DCs (specifically plasmacytoid-derived dendritic cells) play an important role in antiviral immunity through the production of type 1 interferons (IFNs). IFNs, such as IFN-alpha, are not only antiviral cytokines, but also important modulators of the adaptive immune response. It is reported that IFN-alpha enhances both the effector functions of T cells and the expression of proteins involved in viral antigen presentation. It also plays a role in the clonal expansion and differentiation of CD8+ T cells (18, 23-25).

In vitro studies suggest that HCV infects both mature and monocyte-derived DCs, which may lead to the dysfunction of DCs and altered adaptive immune response signaling. These include reduced production of IFN-alpha, impaired allo-stimulatory abilities to CD4+ T cells, and impaired secretion of IL-12, an important cytokine that aids in the development of CD4+ T cell responses (26-30). Furthermore, chronic HCV infection is associated with a decrease of DCs in peripheral blood. These findings indicate that persistent HCV-infection may be due to a defective adaptive immune response mediated by HCV-induced DC dysfunction. Still, the exact role DCs play in HCV-specific immunodeficiency is unclear (18,31).
Another group of innate immune cells that plays an important role HCV control and clearance are natural killer (NK) cells. These aggressive lymphocytes are commonly found in the liver and play a critical role in the destruction of virally infected cells by directly inducing programmed cell death in the infected cell. NK cells also play an important role in immunoregulation by crosstalking with dendritic cells. NK cells and DCs reciprocally activate each other in a complex receptor/ligand pair reaction. This indicates the important role NK cells have in regulating the adaptive immune system against infections (32).

Given the role NK cells have in activating DCs, it is believed that the modulation of NK cells could be another potential pathway for HCV to affect the innate and adaptive immune system. In vitro studies of HCV-infected patients demonstrated that NK cells overexpressed inhibitory receptors and produced cytokines that are known to attenuate the adaptive immune response (2). Still, it is unknown whether or not HCV affects NK cell activity in vivo.
The Adaptive Immune Response

The adaptive immune system functions by targeting and destroying invading pathogens and the toxins they produce (33). Unlike the innate immune system, it is highly specific against the pathogen that induced it, and can provide long-lasting protection (33). One major class of the adaptive immune system is the cellular mediated response, which is comprised of a group of lymphocytes called T cells. Different subsets of T cells play various roles in pathogenic control and clearance. Two well-known T cell types are CD8+ cytotoxic T cells and CD4+ helper T cells. CD8+ cytotoxic T cells induce cell death on virally infected cells. CD4+ helper cells have no cytotoxic or phagocytic activity, but instead work by maximizing the capabilities of other immune cells and directing them to control and clear infected cells or pathogens (34).

Studies of self-resolving HCV infection indicate a strong, HCV-specific CD4+ T cell response that was detected early during the acute phase and sustained for many years after clearance of HCV (35-38). In contrast, patients with chronic HCV infection demonstrated weak or absent T cell responses against chronic HCV infection as indicated by low T cell frequencies, short-lived responses, and defects in effector functions of specific T cells (39-44). Together, these studies strongly confirm the critical role T cells play in HCV infection.

It is still unclear as to why T cell immune response fails to clear HCV infection in 50-80% of cases; however, additional studies that compared HCV to other persistent viruses (HIV, EBV, and CMV) presented interesting findings.
Results indicated that HCV specific CD8+ T cells, in peripheral blood, presented markers associated with an early differentiation phenotype, similar to resting influenza A virus-specific memory T cells which had not been exposed to the virus since the clearance of the last acute case of influenza. Thus, HCV specific CD8+ T cells (in vivo) in patients appear to be in a dormant state, ignoring the HCV virus. Other studies of patients co-infected with both HCV and CMV suggested that a decline in CD8+ T cells in HCV infected patients result from the early loss of mature T cells, or through the impairment or regulation of T cell stimulation (19,45).

Figure 1: Potential mechanisms for which HCV can interfere with the activation of the adaptive immune system. Studies suggest that HCV may affect the adaptive immune system by modulating NK cells, DCs, and T cells.
HCV Life Cycle

HCV is primarily transmitted by blood, mostly through the use of intravenous drugs and blood transfusions (6). Though the lifecycle of HCV is only partially understood, there is a general understanding of the virus’ lifecycle. The entry of HCV is triggered by a felicitously coordinated interaction between cell surface receptors and viral particles (46) (Figure 2). Though the exact process is not fully understood, it is believed that entrance into the hepatocyte requires tetraspanin CD81 (2), the scavenger receptor class B type 1 (47), and tight junction proteins claudin and occludin (48-50). In addition, envelope glycoprotein complexes E1 and E2 located on the surface of the virus bind to LDL and VLDL surface receptors of hepatocytes, aiding in clathrin receptor-mediated endocytosis and release of the viral capsid into the cytoplasm of the cell (6).

Figure 2: HCV receptor mediated endocytosis into hepatocyte.
HCV does not enter the nucleus; rather, it stays in the cytoplasm where its ssRNA serves as mRNA for HCV protein synthesis and a template for viral replication (6). HCV ssRNA consists of a single open reading frame that codes for a large poly-protein (3,000 amino acids) that undergoes co and post-translation cleavage by both viral and host proteases located in the endoplasmic reticulum to yield ten separate and mature viral proteins (Figure 3) (2,4).

Figure 3: HCV genome organization. HCV comprises of a 9.6 kb, single-stranded RNA molecule which consists of a single open reading frame. It codes for a large poly-protein, which is later cleaved into individual proteins that can be classified into 2 groups – structural proteins and non-structural proteins.
These ten proteins can be further classified into two groups: structural and nonstructural (NS) proteins. The structural proteins consist of Envelope 1 and 2 (E1 and E2), Core, and p7 proteins. E1 and E2 heterodimerize to form the glycoprotein envelope which encapsulates the nucleocapsid, which itself is composed core proteins. P7 is believed to form an ion channel that allows the release and maturation of viral particles. The nonstructural proteins are believed to play a role in the pathogenesis of HCV infection and related liver disease by assisting in viral replication (6). They consist of NS2, NS3, NS4A, NS4B, NS5A, and NS5B (6). NS2, NS3, and NS4A are involved in the processing of the presumed NS region of the polyprotein (4). NS3 functions as both a helicase and a proteolytic cleavage enzyme, while NS5b is a RNA dependent RNA polymerase needed for viral replication that is regulated by NS5a (4, 6). Although there is a general understanding of the role and function of structural and nonstructural proteins, further research is yet to conclude the exact role these proteins play in the pathogenesis of HCV infection (6).
HCV pathophysiology

Acute infection by HCV is uneventful in most cases due to the paucity of early symptoms, but runs a chronic course in an estimated 50-80% of patients (7, 51). Chronic hepatitis results in liver damage, leading to inflammation, the onset of fibrosis, and the progression to cirrhosis in 10-20% of cases after 10 - 20 years post infection (Figure 4) (6, 7).

* 10-20% of cases will progress to cirrhosis


Figure 4: Liver degeneration due to HCV infection. 80% of patients infected with HCV will develop chronic infection. Of those, 10-20% will develop cirrhosis within 10-20 years.
The exact mechanisms behind the onset and progression of hepatic degeneration are not fully understood; however, it is clear that the onset and progression of hepatic lesions is a result of the host immune response (6). HCV infects hepatocytes and leads to their destruction. Whether the virus is cytopathic or not remains questionable. What is known is that persistent hepatocyte death leads to the production cytokines/chemokines and growth factors (6, 13). This triggers the recruitment of inflammatory cells, which includes neutrophils, dendritic cells, natural killer (NK) cells, eosinophils, basophils, macrophages, and by residential macrophages called Kupffer cells, to the liver. Macrophages and NK cells synthesize and produce the pro-inflammatory cytokines TNF-alpha and IFN-gamma, which leads to the altered expression of cell adhesion molecules on sinusoidal endothelial cells and allows for further recruitment and chemotaxis of inflammatory cells. Regardless of noxae, these immune cells try to eliminate virally infected cells by inducing apoptosis, which leads to even more tissue damage (6,13).

In response to tissue damage caused by both HCV and host immune response, hepatic stellate cells initiate the wound-healing process through a dynamic process known as fibrogenesis. The production of proinflammatory cytokines activates hepatic stellate cells (HSCs) to produce platelet derived growth factor (PDGF) and interferon growth factor 1 (IFG-1), leading to the trans-differentiation and proliferation of myofibroblasts (MFBs). In addition, HCV core and NS5a proteins alter signal transduction pathways in hepatocytes leading to the production of profibrogenic mediator TGF-B1, which has a predominant role
in the conversion of HSCs to MFBs. TGF-B, the most potent known inducer of fibrogenesis in hepatic stellate cells, stimulates the synthesis of extracellular matrix (ECM) proteins and also inhibits its degradation (13). ECM proteins consist mainly of collagen, but also laminin, fibronectin, and proteoglycans (6). Under most homeostatic circumstances within the body, fibrogenesis is balanced with fibrolysis; however, because of constant tissue damage by the host immune response to HCV, fibrogenesis dominates over fibrolysis, leading to the excess synthesis and deposition of ECM proteins in the liver (6, 13). This “scarring” process leads to the involvement of neighboring mesenchymal structures and the formation of fibrous septa and is called fibrosis.

Fibrosis is the abnormal accumulation of ECM proteins in the liver, and is the main complication of chronic HCV infection. It is unclear what role viral factors play in the progression of fibrosis; however, exogenous factors seem to play a significant role. Immunodepressive factors such as co-infection of HIV and other hepatitis viruses and chronic injury from moderate alcohol consumption, obesity and diabetes (6, 13) seem to play a role in progression of fibrosis to a disease state known as cirrhosis.

Cirrhosis is characterized by hepatocellular dysfunction and increased intrahepatic portal blood pressure due to diffuse fibrosis, parenchymal necrosis, and nodular regeneration of hepatocytes (4). It is at this point that the liver fails to function properly, and the patient needs a liver transplant.
HCV treatment

Currently, no vaccine exists for HCV infection. This is because HCV comes in many forms and is constantly mutating to form different quasi-species. This highly mutable nature of HCV makes it difficult to manufacture antibody-based vaccines (2,52-54). Current treatment for HCV is antiviral therapy, which consists of a combination of pegylated interferon (IFN) alpha and ribavirin (6). Ribavirin is a guanosine analog that is believed to increase IFNs effect and prevent late relapse by increasing the mutation rate of HCV toward “error catastrophe” (6). Though these treatments have proven successful against certain genotypes of HCV, liver transplantation (LT) is the principal surgical procedure recommended for treatment of Chronic HCV patients (7). After transplantation, many patients undergo prophylactic interferon based antiviral therapy to prevent graft infection and fibrosis development. Unfortunately, this treatment option has shown low response rates and high rates of adverse affects, hence there is an almost universal recurrence of HCV infection (7). Treatment for recurrent HCV infection is a combination of pegylated interferon and ribavirin, which has shown a 10-59% sustained virological response (7). Though HCV treatment has shown to be effective in controlling inflammation after viral eradication, fibrosis regression or stabilization has proven to be less predictable (7).

In addition to antiviral therapy, patients who undergo OLT must also receive immunosuppressive therapy to prevent graft rejection by the host.
Currently, there are many immunosuppressive treatments available to patients undergoing OLT, these include calcineurin inhibitors (CNI), corticosteroids, antimetabolites, and antibody therapy (55). CNIs inhibit T cell activation by binding to calcineurin, a calmodulin dependent phosphatase that plays a critical role in transcription and activation of T cell cytokines, including IL2 (55). Similarly, Corticosteroids work by blocking T cell-derived and APC-derived cytokine expression, which includes IL1, IL2, IL3, and IL6. Antimetabolites function by inhibiting RNA, DNA, and protein synthesis, either by antagonizing purine and pyrimidine nucleotide metabolism. This results in the blockage of DNA replication in T lymphocytes (55). Lastly, antibody therapy works by targeting multiple different epitopes on T cells and APCs with monoclonal antibodies. A common Anti-T cell antibody is OKT3, which binds to CD3 on the surface of T cells. This prevents the activation T cells and results in the decline of mature T cells (55).

It is important to note that almost all immunosuppressive therapies work by depleting or hindering T cells, which are known to play a critical role in resolving acute HCV infection. However, if T cell activity is not controlled, the host will reject the new graft. Hence a delicate balance of antiviral and immunosuppressant therapies must be administered to patients to allow for both graft survival and viremic control.
Post-liver transplantation disease progression

HCV-related end-stage liver disease constitutes the leading indication for orthotopic liver transplantation (LT) in the USA, Europe and Japan. After OLT, there is a universal recurrence of HCV infection in virtually all patients at the time of reperfusion (7). After week 2 post-LT, serum HCV RNA levels increase rapidly and peak around 1 to 3 months. Within a year, HCV viremia plateaus at 1-2 logs higher than pre-LT levels (7). Even with antiviral treatment (combination pegylated interferon and ribavirin), approximately one-third of patients will develop accelerated fibrogenesis, which will progress to severe fibrosis (stage ≥ 3) at 5 years post-LT. Hence, approximately 10-25% of HCV recurrent patients will die or require retransplantation within 5 years post-LT (7) (Figure 5). However, very few patients will undergo retransplantation, subsequently because many centers are reluctant to relist patients with previous graft failure due to HCV recurrence (51). Patients that are relisted experience a high wait list mortality (50-80%) due to rapid deterioration following liver decompensation (51,56).
Figure 5: HCV disease progression pre-LT and post-LT. Post-LT disease progression resembles that of Pre-LT disease progression, except at a more rapid pace.

Though the understanding of HCV has steadily progressed in the last few decades, little is still known regarding specific pathogenesis in recurrent HCV infection. What is known is that the immune response in recurrent chronic HCV infection resembles that of chronic HCV infection in immunocompetent patients, except at a more rapid pace (6,13). The immune response against recurrent HCV infection is characterized by activation of the innate immune system and the recruitment of the adaptive immune response cells (57-58). These consist of T lymphocytes (particularly regulatory T cells, cytotoxic T cells, T helper cells) and also B cells. Significant research in the last ten years has shown the importance of the adaptive immune response in controlling HCV disease progression. CD4+
T cells have been linked to the control and clearance in acute HCV infection and also a protective effect against progressing liver disease in chronic HCV infection (18).

The fibrosis progression severity in HCV recurrence disease is variable. The molecular mechanisms involved in accelerated fibrogenesis progression in HCV-infected recipients have been recently studied (59). However, despite the well-known presence of T cells infiltrates into the new graft, considerable gaps still exist in our understanding of the role the adaptive immune response plays in severe fibrogenesis progression in HCV recurrence.

With more than 2,000 liver transplants done every year in the United States due to HCV alone, combined with the significant rise in cirrhosis development in untreated HCV patients and an already limited supply of organs, there is an almost impossible burden of supplying patients with new livers (7). Because of this, it is of utmost importance to understand the role the adaptive immune response plays in the progression of fibrosis, the main complication of HCV infection, and to investigate biomarkers that may help predict and prevent severe fibrosis progression in HCV recurrent patients.
Study rationale: Preliminary data conducting the present research:

Molecular analysis of HCV recurrence disease

Previously in our lab, we studied the molecular biology of HCV recurrence using microarray technology in frozen liver biopsy samples. From these preliminary results, a pairwise comparison analysis of normal and HCV re-infected grafts was performed to identify molecular pathways involved in HCV recurrence. Gene ontology analysis identified T lymphocytes (CD4+ and CD8+) Antigen Presentation Signaling cascade as the top canonical pathway (Figure 6). Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells pathway were also identified. These results are in accordance with previous reports demonstrating high T cell-mediated immune response in HCV recurrence disease (60,61).
Figure 6: Antigen presentation canonical pathway. Oval shapes in color represent differentially expressed genes in HCV recurrence disease versus normal allograft. Red ovals: up-regulation; Green ovals: down-regulation. The color intensity indicates the gene deregulation level. Total RNA was extracted from frozen tissue samples using Trizol following manufacturer’s protocol. RNA quality control criteria included: 28S/18S ratios >1.5, A260/A280 ratios of 1.9-2.1, and RIN ≥7. Microarray generation and analysis: Labeled cRNA synthesis, in vitro transcription, chemical fragmentation, and hybridization cocktail preparation for HG-U133A v2.0 GeneChips® (Affymetrix) were performed according to the manufacturer protocol. Microarray image was generated using high-resolution Affymetrix GeneChip Scanner 3000. Data were analyzed with Affymetrix Microarray Suite Software v5.0 and Bioconductor packages (70, 71). Sample groups were compared using moderated t-test with Holm’s method. A p-value ≤ 0.05 was considered significant. Ingenuity Pathway Analysis tool v8.6 (www.ingenuity.com) was adopted for gene ontology and gene interaction analysis.
Hypothesis and Aims

Our current study intends to identify T cell related genes in varying levels of fibrosis progression in post-LT, HCV recurrent patients, as well as other various immune cells that have been known to play a role in viral clearance.

Graft injury in HCV recurrence disease arises as a result of the immune response to control the acute HCV infection and disease progression. It is characterized by activation of the innate immune system and the recruitment of the adaptive immune response cells (13, 62). Thus, it is hypothesized that the elucidation of the early immune response involved in the liver graft at the time of HCV recurrence might help to identify those patients with high risk of fibrosis development at early post-liver transplantation stage.

Though studies have been done on T cell markers in HCV recurrent patients with differential fibrosis, this will be the first retrospective study, to the best of our knowledge, aimed to predict accelerated hepatic fibrogenesis in HCV recurrent patients based on the expression of certain adaptive immune cells, particularly T cells, at the time of HCV recurrence. In this project, we aimed:

- To examine the association of expression of T cell genes at the time of HCV recurrence and the severity of liver fibrosis progression in the new graft
- To identify the immune cells that associate with accelerated fibrogenesis post-liver transplantation
- To validate these results using an independent set of samples
Methods and Materials

Study Design

In this study, T cell gene expression analysis, using qPCR, was done on RNA isolated from FFPE biopsy samples (n = 27) taken at the time of HCV recurrence. Biopsy samples taken at 36 months post-LT were assessed for fibrosis progression using Metavir scoring. The expression level of those genes was analyzed with respect to the fibrosis grade assessed at 36 months post-LT in order to predict accelerated hepatic fibrogenesis events in response to HCV recurrence disease. For our validation study, T cell gene expression analysis was also done on RNA isolated from frozen tissue biopsy samples (n = 7) taken based on fibrosis severity progression. A flow chart indicating our study can be seen in figure 9, below.

Figure 9: Study design flow chart.
Samples and Patients

The Institutional Review Board at the Favaloro Foundation (Buenos Aires, Argentina) and Virginia Commonwealth University has approved the study protocol. The study included 27 adults who underwent OLT for HCV induced cirrhosis at the Favaloro Foundation (Buenos Aires, Argentina) between 1995 and 2006. All patients completed at least 3 years of follow-up post-OLT and underwent liver biopsy at that time of clinical HCV recurrence, defined by increased ALT level and positive HCV viremia, and at 36 month post-OLT for liver fibrosis progression assessment as marker of recurrent disease severity. Furthermore, an independent set of 8 patients who underwent OLT for HCV induced cirrhosis at Hume-Lee Transplant Center at Virginia Commonwealth University were also evaluated as a validation set.

Patient enrollment considered and collected: 1) Medical history, 2) Demographic information (age, gender, race, duration of HCV infection, alcohol, use of intravenous drugs, genetic factors (i.e., HLA), diabetes), 3) HCV RNA quantitative assay, 4) HCV genotype.

Patient inclusion criteria consisted of: 1) Age: >21-<70 years old, 2) Patients listed for OLT, 3) Anti-HCV positive and HCV RNA positive in serum prior to and after undergoing OLT, 4) HCV recipients without HCV treatment post-OLT are included in the study (to assure evaluation of natural HCV progression post-OLT). On the other hand, patients were excluded by: 1) Re-transplantation (any cause) and/or multiple-organ transplantation, 2) HCV RNA
not done or undetectable prior OLT, 3) Pre-operative diagnosis of fulminant hepatitis, cryptogenic cirrhosis, primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis, or metabolic liver diseases, 4) Recipient of anti-HCV positive organ, 5) Co-diagnosed chronic hepatitis B and/or HIV, 6) Pregnancy, 7) HCV RNA repeatedly negative (by PCR) post-OLT, 8) Presence of acute rejection in the allograft biopsy (by clinical and histological criteria) at the time of clinical HCV recurrence (as defined by detectable HCV RNA in serum and elevated ALT), 9) Vascular and biliary complications post-OLT resulting in the need for interventions.

Post-OLT immunosuppression consisted of calcineurin inhibitors [cyclosporine A and tacrolimus] and corticosteroids. Certain patients received induction therapy [OKT3, anti-thymocyte globulin, or basiliximab], and some received a third immunosuppressive agent at some time during follow-up [mycophenolate or azathioprine]. Patients with biopsy-proven acute rejection received 1 intravenous bolus of 1 g of methylprednisolone, which was followed by an oral recycle of prednisone with a starting dose of 200 mg/day.

Postoperatively, all patients underwent clinical and biochemical monitoring at regular but variable intervals (biweekly, weekly, every other week, and monthly thereafter) according to individual outcomes. In order to provide a natural way of infection, no patient received antiviral therapy for HCV for the duration of the study.

Patient information and laboratory results at pre and post-OLT times that have been prospectively collected were retrospectively evaluated. This includes:
Transplantation time assessments and data collection: 1) Clinical scores, including the “model for end-stage liver disease” (MELD) score and Child-Turcotte-Pugh (CTP) score (22), both methods used by United Network of Organ Sharing (UNOS) to select patients for transplantation 2) HCV viral load at transplantation time, 3) Donor demographic information (age, race, sex), donor cause of death, 4) Cold ischemia time, warm ischemia time, 5) Type of donor (deceased donor/liver donor), 6) Donor demographics and characteristics (age, race, gender, history of alcohol/drug abuse, cause of death) 7) HCV genotypes.

Post-transplant assessments and data collection: 1) HCV viral load at week 1, Month 3, Year 1, 2, and 3: HCV RNA quantitative analysis, 2) FFPE liver biopsies at clinical HCV recurrence time or first protocol biopsy (6 months post-OLT) (for molecular studies) and at 60 months post-OLT (for histological fibrosis score evaluation) (All the biopsy times are in concordance with our clinical biopsy protocol biopsy), 3) Histological evaluation of biopsies, 4) Immunosuppressive drugs monitoring, 5) Episodes of acute rejection, 6) Treatment of acute rejection, 7) CMV infection, 8) Liver function panel: AST, ALT, total bilirubin, albumin, fibrinogen, and platelets at the biopsy times.
Liver biopsy samples

Formalin-Fixed Paraffin-Embedded (FFPE) liver biopsies at the time of HCV recurrence diagnosis (in an average time of 6 months post-OLT) defined as increased alanine aminotransferase levels (ALT) and positive HCV viremia. A retrospective analysis on gene expression was conducted on these samples using QPCR based on the fibrosis progression of biopsy samples from the same patients taken 36 months post-LT. Frozen tissues sample biopsies were taken according to the severity of fibrosis progression post-LT.

Biopsy samples were classified based on fibrosis progression using the METAVIR scoring system (Figure 7). This system has been specifically designed to assess fibrosis staging of fibrosis from biopsy samples taken from HCV-infected patients (7). The level of fibrosis is graded on a 5-point scale from F0 to F4: F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis), or F4 (cirrhosis).

![METAVIR SCORING SYSTEM](image)

Figure 7: METAVIR score system used to assess fibrosis progression.
In addition, necroinflammation activity is graded as A0 (absent), A1 (mild), A2 (moderate), or A3 (severe). According to the severity of fibrosis progression 3 years post-OLT, the patients were placed in three groups: (1) G1 for mild fibrosis (F0-F1), (2) G2 for moderate fibrosis (F2), and (3) G3 for severe fibrosis (F3-F4) (Figure 8).  

![Diagram showing the classification of fibrosis progression and corresponding groups]

**Figure 8:** The classification of our samples into 3 groups (G1, G2, G3) based on fibrosis progression using METAVIR scoring.

### RNA Isolation

Total RNA from FFPE samples was isolated using Recover All™ Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA). Briefly, FFPE samples were incubated in warm xylene in order to solubilize and remove the paraffin wax
from the tissue. Then, the deparaffinized samples are washed with alcohol to remove excess xylene, and then subjected to protease digestion. This removes any proteins that may be covalently bound to RNA. RNA is purified and captured by glass-fiber filtration, washing, and elution. Lastly, a series of high ethanol washes ensure the recovery of smaller RNA fragments.

Total RNA from frozen tissue samples was extracted using TRIzol® (Life Technologies, Carlsbad, CA, USA) reagent following manufacturer’s protocol. TRIzol is a monophasic solution of phenol and guanidine isothiocyanate that maintains RNA integrity and assists in the disruption of cell membranes. RNA quality control criteria included: 28S/18S ratios >1.5, A260/A280 ratios of 1.9-2.1, and RIN ≥7.

**Quantitative Real time Polymerase Chain Reaction**

The expression of selected T cell-related genes was studied on both FFPE liver biopsies collected at the time of HCV recurrence disease diagnosis, and defined as the Training study group, and also on frozen tissue biopsies collected based on post-LT fibrosis severity as Validation group for the expression of CD4-encoding gene (see below). Relative gene expression levels was evaluated using Taqman® Gene expression Assays (Applied Biosystems). Gene quantitation assays were performed in a two-step real time PCR. The first step consists of reverse transcription of total RNA into cDNA. In the second step, PCR products are amplified and probed with specific fluorescent reporter dyes.
This allows for the simultaneous quantification and amplification of a target gene. Proposed target genes for expression evaluation were selected based on specific immune cells (Table 1).

In an attempt to identify the presence of helper T cells, probes specific for helper T cell activation markers IL2 (Hs00174114_m1), IL4 (Hs00174122_m1), and IL17 (Hs00174383_m1), and cell surface marker CD4 (Hs00181217_m1) were used. To identify the presence of cytotoxic T cells, probes specific for cytotoxic T cell surface markers CD3 (Hs01062241_m1) and CD8 (Hs00233520_m1) were used. To identify the presence of regulatory T cells, probes specific for regulatory T cell activation markers FOXP3 (Hs01085834_m1), CTLA4 (Hs03044418_m1), LLRC32 (Hs00194136_m1), and TNSFR1 (Hs00188346_m1), as well as surface markers CD4 and CD25 (Hs00907779_m1) were used. To identify B cells, a probe specific for common B cell surface marker CD19 (Hs00174333_m1) was used. In addition, IL28RA is a receptor subunit that is associated with viral infection, and its expression was also be analyzed.

B2M gene was used as endogenous control for gene expression normalization. The expression level of selected T cell-related genes was analyzed with respect to the fibrosis grade assessed at 36 months post-LT used to predict accelerated hepatic fibrogenesis events in response to HCV recurrence disease.
<table>
<thead>
<tr>
<th>Immune Cell</th>
<th>Target Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helper T Cell</td>
<td>CD4 (Hs00181217_m1): Surface Marker</td>
</tr>
<tr>
<td></td>
<td>IL2 (Hs00174114_m1): Activation Marker/Interleukin</td>
</tr>
<tr>
<td></td>
<td>IL4 (Hs00174122_m1): Activation Marker/Interleukin</td>
</tr>
<tr>
<td></td>
<td>IL17 (Hs00174383_m1): Activation Marker/Interleukin</td>
</tr>
<tr>
<td>Cytotoxic T Cell</td>
<td>CD3 (Hs01062241_m1): Surface Marker</td>
</tr>
<tr>
<td></td>
<td>CD8 (Hs00233520_m1): Surface Marker</td>
</tr>
<tr>
<td>Regulatory T Cell</td>
<td>CD4* (Hs00181217_m1): Surface Marker</td>
</tr>
<tr>
<td></td>
<td>CD25 (Hs00907779_m1): Surface Marker</td>
</tr>
<tr>
<td></td>
<td>FOXP3 (Hs01085834_m1): Activation Marker/Transcription factor</td>
</tr>
<tr>
<td></td>
<td>CTLA4 (Hs03044418_m1): Activation Marker/Cytokine</td>
</tr>
<tr>
<td></td>
<td>TNSFR18 (Hs00188346_m1): Activation Marker/TNF receptor</td>
</tr>
<tr>
<td>B Cell</td>
<td>CD19 (Hs00174333_m1): Surface Marker</td>
</tr>
</tbody>
</table>

Table 1: Immune cells and their corresponding target genes. * Indicates repeated gene CD4 which was used as an identification marker for both Helper T cells and Regulatory T cells.
Data Analysis

The expression level of these specific genes were analyzed with respect to the fibrosis grade assessed at 36 months post-LT, and aimed to predict accelerated hepatic fibrogenesis events in response to HCV recurrence disease. Relative gene expression levels were calculated using the ΔΔCt method and expressed as fold changes. Delta Ct value (ΔCt) were obtained by normalizing the target gene Ct value with respect to the β2M gene as endogenous control (ΔCt = target Ct – β2M Ct) for each sample. The average ΔCt was calculated for each study group. Differential gene expression among groups was expressed as fold changes with respect to “Benign” fibrosis as 1. Thus, the ΔΔCt value was calculated for each group (ΔΔCt = ΔCt G1,G2 or G3 - ΔCt G1). Fold changes were calculated as $2^{-\Delta \Delta Ct}$.

Statistical Analysis

Patient data regarding clinical and pathological characteristics and was analyzed by chi_square statistic (X2, contingency tables) and Analysis of Variance (ANOVA). QPCR data for the three different subpopulations was analyzed using Analysis of Variance (ANOVA) and Tukey’s Honest Significant Difference Test. A p-value of <0.05 was considered significant.
Results

T cell-related gene expression and its association with fibrosis progression severity were retrospectively evaluated in a prospective cohort study of 27 HCV-infected recipients. In addition, a validation group constituted by 7 patients was also retrospectively evaluated. Gene expression was determined in biopsy samples taken at the time of HCV recurrence disease defined as increased ALT level and positive HCV viremia. The expression level of those genes was analyzed with respect to the fibrosis grade assessed at 36 months post-LT aimed to predict accelerated hepatic fibrogenesis events in response to HCV recurrence disease (Figure 10).

![Biological pathway diagram](image)

**Figure 10: Retrospective research study scheme.** T-cell related gene analysis done on our patient population was used to possibly predict the severity of fibrosis progression.
Clinico-pathological characteristics of patient groups:

Clinico-pathological parameters of HCV-positive recipients from FFPE samples are described in Table 2. No patients received antiviral therapy for HCV infection control during the study. Most of recipients were infected with HCV genotypes 1a/b (a = 4; b = 22). One patient was infected with HCV genotype 2a (Mild group). The mean HCV recurrence times were 5.8±5.0, 4.3±2.7, and 4.1±2.0 months for Mild, Moderate and Severe fibrosis groups, respectively, without a significant difference among groups. Out of the 27 patients in our study, 12, 6, and 9 patients were classified for having mild, moderate, and severe fibrosis development, respectfully, at 36 months post-LT. No significant differences were found among groups regarding age, gender, liver donor type, ALT blood levels, as well as administrated immunosuppressant type, acute rejection episodes, and treatment. Patients showed significant decreases in follow-up time post-LT as fibrosis severity worsened. This can be attributed to the fact that HCV recurrent patients with severe fibrosis experience higher mortality rates (7). This same trend can be seen in patient deaths, which significantly increased with fibrosis severity progression. Re-transplantation increased toward the development of severe fibrosis.
<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (n)</td>
<td>12</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Age in years (Interval)</td>
<td>51 (37 – 65)</td>
<td>53 (41 – 59)</td>
<td>57 (47 – 66)</td>
<td>0.51</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>(8/4)</td>
<td>(2/4)</td>
<td>(6/3)</td>
<td>0.34</td>
</tr>
<tr>
<td>HCV genotype 1/2-3</td>
<td>(11/1)</td>
<td>(6/0)</td>
<td>(9/0)</td>
<td>0.76</td>
</tr>
<tr>
<td>Liver donor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Diseased (%)</td>
<td>10 (83)</td>
<td>3 (50)</td>
<td>5 (56)</td>
<td>0.25</td>
</tr>
<tr>
<td>• Living (%)</td>
<td>2 (17)</td>
<td>3 (50)</td>
<td>4 (44)</td>
<td></td>
</tr>
<tr>
<td>Time to recurrence in month</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• &lt;6</td>
<td>7</td>
<td>4</td>
<td>7</td>
<td>0.54</td>
</tr>
<tr>
<td>• 6-12</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>• &gt;12</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ALT at recurrence in IU/L (mean ± SD)</td>
<td>228 ± 118</td>
<td>221 ± 77</td>
<td>329 ± 192</td>
<td>0.22</td>
</tr>
<tr>
<td>Cyclosporine (%) / Tacrolimus</td>
<td>6 (50) / 6</td>
<td>3 (50) / 3</td>
<td>8 (89) / 1</td>
<td>0.14</td>
</tr>
<tr>
<td>Acute rejection (%)</td>
<td>5 (42)</td>
<td>2 (33)</td>
<td>4 (44)</td>
<td>0.90</td>
</tr>
<tr>
<td>Treated acute rejection (%)</td>
<td>4 (33)</td>
<td>2 (33)</td>
<td>4 (44)</td>
<td>0.85</td>
</tr>
<tr>
<td>Steroid boluses (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 1 Bolus</td>
<td>4 (33)</td>
<td>2 (33)</td>
<td>4 (44)</td>
<td>0.85</td>
</tr>
<tr>
<td>• 2 Bolus</td>
<td>1 (25)</td>
<td>1 (50)</td>
<td>1 (25)</td>
<td>0.21</td>
</tr>
<tr>
<td>• 3 Bolus</td>
<td>3 (75)</td>
<td>0 (0)</td>
<td>3 (75)</td>
<td></td>
</tr>
<tr>
<td>Follow up post-LT in years (Interval)</td>
<td>10.0 (5 – 12)</td>
<td>7.0 (3 – 10)</td>
<td>6.8 (5 – 10)</td>
<td>0.03</td>
</tr>
<tr>
<td>Re-transplantation (%)</td>
<td>0 (0)</td>
<td>1 (17)</td>
<td>3 (33)</td>
<td>0.18</td>
</tr>
<tr>
<td>Death (%)</td>
<td>0 (0)</td>
<td>1 (17)</td>
<td>4 (44)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 2: Clinico-pathological parameters of HCV recurrence diagnosed patients (FFPE samples).
Expression analysis of immune response-related genes:

In order to identify and define the immune response, specific markers were selected for each immune cell subset. To identify helper T cells, the expression of those genes encoding CD4, IL2, IL4, and IL17 were determined by the analysis of transcript (mRNA) levels by qPCR. In a similar manner, cytotoxic T cells were evaluated through the CD3 and CD8-encoding gene expression analysis. Genes encoding for CD4, CD25, FOXP3, LLRC32, CTLA4, and TNFRSF18 were analyzed to identify regulatory T cells. The B cells presence was evaluated by the CD19 encoding gene expression. Gene expression analysis for validation samples was performed only on CD4.

PCR amplification was observed for CD4, IL28RA, CD8, FoxP3, LRRC32, and CD3 in 100%, 92%, 67%, 56%, 37% and 22% of samples, respectively (Table 3). In addition, any amplification signal was detected for IL2, IL4, IL17, CD19, CD25, CTLA4, and TNFRSF18 over all samples (Table 3).
Table 3: T cell-related gene expression analysis by sample groups. PCR amplification was observed in FFPE samples for CD4, IL28RA, CD8, FoxP3, LRRC32, and CD3 in 100%, 92%, 67%, 56%, 37% and 22% of samples, respectively (table 3). All samples were negative for IL2, IL4, IL17, CD19, CD25, CTLA4, and TNFRSF18. (+) Indicates positive amplification, (-) indicates no amplification.

Gene expression analysis of target genes for particular immune cells showed the following results: CD4 gene expression showed down regulation trend amongst the three groups (G1, G2, G3), however, this was borderline significant (using ANOVA test: \( p = 0.053 \)) (Figure 11). A pairwise comparison was done between G3 (severe) vs. G1 (mild), and there was 2.9 fold down regulation that was also borderline significant (\( p = 0.052 \)). Validation group
samples presented a similar trend, with a 1.8-fold decrease CD4 expression between G3 vs. G1, although not significant.

Similarly, FoxP3 expression decreased 3.1-fold in G3 with respect to G1, although these were not significant. LRRC32 expression also decreased in G3 samples compared to G1. CD8 expression showed a 1.4 fold decrease between G3 vs. G1. CD3 expression showed a 0.6 fold decrease between G2 vs. G1, and no amplification in G3.

**Figure 11. CD4 Gene Expression Analysis.**
Gene expression is shown as fold changes referred to Mild fibrosis as 1. Fold change values are indicated below each bar. Patient groups named as the fibrosis severity grade are detailed in the X-axis. * Indicates that there was no significant decrease in CD4 amongst the three groups.
**Validation Study Results**

To validate these results, the CD4 gene expression was validated in an independent set of HCV recurrence diagnosed patients with no fibrosis development (n = 3) and moderate to severe fibrosis development (n = 4). Liver biopsy samples were obtained from each patient and fibrosis progression was classified accordingly with Metavir scoring system as described previously. Clinico-pathological and demographic patients characteristics are described in Table 4. Briefly, patient age, gender, race, was taken into account, as well as cases of acute rejection, HCV recurrence, and Metavir score. Total RNA was isolated from frozen liver biopsy samples (n = 7) as described in the Patients and Methods section. Following, the CD4 gene expression was analyzed using qPCR. From the results analysis, a specific and significant amplification was observed for CD4 in all analyzed samples. Interestingly, the CD4 expression was found to be 1.8 fold down-regulated between G3 (n = 4) and G1 (n= 3), although this differential expression was found to be not significant (Figure 12). Nevertheless, it is important to remark that the obtained results coincide with the CD4 expression levels observed in the training group of samples. Importantly, it permits postulate a negative regulation of the CD4 encoding gene in those HCV recurrence cases with severe fibrosis progression.
Table 4: Clinico-pathological parameters for frozen tissue samples used in validation study.

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Sample</th>
<th>Age</th>
<th>Gender</th>
<th>Race</th>
<th>Acute Rejection</th>
<th>HCV Recurrence</th>
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</thead>
<tbody>
<tr>
<td>Mild</td>
<td>1</td>
<td>40</td>
<td>F</td>
<td>white</td>
<td>none</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>62</td>
<td>F</td>
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<tr>
<td></td>
<td>3</td>
<td>57</td>
<td>F</td>
<td>white</td>
<td>none</td>
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<tr>
<td>Severe</td>
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<td>59</td>
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<tr>
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<td></td>
<td>6</td>
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<td>Yes</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>53</td>
<td>M</td>
<td>black</td>
<td>none</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Figure 12: CD4 Gene Expression Analysis for Validation Study. Fold change values are indicated below each bar. Patients groups named as the fibrosis severity grade are detailed in the X-axis.
Discussion

HCV infection is a major public health concern, with over 170 million people around the world affected by the virus (7). Though the clinical course of HCV is highly variable, 85% will develop chronic infection, which is characterized by the development fibrosis, then cirrhosis, and then hepatocellular carcinoma. Because of this, HCV is the leading cause for liver transplant in Europe and the USA (7). Unfortunately, graft HCV infection is a universal phenomenon and is characterized by accelerated fibrosis progression and the development of cirrhosis within 5 years of transplantation in 30% of patients (7). With the increasing difficulty to produce long lasting and effective vaccines, combined with the failure of antivirals to successfully clear the virus, much attention is now focused on the adaptive immune system, particularly T cell immunity, in controlling HCV infection. In an attempt to better understand accelerated fibrosis development in HCV recurrence disease and find potential biomarkers, we investigated the presence of particular lymphocytes of the adaptive immune system.

The adaptive immune system plays a significant role in determining the outcome of HCV infection. Both a strong CD8+ T cell response and a vigorous and sustained CD4+ T cell response can be seen in non-transplant patients who resolve acute HCV infection (63). However, in chronic HCV infection, CD4+ T cell responses are weak or absent, and have been linked to the development of fibrosis and cirrhosis (63). Similarly, a study on the peripheral blood of HCV
recurrent patients demonstrated detectable CD4+ specific T cell responses in patients with mild or no histological recurrence; whereas patients with severe histological recurrence were hyporesponsive to HCV antigens (64). Our findings are consistent with these concepts as our results show that patients with down regulated T cell-related genes at the time of HCV recurrence developed more severe fibrosis 36 months post-LT.

In our analysis, T cell-related gene expression and its association with fibrosis progression severity were retrospectively evaluated in a prospective cohort study of 27 HCV-infected recipients. Gene expression was determined in biopsy samples taken at the time of HCV recurrence disease defined as increased ALT level and positive HCV viremia. The expression level of those genes was analyzed with respect to the fibrosis grade (Mild: G1, Moderate: G2, Severe: G3) from liver biopsies assessed at 36 months post-LT aimed to predict accelerated hepatic fibrogenesis events in response to HCV recurrence disease. Our study showed a 2.9-fold down-regulation of the CD4 gene in patients with severe fibrosis development (G3) with respect to patients with mild fibrosis (G1). Similarly, down regulation trends were also seen observed in cytotoxic T cell related genes (CD3 and CD8) as well as regulatory T cell-related genes (FOXP3, and LLRC32) in patients with severe fibrosis in respect to mild fibrosis; although, all of these were not significant.

The exact reasons underlying the weakened or absent T cell-mediated response in chronically HCV infected patients is unknown. One theory based on a study done on lymphocyte chloromeningitis virus (LCMV) suggests that HCV-
specific T cells may experience “exhaustion.” The study showed that mice persistently infected with LCMV resulted in chronic T cell activation followed by sequential loss of T cell function (2). Likewise, chronic HCV infection has also been associated with continuous activation of HCV-specific T cells but with impaired function (65,66). Mice models affected with LCMV showed a decrease in IL2 production, followed by a loss of cytotoxicity, TNF-a production, and IFN-y production. These results not only coincide with previous studies that have shown impaired effector functions of CD4+ and CD8+ T cells in chronic HCV infection (51), but also with the results from our study. T cell exhaustion could provide the rationale for the down regulation of CD4 and CD8 genes and the lack of IL2 amplification in our study.

Accumulating data also suggests that an absent T cell response in chronic HCV patients might be due to impaired dendritic cell function. Dendritic cells play a critical role in the priming and activation of T cells. Failure of DCs to activate T cells in chronic HCV infection could explain the down regulation of T cell-related genes in our study. Studies on peripheral blood DCs in chronic HCV infection demonstrated impaired ability of DCs to activate allogeneic T cells (40). The exact mechanism behind this is not fully understood, but this could be due to a lack in DC maturation. A study performed by Aufferman-Gretzinger (27) on the peripheral blood of patients who successfully resolved the HCV virus showed that monocyte-derived DCs from chronic HCV patients failed to respond to maturation stimuli. Unlike DCs from healthy donors, they failed to upregulate cell surface proteins for antigen presentation. Since mature DCs aid in the
development of naïve T cells to CD4+ TH1 cells and promote the maturation of cytotoxic T cells (via IL12), a lack of mature DCs could account for the down regulation of CD4, CD3, and CD8 genes and the lack of IL4 and IL17 amplification in our study.

Similarly, a study of DCs in peripheral blood in a small group of HCV recurrent patients found a transient decrease seven days after liver transplantation in the relative and absolute number of blood plasmacytoid DCs in both HCV and non-HCV infected patients (51). A similar trend was also found in the relative number of myeloid DCs. Researchers believe this incident could be due to the use of immunosuppressive drugs administered after transplantation. However, immunosuppressive drugs are known not only to affect DCs but also T cells as well.

When examining the risk factors associated with accelerated fibrosis development in HCV recurrent patients post-LT, the role of immunosuppression is of particular interest because it is the only factor that can be modified (67). Two immunosuppressants used on the patients in our study – cyclosporine and tacrolimus – are inhibitors of calcineurin, a protein phosphatase that activates the expression of IL2, which in turn stimulates the growth and differentiation of T cells. This diminishing effect of CNIs on the adaptive immune system may contribute to the universal recurrence of HCV infection, the accelerated progression of fibrosis (67), and could possibly account for the down regulation of T cell related genes and the lack of IL2 amplification in our study.
The presence of B cells was also examined by analysis of the CD19 encoding gene, as a specific cell population marker, in differential fibrosis progression in HCV recurrent patients. Interestingly, no specific amplification was observed for CD19 throughout all analyzed biopsy samples. These results could potentially be explained by recent evidence that suggests that peripheral blood mononuclear cells, such as B cells, T cells, macrophages, and monocytes could also be potential reservoirs for the HCV replication (68). A study by Zehender et al (69) also confirmed the significant presence of HCV RNA in CD19+ peripheral blood mononuclear cells in patients with chronic HCV. If B cells and T cells are sites of HCV replication, this could explain a down regulation in T cell related genes and the absence of CD19 amplification in our results.

Little is known regarding the specific pathogenesis of HCV recurrence disease. For immunocompetent patients, the gold standard for hepatic fibrosis assessment of and disease progression, post-LT, is liver biopsy. However, this costly and painful procedure is invasive and has been known to have serious, though infrequent, complications such as internal bleeding (7). Furthermore, liver function tests lack the sensitivity and specificity disease severity (63). Therefore, the development of biomarkers for identifying patients at a higher risk for developing accelerated fibrosis could be a very useful clinical tool (7). Presently, there are no means to predict patients at risk for accelerated fibrosis development (63). Our study, to the best of our knowledge, is the first study to demonstrate that gene expression analysis of CD4, near the time of HCV recurrence, could be used to predict accelerated fibrosis progression in HCV.
recurrent patients. These findings could have significant clinical implications. The ability to identify patients at higher risk of accelerated fibrosis development could lead to earlier administration of antiviral therapy. It also could potentially help clinicians regulate immunosuppression therapy to ensure an adequate CD4+ T cell presence is available to help resolve HCV infection.

In conclusion, the present study demonstrated that HCV recurrent patients who developed severe fibrosis 36 months post-LT showed a borderline significant down regulation in CD4 gene expression at the time of HCV recurrence ($p = 0.052$). This down-regulation of CD4 is consistent with other studies that show a weakened CD4 T cell response in chronic HCV patients. Gene expression analysis of CD4 early on in patients with HCV recurrence could potentially aid in identifying patients with greater risks for accelerated fibrosis development, allowing clinicians to regulate both antiviral and immunosuppression therapy. However, we recommend that further studies of CD4 expression in HCV recurrent patients be done, using a larger population of patients.
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