HOST GENE VARIANTS AS BIOMARKERS FOR STRATIFICATION OF PATIENTS WITH VIRAL AND NON-VIRAL LIVER DISEASES

by

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DEDICATION

This is dedicated to my supportive family and friends.

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LIST OF ABBREVIATIONS AND/OR SYMBOLS

Hepatitis C Virus.....HCV

Reverse Transcriptase Polymerase Chain Reaction..... RT-PCR

Peripheral Blood Mononuclear
Cells…..... PBMCs

Interferon Lambda Four.....IFNL4

Interferon .....IFN

World Health
Organization.....WHO

Non-Alcoholic Fatty Liver Disease.....NAFLD

Nonalcoholic Steatohepatitis.....NASH

Reverse Transcriptase Polymerase Chain Reaction..... RT-PCR

Double stranded RNA.....dsRNA

MicroRNA.....miRNA
Complementary DNA.....cDNA

Mitochondrial mRNA..... .....mRNA

Mitochondrial Trifunctional Protein.....MTP

Interferon-α.....IFN-α

Interferon-γ.....IFN- γ

Interferon-λ.....IFN-λ

Interferon-β.....IFN- β

Natural Killer Cells.....NKs

Interferon Alpha Receptor.....IFNAR

Janus activated kinase-signal transducer..... JAK-STAT

Interleukin.....IL

Center for Disease Control and Prevention.....CDC

Ribavirin..... RBV

Pegylated interferon Alpha .....PEG-IFN

ABSTRACT

HOST GENE VARIANTS AS BIOMARKERS FOR STRATIFICATION OF PATIENTS WITH VIRAL AND NON-VIRAL LIVER DISEASES

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George Mason University, 2016

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The incidence of non-alcoholic fatty liver disease (NAFLD) including nonalcoholic steatohepatitis (NASH) is increasing and has become a global health concern. Another important healthcare challenge is chronic infection with hepatitis C virus; this condition is often accompanied by hepatic steatosis. In both types of fatty liver disease, specific hepatic gene expression patterns contribute to individual predispositions to develop liver steatosis and its further progression to NASH. These expression patterns are influenced by specific gene variants carried in the genome of particular patients. The development of novel robust approaches to stratification of patients according to their individual predispositions is critical to maximize the effects of preventive and therapeutic interventions.

Previous studies show that single-nucleotide polymorphism in IFNL3 gene (rs12979860) define probabilities of spontaneous and therapy-induced HCV clearance. Interestingly, IFN-λ4 protein can be generated only by individuals who carry ancestral, functional *IFNL4-ΔG* allele (rs368234815, or ss469415590). With a cohort of 40 patients, this study demonstrated an overall suppression of the transcription of genes associated

with an inflammatory and interferon response in HCV infected patients who are carriers of beneficial, pseudogenic *IFNL4-TT* allele. This suppression was observed at the baseline, suggesting an intrinsic, pre-existing deregulation of the antiviral response in *IFNL4* expression in carriers of *IFNL4*-ΔG allele. Additionally, we showed that the Day 7 course of treatment, patients with genotype *IFNL4-TT* undergo treatment-associated suppression of immune cells apoptosis, implying that more vigorous response to therapy may be mounted in this cohort. These findings provide additional insights into previously noted *IFNL4-ΔG* associated failure to achieve virological end-points in response to antiviral therapies. In the treatment-responsive carriers of beneficial *IFNL4*-TT allele, the most prominently suppressed pathway was "Glucocorticoid Receptor Signaling". Additionally, Metacore-guided pathway analysis led us to hypothesize that patients with *IFNL4*-TT genotype may be less susceptible to the development of liver fibrosis due to the subsequent suppression of the expression of TGFβ receptor RIII. The latter prediction is independent of the presence or absence of HCV infection, and may be relevant to progression of both infectious and non-infectious liver diseases.

A comparison of the disease-specific gene expression profiles from African American and Caucasian patients demonstrated that substantial differences between human races occur at the level of advanced chronic liver disease (NASH), but not at the level of simple steatosis which is known to be relatively benign in all ethnicities. In particular, we report intrinsic differences in the levels of compensatory anti-oxidative response as could be seen from the coordinated changes in the levels of Glutathione S-transferases 2, 4, and 5 in patients with NASH. We also compared gene expression

patterns in NAFLD affected livers of African American and Caucasian patients and identified several potentially important hepatic genes over-expressed in African American patients. Many genes highlighted in our analysis contain polymorphic positions with allele frequencies substantially different between African American and Caucasian cohorts. Differences in distribution of genome variants in these populations may contribute to a degree of protection from the development of advanced non-infectious forms of chronic liver diseases observed in African American patients.

CHAPTER ONE

Introduction:

Comparative overview of the association of liver steatosis to HCV infection and obesity

Fatty liver, also known as hepatic steatosis, is one of the most common diseases globally (WHO, 2014). The origin of hepatic steatosis can be viral, for example, hepatitis B (HBV) or hepatitis C (HCV) (Machado MV et al., 2011), or non-viral. Non-viral causes include excessive consumption of alcohol (Mahli, Hellerbrandt, 2016), hereditary disorders known to produce hepatic lipidosis (Vatier C et al., 2013), the use of certain medications (Amacher, Chalasani, 2014). When hepatic fatty infiltration is observed in absence of excessive alcohol consumption (defined as no or limited daily alcohol intake (less than 20g for women and 30g for men) (Hashimoto E. et al., 2015), the condition is known as non-alcoholic hepatic fatty liver disease (NAFLD).

Pathogenesis of viral and non-viral related liver disease

Hepatitis C virus (HCV) is part of genus Hepacivirus of the Flaviviridae family (Olaby RR, 2014; Dubuisson and Wahid J, 2013; Scarselli E, 2002). It has a single stranded positive strand RNA genome wrapped in the lipid envelope with highly glycosylated E1 and E2 proteins (Olaby RR, 2014; Dubuisson and Wahid J, 2013; Scarselli E, 2002; Khan AG, 2014). Since its discovery in 1989, HCV infection has

become a major health concern, with approximately 160 million people infected worldwide World Health Organization (WHO), 2014; Lanvancy D, 2011; Giang E et al, 2012; World Gastroenterology Organization, 2012; CDC.gov, 2014). The WHO states that three percents of the human population carry the HCV virus, and that 3-4 million new infections occur each year (WHO, 2014.) Massive resources are devoted to the research and treatment of HCV, but much work is left to be done to eradicate this disease.

Acute HCV is often asymptomatic (80% of cases) or produces mild flu-like symptoms within two weeks to six months of infection. Individuals are unaware of infection until 20-30 years after the onset of the infection, by which time the liver is severely damaged. Chronic HCV infection is associated with a variety of life-threatening conditions including cirrhosis of the liver, which occurs in 10–20% of patients, and hepatic fibrosis. Hepatic fibrosis is associated with a 1–5% annual risk of hepatocellular carcinoma, and a 3–6% risk of functional decompensation of the liver. Hepatocellular carcinoma remains the primary reason for liver transplants (WHO, 2014; Lanvancy D, 2011; Giang E et al, 2012).

Amini et al. (2012) showed that approximately 10-45% of infected individuals are capable of the self-clearance of HCV. Most do so within 6 months after initial exposure, but some individuals have been shown to clear the disease up to three years post-exposure.

Hepatitis C virus is represented by seven viral genotypes (Messina P et al. 2015) The prevalences of HCV genotypes differ by geographic region, with an emphasis on genotypes 1-3 worldwide. (Figure 1) (Messina P et al., 2015; Giang E et al., 2012).

Genotype 1 is found in more than 50% of HCV samples collected North America, parts of South America, Africa, Europe, Asia, and Australia; Genotype 2 is prevalent in parts of North America, parts of South America, Northern Europe, Southeast Asia, and a few northern countries in Australia; Genotype 3 is the most common in India and Myanmar (Messina P et al. 2015). In Egypt, the predominant HCV strain is genotype 4, while genotypes 5 and 6 are found primarily in South Africa and Southeast Asia, respectively (Giang E et al., 2012). Genotype 7 was isolated relatively recently from a patient in the Congo (Smith DB et al., 2014).

Infections with HCV Genotype 3 are associated with higher rate of treatment success (~70%) than that for other HCV genotypes, when combinations of pegylated interferon-α and ribavirin are used (Goossens N 2014). On the other hand, when delaying treatment, individuals infected with Genotype 3 virus may develop more severe steatosis with accelerated progression of fibrotic disease and an increased incidence of liver carcinogenesis.

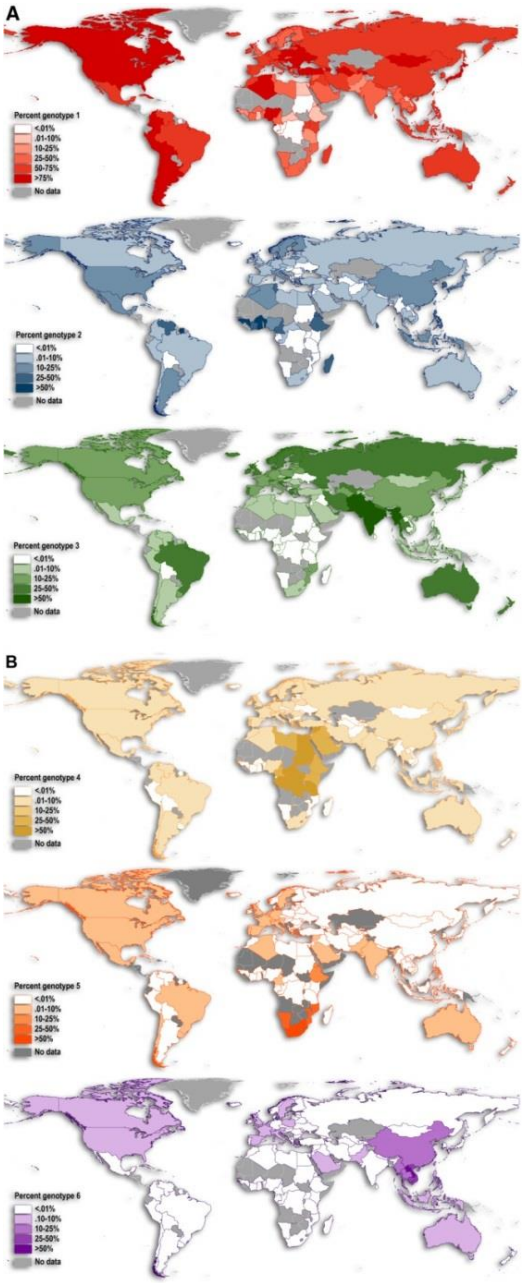

Figure 1: Relative prevalence of each genotype across all virus samples by country. Panel A: genotypes 1–3. Messina et al. 2015

The accumulation of fatty acids in liver parenchyma (liver steatosis) is associated with marked morbidity and mortality burden (Byass P. et al. 2014). As seen in HCV patients, liver steatosis serves as a precursor to fibrosis. In fact, liver steatosis is a precursor to fibrogenesis. From a pathophysiology perspective, hepatic fibrosis is thought to develop as a result of repetitive injury to liver cells resulting from HCV replication and the immune response to the virus and, possibly, to the injured cells. A contributing factor to the development of steatosis may be the cellular release of HCV particles that compete for the components of export machinery with very low density lipoprotein particles which has to be normally transported from the liver to peripheral tissues (Kawaguchi and Mizuta, 2014). In addition, replication of HCV has been shown to upregulate transcription of steatosis-related genes. For example, non-structural protein 2 (NS2)-dependent up-regulation of the expression of fatty acid synthase and sterol regulatory element-binding protein 1c (SREBP-1c) has been observed (Jackel-Cram C et al., 2007; Oem JK et al., 2008). Other studies have directly linked physiological processes taking part in HCV-infected human livers, including liver steatosis with the development of the resistance to the antiviral treatment (Amako Y, 2015).

HCV proteins promote oxidative stress and reduce fatty acid oxidation in mitochondria and related lipotoxicity. In particular, HCV core protein interacts with both the α- and β-subunits of the mitochondrial trifunctional protein (MTP), an enzyme complex which catalyzes the last 3 steps of mitochondrial lipid β-oxidation. In HCV infected hepatocytes, both MTP protein and mRNA levels are down-regulated, and β-oxidation of lipids is suppressed. In turn, the depletion of hepatic cells from MTP impairs

IFN-stimulated gene expression and renders infected cells less responsive to interferon-α (IFN-α) therapy (Amako Y, 2015).

Liver steatosis can also develop as the result of non-viral conditions. Non-viral non-alcoholic hepatic fatty liver disease (NAFLD) occurs in patients of both genders, all ethnicities and in all age groups, including children. The prevalence of NAFLD in the general population is estimated at 20%; while that in the morbidly obese population at ~75-92%, and in the pediatric population, ~13-14% (Erickson, K, 2009; Vernon et al., 2011). In children and teenagers, NAFLD is considered to be a complication arising from obesity.

The incidence of the progressive form of NAFLD, nonalcoholic steatohepatitis (NASH) is estimated at 3.5% (Erickson, K 2009). Similar incidence rates have been reported from other regions globally, to include Mexico, Middle East, Europe and Pacific region (Marchesini G et al., 2003; Farrell et al., 2007; Lazo and Clarck 2008; Uchil D et al., 2009; Fan JG et al., 2009).

NAFLD also known as simple steatosis follows a benign course with little or no progression. There is increasing evidence now that only NASH can be potentially progressive, whereas patients with simple steatosis seem to follow a more benign course with little or no progression (Vernon et al., 2011, Matteoni CA, 1997). However, liver biopsies of patients with NASH show hepatic steatosis in conjunction with hepatocellular damage (Bondini et al., 2007). Approximately 10–15% of patients with histologically proven NASH progress to cirrhosis (Rafiq N et al., 2009) with a portion of these patients

develop further complications from cirrhosis such as liver failure and hepatocellular carcinoma (Marengo A et al., 2016).

Both NAFLD and NASH significantly contribute to overall mortality (Ong JP et al., 2008). They are frequently associated with insulin resistance, impairment of glucose metabolism, metabolic syndrome, and obesity (Yki-Jarvinen H, 2014). They are commonly recognized as the risk factors for type 2 diabetes mellitus (Ballestri et al., 2016), cardiovascular diseases (Fracanzani et al., 2016), polycystic ovary syndrome (Baranova A et al., 2011). The most prominent hallmark of NAFLD is a build-up of triglycerides as microscopically observed in greater than 5% of hepatocytes (Paschos P and Paletas K, 2009; Mehta R et al., 2013). Microscopically similar liver steatosis also develops in patients infected with Hepatitis C virus.

Genetic contributions to viral and non-viral liver disease

Steatosis-contributing organismal and cellular processes include any one or a combination of the following factors: starvation or diet associated increase of the lipolysis in adipose; increased intake of dietary fat; insulin resistance associated impairment of β-oxidation of fatty acids; an increase in *de novo* lipogenesis in the liver; a reduction in lipid droplet clearance due to suppression of autophagy (Koo SH et al., 2013).

Each of these pathophysiological components depends on fine-tuned networks of proteins encoded by gene, many of which may have two or more variants (genotypes) that differ either in their levels of expression or in the activity of their functional products. These

variants are differentially distributed in human populations, which have different propensities to develop hepatic steatosis, spontaneously clear HCV, and respond to HCV treatment. Unfortunately, any predictive associations (e.g., the ethnic genotype versus treatment outcome) are notoriously difficult to assess as they require enrollment of extremely large study cohorts. However, because NAFLD and NASH are among the most frequent metabolic consequences of obesity, these conditions can be used as models for studying gene-environment interactions. More than 90% of patients undergoing bariatric surgery have NAFLD, and 25–30% of these patients have histologic NASH (Stephen et al., 2012). This makes possible an enrollment of relatively large cohorts with relatively similar clinical characteristics (e.g., populations with uniform body mass index and conditions such as marked insulin resistance) because of that, obese populations affected by chronic liver diseases have been instrumental in providing data related to the incidence of liver steatosis and its risk of its progression to NASH and fibrosis in ethnic populations (Younossi ZM et al., 2012).

Interestingly, despite high prevalence of obesity and other metabolic risk factors such as type 2 diabetes in the African American population, the prevalence of NASH is lower than that in Caucasians and Hispanics (Kallwitz ER et al., 2009). It has been speculated that both genetic and lifestyle factors such as an unhealthy diet may explain ethnic differences in the conversion of NAFLD to NASH (Stepanova M et al., 2010).

The use of biomarkers in infectious and non-infectious viral disease

The term “biomarker” refers to objective, quantifiable, clinically relevant indicators which can be measured accurately and reproducibly. According to The

National Institutes of Health Biomarkers Definitions Working Group, a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” (Biomarkers Definitions Working Group, 2001). Examples of biomarkers include physiological parameters, such as pulse and blood pressure, and the results of complex laboratory evaluations of blood and other tissues. Biomarkers of the extent of disease may not necessarily correlate with a patient's symptoms.

In clinical practice, biomarkers are indispensable for early diagnosis of infectious and non-infectious diseases, identification of patient populations at high risk of complications, and monitoring the progression of illness. Moreover, biomarkers aid in making optimal therapeutic decisions, and, ultimately, in predicting and tracking patient outcomes. Several biomarkers are already in clinical use in diagnosing and monitoring the course of chronic and the acute viral infections, and in the stratification of patients with non-viral liver diseases. However, in general, biomarkers and biomarker panels lack specificity and sensitivity. This makes them unable to dissect the complexity of inflammatory and immune processes associated with organ-specific pathophysiological processes.

Current advances in molecular biotechnology have substantially facilitated the discovery of novel biomarkers, which can range from small metabolites to DNA variants, to certain mRNA or miRNA species and proteins or their fragments.

For both infectious and non-infectious diseases, gene variants can be seen as biomarkers as they relate to the detection of their absence or presence in one's genome that can be associated with disease risk and used to stratify certain populations.

Generally speaking, the relative medical risks conferred by individual genome variants are not sufficiently large, or sufficiently understood to be useful in risk prediction for each individual patient (Khoury et al., 2004). However, formulas that combine effects of multiple individual variants are proven to be capable of patients' stratifications into the groups that significantly differ from clinical management point of view. As an example, many population-based programs of breast cancer prevention and early detection commonly include an assessment of genetic susceptibility (Burton H et al., 2013).

In 2015, Mavaddat et al. profiled 77 gene variants predisposing to or protecting individual from the development of breast carcinoma and constructed polygenic risk scores (PRSs) that stratified the general population of women of European ancestry into the genotype-specific risk groups. He showed that PRS-based stratification may facilitate early detection of cancers in younger women or aid in targeted modification of environmental factors exposures in women at higher risk of breast cancer.

It is conceivable that concerted efforts of scientific community may lead to the development of similarly constructed PRS scores for both infectious and non-infectious diseases. This includes the scores based on human gene variants (also known as polymorphisms) contributing to the development of liver steatosis in infectious liver

disease and non-infectious liver disease patients with no history of alcohol overuse. It is known that some polymorphisms strongly contribute to HCV clearance, the progression of viral hepatitis, and patient response to treatment (Table 1).

Table 1. Variants affecting HCV disease progression and viral clearance. Prepared by Cameron Alberts, M.S.

	SNP Name	SNP Description	Function	Importance
IL-28B C/T	rs12979860	Transversion of C to T 3kb upstream of *IL-28B* on Ch19q13.13	Expression of mutated IFNL3 associated with spontaneous clearance	Spontaneous clearance of HCV in 32% of patients homozygous for TT allele compared to 5% for CC or CT
IL-28B T/G	rs8099917	Transition of T to G 9kb upstream of *IL-28B* in Ch19q13.13		Associated with poor response to PEG+RBV
INFL4	ss469415590	Substitution of G upstream of *IL-28B* in Ch19q13.13	Transiently expressed protein that shares 41% sequence similarity with IFNL3, activates the STAT signaling and induces unique set of ISGs	Novel predictor for clinical therapy and spontaneous clearance of HCV. Largest gene product, p179, inhibits viral replication *in vitro.*
IL-6 -174C/G	rs1800795	Transversion of C at -174 in the promoter region in Ch7.22	Reduces expression of IL-6	Associated with spontaneous clearance of HCV

The role of human interferons/interferon lambda 4 (IFNL4) in viral and non-viral liver disease

Interferons are a class of cytokines that mediate the host immune response to infection by viral and non-viral pathogens (Prinz M et al., 2004; Bogdan C et al, 2004; Seliger B et al., 2008). They are categorized into three types based on their protein sequence (O'Brien T et al., 2014) (Table 2).

Type I interferons are rapidly produced when viral envelope glycoproteins, CpG DNA, or dsRNA interact with host cell receptors such as mannose receptors, toll-like receptors, and cytosolic receptors (Malmgaard L et al., 2004). Type 1 interferons can directly activate natural killers (NKs), antigen-presenting dendritic cells as well as CD4 and CD8 T cells (Hervas-Stubbs S et al., 2011). All type I interferons signal through a common receptor, interferon alpha receptor (IFNAR) (Table 2). The IFNAR induces the Janus activated kinase-signal transducer and activation of transcription (JAK-STAT) (Figure 3) pathway that control a large collection of genes through regulated expression of various signaling intermediaries (Olex Al et al., 2016; Messina NL et al., 2016; Guan J et al., 2014). In T cells, the signaling through the IFNAR is critical for the acquisition of effector functions (Kole A et al., 2013)

Type II interferons are represented by pleiotropic Th1-type cytokine interferon-γ (Table 2). The IFN-γ is induced in response to a variety of cytokines, including interleukin-2 (IL-2), IL-18, Type I IFNs alpha/beta, or by stimulation through T cell receptors (TCRs) or NK cell receptors (Malmgaard L, 2004). Similar to Type I

interferons, IFN-γ stimulates JAK/STAT pathway. In addition, a number of other pathways, including MAP kinase, PI3-K, CaMKII, and NF-κB cross-talk with JAK-STAT signaling to fine tune the multifaceted effects of IFNγ, which are exerted in a gene- and cell type-specific manner (Gough DJ, et al., 2008).

The type III family of interferons are comprised IFN-λ1, IFN-λ2, and IFN-λ3 or IL-29, IL-28A, and IL-28B, respectively (O'Brien T et al,2014; American Association for the Study of Liver Diseases, 2014; Kotenko SV et al., 2003; Lin FC and Young,2014; Gad HH et al, 2009). These interferons signal through a receptor complex composed of the IFN-λR1 chain (also known as IL-28RA) and the IL-10R2 chain, which is also a part of the receptor complexes for IL-10, IL-22, and IL-26 (Sheppard P et al., 2003; Donnelly RP and Kotenko SV, 2011; Gad HH et al., 2009; Lopušná K et al., 2013).

In 2013, a new member of the interferon λ (lambda) family, IFN-λ4, was described which signals through the IFNλR1 and IL-10R2 receptor chains (Hamming OJ et al., 2013). The IFN-λ4 is encoded by gene IFNL4, whose expression has been shown to be upregulated in response to HCV infection, but not to HBV infection (Estep M et al., 2014).

Table 2. Classification of Interferons

Interferon (IFN) Type	Receptor Type	Protein Structure	Genes	Gene Location	Tissue Expression Pattern
Type I	IFN α receptor that consists of IFNAR1 and IFNAR2 chains	α–helix	IFN-α 2a and 2b	Chr. 9	Leukocytes, macrophages, endothelial cells, tumor cells, keratinocytes, and mesenchymal cells
			IFN-b		Fibroblasts, endothelial cells, macrophages, and epithelial cells
			IFN-ω		T lymphocytes
			IFN-ε		Cerebral tissues
			IFN-κ		Not known
Type II	IFNGR consisting of IFNGR1 and IFNGR2 chains	Core of six α– helices and an extended unfolded sequence in the C-terminal region	IFN-γ	Chr. 12	T and Natural Killer cells
Type III	Receptor complex consisting of IL10R2 and IFNLR1 chains	Structurally similar to the IL-10, despite functionally being an IFN	IFN-λ		Dendritic cells and macrophages

<u>The IFNL4 locus</u>

The *IFNL4* gene is located on chromosome 19q13, just over 1kb upstream of, and in the same orientation as, the gene encoding IFN-λ3 (Figure 2). It is extremely conserved in all mammals, indicating its functional importance (Key FM et al., 2014). The ancestral allele of *IFNL4,* contains a guanine residue at position 342 of the coding sequence (referred to as "ΔG"). It encodes a functional IFN-λ4 peptide.

The *IFNL4* locus is known to contain a number of medically relevant single nucleotide polymorphisms (SNPs). One of these, rs368234815 or ss469415590 (TT), is characterized by the substitution of the G nucleotide with two thymine residues (TT) resulting in a nonsense mutation. As a result, IFN-λ4 can be generated only by individuals, who carry the ΔG allele.

To date, the majority of the studies of *IFNL4* locus have been performed in the context of HCV. The ΔG allele is associated with adverse outcomes of infection and interferon-based treatments (Franco S et al, 2014 AIDS; Aka, P et al., 2014; Nozawa Y et al., 2014; Amanzada A et al., 2013; Jouvin-Marche E et al., 2014; NIH, 2013; Stättermayer A et al., 2014) while the TT allele is associated with the spontaneous clearance of HCV and interferon responsiveness. It is presently the strongest known host factor for predicting clearance of HCV (O'Brien T et al., 2014). Another SNP, known as rs12979860, located within the intron of IFNL4 gene, is closely linked to the rs368234815 allele and is significantly associated with sustained viral response (SVR) in HCV patients (Younossi Z et al., 2012).

In a genome-wide association study published in 2009, the presence of a rs12979860 with a "C" allele was strongly associated with spontaneous viral clearance and treatment response. Patients who were homozygous for the presence of "C" allele had a greater than 2-fold increase in rates of SVR as compared to patients with the heterozygozity of this locus (C/T allele combination) and homozygous state T/T (Stättermayer, A et al., 2014, Younossi Z et al., 2012; Meissner EG et al, 2014). In

addition to increased SVR rates, patients homozygous for C allele (C/C) were more likely to demonstrated spontaneous clearance of HCV (Thomas DL et al., 2009). Additionally, the presence of SVR-promoting rs12979860 allele of *IL28B* locus was associated with lower baseline inflammation and possible suppression of apoptosis in peripheral blood mononuclear cell (PBMCs) evaluated during early phase of the treatment as compared to the presence of deleterious allele (Younossi ZM et al., 2012).

Interpretation of these finding relies on the proximity linkage of rs12979860 (*IL28B*) to rs368234815 *(IFNL4*) that is functionally responsible for effects of both variants. Due to shorter average size of haplotype blocks in individuals of African ancestry, rs368234815 is more strongly associated with HCV clearance in these ethnicities, whereas in Europeans and Asians it performs similarly to rs12979860 (Prokunina-Olsson et al., 2013).

Non-functional rs368234815-TT allele is specific for humans and is common in all human populations. In HapMap collection, it is detected in 93% of Asians genomes, 68% of European genomes and 23% of Africans genomes (Prokunina-Olsson et al, 2013). It is theorized that it emerged right before the onset of the “out-of-Africa” migration and was immediately supported in its spread by positive selection in European and Asian populations resulting in the high frequency observed today (Key FM et al., 2014).

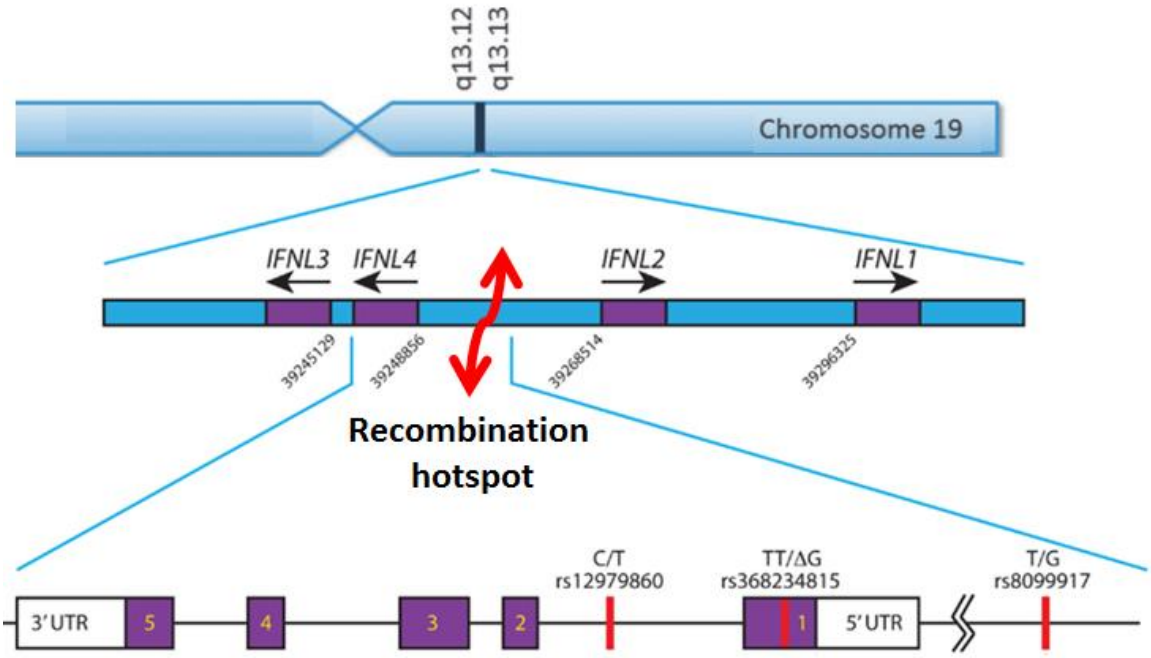

Figure 2: Location of common SNPs in IFNL4 Locus on Chromosome 19, and the map of IFNL4 exons. From: Stephen M. Laidlaw and Lynn B. Dustin, 2014, with changes.

It is most likely that the selective force that was driving elimination of IFN-λ4 in a majority of human populations was an exposure to certain pathogen (or pathogens), most likely, a virus. However, this pathogen is unlikely to be HCV, which is known for its relatively slow progress toward symptomatic phase. (Fan JH et al, 2016) There is also no association between *IFNL4* polymorphisms and HBV susceptibility or natural clearance (Fan JH et al., 2016). The advantages or disadvantages of IFN-λ4 expression in case of infection with a majority of non-HCV non-HBV viruses remain unknown.

A number of recent studies showed that IFN-λ4 possesses strong antiviral activity toward HCV and coronaviruses (Hamming OJ et al., 2013; Prokunina-Olsson et al, 2013). When overexpressed in hepatoma cells, *IFNL4* induces STAT1/STAT2 phosphorylation and expression of interferon-stimulated genes (Prokunina-Olsson et al., 2013; O'Brien T et al., 2014; Randall R and Goodburn S, 2008; Ank N et al., 2006). Interestingly, the antiviral activities of recombinant IFNλ3 and IFNλ4 were similar when studied against either HCV or coronavirus (HCoV-229E and MERS-CoV) challenges tested in either human ciliated airway epithelial cell (HAE) or hepatocyte cultures, (Hamming OJ et al., 2013). Another recent comparative study of Type III interferons, performed using transcriptome sequencing, failed to reveal any crucial differences between particular members of this family, with the majority of the identified genes being similarly regulated in hepatocytes as well as airway epithelial cells (Lauber C et al., 2015). Hence, it looks like the differences in mode of action for various IFN-λ may be due to their direct binding to some cellular or viral targets rather than to the transcription programs they stimulate.

In contrast, Prokunina-Olsson proposed the hypothesis that functional IFN-λ4 protein may compete with the IL28B/IFN-λ3 receptors and cause a pre-activation of the interferon-dependent genes, thus reducing overall responsiveness to Type I and III interferon (Prokunina-Olsson et al, 2013). This hypothesis agrees with previous findings that SVR-promoting alleles are associated with lower baseline inflammation (Younossi ZM et al, 2012).

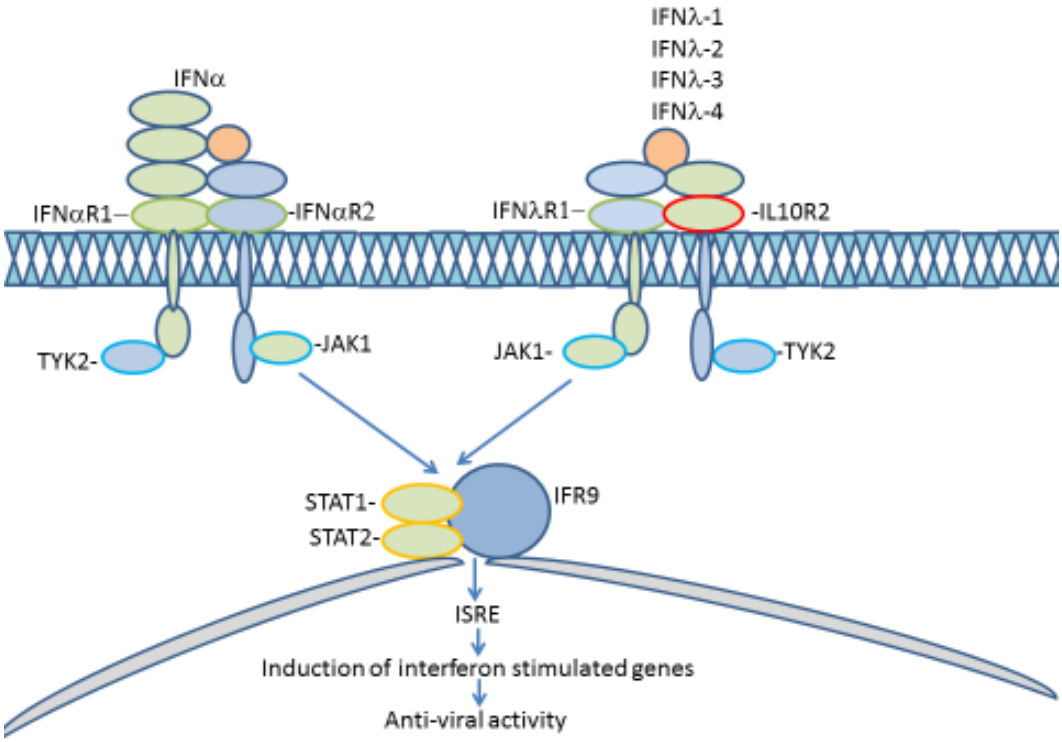

Figure 3: Schematic map of Jak-STAT pathway during an immune response with type 1-3 interferon antiviral activity.

There are some evidences that the polymorphisms in IFNλ4 may influence outcomes of non-HCV non-coronavirus types of acute and chronic infections. In particular, solid-organ transplant recipients homozygous for the active, ancestral rs368234815 allele (ΔG) are more susceptible to CMV replication, especially in absence of antiviral prophylaxis (Manuel O et al., 2015; Egli A., 2014). Another study showed that the same allele is associated with increased susceptibility to AIDS-related CMV retinitis (Bibert S et al., 2014).

Findings related to infection with HIV virus remain controversial. One study showed that that in Caucasian populations favorable CC genotype of rs12979860 is also associated with spontaneous control of human immunodeficiency virus (HIV) viremia (Machmach K et al., 2013), while in cohorts of African Americans these findings were not replicated (Sajadi MM et al., 2011; Salgado M et al., 2011). In a study of Real and co-authors, pseudogenized allele rs368234815-TT that protects against infection with HCV was also associated with decreased likelihood of HIV-1 infection in male intravenous drug users (odds ratio (OR): 0.3; P=0.006), and this association was not modified by the genotype of CCR5 (Real LM et al., 2015). Another recent study of rs368234815 variant showed that carriers of its active, ancestral variant ΔG have higher occurrence of AIDS-defining illnesses and lower CD4 T cell counts (Machmach K et al., 2015). These results suggest that genetic susceptibility to HCV and HIV-1 infection shares common molecular pathway (Real LM et al., 2015).

Specific Aims:

Aim 1: To determine the mechanisms how IFNL4 host genotypes influence the response to therapy in chronic HCV patients.

Aim 2: To identify differential gene expression biomarkers in African American and Caucasian patients with obesity-related NAFLD.

CHAPTER TWO

The Mechanisms of IFNL4 Host Genotype Influence on the Response to Anti-HCV Therapy

Background

The success of therapeutic approaches to HCV is measured by both HCV viral clearance and improved clinical outcomes. In clinical practice, the sustained virologic response (SVR) is defined as the inability to detect HCV RNA in the peripheral blood after the completion of the treatment. In patients who achieved SVR, clinical outcomes are significantly improved compared to patients who have not achieved SVR, including lower rates of the development of hepatocellular carcinoma, liver cirrhosis, and all-cause mortality (van der Meer AJ and Berenguer M, 2016).

Over the past 25 years, the treatment of HCV has undergone revolutionary changes. Prior to 1998, HCV patients were treated with recombinant interferon only, which was administered three times per week for 6 months. Under this regimen, HCV Genotype 1, the most prevalent type of HCV infection in Western world, had a SVR rate of approximately 6%. In 1998 the first combination therapy, IFN and ribavirin (RBV), became available. Ribavirin is a synthetic guanosine (ribonucleic acid) analog which inhibits viral RNA synthesis and viral mRNA capping. By combining RBV with interferon, and prolonging the duration of therapy to 48 weeks, the SVR rate for HCV

Genotype 1 increased to more than 30%. Further improvement in SVR rates was observed with the introduction of pegylated interferon (polyethyleneglycol molecules added to interferon). Patients treated with pegIFN demonstrated a SVR rate of 43%. In 2011, the use of the combination therapy consisting of the NS3/4A protease inhibitors telaprevir (Incivek) and boceprevir (Victrelis) with of pegIFN and RBV saw the SVR rate rise to approximately 70%. This combination, however, was associated with pronounced side effects, including severe anemia and serious skin rashes. In 2013, telaprevir/ boceprevir were replaced by simeprevir (Olysio) and sofosbuvir (Sovaldi). The use of sofubusvir, when used in combination with pegIFN+RBV for 12 weeks resulted in a SVR rate of 90%. In 2014, IFN-free HCV direct antiviral agents (DAA) therapies were introduced. The use of the combination regimens of ledipasvir/sofosbuvir (Harvoni) and the '3D' regimen ombitasvir/paritaprevir/ritonavir tablets in combination with dasabuvir (Viekira Pak) demonstrated a SVR of 90% (Zhang et al., 2016; Lam B. et al., 2016). The most effective DAA therapy is a combination of sofosbuvir with velpatasvir (Epclusa), which demonstrated a treatment efficiency of 95-100% across all of the six major HCV genotypes after a 12 weeks regimen (Chanine EB et al., 2016). This includes infections with HCV genotype 3, in whom previous standard of care therapies were not always sufficient to achieve SVR especially in those treatment-experienced patients with cirrhosis (Chanine EB et al., 2016).

An availability of a variety of HCV therapies provide an opportunity for individualized treatments that take into account multiple viral and host factors. These include, but are not limited to HCV genotype, prior exposure to antiviral treatments,

presence of cirrhosis, liver transplantation, the presence of renal disease, HIV co-infection, or resistance-associated HCV variants at baseline, and the potential for significant drug-drug interactions.

Although the introduction of DAA therapies made interferon-based antiviral regimens outdated, the understanding of the cellular and molecular mechanisms of interferon response remains important for the development of other host-targeting antivirals. Moreover, this understanding may aid in the development of novel types of vaccines, or preventive medications that would be used for early clearance of the virus rather than for treating patients already developed chronic viremic state.

A majority of the evidence associating *IFNL4* genotype with treatment outcomes come from observations in cohorts treated with interferon-based regimens. However, there is growing evidence that the functional ΔG allele of IFNL4 is also associated with decreased efficacy of direct-acting antivirals and recently developed interferon-free regimens of chronic HCV infection treatment (Meissner EG, 2014; Fujino, 2013). Additionally, the association of *IFNL4* non-expression with natural clearance of HCV may also be associated with innate IFN production during acute HCV infection. An association of *IFNL4* alleles and its expression with the expression levels of interferon-dependent and inflammatory genes shortly after exposure to interferon treatment have not been yet determined.

In this study, the goal was to determine if *IFNL4* host genotypes can be used as predictors of response to pegylated interferon alpha (PEG-IFN) and RBV therapy. We

assessed the expression levels of 153 genes relevant to interferon response and correlated gene expression with *IFNL4* genotypes in patients diagnosed with HCV genotype 1. Gene expression was examined at baseline and during various durations of treatment with PEG-IFN and RBV.

Materials and Methods

This study, including gene expression and genotyping, was approved by the Inova Health Systems Institutional Review Board.

HCV Patient Samples

Patient samples used for this research collected were at part of the IRB 13-1383 protocol.

Whole blood with a total volume of 5 ml was collected from each patient at baseline (prior to treatment- day 0), and after day 1, day 7, day 28, and day 56 after the first therapeutic dosing into PAXgene™ RNA blood tubes (PreAnalytiX GmbH, Switzerland).

Buffy coat peripheral blood mononuclear cells (PBMC) and serum were separated according to standard laboratory operating instructions and stored along with whole blood aliquots in the INOVA Fairfax biorepository -80 C.

Patient Selection

From the biorepository, we retrieved 40 peripheral blood mononuclear cells samples. All the samples were from patients diagnosed with HCV genotype 1 and treated with a course of the standard doses of PEG-IFN-α2a or PEG-IFN-α2b and a weight-based dose of RBV. Relevant clinical data were obtained from protocol records.

qRT-PCR and Gene Expression

PBMC samples were stored on dry ice and sent to Celera, Alameda, CA. There, qRT-PCR analysis of 153 genes belonging to various IFN-inducible and immune response related pathways (Table 3) was performed in the collaborating laboratory of Dr. Sheng-Yung P. Chang. Briefly, amplifications were performed with SYBR Green using 5 ng of total RNA as a template in 384-well format with a duplicate of each 15-µl reaction using Prism® 7900HT Sequence Detection System (Applied Biosystems). The mRNA expression levels were normalized by using six housekeeping genes (*PPP1CC, PPP1CA, RPL12, RPL37A, KIAA0174*, and *SNRP70*), a reference RNA and the ΔΔCt method (Garcia et al., 2005). Briefly, the expression level of each gene was first normalized with the average of the expression levels of six housekeeping genes, and then further normalized to a control "calibrator," Universal Human Reference RNA (Stratagene, La Jolla, CA). [ΔΔCt = ΔCt of sample (Ct of TOI - Average Ct of HSKs) – ΔCt of control (Ct of TOI - Average Ct of HSKs) (Livak et al., 2001). The final ΔΔCt values of 148 mRNAs were used for statistical analyses. The positive and negative fold change values represent increased and decreased gene expression levels in patients who

achieved SVR. Based on our previous assays which have been performed in a similar fashion, an average intra-assay CV was of 1.2% and Ct was in a range from 15 to 29. The results of qRT-PCR analysis were provided in the form of Excel spreadsheets.

Table 3. A list of 153 human genes belonging to various IFN inducible and immune response related pathways profiled on 153 study samples by qRT-PCR.

Gene name	**Name**
OAS1	2'-5'-oligoadenylate synthetase 1, 40/46kDa
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa
OASL	2'-5'-oligoadenylate synthetase-like
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
MX2	myxovirus (influenza virus) resistance 2 (mouse)
IFI16	interferon, gamma-inducible protein 16
IFI27	interferon, alpha-inducible protein 27
IFI30	interferon, gamma-inducible protein 30
IFI35	interferon-induced protein 35
IFI44	interferon-induced protein 44
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
IFIT2	interferon-induced protein with tetratricopeptide repeats 2
IFIT3	interferon-induced protein with tetratricopeptide repeats 3
IFIT5	interferon-induced protein with tetratricopeptide repeats 5
IFITM1	interferon induced transmembrane protein 1
IFITM2	interferon induced transmembrane protein 2
IFITM3	interferon induced transmembrane protein 3
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2
PRKRIR	protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of (P58 repressor)
PRKRA	protein kinase, interferon-inducible double stranded RNA dependent activator
ISG15	ISG15 ubiquitin-like modifier
IFI6	interferon, alpha-inducible protein 6
GBP1	guanylate binding protein 1, interferon-inducible
GBP2	guanylate binding protein 2, interferon-inducible
ISG20	interferon stimulated exonuclease gene 20kDa
JAK1	Janus kinase 1
JAK2	Janus kinase 2

LIPA	lipase A, lysosomal acid, cholesterol esterase
NMI	N-myc (and STAT) interactor
STAT1	signal transducer and activator of transcription 1, 91kDa
STAT2	signal transducer and activator of transcription 2, 113kDa
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)
STAT4	signal transducer and activator of transcription 4
STAT5A	signal transducer and activator of transcription 5A
STAT5B	signal transducer and activator of transcription 5B
STAT6	signal transducer and activator of transcription 6, interleukin-4 induced
ADAR	adenosine deaminase, RNA-specific
AIM2	absent in melanoma 2
BST2	bone marrow stromal cell antigen 2
CTLA4	cytotoxic T-lymphocyte-associated protein 4
IRF1	interferon regulatory factor 1
IRF2	interferon regulatory factor 2
IRF3	interferon regulatory factor 3
IRF4	interferon regulatory factor 4
IRF5	interferon regulatory factor 5
IRF7	interferon regulatory factor 7
MNDA	myeloid cell nuclear differentiation antigen
PLAUR	plasminogen activator, urokinase receptor
PLSCR1	phospholipid scramblase 1
RNASEL	ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase
TLR2	toll-like receptor 2
TLR4	toll-like receptor 4
TLR7	toll-like receptor 7
TRIM14	tripartite motif containing 14
TRIM26	tripartite motif containing 26
TRIM34	tripartite motif containing 34
IL10	interleukin 10
PF4	platelet factor 4
WARS	tryptophanyl-tRNA synthetase
YARS	tyrosyl-tRNA synthetase
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)
TARS	threonyl-tRNA synthetase
TGFB1	transforming growth factor, beta 1
IFNAR1	interferon (alpha, beta and omega) receptor 1
IFNAR2	interferon (alpha, beta and omega) receptor 2

IFNAR2	interferon (alpha, beta and omega) receptor 2
IFNGR1	interferon gamma receptor 1
IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)
TRADD	TNFRSF1A-associated via death domain
EP300	E1A binding protein p300
BAG1	BCL2-associated athanogene
CCL2	chemokine (C-C motif) ligand 2
CCL3	chemokine (C-C motif) ligand 3
CCL4	chemokine (C-C motif) ligand 4
CCL5	chemokine (C-C motif) ligand 5
CXCL10	chemokine (C-X-C motif) ligand 10
CCR1	chemokine (C-C motif) receptor 1
FAS	Fas (TNF receptor superfamily, member 6)
PML	promyelocytic leukemia
ICAM1	intercellular adhesion molecule 1
IRF8	interferon regulatory factor 8
CIITA	class II, major histocompatibility complex, transactivator
SOCS1	suppressor of cytokine signaling 1
SOCS6	suppressor of cytokine signaling 6
PSME1	proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)
PSME2	proteasome (prosome, macropain) activator subunit 2 (PA28 beta)
IL15	interleukin 15
IL15RA	interleukin 15 receptor, alpha
IL18	interleukin 18 (interferon-gamma-inducing factor)
IL1B	interleukin 1, beta
IL8	interleukin 8
NFIL3	nuclear factor, interleukin 3 regulated
SELL	selectin L
LCK	lymphocyte-specific protein tyrosine kinase
SP110	SP110 nuclear body protein
SP100	SP100 nuclear antigen
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase β
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase ε
IKBKG	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase γ
TANK	TRAF family member-associated NFKB activator
CD81	CD81 molecule
LBA1	TRANK1 tetratricopeptide repeat and ankyrin repeat containing 1
GTPBP2	GTP binding protein 2

GMPR	guanosine monophosphate reductase
CD47	CD47 molecule
COX17	COX17 cytochrome c oxidase assembly homolog (S. cerevisiae)
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
NUB1	negative regulator of ubiquitin-like proteins 1
SDCBP	syndecan binding protein (syntenin)
SHFM1	split hand/foot malformation (ectrodactyly) type 1
SSBP1	single-stranded DNA binding protein 1, mitochondrial
CFLAR	CASP8 and FADD-like apoptosis regulator
LAP3	leucine aminopeptidase 3
LGALS9	lectin, galactoside-binding, soluble, 9
AIF1	allograft inflammatory factor 1
FYN	FYN oncogene related to SRC, FGR, YES
GPX1	glutathione peroxidase 1
PIAS1	protein inhibitor of activated STAT, 1
PTEN	phosphatase and tensin homolog
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
CREB1	cAMP responsive element binding protein 1
SMAD3	SMAD family member 3
PRDM1	PR domain containing 1, with ZNF domain
TMSB4X	thymosin beta 4, X-linked
ADAM9	ADAM metallopeptidase domain 9
AP3M2	adaptor-related protein complex 3, mu 2 subunit
RHOC	ras homolog family member C
ATP6V0B	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b
BTG1	B-cell translocation gene 1, anti-proliferative
DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
CD58	CD58 molecule
PDGFA	platelet-derived growth factor alpha polypeptide
PDGFB	platelet-derived growth factor beta polypeptide
BCL2	B-cell CLL/lymphoma 2
IFI44L	interferon-induced protein 44-like
C1ORF38	chromosome 1 open reading frame 38
B2M	beta-2-microglobulin
TRIM22	tripartite motif containing 22

Extraction of DNA and Genotyping

Whole blood samples were removed from the -80 degrees, thawed and DNA extracted using QIAamp DNA Blood Mini Kits as described in the kit manual. *IL28B* and *IFNL4* genotyping was performed using custom TaqMan™ probes designed to determine allelic states of rs12979860 (IL28B) and rs368234815 (IFNL4) using a standard two step protocol.

Statistical analyses

For each time point, namely, prior to treatment (day 0), day 1, day 7, day 28, and day 56 after the first dosing, the Spearman's correlation coefficients were calculated for expression levels of each gene and the presence/ absence of the functional ΔG allele of rs368234815 (IFNL4). Analysis for the rs12979860 (IL28B) was performed in essentially similar way. For each gene, the expression levels were compared in the cohort of TT homozygotes (no expression of IFNL4) and in the cohort with heterozygosity ΔG / TT in *IFNL4* locus or with the presence of two functional alleles (ΔG/ΔG) by non-parametric Mann-Whitney test. For each cohort, means and variances of gene expression levels for each gene were calculated. Additionally, for each time point, gene expression data were correlated with SVR. Only p-values of <0.05 were considered significant (unless noted otherwise).

Functional analysis of gene expression patterns

Differential gene expression values were used as inputs for knowledge based, integrative analysis of the pathways, which was performed using pathway analysis

software packages MetaCore (GeneGo/Thompson Reuters) and Pathway Studio (Ariadne/Elsevier). MetaCore and Pathway Studio are text mining technologies that employ curated ontologies to process vast collection of published data, automatically extracting biological terms, concepts, and relationships from NCBI's GenBank®, RefSeq, Online Mendelian Inheritance in Man® (OMIM) (http://www.ncbi.nlm.nih.gov/omim), and the Human Genome Organization (HUGO) (http://www.genenames.org), and augment these extractions with manually curated entries from the full-text manuscripts reflected in PubMed. Both software packages import lists of gene IDs such as gene symbol, Entrez Gene, RefSeq, GenBank and Affymetrix probesets (PID), with or without expression data. In return, PAS provide the network analysis, that is performed by the assignment of genes that have direct (or both direct and indirect) interactions into groups or networks of manageable size (typically 35–50 genes) and functional analysis, that allows the assessment of key functions enriched within the input gene list. In canonical pathway analysis, the enrichment of genes within well-known biochemical and signaling pathways is determined. The comparative analysis of the performance of these PAS packages could be found in (Henderson-Maclennan NK et al, 2010; Ekins S et al., 2007).

Results

The Frequency and the Distribution of rs368234815 alleles in Study Cohort

For each patient, rs368234815 and rs12979860 genotypes were determined using custom TaqMan probes (Table 4). Both the frequency and ethnic distribution of rs368234815 and rs12979860 alleles were consistent with these previously reported and archived in HapMap and dbSNP databases (Table 4). In table 4 observance of SVR was reported in patients as a value of one and no SVR was reported as zero.

Of the 40 patients profiled, 21 (52.5%) were homozygous for the rs368234815 TT allele, 14 (35%) were heterozygotes, and 5 (12.5%) were homozygous for the ΔG allele. The presence of at least one functional *IFNL4*-ΔG was modestly but significantly correlated with African American ethnicity (rho = 0.439, P<0.01). As expected, the presence of the IFNL4-TT allele showed strong linkage to the *IL28B* allele C (rho = 0.837, P<001).

Table 4: Ethnic distribution of rs368234815 and rs12979860 alleles from sample tested.

Patient Number	Gender	African American	Sustained Viral Response	rs368234815 Genotype	rs12979860 Genotype
1	Male	no	yes	TT/TT	C/C
2	Male	no	yes	TT/TT	C/C
3	Male	no	no	TT/TT	C/C
4	Female	no	no	TT/TT	C/C
5	Male	no	yes	TT/TT	C/C
6	Male	no	yes	TT/TT	C/C
7	Male	no	no	TT/TT	C/C
8	Male	no	no	TT/TT	C/C
9	Female	no	no	TT/TT	C/C
10	Female	no	no	TT/TT	C/C

11	Female	no	no	TT/TT	C/C
12	Male	no	yes	TT/TT	C/C
13	Male	no	yes	TT/TT	C/C
14	Male	no	yes	TT/TT	C/C
15	Female	no	no	TT/TT	C/C
16	Male	no	yes	TT/TT	C/C
17	Male	yes	yes	TT/TT	C/C
18	Female	no	no	TT/TT	T/C
19	Female	no	yes	TT/TT	T/C
20	Female	no	no	TT/TT	T/C
21	Male	no	no	TT/TT	T/C
22	Female	no	no	TT/ΔG	T/C
23	Female	no	no	TT/ΔG	T/C
24	Male	no	no	TT/ΔG	T/C
25	Female	no	no	TT/ΔG	T/C
26	Female	no	yes	TT/ΔG	T/C
27	Male	no	yes	TT/ΔG	T/C
28	Female	no	no	TT/ΔG	T/C
29	Male	no	no	TT/ΔG	T/C
30	Male	no	no	TT/ΔG	T/C
31	Male	no	yes	TT/ΔG	T/C
32	Male	yes	no	TT/ΔG	T/C
33	Female	yes	no	TT/ΔG	T/C
34	Male	yes	no	TT/ΔG	T/C
35	Male	yes	no	TT/ΔG	T/C
36	Female	no	no	ΔG/ΔG	T/C
37	Female	yes	no	ΔG/ΔG	T/C
38	Male	no	no	ΔG/ΔG	T/T
39	Male	yes	yes	ΔG/ΔG	T/T
40	Female	yes	no	ΔG/ΔG	T/T

The Analysis of Gene Expression in PBMCs of patients with different IFNL4 genotypes

Differential expression of the 153 genes listed in Table 3 was evaluated both by pairwise analysis of the cohorts differing by their genotypes as well as by analysis of correlation between the presence/absence of each allele in entire cohort and the expression value for the gene of interest. For pairwise comparisons, expression levels of

each gene were compared between the cohort of patients with the *IFNL4*-TT/TT genotype and the cohort with any other genotype in this locus.

Additionally, similar comparisons were performed for *IFNL4*-TT/ΔG patients and *IFNL4*-ΔG/ΔG patients. Finally, gene expression levels for *IFNL4*-TT/TT patients were compared to that of *IFNL4*-ΔG/ΔG patients (comparison of extremes). Due to the low number of patients homozygous for the *IFNL4*-ΔG allele (N = 5), only comparisons between patients with the *IFNL4*-TT/TT genotype (N = 21) versus *IFNL4*-TT/ΔG patients (N = 14) or *IFNL4*-TT/TT genotype (N = 21) versus all non-TT/TT *IFNL4* genotypes (N = 19) have yielded statistically significant results (Table 5).

In patients homozygous for the *IFNL4*-TT allele, the strongest divergence of observed gene expression patterns was observed before at the pretreatment baseline and at Day 7 of treatment, with at days 1, 28, and 58, little or no differential expression was observed. Gene specific pairwise comparisons showed that for a majority of differentially expressed genes, lower expression values were observed in patients homozygous for the *IFNL4*-TT allele. However, one notable exception to this tendency was SMAD3, which expression levels were significantly higher in patients with *IFNL4* (TT/TT) genotype both at baseline and at Day 7 of treatment with pegylated interferon and ribavirin. Other exceptions include *AP3M2*, which expression levels were increased at the treatment Day 1, and *BCL2*, whose expression levels were increased at the treatment day 7 in patients homozygous for the *IFNL4*-TT allele.

Table 5: The list of genes with significant differential expression between groups of patients differing by their IGNL4 genotypes. Data are shown for those measurements that are statistically significant with a Mann-Whitney cutoff of p-values < 0.05. Positive values indicate downregulation, and negative values indicate upregulation. No differences were detected for genes expressed on Treatment Days 28 and 56.

Gene	Treatment Day 0		Treatment Day 1		Treatment Day 7	
	All Genotypes vs. IFNL4-TT/TT	IFNL4-TT/ΔG vs. IFNL4-TT/TT	All Genotypes vs IFNL4-TT/TT	IFNL4-TT/ΔG vs. IFNL4-TT/TT	All Genotypes vs. IFNL4-TT/TT	IFNL4-TT/ΔG vs. IFNL4-TT/TT
AP3M2				-1.4		
BCL2					-1.28	-1.31
CCL4					1.47	
CD58					1.15	
CEBPB	1.25					
GBP1					1.27	
IRF8	1.33					
PRKRIR		-1.45				
RHOC		1.4	1.3	1.44	1.16	1.32
SDCBP	1.22					
SMAD3	-1.47	-1.76			-1.32	-1.37
TLR2	1.34					

When analysis of correlation between the presence/absence of each *IFNL4* allele in entire cohort and the expression value for the gene of interest was performed, similar trends were observed (Table 5). The number of genes whose expression significantly correlated with *IFNL4*-TT allele presence was greatest at the baseline and at treatment Day 7. For the majority of studied genes, their expression levels negatively correlated with the presence of *IFNL4*-TT allele, with notable exceptions of *SMAD3* and *BCL2*, which demonstrated positive correlation at the treatment Day 7.

Pathways analysis in untreated patients differing by their IFNL4 genotypes

To examine baseline gene expression pattern, a pathway analysis using Ariadne Pathway Studio (Elsevier) was performed. Notable decrease in intensity of inflammation related processes was associated with the presence of *IFNL4*-TT allele. The highest ranking pathways enriched in differentially expressed genes in cohorts of patients with the TT allele of *IFNL4* were those involved in "Apoptosis" ($P<0.0001$), "B cell activation" ($P<0.001$), "Macrophage Receptor/CEPB signaling" ($P<0.01$), "CD19/NF-κB signaling" ($P<0.5$), "IL7R/FOXO signaling" ($P<0.05$) and "IFNG/STAT" signaling ($P<0.001$).

A follow-up analysis using MetaCore software (Thomson Reuters) to analyze pretreatment pathway expression yielded similar results; several of the top ten differentially expressed pathways are directly associated with immune responsiveness including "Immune response/IFN gamma signaling pathway" ($P<0.001$), "Immune response/ IL-17 signaling pathway" ($P<0.001$), "Immune response/ CD40 signaling" ($P<0.001$), and "Immune response/ IL-1 signaling pathway" ($P<0.001$).

Of particular interest was the finding that, in patients with the *IFNL4*-TT allele, the IFNγ pathway shows decreased expression of genes both upstream and downstream of STAT1(Figure 4).

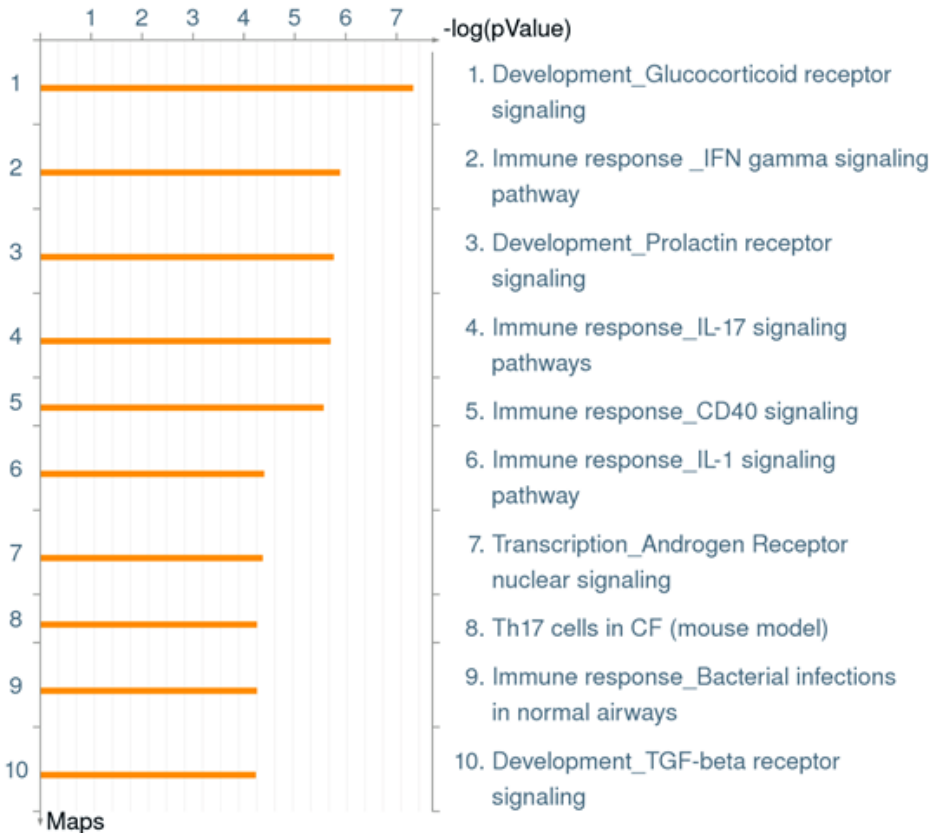

Figure 4: Top Ten Metacore-identified pathways differentially expressed in non-treated patient groups differing by their IFNL4 genotypes.

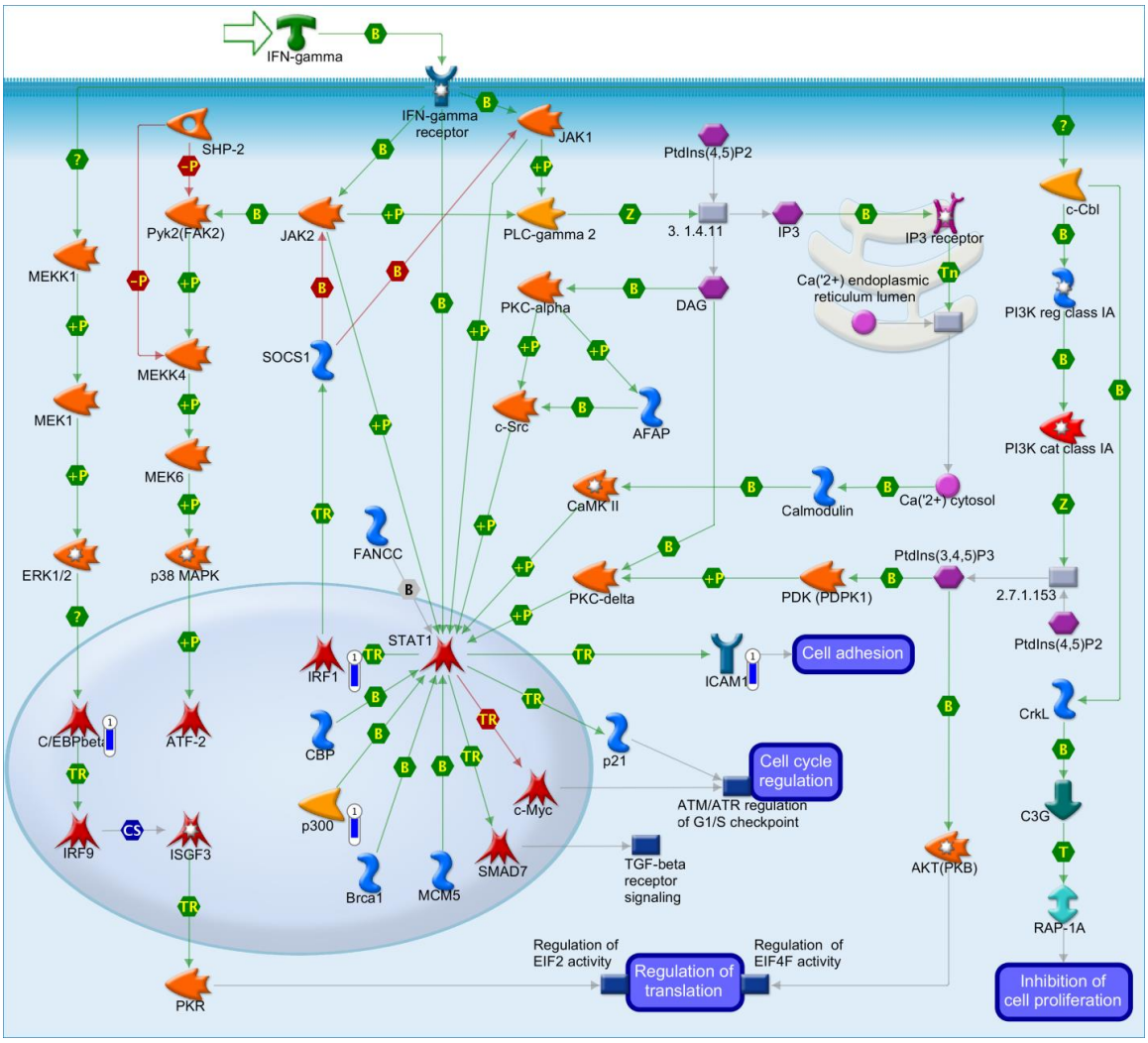

Figure 5: Metacore depiction of IFN-gamma signaling pathway

<u>Gene expression–based pathways analysis in interferon/ribavirin-treated patients differing by their IFNL4 genotypes</u>

At Day 7 of treatment, the most prominently highlighted pathways were those involved in apoptosis and survival (Figure 6). Nevertheless, inflammation and immune response pathways remained among the ranked list of most significant differentially expressed pathways. Combined, the four highest ranking pathways suggest the *IFNL4*-

TT allele is associated with a decrease of intensity of apoptosis that is manifested through a variety of relevant mechanisms, including BCL-2 dependent increase in survival (Figure 6).

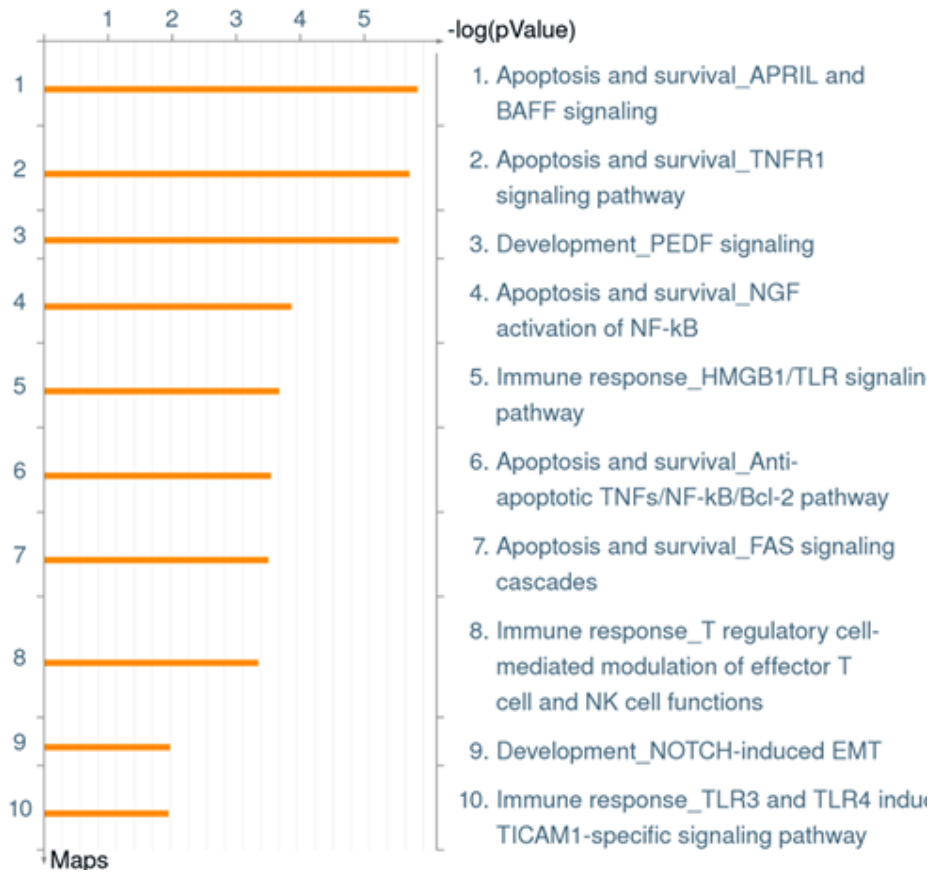

Figure 6: Top Ten Metacore-identified pathways differentially expressed in course of interferon/ribavirin treatment in patient groups differing by the presence or absence of functional allele of IFNL4 genotypes.

Discussion

This study compared the patients with chronic HCV/Genotype 1 which differ by presence or absence of functional allele of IFNL4 genotypes in term of expression levels of 153 interferon related genes before onset of the treatment (baseline), and at various times post treatment with PEG-IFN and RBV. In patients with ancestral rs368234815 allele (ΔG) and favorable non-functional TT allele of *IFNL4* locus, most significant gene expression differences were observed between at the baseline and at Day 7. At later time points post treatment, the differences in gene expression patterns between these two groups of patients were minimal.

At the baseline, in patients homozygous for the *IFNL4*-TT allele, the expression of several genes related to inflammation and interferon was suppressed. These findings are consistent the results of Xiao and co-authors (Xiao C et al., 2012), who studied interferon response in the livers of HBV infected patients who achieved or not achieved SVR after interferon-α therapy, and with previously published reports of hepatic expression patterns in patients with various *IFNL4* and/or *IL28B* genotypes (Urban TJ et al., 2010; Amanzada A et al., 2013)

According to MetaCore pathway analysis, in the treatment-responsive *IFNL4*-TT genotype group, the most prominently suppressed pathway was "Glucocorticoid Receptor Signaling" (Figure 5). Interestingly, treatments with a potent glucocorticosteroid, dexamethasone, or its less potent natural analogue, hydrocortisone, cause an increase in expression of TGFβ receptor RIII (Wickert et al, 2004), indicating that expression of this

receptor is controlled by these hormones, and that the glucocorticosteroids are capable of modulating TGF-β signaling, one of the most important events at the beginning of liver fibrosis (Fabregat I et al., 2016). Therefore, in treatment-responsive *IFNL4*-TT cohort, homozygous for ancestral allele, the hepatic fibrogenesis may be decreased through the suppression of glucocorticoid signaling and, therefore, lesser availability of TGFβ receptor RIII followed by diminished responsiveness to TGFβ signal (Figure 5). Although this link is tenuous, a possible connection between *IFNL4* genotype variants and hepatic fibrosis may be worth exploring.

Between the first and second week of the treatment with the PEG-IFN/RBV regimen, the differences in gene expression patterns between the specimens collected from patients with ancestral rs368234815 allele (ΔG) and non-functional TT allele in *IFNL4* locus diminish. This implies that the patients with the TT/TT genotype in *INFL4* locus undergo a more robust initial response to the treatment than those with the *IFNL4*-ΔG allele. In other words, at the initiation of treatment, genes for interferon-responsive and inflammation-related pathways such as the IFN-γ pathway undergo a significant increase in expression in the *IFNL4*-TT group while patients with at least one functional *IFNL4*-ΔG allele would only experience a modest increase, suggesting an intrinsic, pre-existing deregulation in the antiviral response mechanism in presence of *IFNL4* expression.

Patients with functional *IFNL4*-ΔG allele(s) may be handicapped by their antiviral and anti-inflammatory response being in persistent overdrive that, in turn, may reflect

higher levels of systemic inflammation within the patient's body, and therefore, more pronounced complications of chronic infection with HCV, both liver-limited (fibrosis, cirrhosis and hepatocellular carcinoma) and extrahepatic (lymphoproliferative disease, atherosclerosis, cardiovascular and brain disease).

Although our study examined only patients undergoing what is now outdated treatment with PEG-IFN/RBV, these findings can offer additional insights into *IFNL4*-ΔG associated decrease in SVR in patients treated with direct acting antivirals. Indeed, slower loss of free virus and decreased drug efficacy were previously noted in *IFNL4*-ΔG patients treated with the direct antiviral agents (DAAs), i.e., sofosbuvir [Meissner EG et al, 2014). These findings may be explained either by pre-existing inflammatory overdrive or by substantially more severe manifestations of the HCV disease seen in *IFNL4*-ΔG group. To discern between these possibilities, further studies of DAAs in cohorts stratified by *IFNL4* genotypes are warranted.

At the Day 7 of treatment, the most marked genotype-dependent divergence of gene expression profiles was noted for in apoptosis associated gene networks, while there also was a concomitant increase in expression of genes associated with survival. Several recent studies have associated HCV infection with alterations in apoptosis signaling (Schiavon, 2011; Brost, 2014) including HCV-guided deletion of cytotoxic T cells (Larrubia GR et al, 2013). In the current study, we have demonstrated for the first time that in patients carrying at least one copy of non-functional allele *IFNL4*-TT, there is a

treatment-associated suppression of apoptosis in immune cells of the blood; implying that more vigorous immune response may be mounted in *IFNL4*-TT cohort.

Our study has several weaknesses that could affect the interpretation of our results. The most obvious of these is relatively limited sample size (N=40), that reflected upon the number of available *IFNL4*-ΔG homozygotes. However, this weakness is partially mitigated by avoidance of possible confounders by limiting enrollment to patients infected with HCV genotype 1. Another limitation was that some patients were hadn't received prior treatment, while others were not. As of now, we have no reason to believe that our data would have been different if treatment-homogenous groups were analyzed. However, at least in theory, patients with two beneficial *IFNL4*- TT alleles who undergone antiviral treatment and failed to achieve an SVR, may represent a distinct subgroup, possibly differing by presence of some other viral or host factor, i.e. some other genomic variant directly affecting their response to interferon.

To summarize, this study has revealed an overall increase in expression levels of genes associated with inflammatory and interferon response in carriers of *IFNL4*-ΔG allele. This increase is observed at the pretreatment baseline, suggesting an intrinsic, pre-existing deregulation in the antiviral response mechanism in presence of *IFNL4* expression. Additionally, we showed that later in course of treatment, patients with a beneficial genotype *IFNL4*-TT undergo treatment-associated suppression of immune cells apoptosis, implying that more vigorous immune response may be mounted in this cohort. These findings can provide additional insights into previously noted *IFNL4*-ΔG

associated decrease in virologic end-points that reflect the response to direct acting antivirals. In the treatment-responsive *IFNL4*-TT group, the most prominently suppressed pathway was “Glucocorticoid Receptor Signaling”. We hypothesize that patients with *IFNL4*-TT genotype may be less susceptible to the development of liver fibrosis due to the subsequent suppression of the expression of TGFβ receptor RIII.

CHAPTER THREE

Differential Gene Expression Biomarkers in the Livers of African American and Caucasian Patients with various types of Obesity-Related NAFLD

Background

Non-Alcoholic Fatty Liver Disease (NAFLD) and its progressive form, non-alcoholic steatohepatitis (NASH), are among the most frequent and least understood metabolic consequences of obesity (Ong JP and Younossi ZM, 2007). Demographic analysis of obese populations has provided evidence of ethnic variation in the incidence and the risk of progression of NAFLD to NASH. In the Hispanic population, for example, NASH is more common than in the Caucasian or African American populations (Clark JM, 2006; Kallwitz ER et al, 2009). In African Americans, this low incidence prevails despite a high rate of obesity and other metabolic risk factors such as type 2 diabetes (Kallwitz ER et al, 2009, Mohanty SR et al, 2009; Rivera CA 2008).

This differential manifestation of NAFLD and NASH amongst different ethnicities may be potentially explained by interplay between genetic and environmental factors, but currently there is no data on any biological mechanism that supports this.

A number of ethnically distributed gene variants have been tied to the incidence of liver steatosis and the progression of NAFLD to NASH. The most clinically significant of these variants is rs738409C→G (I148M), which is located in adiponutrin, a

patatin-like phospholipase domain-containing protein 3-encoding gene *PNPLA3* (Zain SM et al., 2012; Sookoian S et al., 2011; Sookoian S et al., 2014; Speliotes EK et al., 2010). The physiologic impact of its deleterious G allele may be exacerbated in patients with severe obesity (Guichelaar MM et al., 2013). The frequency of the I148M allele matches the prevalence of NAFLD in various ethnicities (Romeo et al., 2008). Hispanics have a higher frequency of the G allele (49%) as compared to European Americans (23%) and African Americans (17%) (Romeo et al., 2008). Similarly, one can find genetic contributors to the progression of NAFLD and NASH to liver fibrosis that also have differential prevalence in patients of different ethnic origins.

In this study, we compared frequency of gene expression in the livers of Caucasian and African American patients with obesity-related NAFLD. Our primary goal was to determine if these frequencies could be used as biomarkers of disease progression. Our secondary goal was to identify any pathophysiological mechanisms that might explain different rates of the progression NAFLD past liver steatosis stage in these populations.

Materials and Methods

This study, including gene expression and genotyping, was approved by the Inova Health Systems Institutional Review Board.

Patient Samples

The samples used in this study were originally collected at part of the protocol Epidemiology of NAFLD (EPI-NAFLD). This protocol enrolled patients undergoing bariatric surgery at Inova Fairfax Hospital (Falls Church, VA) from 2001 to 2009. The EPI-NAFLD study, patients with evidence of excessive alcohol use (>10 g/day) or other causes of liver disease were excluded. Insulin resistance was defined as a homeostatic model assessment (HOMA) index more than 2.2, and diabetes mellitus was defined as a clinically established diagnosis in a patient treated with diet control or anti-diabetic medications or both. Metabolic syndrome was defined using Adult Treatment Panel III (ATP III) criteria (Expert Panel, 2001).

For each of the patients, two specimens of hepatic parenchyma were obtained at the time of liver biopsy. One specimen was sent for routine pathologic assessment and the other was immediately snap-frozen with liquid nitrogen and stored in the biorepository at Inova Fairfax at minus 80 C degrees. The hepatopathologist reviewed specimen slides according to a predetermined histologic grading system (Gramlich T et al., 2004). The degree of steatosis was scored on hematoxylin and eosin stained slides as an estimate of the percentage of tissue occupied by fat vacuoles using the values: 0 = none, 1 = 0-5%, 2 = 6-33%, 3 = 34-66%, 4 = >66%. In addition, Masson trichrome stained slides were examined to determine the presence and extent of fibrosis. The presence of NASH was defined as steatosis with lobular inflammation and ballooning degeneration of hepatocytes with or without Mallory-Denk bodies or pericellular fibrosis.

Sample Processing

For the current study, 94 patients with the histological diagnosis of NASH and an ethnic origin of either African American or Caucasian were identified from EPI-NAFLD protocol records. These samples were retrieved from the Inova Fairfax biorepository, and subjected to RNA extraction and microarray analysis.

RNA Preparation

Total RNA was extracted from 20 to 30 mg of liver tissue by using the RNeasy® Mini Kit (Qiagen, Valencia, CA) and treated by DNA-free™ (Ambion, Austin, TX). Abundance and ratio between 28S and 18S rRNA were monitored both by agarose gel electrophoresis and Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). Total RNA pooled from 10 different human cell lines (Universal Human Reference, Stratagene, La Jolla, CA, Cat # 740000) was used throughout the experiment as a reference RNA sample. Liver RNA and reference RNAs (1.5 μg) were amplified with the MessageAmp aRNA Kit (Ambion).

Microarray Printing and Processing

Microarray printing and processing was performed at George Mason University by Dr. Luca del Giacco with assistance of Francesco Gorreta.

A custom microarray "CARTNASH chip" was produced from 5,220 human cDNA clones (Research Genetics, Carlsbad, CA) representing genes expressed in hepatocytes and other cellular components of the liver. A complete list of genes with

their accession numbers is available at: http://www.gmu.edu/centers/genomics/research/keys. aRNA (4 μg) was reverse transcribed and labeled according to The Institute for Genomic Research protocol (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml). The amount of available RNA allowed the testing of duplicate or triplicate arrays for most of the samples, yielding 343 total arrays. Prehybridization and hybridization of the glass microarray slides were performed as described by Younossi ZM et al. (2005). Then, slides were scanned with the confocal laser scanner ScanArray™ Express HT (Packard BioScience, Boston, MA). Images were acquired by ScanArray™ Express 2.0 software (PE Life Sciences, Boston, MA) and processed with QuantArray 3.0 software (PE Life Sciences) to quantify intensity levels and local background for both Cy3 and Cy5 channels of each spot.

Microarray Plate Quality Control Assay

The quality of all microarrays was assessed with a series of controls at INOVA Fairfax Hospital by Dr. Karen Schlauch and Dr. Ancha Baranova. This assessment included a determination of dye bias and generation of heatmap images of each microarray. Arrays with any identifiable problems were excluded from the study, resulting in 183 arrays of good quality for 94 patients. After subtraction of local background, each microarray was filtered to determine which intensity levels were significantly greater than background values, and thereby deemed detected. A per-chip background threshold for each channel defined as the mean negative control intensity of each channel on the array provided a useful threshold for these customized microarrays. Intensity values lower than the threshold in either channel was set equal to the threshold in that channel to avoid the

computation of artificially large expression ratios. Expression ratios (Cy5/Cy3) were computed, and normalized using a linear median normalization (division by the per-chip median ratio). Average ratios were then computed across the set of replicated arrays of each hepatic tissue sample. For each gene, mean value obtained after averaging two replicated experiments were subjected to further analysis.

Results Real-Time Polymerase Chain Reaction (RT-PCR) Quality Control for Microarray Probes

The differential expression of four genes, *HMGCS2, ACSL4/FACL4, CAT* and *ATF3*, was validated by real-time RT-PCR performed in a 96-well format in the BioRad iCycler iQ Real-Time Detection System (BioRad Laboratories, Hercules, CA) on a random selection of 23 samples as described previously (Younossi ZM et al., 2005). For each gene, three independent PCR experiments from the same reverse transcription sample were performed. The presence of a single specific PCR product was verified by melting curve analysis and confirmed on agarose gel.

Statistical Analyses

Microarray statistics were assessed at Inova Fairfax Hospital by Dr. Maria Stepanova. Statistical analysis of the differential gene expression was performed using SAM (Significance Analysis of Microarrays) algorithm (Tusher VG et al., 2001). The SAM algorithm is a permutation-based method that relies on variance information present in measurements obtained from all probes on a microarray. In SAM, the relative gene expression difference di for each gene is compared to the distribution of d following

random permutation of the sample categories (e.g., NASH and non-NASH). As a result of series of permutations, a certain proportion of all genes in the permutation set are found to be 'significantly' differentially expressed by chance. This proportion is then used to calculate a false discovery rate presented as a q value for each gene in the final list of significantly differentially expressed genes. The R implementation of the SAM algorithm was obtained from http://www-stat.stanford.edu/~tibs/SAM/.

Results

Table 6 summarizes the clinical and demographic data for the two cohorts tested. The p-values reflecting inter-cohort variability were calculated using Mann-Whitney non-parametric test for numerical parameters and using chi-square test for independence for binary parameters (such as presence of a condition or a risk factor). Caucasian patients had a significantly higher rate of metabolic syndrome than African Americans (66% vs. 33%, p-value = 0.0069) which can be linked to a higher prevalence of hyperlipidemia (fasting serum cholesterol: 202±34 mg/dL vs. 178±41 mg/dL, respectively; p-value = 0.0073, and fasting serum tryglicerides: 169±72 mg/dl vs. 126±87 mg/dL, respectively; p-value = 0.00093).

The African American cohort consisted of only female patients, while Caucasian cohort had 80% female and 20% male patients. In order to avoid introduction of gender-specific factors into the ethnicity-based comparisons of gene expression levels, all

analyses were run twice; once for all patients, and once for female patients only. Outcomes of "all genders" and "females only" analyses were essentially similar.

Table 6: Comparison of Clinical Characteristics of African American and Caucasian Patients

	African Americans	**Caucasians**	**p-value**
	N=21	N=73	
Age	36.7±9.2	41.1±10.0	**< 0.069**
Hip-to-Waist ratio	1.14±0.12	1.07±0.11	NS
Body mass index	47.8±9.7	49.1±8.2	NS
Alanine transaminase (ALT), µ/l	21.3±11.3	27.7±15.6	**< 0.023**
Aspartate transaminase (AST), µ/l	20.8±7.7	22.8±10.9	NS
ALT/AST	1.02±0.33	1.21±0.54	**< 0.074**
Fasting serum glucose, mg/dl	115±60	105±26	NS
Fasting serum cholesterol, mg/dl	178±41	202±34	**< 0.0073**
Fasting serum triglyceride, mg/dl	126±87	169±72	**< 0.00093**
Fasting serum insulin, µU/ml	10.4±9.0	11.7±9.8	NS
Homeostatic model assessment score	3.2±3.4	3.8±5.2	NS
Female (%)	100	79	**< 0.025**
Type II diabetes (%)	14	16	NS
Hyperlipidemia (%)	33	44	NS
Metabolic syndrome (%)	33	66	**<0.0069**
Histologic NASH	5 (24%)	19 (26%)	NS
Histologic simple steatosis	12 (57%)	45 (62%)	NS
Finding of normal liver on a biopsy	4 (19%)	9 (12%)	NS

Differential Gene Expression -- African American and Caucasians

Five genes were found to be over expressed in livers of African American patients as compared to that in Caucasian cohort (q-value = 0): erythrocyte membrane protein band 4.1-like 1 (*EPB41L1*), insulin-like growth factor 2 (somatomedin A) (*IGF2*), fumarylacetoacetate hydrolase (fumarylacetoacetase) (*FAH*), Acyl-CoA synthetase long-chain family member 4 (*ACSL4*), and fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific) (*FUT4*).

The testing was repeated on the female patients of the two cohorts. In this gender-balanced comparison, four of the five genes remained significantly over expressed in African Americans. The exception was *FAH*, whose q-value increased to 24.4%. This represented the lowest non-zero q-value for this comparison. The same set of genes remained significantly differentially expressed even after further exclusion a group of patients without NAFLD, with the only exception of *IGF2*.

Simple Steatosis and NASH subcohorts-specific comparisons of Gene Expression in hepatic tissue of African Americans and Caucasians

In order to determine ethnicity-specific NASH-related molecular processes, we separately repeated the gene expression analysis for the African American and Caucasian cohorts with simple steatosis, and, separately, with NASH.

In patients with simple steatosis, there was no significant difference between genes differentially expressed in the African Americans and Caucasians samples.

In NASH patients, we found 5 differentially expressed genes with q-value=0: Glutathione S-transferases 2, 4, and 5 (*GSTM2, GSTM4, GSTM5*), fumarate hydratase (*FH*), and acyl-CoA synthetase long-chain family member 4 (*ACSL4*). Three of these genes remained significantly differentially expressed when gender-balanced comparison was performed (*GSTM4, GSTM5, FH*), while the other two genes gained in their q-values which, however, remained minimal (22.8%).

Differential Gene Expression - African Americans and Caucasians with and without NASH

Differentially expressed genes detected in comparisons of patients with and without NASH in the cohorts of African Americans and Caucasians were analyzed separately. In Caucasians, 9 genes had q-value of 0 (*AGXT, SLC6A13, FAAH (2 ESTs), CYP2B6, ACADS, CHAD, C10orf116, RALGDS, ERAP1*) and 3 genes had q-value = 6.9% (*RARRES2, SLC6A12, EPHX2*).

In African Americans, no genes were assigned zero false discovery rates, most likely due to the small sample size. However, for the highest ranking 64 genes, the false discovery rate was kept as low as 2.0%. In both African Americans and Caucasian cohorts, all significantly differentially expressed genes were under-expressed in NASH patients. The only gene in common between NASH-related gene lists obtained from African American and Caucasian cohorts was *CYP2C8* gene encoding cytochrome P450, family 2, subfamily C, polypeptide 8 (in African Americans, average gene expression

level was 0.73 and lower in NASH patients in comparison with non-NASH; in Caucasians, this ratio was 0.74).

Differential gene expression levels that reached statistical significance are summarized in Tables 7 and 8.

Table 7: Genes that were differentially expressed in hepatic tissue specimens of African Americans vs. Caucasians with NAFLD.

Full and Abbreviated Gene Names	**Differential Expression (folds change)**	**Description**
Erythrocyte membrane protein band 4.1-like 1 (*EPB41L1*)	**1.46**	Mediates interactions between the cytoskeleton and plasma membrane in erythrocytes and neurons. Stabilizes some receptors. Binds and inhibits myosin in the liver
Insulin-like growth factor 2 (somatomedin A) (*IGF2*)	**1.55**	Member of the insulin family of polypeptide growth factors. Candidate gene for eating disorders. Mediation of the profibrotic TGF-β signal. Increased in steatohepatitis. Expression altered in heptocellular carcinoma (HCC)
Fumarylacetoacetate hydrolase (*FAH*)	**1.54**	Catalyzes the final step of tyrosine catabolism to produce fumarate and acetoacetate. Mutations result in death within a few months due to hepatic failure
Acyl-CoAsynthetaselong-chain family member 4 (*ACSL4*)	**2.02**	Central enzyme controlling the unesterified arachidonic acid (AA) level in cells
Fucosyltransferase 4 (*FUT4*)	**1.33**	Glycosylation enzyme. Important contributor to selectin-dependent leukocyte recruitment in context of inflammation

Table 8: Genes that were differentially expressed in hepatic tissue specimens of African American and Caucasians with NASH.

Full and Abbreviated Gene Names	Differential Expression (folds)	Description
Glutathione S-transferase M5 (*GSTM5*)	**3.31**	Detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione
Fumarate hydratase (*FH*)	**2.73**	An enzymatic component of the tricarboxylic acid (TCA) cycle or Krebs cycle, and catalyzes the formation of L-malate from fumarate. Dietary controlled. Mutated in multiple cutaneous and uterine leiomyomatosis
Glutathione S-transferase M4 (*GSTM4*)	**2.59**	Detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione. Cellular defenses against the oxidative stress
Glutathione S-transferase M2(muscle) (*GSTM2*)	**2.17**	Detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione
Acyl-CoAsynthetaselong-chain family member 4 (*ACSL4*)	**3.15**	Central enzyme controlling the unesterified arachidonic acid level

Discussion

This study analyzed the differential gene expression in liver biopsy samples from 94 Caucasian and African American patients with various forms of NAFLD. Furthermore by using an integrative approach; which combines gene expression pattern with clinical

and pathologic data, we have generated a number of important insights into ethnic differences of the pathophysiology of NAFLD.

Five potentially important genes were found to be over-expressed in the African American patients as compared to Caucasians. These are: *IGF2*, *ACSL4*, *FAH*, *FUT4*, and *EPB41L1*. Of these, only EPB41L1 has not been previously described as the components of the pathways specifically activated in fatty livers. An increase in the levels of *IGF2* gene, which is known for its involvement in the mediation of the profibrotic TGF-β signal (El-Shewy HM 2009), has already been reported to be NASH-specific (Chiappini F et al., 2006). Similarly, up-regulation of the fatty acid metabolism enzyme encoded by *ACSL4* has been seen in livers of the patients with NAFLD, independent of obesity (Westerbacka J et al., 2007). *FAH*-encoded fumarylacetoacetase (FAH), the last enzyme in a pathway responsible for the catabolism of tyrosine, prevents oxidative damage of hepatocytes. It has been reported that *FAH* mutations lead to stress-induced failure of cell death programs and cancer promoting accumulation of damaged cells (Vogel A et al., 2004). Expression of the fucosyltransferase gene *FUT4* has been shown to correlate with cellular apoptosis (Azuma Y et al., 2004). All these functional connections indicate plausibility that the genes differentially expressed in hepatic tissues of African Americans and Caucasians are, indeed, play a role if pathophysiology of NAFLD.

Importantly, comparison of the disease-specific gene expression profiles from African American and Caucasian patients demonstrated that substantial differences

between these two ethnic groups may differentially occur at the level of advanced chronic liver disease (NASH), but not at the level of simple steatosis which is known to be relatively benign in all ethnicities. This comparison highlights possible intrinsic differences in the levels of ability to detoxify xenobiotics in African Americans and Caucasian. In NASH-specific inter-ethic comparison, we have observed a coordinated change in the levels of Glutathione S-transferases 2, 4, and 5, Phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) to electrophilic compounds and, therefore, detoxify many environmental and endogenous agents that may otherwise injure liver parenchyma (Serviddio et al., 2013; Rolo et al., 2012).

It is worthwhile to mention that many genes highlighted in our analysis contain polymorphic positions with allele frequencies that are substantially different between African American and Caucasian patients. For example, an analysis of *GSTM5* gene revealed C/T polymorphism that leads to the substitution of the Leucine to Proline in the C-tail of the respective Glutathione S-Transferase. An analysis of Human Diversity Panel (Jakobsson M, 2008) showed that a heterozygotic state of this polymorphism present in 29.2-57.3% of individuals of African Americans and only in 6.5 - 18% of Caucasians. Similarly, ethnicity-specific shifts in polymorphic allele frequencies were noted in *SLC6A13, CYP2B6, AGXT, CHAD, C10ORF1116* and others. Differential distribution these and other polymorphic variants located in vicinity of or within NAFLD pathogenesis related genes could potentially provide some biologic explanation for better disease outcomes of NAFLD in African American patients as compared to Caucasians.

The limitations of our study include absence of male patients in the African American cohort, thus leading to a potential bias in our observations. However, by performing independent analysis in “all patients” and in “females only” cohorts, we demonstrated that the resultant differentially gene lists are stable. Hence, the bias due to relative lack of African males with NAFLD in our cohort is not substantial. Using the SAM algorithm for gene expression analysis also introduces a number of additional limitations. For example, choosing one or another arbitrary selection threshold influences the output from SAM, and, therefore, may critically alter the conclusion of a study (Larsson O. et al., 2005; Subramanian A, 2005) However, the strength of our analysis is confirmed by the fact that the balancing the cohorts by the demographic parameters led to minimal oscillation of q-values, thus confirming the specificity of our ethnicity-based comparisons. Most likely, these minimal oscillations were caused by variation in sample sizes: while larger comparison of NASH patients of different ethnicities included 19 Caucasians, the gender-balanced comparison was profiled for only 12 Caucasian females. None of the genes highlighted by this analysis was previously identified as obesity associated gene using the same microarray platform (Younossi ZM et al., 2005). Interestingly, the gene *EPB41L1* (upregulated 1.46 fold) is well known for its involvement in the susceptibility to infection by Plasmodium falciparum in African population (Facer CA et al., 1995). Since this ethnicity-based comparison involved all subjects including those without advanced liver disease, this finding directly confirms specificity of the observed gene expression differences.

Although it might have been useful to include Hispanic patients with more progressive NASH, we did not have enough adequate sample size of Hispanic patients with NAFLD to include in our analysis.

In the future, we anticipate substitution of the gene-oriented approaches exploited by the pathway-specific analysis that will possibly highlight race-specific differences in the liver metabolism and maintenance. The ethnicity dependent mechanisms impacting the initiation of NAFLD and its progression to NASH and to liver fibrosis may be helpful in the proper management of NAFLD in these patients as well as in guiding the development of novel treatment strategies. Progression of both infectious and non-infectious chronic liver diseases follows the same route terminates in liver fibrosis and cirrhosis. The ethnicity-specific differences in course of non-infectious chronic liver disease may be paralleled by differential infectivity for known liver pathogens, including HCV. Gene expression biomarkers that differentiate between ethnic populations with non-viral chronic liver disease should be also studied in a context of viral infections.

CHAPTER FOUR

Conclusions and Future Directions

The purpose of this study was twofold: to determine the mechanisms how *IFNL4*-defined host genotypes influence the response to therapy in chronic HCV patients and to identify differential gene expression biomarkers in African American and Caucasian patients with obesity-related NAFLD.

In particular, we have demonstrated an overall suppression of the transcription of genes associated with an inflammatory and interferon response in HCV infected patients who are carriers of beneficial, pseudogenic *IFNL4-TT* allele. This suppression was observed at the baseline, suggesting an intrinsic, pre-existing deregulation of the antiviral response in *IFNL4* expression in carriers of ancestral *IFNL4*-ΔG allele. Additionally, we showed that later in course of treatment, patients with beneficial genotype *IFNL4-TT* undergo treatment-associated suppression of immune cells apoptosis, implying that more vigorous response to therapy may be mounted in this cohort. These findings provide additional insights into previously noted *IFNL4*-ΔG associated failure to achieve virological end-points in response to antiviral therapies.

Additionally, Metacore-guided pathway analysis led us to hypothesize that patients with *IFNL4*-TT genotype may be less susceptible to the development of liver fibrosis due to the subsequent suppression of the expression of TGFβ receptor RIII. The

latter prediction is independent of the presence or absence of HCV infection, and may be relevant to progression of both infectious and non-infectious liver diseases.

Exact mechanisms for *IFNL4*-deletion driven HCV clearance have not yet been elucidated. However, one could postulate that anti-host factor antibodies or siRNAs that specifically inhibit expression of *IFNL4* may be developed as molecular aids to abate HCV epidemics. Moreover, an introduction of novel but very efficient and also expensive treatments for HCV suggests an interesting field for IFNL4 genotype testing, namely, the stratifications of out-of-pocket paying patients into the differentially treated groups. In particular, some patients with beneficial *IFNL4*-TT may be efficiently treated with older, cheaper drugs, i.e., PEG-IFN/RBV combination, while carriers of ancestral *IFNL4*-ΔG allele require substantially more expensive treatment with direct antiviral agents including sofubusvir, boceprivir or telaprivir.

In 2011, the American Association for the Study of Liver Diseases proposed a guideline to test for a C or T SNP at rs12979860 in *IL28B* which is associated with response to treatment with PEG-IFN and RBV. This differential guideline was based upon observation that treatment response rates vary according to *IL28B* genotype: interferon/ribavirin treatments were successful in 69% and 27% of Caucasians for CC and TT genotypes, while in ethnically African American patients, the treatment success rates were 48% and 13% for CC and TT genotypes, respectively. In our opinion, similarly designed screening for the variants in *IFNL4* locus rather than less precisely tests for same trait-linked IL28B variants may aid in more efficient, differential

distribution of anti-HCV treatments and, possibly, prevent wasting of resources which is a major concern in economically disadvantaged regions of the world.

In this study, we also looked into the ethnic-specific patterns of gene expression in livers of obese patients susceptible to the development of NAFLD and its advanced form known as NASH. Despite obvious differences in obesity-related NAFLD and HCV-related fibrosis etiology, the pathophysiological mechanisms, including chronic inflammation, consequent cell damage and regenerative proliferation are shared between these two conditions. Therefore, insights extracted by us from studying NAFLD model are transferable to chronic hepatitis cases due to infection with HCV and other viruses affecting the liver.

Importantly, comparison of the disease-specific gene expression profiles from African American and Caucasian patients demonstrated that substantial differences between human races occur at the level of advanced chronic liver disease (NASH), but not at the level of simple steatosis which is known to be relatively benign in all ethnicities. In particular, we report intrinsic differences in the levels of compensatory anti-oxidative response as could be seen from the coordinated changes in the levels of Glutathione S-transferases 2, 4, and 5 in patients with NASH. Many genes highlighted our analysis contain polymorphic positions with allele frequencies substantially different between African American and Caucasian cohorts. Differences in distribution of genome variants in these populations may contribute to a degree of protection from the development of advanced non-infectious forms of chronic liver diseases observed in at-risk populations such as African Americans.

From this research perhaps future strides can be made to improve treatment and vaccine development for HCV infection. *IFNL4* variant screening could be incorporated into existing HCV screening to mitigate failed or non-responsive treatment and increase long-term economic benefits. *IFNL4* variant screening can be expanded to other viruses to further improve an infected patient's quality of life. This dual screening can have a positive impact on diagnosing and treating infected patients more importantly patients that due to economic constraints would not have access to the said screening.

It might be also worthwhile to compare study the genes differentially expressed in patients with *IFNL4* variants in NAFLD and NASH patients, and investigate whether the changes in their expression levels are associated with either steatosis or liver inflammation, or mechanistically related to insulin resistance.

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BIOGRAPHY

Kellie Perry received her Bachelor of Arts degree in Biology from the University of Louisville, KY in 1999. In 2004 she graduated from MS program in Medical Research and Technology with a concentration in Biomedical Research at The University of Maryland, MD. During this time, she also worked at Johns Hopkins Applied Physics Lab. In 2007, Kellie entered the Biosciences program in the School of Systems Biology, George Mason University, while also serving as a Senior Health Services Officer in the United States Commissioned Corps. During her time as a doctoral student at GMU, Kellie worked at several government and DoD entities, combating bioterrorism and public health threats. Kellie has also been an adjunct at Northern Virginia Community College and The University of Maryland University College. In 2010, Kellie gave birth to her son Kolton.

Recently, Kellie has been accepted in the American Society of Microbiology Science Teaching Fellowship Program. She plans on continuing her efforts in combating bioterrorism and public health threats in addition to college-level teaching.