INVESTIGATIONS INTO THE ASSOCIATION OF IL28B GENOTYPE AND THE METABOLIC PROFILE OF PATIENTS WITH AND WITHOUT CHRONIC HEPATITIS C

by

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Investigations into the Association of IL28B Genotype and the Metabolic Profile of Patients With and Without Chronic Hepatitis C

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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ABSTRACT

INVESTIGATIONS INTO THE ASSOCIATION OF IL28B GENOTYPE AND THE METABOLIC PROFILE OF PATIENTS WITH AND WITHOUT CHRONIC HEPATITIS C

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Hepatitis C Virus (HCV) is a viral infectious disease that affects an estimated 130-170 million people worldwide. Most of those infected (80%) go on to develop a chronic infection. Until recently, the standard treatment for those with chronic HCV was a combination of pegylated interferon-α (PEG-IFN-α) and the antiviral ribavirin (RBV). In treatment-naive genotype 1 HCV patients, the combination of PEG-IFN-α and RBV typically leads to Sustained Virological Response (SVR) rates between 47% and 54%. In a number of recent studies, the *IL28B* gene has been shown to play an important role in the outcome of HCV treatment. A particular single nucleotide polymorphism (SNP) on chromosome 19q13 (rs12979860), commonly referred to as the "*IL28B* variant," is strongly associated with SVR or lack of it. Metabolic syndrome (MetS) is a group of medical disorders usually associated with obesity. Some evidence suggests that HCV infection exacerbates this condition, possibly by causing increased insulin resistance (IR)

and promoting visceral obesity even further. Metabolic abnormalities have also been shown to influence patients' response to HCV treatment. Recent, but not conclusive, evidence suggests that, in addition to influencing response to HCV treatment and SVR rates, the *IL28B* genotypes may be associated with metabolic confounders of HCV such as insulin resistance and metabolic syndrome. This study investigates a possible connection between *IL28B* genotype and MetS components in patients with and without chronic HCV. As expected, correlations between *IL28B* genotype and SVR, as well as between metabolic profile and SVR, were revealed. Further studies of a connection between *IL28B* genotype and metabolic outcomes are warranted.

CHAPTER 1: INTRODUCTION

1.1 Hepatitis C

Hepatitis C Virus (HCV) is a viral infectious disease that affects an estimated 170 million people worldwide (Houghton, 2005). Most of those infected (80%) develop chronic infection known as chronic hepatitis due to HCV (CH-C) (Nelson et al., 2011). HCV is the most common cause of chronic liver disease, as it accounts for an estimated 27% of cirrhosis and 25% of primary hepatocellular carcinoma (Alter, 2007). HCV-related cirrhosis is the most common cause of liver transplantation, however, the virus usually reappears in the transplant. In fact, recent data suggests that ten percent of transplant recipients progress to cirrhosis within 3 years of transplant (Berenguer, 2002). HCV can also cause gastric varices, and, in some cases, esophageal cancer (Fraquelli et al., 2012).

In general, HCV causes inflammation of the liver that can later lead to fibrosis and ultimately, to the development of cirrhosis. Hepatitis C progresses through several stages of infection. The first stage, acute infection, causes symptoms in about 15% of cases (Maheshwari et al., 2008). Initial symptoms are generally mild, involve overall aches and malaise, and usually do not include jaundice. The virus spontaneously clears in up to 20% of acute infections, most often in patients who are females (Rao et al., 2012). On the

other hand, 80% of those infected with HCV who do not spontaneously clear the virus progress to the next stage of chronic infection. Oftentimes there are minimal or no symptoms for many years, or even decades, at the chronic stage. The most common symptom is fatigue, which can be associated with any number of illnesses, making early diagnosis more difficult.

Occasionally, infection with HCV can progress to extrahepatic diseases, including include type 2 diabetes, thrombocytopenia, Sjögren's syndrome, and B-cell lymphoproliferative disorders. Thrombocytopenia, a decrease in amount of platelets in the blood, is estimated to occur in up to 45% of those infected with chronic HCV (Louie et al., 2011). HCV infection is also associated with mixed cryoglobulinemia, which involves inflammation of small to medium sized blood vessels due to deposits of cryoglobulins, an immunoglobulin that precipitates at low temperatures and dissolve upon rewarming (Iannuzzella et al., 2010). The mechanisms that link some of these diseases with HCV remain unknown; what is clear is that HCV's effects are not limited to only the liver.

With time, HCV infection commonly results in cirrhosis. Up to 30% of patients with chronic HCV, most commonly, males, develop cirrhosis over 30 years (Rosen et al., 2011). Cirrhosis is also more common in those co-infected with Hepatitis B, HIV, or alcoholics. Liver cirrhosis is associated with a 20-fold greater risk for hepatocellular carcinoma; these risks increase to 100-fold in cases of simultaneous excessive alcohol use

(Mueller et al., 2009). Cirrhosis is commonly associated with severe symptoms of portal hypertension, abdominal accumulation of fluid (ascites), jaundice, varices, and hepatic encephalopathy (Kuske et al., 2012).

The HCV virus is a small, enveloped, single-stranded positive sense RNA virus, and is a member of the *Flaviviridae* family. There are seven major genotypes of HCV, which are indicated by the numbers 1-7. In the United States, the majority of cases, about 70%, are caused by genotype 1, followed by 20% caused by genotype 2. Genotype 1 is also the most prevalent in South America and Europe. The remaining genotypes are responsible for about 1% of U.S. cases each (Nakano et al., 2011).

In the 20th century, due to the increased use of recreational drugs and injectable medical therapies, the spread of HCV increased to an unprecedented level (Alter, 2007). HCV infection is often asymptomatic for years, or even decades, making transmission even more likely. An estimated 3-4 million people are infected with HCV every year, and there is currently no vaccine available (Soriano et al., 2007).

1.2 Therapies For Chronic Hepatitis C

Until recently, the standard treatment for those with chronic HCV was a combination of pegylated interferon-α (PEG-IFN-α) and the antiviral ribavirin (RBV). Pegylation involves the addition of a polyethylene glycol molecule, which gives the drug a longer half-life, allowing for the convenience of weekly dosing (Glue et al., 2000). Pegylation

also has the added benefit of increasing the solubility of the drug while keeping it biologically active, and in some cases helping to prevent a host immune response to the drug (Novikov et al., 2010). The initial therapy period is normally 24 to 48 weeks. Sustained Virologic Response, or SVR, is defined as the absence of detectable HCV RNA in blood serum after 24 weeks. Initial studies demonstrated that patients that received pegylated interferon alone for 48 weeks achieved SVR rates approximately double that of standard interferon, although relapse rates remained high, especially for those with genotype 1 HCV (Lindsay et al., 2001). The addition of ribavirin to the treatment substantially decreased the rate of relapses. In previously untreated patients infected with genotype 1 HCV, the combination of PEG-IFN-α and RBV typically leads to SVR rates between 47% and 54% (Manns et al., 2001).

1.3 New Treatment Modalities

The recent approval of direct-acting antivirals (DAA) is changing the way HCV is treated. Treatments modalities are moving away from poorly tolerated subcutaneous interferon treatments and towards protease inhibitors. DAAs are now commonly used in conjunction with PEG-IFN-α and RBV. This combination is currently recommended for patients infected with genotype 1 HCV. Early evidence suggests that these can lead to SVR rates of over 75% (Soriano et al., 2007).

Bocepevir and telaprevir are two DAAs already approved for HCV treatment. Boceprevir is an antiprotease that works by directly blocking HCV replication (Habersetzer et al.,

2012). Telaprevir works by inhibiting the viral NS3/4A protease, which is key in viral evasion of the host's immune system (Kong et al., 2012). Although the new treatments are very promising, anemia is still a significant obstacle, occurring in up to 50% of patients, and additional side effects, including fatigue, nausea, and depression, have also been reported (Habersetzer et al., 2012). Other downsides of multi-component treatment regimes include increased number of pills for patients to take, more opportunity for side effects, and increasing costs. These issues highlight the need to develop safer drugs with greater antiviral activity. It is expected that the first generation DAA's will soon be replaced with safer, more effective drugs.

1.4 Metabolic Syndrome

Although treatments for HCV already are promising and continue to improve, the success of treatment depends on a variety of factors. Some of these include gender, age, presence or absence of obesity, and ethnicity. Another factor that greatly influences the outcome of HCV treatment is metabolic syndrome (MetS), a group of medical conditions usually associated with an increase in visceral adiposity (Table 1, Table 2). MetS components include high BMI (body mass index), type 2 diabetes, fasting glucose levels greater than 110 mg/dL, high cholesterol, blood pressure greater than 140/90, hepatic steatosis, and elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Chronic low-grade inflammatory activity is also commonly seen in MetS patients, especially those who are obese and/or have type 2 diabetes (di Lorenzo et al., 2013). One weakness in the current definition of MetS is the lack of consideration of measures of this

inflammatory activity. The inflammatory marker C-reactive protein has been shown to be strongly associated with a number of metabolic disorders (Festa et al., 2000). Several other proinflammatory markers are known to be elevated in MetS patients, including the cytokine interleukin-6, tumor necrosis factor-α, and fibrinogen (Pickup et al., 1998). Importantly, biomarkers of proinflammatory state and oxidative stress do exist, but currently are not taken into account when MetS is diagnosed by definitions recommended by ATP III and the WHO.

MetS is considered pandemic, and roughly 25% of people in the United States are thought to be affected by it (McCullough, 2011). The clinical features of MetS all have insulin resistance as a common pathogenic mechanism. Importantly, MetS can lead to other complications including type 2 diabetes, nonalcoholic fatty liver disease (NAFLD), cardiovascular disease, and multiple types of tumors including hepatocellular carcinoma (Negro, 2012). Some evidence suggests that chronic HCV infection exacerbates this condition, possibly by causing increased insulin resistance (IR) and promoting visceral obesity (Younossi et al., 2009). These metabolic abnormalities have also been shown to influence patients' response to HCV treatment. In fact, the presence of IR or obesity reduces rates of attained SVR by over 25% (Romero-Gomez et al., 2005), while, on the other hand, success in achieving SVR reduces IR and improves β-cell function treated patients (Younossi et al., 2009). However, some other studies failed to demonstrate an association between HCV infection and MetS, concluding that these two conditions do not occur together at a higher rate than would occur by chance (Shaheen et al., 2007).

Although HCV has been linked to type 2 diabetes (Kaddai et al., 2011), the serum lipid profile of HCV patients shows decreased levels of cholesterol and triglycerides, which is the opposite to that seen in serum profiles of MetS patients (Bugianesi et al., 2012). Overall, it appears that HCV infection does interact with lipid and glucose metabolism, but it is unclear which changes occur first or the mechanism by which they occur (Negro, 2012).

NAFLD, or nonalcoholic fatty liver disease, refers to a range of disorders from hepatic steatosis to more advanced NASH (nonalcoholic steatohepatitis), which, in turn, can lead to fibrosis, cirrhosis, and liver failure. NAFLD patients typically have elevated AST and ALT concentrations, which can indicate reduced liver function (Younossi et al., 2009). Steatosis is a histological feature of NAFLD impacts outcomes of HCV treatment. It involves the abnormal accumulation of fat in cells. Steatosis is caused by, and further augments, alterations of glucose and fat metabolism, including inhibition of glucose-6-phosphatase or inhibition of fatty acid oxidation; elevated triglyceride levels in the liver (Romero-Gomez et al., 2005).

Table 1: ATP III Clinical Identification of Metabolic Syndrome
(Adult Treatment Panel III, 2002)

Risk Factor		Defining Level
Abdominal obesity (given as waist circumference)	Men	>102 cm (>40 in)
	Women	>88 cm (>35 in)
Triglycerides		≥150 mg/dL
HDL cholesterol	Men	<40 mg/dL
	Women	<50 mg/dL
Blood pressure		≥130/≥85 mm Hg
Fasting glucose		≥110 mg/dL

Table 2: WHO Clinical Criteria for Metabolic Syndrome
(World Health Organization, 1999)

Insulin resistance, identified by 1 of the following:

- Type 2 diabetes
- Impaired fasting glucose
- Impaired glucose tolerance
- Or for those with normal fasting glucose levels (<110 mg/dL), glucose uptake below the lowest quartile for background population under investigation under hyperinsulinemic, euglycemic conditions

Plus any 2 of the following:

- Antihypertensive medication and/or high blood pressure (≥140 mm Hg systolic or ≥90 mm Hg diastolic)
- Plasma triglycerides ≥150 mg/dL (≥1.7mmol/L)
- HDL cholesterol <35 mg/dL (<0.9 mmol/L) in men or <39 mg/dL (1.0 mmol/L) in women
- BMI > 30 kg/m² and/or waist/hip ratio >0.9 in men, >0.85 in women
- Urinary albumin secretion excretion rate ≥20 μg/min or albumin/creatinine ratio ≥30 mg/g

1.5 IL28B Polymorphisms

In a number of recent studies, the *IL28B* gene has been shown to play an important role in the outcome of HCV treatment. A particular single nucleotide polymorphism (SNP) on chromosome 19q13 (rs12979860) is strongly associated with SVR. This SNP is located upstream of the *IL28B* gene, which encodes interferon lambda (IFN-λ3). The CC genotype of *IL28B* is associated with a greater than two-fold higher rate of SVR as compared to the C/T or T/T genotypes (Ge et al., 2009). Numerous studies have associated the *IL28B* gene with higher rates of SVR in patients with genotype 1 HCV, and recent studies have shown a similar impact to SVR rates in patients infected with other HCV genotypes as well (Mangia et al., 2010; Indolfi et al., 2011; Lindh et al., 2011; Yu et al., 2011). Additionally, the CC genotype of *IL28B* has been linked to spontaneous resolutions of HCV infections (Thomas et al., 2009).

Asian patients have the highest C allele frequency at rs12979860, followed by European patients and African patients (Ge et al., 2009), while in African cohorts frequencies of T/T and T/C genotypes are prevalent (Melis et al., 2011). These figures indicate that high frequency of T allele in African populations provide substantial contribution to the different SVR rates observed across different population groups (Fig. 1).

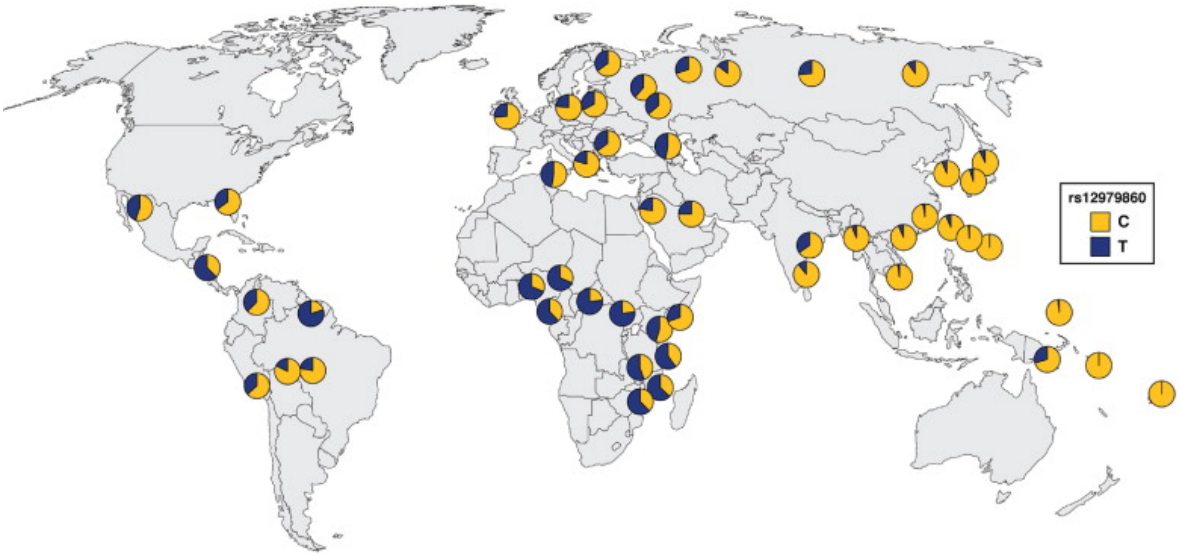

Figure 1: Allele frequencies of the SNP rs12979860 among different ethnic populations (Balagopal et al., 2010)

Although the *IL28B* gene clearly plays an important role in the pathogenesis of HCV, the underlying molecular and cellular mechanism of its action remain unknown. One hypothesis is that interferon lambda may help create a better immunologic profile that aids in viral clearance (Urban et al., 2010). Supporting this theory, some studies have shown that patients with the T allele of *IL28B* appeared to have a baseline activation of the Hepatitis C response pathway, while those with the CC genotype only had activation in this pathway after the beginning of treatment (Younossi et al., 2012). Interestingly, in normal livers of patients with CC genotype, Interferon Stimulated Genes (ISGs) are expressed at highest level, CT at intermediate and TT at the lowest. This is opposite to

the pattern seen in HCV patients. Principal component analysis of *IL28B* genotype and levels of ISG expression revealed that ISG15, HTATIP2, LGALS3BP, IRF2 and BCL2 levels correlate with the presence of C alleles, while IFNα, β, γ, λ3 and CD80 levels correlate with T alleles (Raglow Z. et al., 2013). Recently, a new dinucleotide variant ss469415590 (TT or ΔG), which is in high linkage disequilibrium with rs12979860, was identified upstream of *IL28B* (Prokunina-Olsson L et al., 2013). ss469415590[ΔG] is a frameshift variant that creates a novel gene, designated *IFNL4*, encoding the interferon-λ4 protein (IFNL4), which is moderately similar to IFNL3 (Prokunina-Olsson L, 2013). In hepatoma cells, a transient overexpression of IFNL4 induces the expression of ISGs (Prokunina-Olsson L et al., 2013), which adds yet another piece to the *IL28B* puzzle. Although *IL28B* polymorphisms have been shown to influence the effectiveness of HCV treatments and SVR rates, their effects on overall metabolic profiles are not clearly known.

Studies suggest an existence of a complex interplay between metabolic factors, *IL28B* SNP status and HCV genotypes. Infection with HCV has been indicated as a risk factor for developing both IR and metabolic syndrome (Persico et al., 2009). There is also an association between diabetes, high BMI, and liver fibrosis with steatosis (fat retention in the liver) in chronic Hepatitis C disease (Persico et al., 2009). IR has been independently associated with fibrosis and HCV genotypes 1 and 4 regardless of steatosis or serum HCV RNA levels (Moucari et al., 2008). However, another study shows that HCV is associated with IR, but the prevalence of metabolic syndrome was not, and was actually

higher in patients with non-alcoholic fatty liver disease (Lonardo et al., 2009). One study showed that IR predicts SVR response to treatment independently of *IL28B* genotype (del Campo et al., 2012). Another study, however, showed that a T allele of the *IL28B* gene is associated with IR, and may explain a poor outcome to standard PEG-IFN-α and RBV treatments (Stattermayer et al., 2012). Despite a plethora of studies on this topic, whether *IL28B* alleles affect metabolic syndrome, IR, and overall metabolic profile in addition to response to HCV treatments and SVR rates remains unclear.

Specific Aims

- The Primary Aim of this study is to assess whether there is a relationship between metabolic syndrome and the presence of the variant *IL28B* allele.
- The Secondary Aim of this study is to examine the associations between *IL28B* variant allele and the metabolic profile of patients with HCV.

CHAPTER 2: METHODS AND MATERIALS

Blood Samples

Frozen peripheral blood samples were obtained from a previously collected biobank of the Inova Fairfax Beatty Liver and Obesity Research Center. Study set included 113 HCV positive and 117 HCV negative patients with NAFLD who provided informed consent for the study. The study was approved by the Inova IRB.

DNA Extraction

Qiagen QIAamp DNA Mini Kit (Qiagen, USA) was used, as recommended by the manufacturer, to extract DNA. Manufacturer's protocol was followed with no alterations. This kit separates DNA from other macromolecules by differential solubility followed by "on column" purification and a final elution step. In the final step, Buffer "AE" was used instead of the option of distilled water to help protect the integrity of the DNA. This buffer is slightly basic to prevent acid hydrolysis, and contains EDTA, which minimizes nuclease activity.

The quality of the extracted DNA was checked on a UV-vis spectrophotometer. The protocol recommends an A260/A280 ratio between 1.7 and 1.9; the protocol also states a

DNA yield of 3-12 µg per 200µL of whole blood can be expected. Samples were stored at -80° C.

Tetraprimer PCR

A tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) was used to genotype the *IL28B* alleles. PCR was conducted according to the Galmozzi method (Galmozzi et al., 2011) (Fig. 5). This is a single-tube PCR used to evaluate the presence or absence and allelic state of rs12979860 C>T *IL28B* single nucleotide polymorphism that is located 3 kb upstream of the *IL28B* gene (Fig. 2).

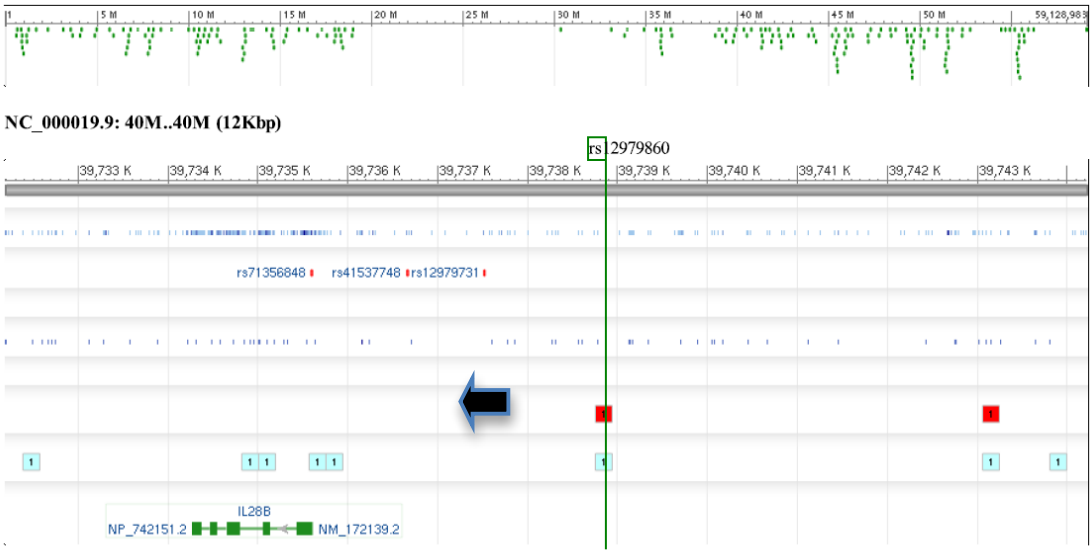

Figure 2: Location of rs12979860 SNP (NCBI, 2013)

The region containing the SNP is amplified by two outer primers that produce a control amplicon that is nonallele-specific; then two allele-specific inner primers are designed in the opposite orientation (Table 3). When used in conjunction with the outer primers, both the C and T allele amplicons can be amplified (Fig. 3). Specificity is conferred by matching the terminal 3' nucleotide with either the C or T allele. The two allele-specific amplicons have different lengths and can be separated using gel electrophoresis (Fig. 4).

Table 3: Primer sequences for T-ARMS-PCR

Direction	Primer Sequence
Outer Forward	5' AACTCAACGCCTCTTCCTCCT 3'
Outer Reverse	5' TTCCCATACACCCGTTCCTGT 3'
Inner Forward (T)	5'AGGAGCTCCCCGAAGGAGT 3'
Inner Reverse (G)	5'GTGCCATTCAACCCTGGTACG 3'

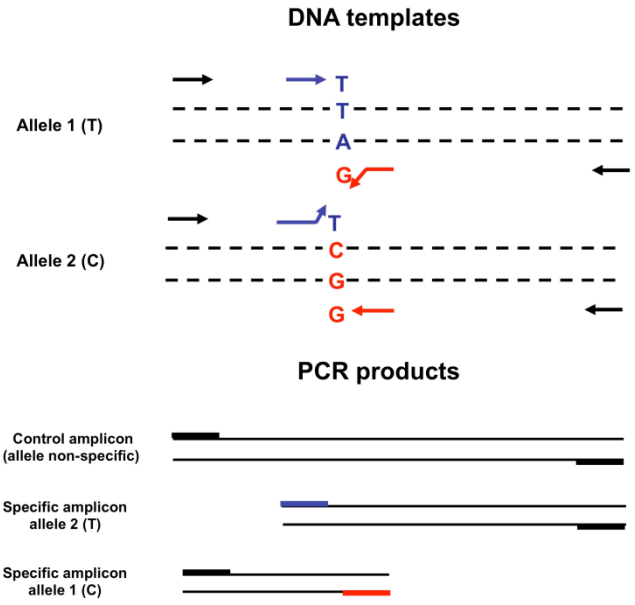

Figure 3: Scheme of T-ARMS-PCR primer amplicons (Galmozzi et al., 2011)

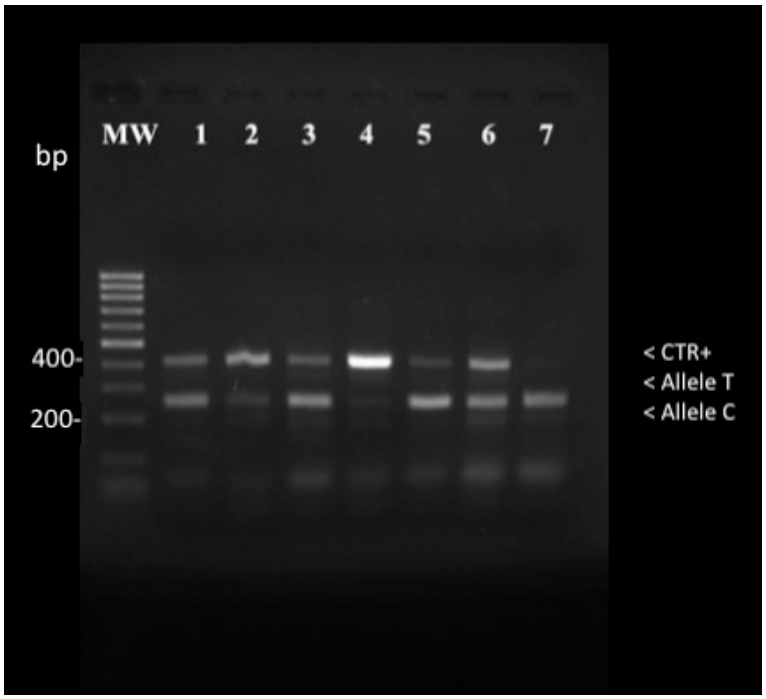

Figure 4: Typical result of *IL28B* alleles separation using gel electrophoresis

To create the tetraprimer mixture, primers were heated to 65° C for 5 minutes. 25µL of each primer was added to create a 5µM primer stock. The PCRs were performed in a volume of 20µL, containing 2µL template of 10-60µg/mL of genomic DNA extracted, 1.6µL of tetraprimer mixture, 10µL HotStarTaq Master Mix (Qiagen, USA), and 6.4µL water. PCR was performed using the BioRad C1000 Thermal Cycler (BioRad Laboratories, USA).

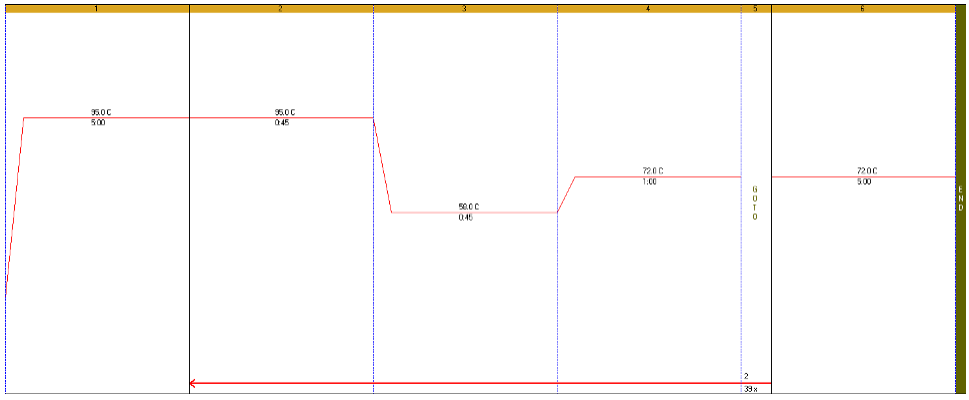

Figure 5: PCR protocol- Step 1: 5 minutes of denaturation at 95° C, Step 2: 35 cycles of 95° C for 45 seconds, Step 3: annealing at 58° C for 45 seconds, Step 4: 72° C for 1 minute, Step 5: final extension at 72° C for 5 minutes

Gel Electrophoresis

PCR products were separated using standard gel electrophoresis using a 30mL 1.7% agarose gel containing 4µL ethidium bromide. 10µL of each sample was added along

with 2μL Blue/Orange 6X Loading Dye (Promega, USA). Size discrimination was accomplished using 8μL of exACT Gene 100bp PCR DNA Ladder (Fisher Scientific, USA). The gel was run at 75 volts for approximately 90 minutes. Genotypes were then discriminated using the number and size of bands.

TaqMan SNP Genotyping Using qPCR

To confirm the accuracy of the genotyping from the tetraprimer PCR, real-time PCR (qPCR) was used with TaqMan GTXpress Master Mix (Applied Biosystems Inc., USA), used according to protocol, and custom designed TaqMan probes for the rs12979860 SNP (Invitrogen, USA). These probes are oligonucleotides with a fluorescent dye at the 5' end of the probe and a quencher at the 3' end. When the probe is intact, the quencher reduces the fluorescence emitted by the dye. When the specific target sequence (the SNP) is present in a sample, the probe anneals. During the extension phase of the PCR, the probe is cleaved by the Taq DNA polymerase. This removes the probe from the target DNA so that primer extension can continue, but also separates the dye from the quencher. This causes a fluorescent signal, which increases in intensity with each repeated PCR cycle. In order to perform the genotyping of the samples, two probes with different dyes are used, in this case VIC and FAM. One is specific for the C allele (VIC), while the other is specific for the T allele (FAM). If the assay results in only one fluorescent color, the sample is homozygous for the corresponding allele. If both fluorescent colors are present, then that sample is heterozygous. Results are shown on an amplification graph and can be easily analyzed (Fig. 6).

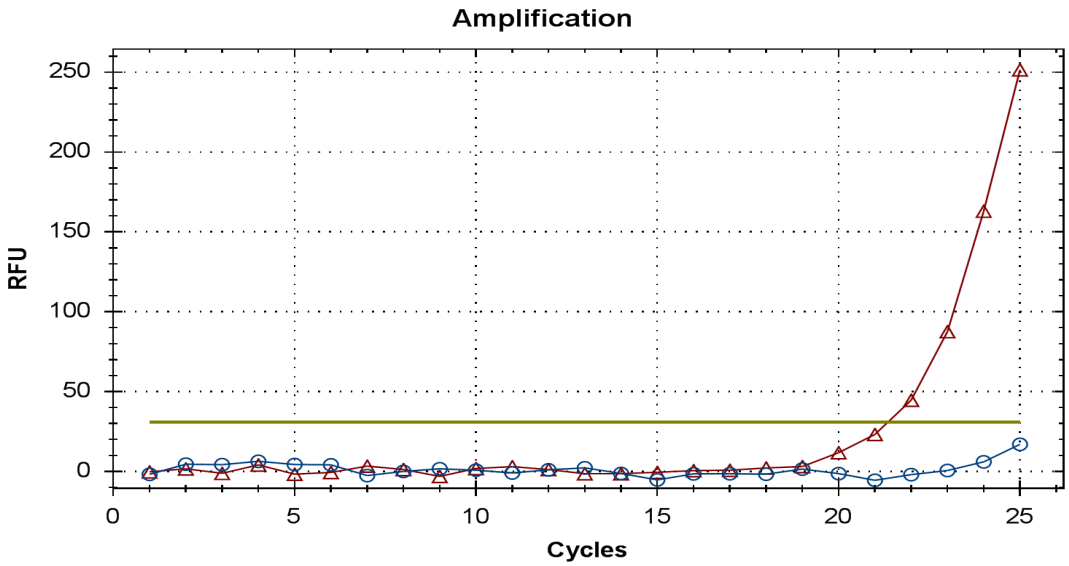

Figure 6: Sample amplification graph from SNP genotyping using real-time PCR. Only VIC fluorescence is seen indicating presence of C alleles only.

Statistical Tests

According to the result of assessment of the qPCR results, HCV infected and non-HCV infected NAFLD patients were divided into CC and non-CC genotype groups. Descriptive, univariate, and multivariate statistical tests were performed. Spearman correlation analysis was performed. A Spearman correlation measures statistical dependence between two variables. A correlation coefficient is calculated that falls between -1 and +1, where -1 and +1 indicate the two variables are perfectly correlated and dependent upon each other. P-values were calculated for significant r-values.

CHAPTER 3: RESULTS

DNA Extraction

Qiagen QIAamp DNA Mini Kit (Qiagen, USA) was used as recommended by the manufacturer to obtain DNA from the samples. The quality of the extracted DNA was then evaluated using a UV-vis spectrophotometer. The protocol recommends an A260/A280 ratio between 1.7 and 1.9; the majority of samples fell within these parameters (Table 4). The protocol also states a DNA yield of 3-12 µg per 200µL of whole blood can be expected. The average total yield of the DNA extracted from each sample was 5.55 µg.

Table 4: Spectrophotometerical quantitation of DNA extracted from blood samples

Number	Sample	260/280 ratio	Concentration (µg/mL)	Total Yield (µg)
1	TR1-001-0202	1.815	36.7	7.34
2	TR1-001-0294-0	1.784	49.5	9.90
3	TR1-001-0301-0	1.792	32.3	6.46
4	TR1-001-0300-0	1.762	27.8	5.56
5	TR1-001-0302-E	1.800	27.0	5.40
6	TR1-001-0299-0	1.857	39.0	7.80
7	TR1-001-0307-0	1.840	34.5	6.90
8	TR1-001-341	1.857	19.5	3.90
9	TR1-001-0308-0	1.852	18.8	3.76
10	TR1-001-0346	1.849	50.6	10.12

11	TR1-001-0347	1.827	27.8	5.56
12	TR1-001-0345	1.870	24.2	4.84
13	015-01-0151	1.839	30.0	6.00
14	16-01-0105	1.954	23.8	4.76
15	015-01-0129	1.826	15.8	3.16
16	016-01-0136	1.871	10.9	2.18
17	015-01-0128	1.962	19.5	3.90
18	16-01-1006	1.881	14.8	2.96
19	016-01-1007	1.857	31.7	6.34
20	015-01-0130	1.864	15.4	3.08
21	016-01-0152	1.875	16.9	3.38
22	16-01-1005	1.864	28.3	5.66
23	016-01-0145	1.887	21.9	4.38
24	015-01-0117	1.800	6.7	1.34
25	015-01-0131	1.876	48.2	9.64
26	TR1-001-0214E	1.840	17.3	3.46
27	003-01-0017	1.860	32.4	6.48
28	TR1-001-0224E	1.857	24.4	4.88
29	TR1-001-342	1.857	17.1	3.42
30	003-01-0023	1.806	12.2	2.44
31	TR1-001-0334E	1.885	46.3	9.26
32	TR1-001-376-0	1.878	26.1	5.22
33	TR1-001-0172E	1.899	35.2	7.04
34	TR1-001-0330-0	1.857	41.4	8.28
35	003-01-0034	1.769	38.8	7.76
36	TR1-001-0257-E	1.733	29.2	5.84
37	003-01-0041	1.750	36.8	7.36
38	TR1-001-0332-0	1.742	21.6	4.32
39	003-01-0005	1.831	26.4	5.28
40	TR1-001-0323-0	1.887	25.1	5.02
41	TR1-001-0256-E	1.794	21.2	4.24
42	TR1-001-0322-0	1.855	43.1	8.62

Gel Electrophoresis

The T-ARMS-PCR used for genotyping *IL28B* alleles showed that 54 (31.8%) samples overall had CC genotype, while the remaining 116 (68.2%) were having CT or TT (referred to as non-CC) genotypes. Among HCV patients, 40.2% were CC genotype, while 59.8% were Non-CC genotypes. Among NAFLD patients, 19.3% were CC genotype, while 80.7% were Non-CC genotypes. This is much lower than the expected frequency of the CC genotype in the general US population, which is roughly 43% (NCBI, 2013). This led to the conclusion that there could have been contamination of the DNA samples during PCR, or human errors when reading unclear gels. To exclude possible contamination, DNA was re-extracted from the blood samples and genotyping was confirmed using qPCR SNP genotyping.

qPCR SNP Genotyping

The TaqMan based SNP genotyping, which is more straightforward and more accurate than the T-ARMS-PCR technique, showed that 88 (38.3%) samples overall were CC genotype, while the remaining 142 (61.7%) were CT or TT (referred to as non-CC genotype). According to TaqMan genotyping, among HCV patients, 50.6% were CC genotype, while 49.4% were Non-CC genotypes. Among NAFLD patients, 48.3% were CC genotype, while 51.7% were Non-CC genotypes. These percentages are in much better agreement with the expected frequency of CC genotype in human populations. This experimental data was compiled with database-extracted data including assessment for metabolic syndrome components as well as demographic factors.

Analysis of association between IL28B genotype and various clinical parameters

Descriptive, univariate, and multivariate statistical tests were performed. Baseline percentages for metabolic and demographic characteristics were calculated for the entire cohort of patients, and also broken down into CC allele patients and non-CC allele patients (Table 5). Average values and standard deviations were calculated for characteristics with continuous data.

Table 5: Metabolic and demographic characteristics of full cohort of patients. Percentages for presence/absence of metabolic and demographic characteristics were calculated, and average values and standard deviations were calculated for characteristics with continuous data (grey).

	Full Cohort	CC Group	Non-CC Group
N (Number of patients)	230	89 (38.7%)	141 (61.3%)
HCV	113 (49.1%)	45 (50.6%)	68 (48.2%)
NAFLD	117 (50.9%)	43 (48.3%)	74 (52.5%)
Female	109 (47.4%)	38 (42.7%)	71 (50.4%)
Type 2 Diabetes	53 (23.0%)	18 (20.2%)	35 (24.8%)
Race, African American	87 (37.8%)	35 (39.3%)	52 (36.9%)
Hyperlipidemia	56 (24.3%)	22 (24.7%)	34 (24.1%)
Age, years	45.9 ±9.7	47.9 ± 9.1	44.7 ± 9.9
ALT (alanine aminotransferase- IU/L)	60.2 ± 54.1	62.0 ± 48.1	59.1 ± 57.7
AST (aspartate aminotransferase- IU/L)	47.3 ± 44.4	46.3 ± 39.0	48.0 ± 47.6
BMI (body mass index- kg/m²)	37.9± 12.1	37.6 ± 11.4	38.1 ± 12.6

A Pearson's chi-squared test was calculated for an association between CC genotype and NAFLD. The frequency of the T allele is 0.339 (38); using the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$) the estimated frequency of a CC genotype was calculated to be 0.437. Using the standard formula $\chi^2 = (\text{Observed-Expected})^2/\text{Expected}$ to obtain a χ^2 value, and using the observed frequency of 48.3% of NAFLD patients having the CC genotype, χ^2 was calculated to be 0.005. Using a standard chi-squared probability table and one degree of freedom, a critical value of 3.84 for $p=0.05$ was found. Since the χ^2 value is far less than 3.84, it showed these results do not depart from expectation. Therefore, presence of CC genotype or T allele in *IL28B* gene is not associated with presence of NAFLD.

The chi-squared test was repeated for the HCV group using .437 as the expected value and the observed frequency of 50.6%; χ^2 was calculated to be 0.011. Again, this is far less than 3.84, so results do not depart from expectation. Therefore, presence of CC genotype or T allele in *IL28B* gene is not associated with presence of HCV infection *per se*.

Spearman correlations were first calculated for the entire cohort of patients connecting the presence of CC genotype and age, albumin levels, ALT and AST levels, BMI, glucose, total bilirubin, and presence or absence of type 2 diabetes, female gender, hyperlipidemia, African American race, and steatosis (Table 6). P-values were not significant for any factor except African American race. However, even with a large sample size, a p-value less than 0.05 may not be a reliable indicator of significance with a

low Spearman correlation coefficient. Therefore, we conclude that our results showed little to no correlation between the metabolic and demographic factors and CC genotype when all samples were considered.

Table 6: Spearman correlation between CC genotype and investigated factors in all patients. All P-values are non-significant (P>0.05) except for African American Race.

Clinical or Demographic Parameter	Spearman's Rank Correlation Coefficient
Age, years	0.1789
Albumin, g/dL	0.1378
ALT, IU/L	0.1066
AST, IU/L	0.0214
BMI	-0.0104
Type 2 Diabetes	-0.0106
Female Gender	-0.0701
Glucose, mg/dL	-0.1251
Hyperlipidemia	-0.0067
African American Race	-0.2452
Steatosis	-0.0214
Total Bilirubin, mg/dL	-0.0645

In the next round of analysis, samples were separated into HCV and non-HCV/NAFLD cohorts, studies of correlation were performed for each of the groups separately. Spearman correlations and p-values were calculated.

The null hypothesis, that the two factors in any given comparison were independent of each other, would be rejected if $p<0.05$. The HCV samples showed significant correlations between the CC genotype and several metabolic factors, including SVR rates, hypertension, BUN, and creatinine (Table 7). Results showed that, as expected, the CC-group patients had significantly higher rates of SVR, but this did not correspond with the other factors examined. The presence of CC alleles positively correlated with hypertension, BUN, and creatinine. In Table 7, statistically significant p-values are highlighted in yellow. In non-CC patients, achievement of SVR negatively correlated with elevated AST, ALT, glucose, BUN, direct bilirubin, and steatosis.

Table 7: Spearman correlation between CC genotype and investigated factors in HCV patients. NS = non-significant factor (P>0.05).

Clinical or Demographic Parameter	Spearman's Rank Correlation Coefficient	P-Value
Age, years	0.0540	NS
SVR	0.4263	2.778E-06
ALT, IU/L	0.0483	NS
AST, IU/L	-0.0341	NS
BMI	-0.0014	NS
BP Diastolic, mmHg	0.1162	NS
BP Systolic, mmHg	0.0791	NS
BUN, mg/dL	0.4296	0.0200
Creatinine, mg/dL	0.3667	0.0463
Diabetes (type 2)	-0.1437	NS
Glucose, mg/dL	-0.1257	NS
Hypertension	0.2428	0.0197
Hyperlipidemia	-0.0015	NS
Steatosis	-0.0201	NS

When Spearman correlations and p-values were calculated for the NAFLD samples, there were no correlations between the presence of the T allele alleles and any metabolic factor. None of the p-values were below the 0.05 cutoff (Table 8).

Table 8: Spearman correlation between CC genotype and investigated factors in NAFLD patients. All P-values are non-significant (P>0.05).

Clinical or Demographic Parameter	Spearman's Rank Correlation Coefficient
Age, years	0.2943
ALT, IU/L	0.1751
AST, IU/L	0.0665
Cirrhosis	-0.1009
Diabetes	0.1042
HDL, mg/dL	-0.0510
LDL, mg/dL	-0.1386
Total Cholesterol, mg/dL	-0.0708
Hyperlipidemia	0.0013
Hypertension	0.0314
Triglycerides, mg/dL	-0.0647

After separating the NAFLD patients into a CC-group and a Non-CC-group, average values for each metabolic factor were calculated (Table 9). 95% confidence intervals were calculated. The data shows no statistically significant differences between the CC and Non-CC groups that fall within a 95% confidence interval (Fig. 7).

Table 9: Average values for each investigated metabolic factor in NAFLD patients with 95% confidence interval

Clinical or Demographic Parameter	CC Group	Non-CC Group
N	43	74
Age, years	46.4±10.3	41.5±10.7
ALT, IU/L	42.3±29.2	35.5±28.8
AST, IU/L	30.8±25.6	27.7±22.9
BMI	46.0±9.1	46.6±11.1
HDL, mg/dL	43.7±11.5	44.1±13.7
Total Cholesterol, mg/dL	172.1±38.9	191.8±56.7
Triglycerides, mg/dL	135.0±84.6	150.3±82.8

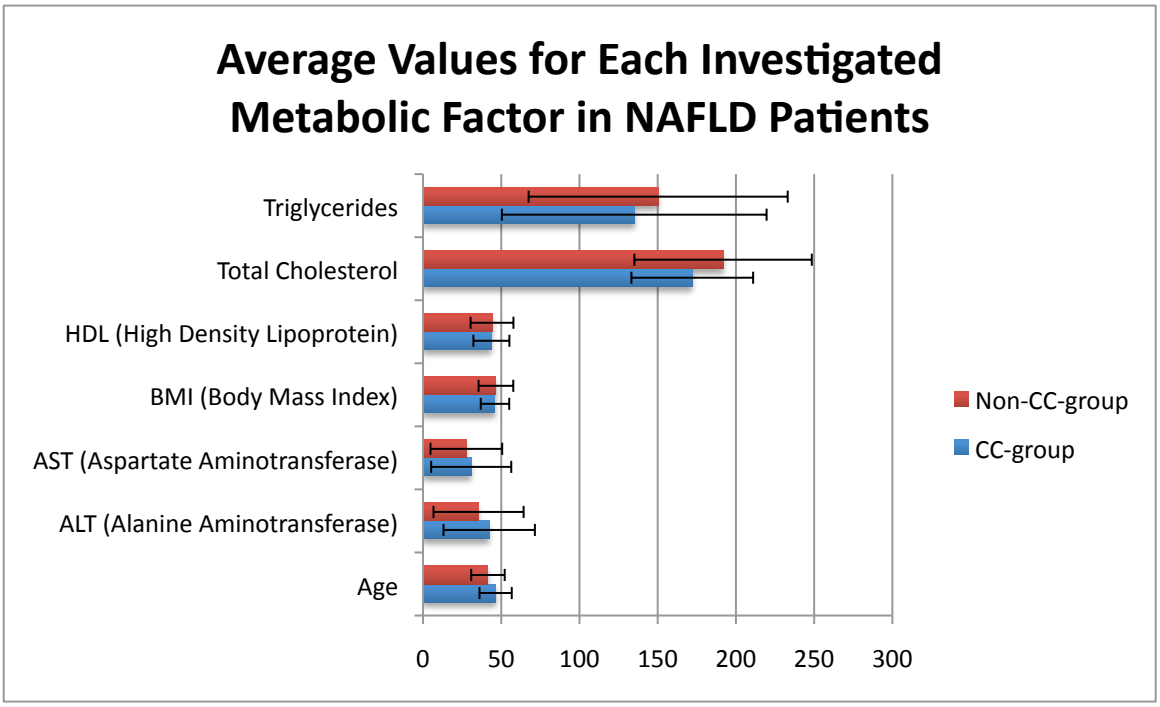

Figure 7: Comparison of average values and standard deviations for investigated metabolic factor in NAFLD patients with

CHAPTER 4: DISCUSSION

Hepatitis C Virus (HCV) is a viral infectious disease that affects an estimated 130-170 million people worldwide. Most of those infected (80%) do not spontaneously clear the virus and go on to develop a chronic infection. In a number of recent studies, the *IL28B* gene has been shown to play an important role in the outcome of HCV treatment. A particular single nucleotide polymorphism (SNP) on chromosome 19q13 (rs12979860), commonly referred to as "*IL28B*" is strongly associated with SVR.

Metabolic syndrome (MetS) is a group of medical disorders usually associated with visceral fat. It is not known whether or not *IL28B* genotypes affect overall patient outcomes, including metabolic syndrome, insulin resistance, and overall metabolic profile in addition to response to HCV treatments and SVR rates. It is also not known whether or not *IL28B* genotype affects metabolic profiles of patients without HCV but having other liver diseases, such as NAFLD.

In the course of this study, 113 HCV positive and 117 HCV negative patients were genotyped for *IL28B* allele by tetraprimer-PCR. These patients were chosen for the purpose of this study due to availability of well-documented, extensive medical history. *IL28B* genotype frequencies were defined experimentally. After genotyping was

complete, 89 samples overall were determined to be CC genotype, while the remaining 141 were CT or TT (referred to as non-CC genotype). Genotypes were correlated to clinical and demographic parameters, such as BMI, presence of type 2 diabetes, and hypertension. Spearman correlation coefficients were calculated and p-values were assessed. This data was compiled with assessment for metabolic syndrome components as well as demographic factors. Results showed little to no correlation between any of the metabolic or demographic factors and CC genotype when all samples were considered. Presence of CC genotype or T allele in *IL28B* gene was not associated with presence of NAFLD or HCV infection *per se*.

When samples were separated into HCV and non-HCV/NAFLD cohorts, the HCV samples showed significant correlation between the presence of CC genotype and several metabolic factors, including SVR rates, hypertension, blood urea nitrogen (BUN), and creatinine (p-values within the 95% confidence interval). Results showed that, as expected, the CC-group patients had significantly higher rates of SVR, but this did not associate with the any other metabolic factors examined. CC alleles positively correlated with hypertension, BUN, and creatinine, possibly indicating reduced liver and renal function in CC carriers. In the non-CC group, achievement of SVR negatively correlated with elevated AST, ALT, glucose, BUN, direct bilirubin, and steatosis. There might be a connection between the degree of liver and, possibly, renal, function and the achievement of SVR, as well as a potential association between *IL28B* alleles and metabolic parameters. All together, our data indicate that when T allele is present, a patient's

metabolic profile is important in determining whether or not they will be able to achieve SVR.

Importantly, an analysis of genotype distribution within the NAFLD cohort failed to reveal any correlation between CC allele and any metabolic factor analyzed. This is surprising given the number of samples and number of factors assessed. When average values for each metabolic factor assessed were calculated for NAFLD patients, again there was no significant difference between CC-group and Non-CC-group. This reinforces the finding that in non-HCV patients, there is no correlation between *IL28B* alleles and components of MetS.

This study has several inherent limitations that should be taken into consideration when interpreting results. The sample size of patients is relatively small given the number of factors being considered. This can result in a type II error during statistical analysis, where the null hypothesis is false but fails to be rejected. The patient population is also biased in that all non-HCV patients are NAFLD patients. An additional weakness of this study is that it consisted of analyzing data and looking for correlations, rather than experimentation on the effects of *IL28B* alleles.

Future directions of this study should include larger groups of non-HCV patients, and possibly non-NAFLD patients as well. A comparison of CC groups versus non-CC groups in healthy population-based cohort with no liver disease would be interesting.

Examining effects of *IL28B* alleles during the course of treatment for MetS components could also provide valuable insight. More studies are warranted to determine the effects of *IL28B* genotypes on treatment outcomes in liver disease patients.

CHAPTER 5: CONCLUSION

In HCV patients, there is a significant correlation between the presence of CC genotype and several metabolic factors. In non-HCV patients with NAFLD, no such correlation was found. The metabolic profiles of the CC and Non-CC groups of NAFLD patients were also not different from each other. The beneficial effects of CC genotype on metabolic profiles may only be conferred after incidental infection with HCV, or after the treatment with antiviral therapy. *IL28B* genotyping in larger groups of non-HCV patients is warranted to determine the true effect of *IL28B* genotypes on metabolic outcomes.

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