Discussion

Chronic HCV infection involves in a complex interaction of virus with host innate and adaptive immunity. It has been observed that the acute hepatitis is less conciliable to therapy. Approximately 80% of HCV infections progress to the chronic stage. Several host factors including gender, co-infection with the human immunodeficiency virus or the hepatitis B virus and age at the time of infection are known to affect disease courses in HCV infection (Thomas et al. 2000; “NIH Consensus Statement on Management of Hepatitis C: 2002” 2002; Shepard, Finelli, and Alter 2005; Bialek and Terrault 2006). Research studies have shown evidences that support contention of involvement of host genetics in immune responses, and may predict treatment response (Rehermann and Nascimbeni 2005b; Lloyd et al. 2007; Szabo and Dolganiuc 2008). HCV has developed a myriad of mechanism by which it can evade the host immune surveillance. These mechanisms include triggering production of viral inhibitory proteins and negative signal regulatory proteins that impede signal transduction pathways of human innate immune system. In addition, Hepatitis C Virus employs multiple evasive strategies including high viral turnover, baseline viral load and viral genetic variability to protect HCV from host humoral and cellular immunity. This study attempts to co-relate the C3 levels and it's SNP that influences its expression in chronic hepatitis C infection and also its relation to treatment. In this study we have observed that in chronic hepatitis C patients there is a significant reduction in the complement C3 levels and that this reduction may be a manifestation of polymorphism that have previously been reported in other pathogenic conditions. This data is in accordance with Majumdar et al (2012) who reported that
there was lowering of C3 levels in chronic hepatitis C infected patients (B. Mazumdar et al. 2012). The study confirms to the contention that complement system component C3 is involved in chronic hepatitis C infection substantiated in sample size larger compared to Majumadar et al. However, this study deviates from a report by Dumestre-Perard et al (2002) who were not able to find any significant difference in the level of C3 component within the chronic hepatitis C patients (Dumestre-Perard et al. 2002b). The study is also in corroboration with study by Teisberg and Gjone (1975) where they had reported reduced C3 and C4 levels in chronic active hepatitis patients (Teisberg and Gjone 1975). This study may provide an insight into C3 role as an indicator of immunological activity.

The study also indicates that the alteration of C3 levels and response to treatment may be dictated by rs2230201 polymorphism. This polymorphism has been reported to be associated with susceptibility to diseases like SLE by Miyagawa et al. (2008). In their study they reported that the C3 SNP rs2230201 'C' allele and rs7951 'T' allele were had significant association with Systemic Lupus Erythematosus. Their study also reports that the mean serum C3 level of carriers of the rs7951 'C' allele was significantly lower than that of non-carriers of the 'C' allele in SLE patients (Miyagawa et al. 2008). However, another study by Yang et al. were not able to report any relation between the C3 levels and rs2230201 polymorphism. This study however indicates that in healthy controls 'C' allele of rs2230201 was associated with significant increase of C3 levels. This study also reports that healthy controls carrying single copy 'C' allele of the rs2230201 had significantly higher levels of serum C3 than healthy controls with no copy of 'C' allele of rs2230201. Our study also revealed that C3 mRNA levels of the CHC group was significantly lower than the healthy and is in agreement with the
study by Mazumdar et al. However, the reduction reported in C3 mRNA expression by Mazumdar et al., (2012), was 4 fold whereas our study revealed that the reduction was 1.55 folds. The difference may be because of the nature of the tissue used which was Liver biopsy tissues in their case and whole blood in this study (B. Mazumdar et al. 2012). Even though the levels of C3 mRNA differed in cases versus controls, none of the polymorphism was found to be associated with the change. This may be because both rs7951 and rs2230201 polymorphism are not in the promoter region and so may not affect mRNA expression directly. The role of rs2230201 and rs7951 effect on transcription and translation, was predicted using online prediction tools, which include using TFSearch (Heinemeyer et al. 1998) for transcriptional regulation, Ensembl-NS (Ng and Henikoff 2006) for protein coding alteration, Human Splice Finder (Desmet et al. 2009) for splicing regulation and sulfinator (Monigatti et al. 2002) for post-translational modification. It was predicted that both rs7951 and rs2230201 had splicing regulatory effect and that they could not regulate protein coding or post-translational modification. Rs2230201 polymorphism is included in a region of exonic splice enhancer (ESE). When this polymorphism was fed in predictor software Human Splice Finder it was observed that ‘C’ allele resulted in breaking of ESE sequence whereas ‘T’ allele incorporation showed formation of new ESE site. But in this study there was no relationship between the rs2230201 genotypes and the C3 mRNA levels which may be because the primer used to amplify C3 mRNA amplifies the region which does not include rs2230201 and so any transcript variant, if produced, by rs2230201 will not alter real time amplification results. Though above mentioned effect was tested on prediction software, the effect of the polymorphism in this context needs to be further evaluated.
In this study a new scoring model was developed to predict the treatment response using binary logistic regression. From the logistic regression it was found that response to Interferon treatment could be predicted from C3 level, Age and rs2230201 of the patient with a diagnostic accuracy of 67.06%. It was found that increased level of C3 levels, low age and rs2230201 CC genotype was associated with positive treatment response. In this study we also compared new scoring system with baseline RNA levels to find out if C3 level is a better predictor of treatment response by utilizing Receiver Operating Characteristics (ROC) curve. Our study revealed that the AUC for new scoring system and baseline RNA were significantly different and that new scoring system occupied more area suggesting its incorporation as a treatment response factor.

The role of rs2230201 polymorphism was also evaluated to assess its role in progression towards fibrosis. A multivariate logistic regression test revealed that Age and non-TT genotype of rs2230201 were at a risk factor for progression towards increased fibrosis. It was found by logistic regression that every unit increase in age would increase the risk of fibrosis by 1.1. There have been many reports which have shown age as a risk factor for fibrosis and have included it as a parameter in their prediction model. In this C3 levels did not correlate with fibrosis and is in concordance with study from Mazumdar et al (2012). The correlation between complement component C3 and fibrosis is sketchy and is full of contradictions (B. Mazumdar et al. 2012). A study by Ali et al.,(2005) found that C3 levels in HCC cases were higher when compared to healthy controls whereas it was lower in liver cirrhosis when compared to HCC cirrhotic patients (Ali, Abo-Shadi, and Hammad 2005). However, another study by Gangadharan et al.,(2011) suggested that there might be increase in fragments of C3 such as C3dg in cirrhosis(Gangadharan et al. 2011). The reason as to
why there was no correlation between fibrosis and C3 levels in this study may be ascribed to the fact that in this study established HCC and decompensated cirrhosis (F>5) cases were not included whereas the above mentioned studies included cases with cirrhosis and HCC.

This study was not able to find any correlation between the alleles of rs7951 and serum C3 levels. However this study reported that chronic hepatitis C patients carrying two copies of the rs2230201 ‘C’ allele had higher levels of serum C3 compared to those carrying CT genotype. This was also observed in case of healthy controls where ‘CC’ genotype carriers had significant higher C3 level than those with ‘CT’ and ‘TT’ genotype. The vast difference between the levels of C3 between CT genotype and non-CT genotype of rs2230201 between the chronic hepatitis C may be because of hepatitis C virus core protein capability in inhibiting C3 promoter expression as suggested by Mazumdar et al (B. Mazumdar et al. 2012). However the added effect of increased C3 levels by the ‘C’ allele cannot be undermined. The above study suggests, as for C3 deficiency, decrease in levels of serum C3 may be associated with HCV persistence and numerous, to an extent confounding, factors may be involved in alteration of C3 concentrations. This condition that’s why becomes very important specifically in case of patients receiving treatment. Our study indicates that the ‘C’ allele may have a protective role which is substantially effective only in homozygous condition. We have observed that in patients receiving treatment ‘CC’ genotype can be a positive predicting factor for the outcome of Interferon and Ribavirin double therapy.

The fourth component of the complement system, C4, plays a critical role in mounting an efficient and proper immune response against infection (Y. Yang et al. 2003). C4 is a polymorphic serum protein consisting of two isoforms, C4A and C4B.
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These isoforms are encoded by two separate genes that are located 10 kb apart within the central portion of the major histocompatibility complex (MHC) on chromosome 6p (Carroll et al. 1984). The promoter regions of the C4 gene are conserved in C4A and C4B (Ulgiati and Abraham 1996). As with other components of the complement system, C4 is expressed primarily in the liver and in macrophages. Its expression is induced in response to acute inflammation or tissue injury. This study attempts to correlate the total C4 levels and its SNP that influences its expression in chronic hepatitis C infection and also its relation to treatment. This data is in corroboration with Banerjee et al., (2011) who found that the C4 levels were significantly lowered in chronic hepatitis C infected patients (Banerjee et al. 2011b). This study is also in accordance with study by Teisberg and Gjone where they found that chronic active hepatitis patients have reduced C3 and C4 levels and study by Dumestre-Perard et al., (2002) who also showed that there was decrease in complement C4 levels in chronic hepatitis C patients when compared to normal healthy controls, however they were not able to find decrease in C3 and C1s-Cinh level (Teisberg and Gjone 1975; Dumestre-Perard et al. 2002b). The above finding led Dumestre-Perard et al., (2002) to suggest that maybe complement system is activated in case of hepatitis C without the involvement of classical pathway. To further investigate this reduction in C4 levels, mRNA expression levels of C4 were investigated in this study. It was found that there was significant difference between C4 mRNA levels between healthy and CHC group. There was 7.51 fold decreases in the C4 mRNA expression levels as compared to healthy. The data was in corroboration with Banerjee et al., (2011) where they had difference in C4 mRNA expression levels between genotype 1a patients and subjects with unrelated liver disease (Banerjee et al. 2011b). Since in this study observation was
made regarding the regulation of C4 at the mRNA level we went on finding potential polymorphism that could affect C4 expression. The SNP rs2857009, located in the 3' end of C4 gene, was selected because a study by Yang et al., (2012) had found that this polymorphism could confer independent effect on affecting the concentration of C4 (X. Yang et al. 2012). We predicted rs2857009 activity on transcription regulation by utilizing prediction software TFSearch which revealed that this polymorphism results in changes in transcription factor recognizing sequence. It was found in this study that CC genotype was associated with decrease in C4 mRNA expression by \( \sim 3.84 \) folds when compared to GC and GG genotypes in healthy cases. In disease group also the CC genotype was associated with decrease C4 mRNA expression; however this time the fold change between CC and GG genotype was \( \sim 1.4 \) fold. This change in mRNA expression between healthy and disease having same genotype of rs2857009 may point to other factors that may be regulating the expression of C4 mRNA during HCV infection. One of the factors may be the HCV NS5A induced reduction in C4 promoter activity as indicated by Banerjee et al., (Banerjee et al. 2011b). The reduction in C4 promoter activity was found to be dose dependent thereby implicating that HCV viral load may affect mRNA expression levels in chronic hepatitis C group. It was found that patients carrying CC genotype were at 2.5 times increased risk of hepatitis C infection when compared to non CC genotype carriers. It was also found that rs2857009 also played an important role in treatment outcome as the CC genotype carriers were at \( \sim 55 \) times of increased risk of not responding to treatment than the non-CC genotype carriers. This indicates that may be rs2857009, which in turn dictates C4 levels, may play an important part in limiting infection. This study showed that there was significant difference in the baseline C4 protein as well as mRNA expression level and
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that CC genotype was associated with lower levels of expression of mRNA as well as C4 protein. Since the study by Dumestre-Perard et al., (2002) showed that the specific activity of C4 was reduced in relapsers compared with sustained responder, it was suggested that C4 can be a potential predictor of treatment response (Dumestre-Perard et al. 2002b).

In view of the studies mentioned above a new scoring model was developed to predict the treatment response using binary logistic regression. From the logistic regression it was found that response to Interferon treatment could be predicted from baseline C4 level of the patient with a diagnostic accuracy of 85.71%. By logistic regression it was found that patients with increased level of baseline C4 would have better probability to respond to treatment. In this study we also compared new scoring system with baseline RNA levels to find out if C4 level is a better predictor of treatment response by utilizing Receiver Operating Characteristics (ROC) curve. Our study revealed that the AUC for new scoring system and baseline RNA were significantly different statistically and that new scoring system occupied more area suggesting its incorporation as a treatment response factor. One important observation was that even though the CC genotype was higher in disease and non responder it was excluded in the logistic regression mode may point to the fact that other confounding factors might be in play.

One of the factors might be because of an interesting revelation by Mawatari et al., (2013) where they have shown that HCV NS3/4A protease cleaves C4γ and leads to inhibition of classical complement pathway which is dose dependent manner (Mawatari et al. 2013a) as well as the study by Banerjee et al., (Banerjee et al. 2011a) indicating involvement of factors other than the polymorphism in play.
The role of rs2857009 polymorphism was also evaluated to assess its role in progression towards fibrosis. A multinominal logistic regression test revealed that Age, non-GG genotype of rs2857009 and HCV non genotype 1 were independent predictors of progression towards increased fibrosis (Table). It was found by logistic regression that every unit increase in age would increase the risk of progressing towards high fibrosis (F≥3) by 1.12 times. This is in accordance with studies where it has been found that increased age was a risk factor for progression towards fibrosis. These prediction models named FibroTest (Imbert-Bismut et al. 2001), Hepascore (Adams et al. 2005), also included age to calculate a predictive score for predicting fibrosis. From regression it was found that the rs2857009 CC and GC genotype were at increased risk of progressing towards high fibrosis (F≥3) compared to those with GG genotype. From the logistic regression model the levels of baseline C4 did not present as a risk factor for fibrosis and this data was in concurrence to the report from (Buğdaci et al. 2012). Banerjee et al., (2011) reported that changes in C4 levels did not correlate with the liver fibrosis ad was in agreement with reports from this study (Banerjee et al. 2011b). In agreement with our results, Dumestre-Perard et al reported that there were no statistical significant correlations between specific C4 activity and each of HCV-RNA, ALT, Metavir histological fibrosis and Metavir histological activity (P = 0.29, 0.9, 0.96 and 0.22 respectively), however in our case Ishak scoring system was used instead of Metavir scoring system (Dumestre-Perard et al. 2002b). However the study by Gangadharan et al., (2011) reported that there was decrease in C3 and fragments for C4 but did not mention if the level of C4 was altered. Another important factor that can be a risk factor is the HCV genotype (Gangadharan et al. 2011). This study revealed that patients carrying HCV genotype 1 were at reduced risk of progressing to high fibrosis.
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(F$\geq$ 3) when compared to genotype 3 and 4. There has been evidence in which it has been observed that HCV genotype 3 patients are associated with accelerated fibrosis (Bochud et al. 2009) which further supports the contention that genotype 1 have a protective role in progression to high fibrosis group. A study by Probst et al.,(2011) also found that the odds ratio for the association of genotype 3 with accelerated fibrosis = 0.007) in single-biopsy studies progression was 1.52 (95% CI 1.12-2.07, P= 0.007) in single-biopsy studies (Probst et al. 2011). However in the study by Poynard et al (Poynard, Bedossa, and Opolon 1997; Poynard et al. 2001) showed that than virological factors in HCV infection was not associated with fibrosis progression. This discrepancy may be because of the nature of subjects included. Their study had included cases who consumed alcohol whereas in this study none of the cases had history of alcohol consumption.

CFH is a member of the regulator of complement activation (RCA) gene cluster on chromosome 1q32 (Rodríguez de Córdoba et al. 2004). The first exon encodes the 5' untranslated region of the mRNA and the N-terminal 18 amino acids that organize the signal peptide. Each SCR in factor H is encoded by a single exon except for SCR2, that is encoded by exons 3 and 4. Exon 10 does not contribute to the factor H transcript. It is used exclusively in the alternative transcript that codes for the FHL-1 molecule. Exon 10 encodes the last four amino acids (Ser-Phe-Leu-Thr) and the 3' untranslated region of FHL-1. From this study it was found that levels of serum CFH levels were higher in healthy controls than chronic hepatitis group. This study is in concurrence with a study by Quin et al., where they found that the CFH levels were significantly decreased in patients versus in controls (S. Qin et al. 2012). It is established that CFH levels controls complement destruction of host cell by blocking binding of Bb to C3b and also by
assisting factor I in cleaving C3b into inert factors C3c and C3d. The reduction in CFH may be because of regulatory factors like micro RNA and IRF. A study by Zhang et al., (2013) found that HCV differentially modulates miRNAs to facilitate entry and early establishment of infection in vitro (X. Zhang et al. 2013). Another study by Amadi-Obi et al., (2012) revealed a novel role of IL-27 in regulating complement activation through up-regulation of CFH and suggest that defects in IL-27 signaling or expression may contribute to the reduction of CFH expression in the retina of patients with AMD (Amadi-Obi et al. 2012). From our study it was revealed that there was 1.55 fold reduction in the mRNA levels of CFH in disease group when compared to healthy suggesting that stress by HCV infection resulted in decrease in expression. This study was not able to find any association between the genotype of rs4658046 and rs10922103 and CFH expression both at protein and mRNA levels in serum. This study indicates that decreased levels of CFH and rs4658046 TT genotype were independent risk factors for increased likelihood of chronic hepatitis C. This study also indicates that rs10922103 AA genotype may be a risk factor for increased likelihood of chronic hepatitis C and negative response to treatment. Even though there have been studies in which the CFH polymorphism has been seen associated with other viral and parasitic infections like Dengue, Neisseria meningitides but there has been no literature on CFH polymorphism in hepatitis C infection. A study by Davila et al., (2010) found that rs1065489 was associated with host susceptibility to meningococcal disease (Davila et al. 2010). Another study by Zhang et al., (2013) showed that rs1065489 and rs3753395 were significantly associated with leprosy and its subtypes (D.-F. Zhang et al. 2013). A study by Pastor et al., (2013) had assessed CFH SNP rs3753394, rs800292, rs3753396, rs1065489 and Dengue infection severity and found that minor alleles at rs3753394 and
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rs800292 were associated with reduced infection severity in the Brazilian patients (Pastor et al. 2013). However another study by Kraivong et al., (2013) were unable to detect a direct interaction between CFH and Dengue infection severity in Thai population (Kraivong et al. 2013). This study also indicates that there is no association between the level of CFH and its polymorphism with the progression of disease.

In this study it was found that 59.52% of HCV infected cases were infected with genotype 3 followed by genotype 1 with 33.33% and genotype 4 with 7.14% of cases. It was found that genotype 3 was with the highest prevalence and was in corroborance with study by Chakravarti et al., (2011) who had shown that the 63% of cases were infected with genotype 3. But in this study there were no genotype 2 infected cases which was in accordance with study by Das et al., (2002) where they couldn’t find any genotype 2 infected cases but genotype 4(Das et al. 2002; Chakravarti et al. 2011)