Clinical importance of pharmacogenetics in the treatment of hepatitis C virus infection

ADINA MARIA KAMAL¹, PAUL MITRUȚ¹, KAMAL CONSTANTIN KAMAL², OANA-SORINA TICA³, MIHAELA NICULESCU⁴, DRAGOȘ OVIDIU ALEXANDRU⁵, ANDREI-ADRIAN TICA⁶

¹Department of Internal Medicine, University of Medicine and Pharmacy of Craiova, Romania
²Department of Family Medicine, University of Medicine and Pharmacy of Craiova, Romania
³Department of Obstetrics and Gynecology, University of Medicine and Pharmacy of Craiova, Romania
⁴Department of Anatomy, University of Medicine and Pharmacy of Craiova, Romania
⁵Department of Medical Informatics, University of Medicine and Pharmacy of Craiova, Romania
⁶Department of Pharmacology, University of Medicine and Pharmacy of Craiova, Romania

Abstract
Globally, over 4% of the world population is affected by hepatitis C virus (HCV) infection. The current standard of care for hepatitis C infection is combination therapy with pegylated interferon and ribavirin for 48 weeks, which yield a sustained virological response in only a little over half of the patients with genotype 1 HCV. We investigated the clinical importance of pharmacogenetics in treatment efficacy and prediction of hematoxicity. A total of 148 patients infected with HCV were enrolled. All patients were treated for a period of 48 weeks or less with pegylated interferon and ribavirin. Four genotypes were investigated: inosine triphosphatase (ITPA) rs1127354, C20orf194 rs6051702, interferon lambda (IFNL)3 rs8099917, IFNL3/4 rs12979860 in the population from southwestern Romania. Genetic variants for rs129798660 and rs6051702 proved once more to represent an indisputable clinical tool for predicting sustained virological response (SVR) (69.23%, p<0.05, respectively). ITPA genetic variants protect against ribavirin-induced hemolytic anemia [7].

Keywords: hepatitis C virus, inosine triphosphatase, single nucleotide polymorphism, ribavirin, interleukin 28B.

Introduction
Hepatitis C virus infection represents a major health problem worldwide, affecting over 180 million people [1]. Since the first discovery of hepatitis C virus, over 20 years ago, two scientific breakthroughs have taken the medical society closer to eradication of HCV infection. The first breakthrough was the discovery of the single nucleotide polymorphism (SNP) near the interleukin 28B (IL28B) gene, strongly associated with HCV clearance, spontaneous or on treatment [2]. The second breakthrough resides in the discovery of interferon (IFN)-free direct antiviral agents, which yield a sustained virological response in up to 90–95% of the cases, depending on HCV genotype [3].

In most countries, the standard of care remains the combined therapy with pegylated interferon (PegIFN) and ribavirin (RBV) for 24–48 weeks [4, 5]. The most important drawback of combination therapy is the development of adverse events, amongst which hematological side effects influence the treatment outcome the most. Ribavirin-induced hemolytic anemia, forces the reduction of start doses, thus diminishing the possibility to obtain a sustained virological response, defined as undetectable viral load 24 weeks after completion of treatment. Using genome-wide association studies, there have been identified a number of single nucleotide polymorphisms located on chromosome 20p13 on the inosine triphosphatase gene, encoding the inosine triphosphate pyrophosphohydrolase, a protein that hydrolyses inosine triphosphate to the monophosphate derivate [6]. Reports show that SNP of ITPA influence ribavirin-induced hemolytic anemia and C20orf194 also proved to be protective against thrombocytopenia. These clinical findings strengthen the belief that pharmacogenetics should play a constant role in treatment decisions for patients infected with hepatitis C virus.

The purpose of this paper is to evaluate, in the population in southwest of Romania, the factors related to the host associated with response to treatment with IFN and RBV for HCV infection. Genetic factors have a huge impact in producing great individual variation in terms of drug efficacy. Thus, arose the concept of pharmacogenetics, defined as the study of individual variations (polymorphisms) of specific genes on the action and effects of drugs. We aim through this paper, to bring more into attention the concept of pharmacogenetics and pharmacogenomics.

Development of pharmacogenomics could allow in the future – using appropriate tests, predictive therapeutic response – drug selection, determining the optimal dose and avoiding adverse reactions or, in short, personalize medication.

Patients, Materials and Methods
Patients
Between January 2012 and August 2015, blood samples...
were collected from 148 patients with HCV infection admitted in the IInd Department of Internal Medicine, Emergency County Hospital, Craiova, or from “Renășterea” Medical Centre, Craiova, Romania. All patients signed the informed consent prior to study initiation. Study protocol was designed in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the Ethics Review Committee of the University of Medicine and Pharmacy of Craiova.

The patients included, tested positive for HCV infection, had compensated liver disease and were willing to participate in the study. Patients with HIV/HBV (human immunodeficiency virus/hepatitis B virus) co-infection were excluded, as well as patients with alcoholic liver disease, decompensated liver cirrhosis, pulmonary tuberculosis, or patients that were in treatment for any malignant tumor. All patients were treated with pegylated interferon alpha-2a or -2b and ribavirin 800/1000/1200 mg, over a period of 48 weeks or less.

We monitored the patients for a period of 18 months, medical examinations being performed at 4, 12, 24 and 48 weeks from the start of the treatment and at 24 weeks after completion of therapy. At each visit, a complete blood count was performed and the patient was handed a questionnaire to record subjective adverse effects that occurred during treatment.

The collected blood samples were stored in an ultra-freezer, at -86°C, until the time of analysis.

### Table 1 – Baseline characteristics of study group

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>ITPA gene</th>
<th>C20orf194 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>AC+CT</td>
</tr>
<tr>
<td>No. of patients (%) (gender)</td>
<td>148 (100%)</td>
<td>134 (90.54%)</td>
<td>14 (9.46%)</td>
</tr>
<tr>
<td>Gender – female / male ratio (%)</td>
<td>(69.59% / 30.41%)</td>
<td>89 / 45</td>
<td>14 / 0</td>
</tr>
<tr>
<td>Age mean±SD [years]</td>
<td>53.12±10.85</td>
<td>52.95±10.62</td>
<td>54.79±13.17</td>
</tr>
<tr>
<td>No. of patients (%) with anemia</td>
<td>87 (58.76%)</td>
<td>83 (61.94%)</td>
<td>4 (28.57%)</td>
</tr>
<tr>
<td>Baseline Hb concentration [g/dL]</td>
<td>13.44±0.88</td>
<td>13.45±0.89</td>
<td>13.32±0.86</td>
</tr>
<tr>
<td>No. of patients (%) with baseline Hb concentration &lt;13 g/dL</td>
<td>50 (33.78%)</td>
<td>46 (34.33%)</td>
<td>4 (28.57%)</td>
</tr>
<tr>
<td>Baseline platelet count [10^3/μl]</td>
<td>158 557.43±62 280.17</td>
<td>155 332.09±69 208.18</td>
<td>189 428.57±63 189.28</td>
</tr>
<tr>
<td>Baseline WBC count [10^3/μl]</td>
<td>3854.32±1571.89</td>
<td>3894.18±1584.81</td>
<td>3361.43±1361.36</td>
</tr>
<tr>
<td>No. of patients (%) with indicated genotypes of gene: IL28B CC CT TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>52 (35.14%)</td>
<td>48 (35.82%)</td>
<td>4 (28.57%)</td>
</tr>
<tr>
<td>CT</td>
<td>75 (50.68%)</td>
<td>65 (48.51%)</td>
<td>10 (71.43%)</td>
</tr>
<tr>
<td>TT</td>
<td>21 (14.19%)</td>
<td>21 (15.67%)</td>
<td>0 (0.00%)</td>
</tr>
<tr>
<td>No. of patients (%) with indicated genotypes of gene: IFNL3 rs8099917</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>12 (8.11%)</td>
<td>12 (8.96%)</td>
<td>0 (0.00%)</td>
</tr>
<tr>
<td>GT</td>
<td>57 (38.51%)</td>
<td>50 (37.31%)</td>
<td>7 (50.00%)</td>
</tr>
<tr>
<td>TT</td>
<td>79 (53.38%)</td>
<td>72 (53.73%)</td>
<td>7 (50.00%)</td>
</tr>
<tr>
<td>Obtained SVR</td>
<td>81 (54.73%)</td>
<td>71 (52.99%)</td>
<td>10 (71.43%)</td>
</tr>
<tr>
<td>No. of cases (%) with ribavirin dose reduction</td>
<td>43 (29.05%)</td>
<td>41 (30.60%)</td>
<td>2 (14.29%)</td>
</tr>
</tbody>
</table>

ITPA: Inosine triphosphatase; C20orf194: Chromosome 20 open reading frame 194; SD: Standard deviation; Hb: Hemoglobin; WBC: White blood cell; HCV: Hepatitis C virus; RNA: Ribonucleic acid; IL28B: Interleukin 28B; IFNL: Interferon lambda; F1: Minimal fibrosis; F2: Fibrosis occurred and spread inside the areas of the liver including blood vessels; F3: Fibrosis spread and connected to other liver areas that contain fibrosis; F4: Cirrhosis or advance liver fibrosis; SVR: Sustained virological response.

### Genotyping

Four genotypes were analyzed in order to investigate the clinical importance of pharmacogenetics in the treatment for HCV infection.

Genotyping was performed with TaqMan Pre-designed SNP Genotyping Assay, small scale, human, using the real-time polymerase chain reaction (RT-PCR) technique on the Corbett Rotor-Gene 6200 RPM platform with interchangeable blocks ViA™ 7 Applied Biosystems and automatic pipetting Thermocycler Eppendorf epMotion 5070 Mastercycler. Sequencing was performed using CEQ™ 8000 Genetic Beckman Coulter sequence analysis system.

### Statistical analysis

All data collected was stored in Microsoft Excel files (Microsoft Co., Redmond, WA, USA) and data processing was performed using ChiTest, Pivot Tables, functions chart analysis; fundamental statistical parameters, mean and standard deviation and coefficient of variation were calculated.

### Results

We included, in this study, 148 patients diagnosed with chronic HCV infection. Baseline features of the study population are presented in Table 1.
Out of the total 148 patients included in the study, 81 (54.27%) obtained SVR, while 67 (45.27%) patients did not. Of the total 67 patients that did not obtain SVR, nine (13.43%) patients were relapsers, one (1.49%) patient was interrupted for tuberculosis occurrence, two (2.98%) patients were interrupted due to decompensated liver cirrhosis and 55 (82.08%) patients did not have undetectable viral load 24 weeks after completion of therapy.

First, we genotyped interferon lambda 3 rs8099917, IFNL3/4 rs12979860, inosine triphosphatase rs1127354 and chromosome 20 open reading frame 194 (C20orf194) rs6051702 in all 148 patients included. Fifty-two and 78 patients presented the major and minor allele, respectively for, rs12979860. Seventy-nine and 69 patients presented the major and minor allele, respectively, for rs8099917.

**Achieving the sustained virological response according to IFNL3 rs8099917, IFNL3/4 rs12979860**

We investigated whether any of the genotypes could predict SVR in the given population as it has previously been reported. For IFNL3/4 rs12979860 interleukin 28B (IL28B), CC genotype proved to have the highest rate for predicting SVR as 36 (69.23%, chi-square p=0.007846, p<0.05) of the patients had undetectable viremia at the end of treatment. Response rates according to IL28B genotype are presented in the graphic below (Figure 1).

**Figure 1 – Response rates according to IL28B genotype.**

Next, we investigated the correlations of rs8099917 genotypes to the rates of response to treatment for chronic HCV infection. The major allele proved to impact de rates of response the most, as patients with TT genotype achieved sustained virological response in 63.29% of the cases (chi-square p=0.007846, p<0.05) (Figure 2).

**Figure 2 – Response rates according to rs8099917 polymorphisms.**

As previously studied, we confirmed the findings in our study cohort, thus strengthened the belief that CC and TT genotypes are able to predict acquisition of SVR especially when used together rather than separate. The rs8099917 SNP was associated with higher rates of thrombocytes drop, although in our study, no such correspondence could be established. There was evidence that major allele could be associated with higher rates of neutrophil reduction under treatment (data not shown).

**Hemoglobin drop >2.5 g/dL during treatment with pegylated interferon and ribavirin**

Next, we investigated all factors connected to a drop in hemoglobin (Hb) levels of over 2.5 g/dL from baseline, at week 4 of treatment and the occurrence of anemia in the studied population.

In our cohort, 48 (32.43%) patients experienced a drop in Hb levels of over 2.5 g/dL at week 4. There was a strong statistical correlation between ribavirin doses and the drop in Hb (p=0.003). Moreover, the reduction of ribavirin doses during treatment, correlated more strongly with the drop in Hb. No further connections could be established in relation to the investigated parameter.

Anemia occurred in 81 (58.78%) of the patients with no evident connection to age, gender, body mass index (BMI) or baseline Hb levels. However, anemia was strongly connected to ribavirin doses as expected, and the connection between anemia and ribavirin dose reduction was statistically significant (p=0.0001). There was no statistical correlation between ribavirin dose reduction and SVR in our study as ribavirin doses did not differ considerably from patients who achieved SVR and patients who did not.

**ITPA rs1127354 variants protect against Hb drop over 2.5 g/dL at week 4 and against anemia throughout the course of treatment for HCV**

We investigated the association between ITPA genetic variants and Hb drop over 2.5 g/dL from baseline at week 4. Minor allele proved to be protective against ribavirin-induced hemolytic anemia. CC genotype correlated significantly to anemia throughout the course of treatment – 83 (64.94%) patients (Figure 3).

**Figure 3 – ITPA correlation with ribavirin-induced anemia over the course of treatment.**

We also tested whether SVR could be correlated to any of ITPA genetic variants (chi-square p=0.392233) but we could not prove there is a statistically significant difference between CC genotype and the other genotypes in regards to responder status (p>0.05).

No other correspondence could be established between ITPA variants and leucopenia, thrombocytopenia or neutropenia.
C20orf194 rs6051702 variants – complex findings

The association between rs6051702 variants and hematotoxicity was further investigated. The homozygous individuals with AA major allele (67 patients, 65.69%, chi-square $p=0.011074$, $p=0.05$) were more prone to develop anemia throughout the course of treatment with pegylated interferon and ribavirin, than AC/CC genotypes (20 patients, 43.48%).

No association was found between C20orf194 variants and Hb drop >2.5 g/dL from baseline at week 4, nor with neutropenia and reduction of white blood cell (WBC) count. Unlike ITPA variants, rs6051702 variant AA proved to be protective against thrombocytopenia, statistical analysis bringing evidence that AC/CC genotypes are more prone to develop thrombocytopenia (28 patients, 60.87%, chi-square $p=0.014425$, $p=0.05$).

Unlike previous reports our data sustain the hypothesis that there is a difference between AA genotype and the other genotypes in regards to responder status (62 patients, 60.78%, chi-square $p=0.027558$, $p=0.05$). Detailed data regarding responder status is listed in Table 2.

Table 2 – Correlations between all four SNP and responder status

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Responder</th>
<th>Non-responder</th>
<th>Chi-square $p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNL3 rs8099917</td>
<td>GG 2 (16.67%) 10 (83.33%)</td>
<td>0.007846 (significant)</td>
<td></td>
</tr>
<tr>
<td>IFNL3 rs12979860</td>
<td>GT 29 (50.88%) 28 (49.12%)</td>
<td>0.000105 (highly significant)</td>
<td></td>
</tr>
<tr>
<td>ITPA rs1127354</td>
<td>CC 36 (69.23%) 16 (30.77%)</td>
<td>0.392233 (non-significant)</td>
<td></td>
</tr>
<tr>
<td>C20orf194 rs6051702</td>
<td>AC+CT 10 (71.43%) 4 (28.57%)</td>
<td>0.027558 (significant)</td>
<td></td>
</tr>
</tbody>
</table>

SNP: Single nucleotide polymorphism; IFNL: Interferon lambda; ITPA: Inosine triphosphatase; C20orf194: Chromosome 20 open reading frame 194.

Discussion

Combined therapy with PegIFN and RBV is known to be associated with a number of treatment-limiting adverse events. As previously reported in several studies, anemia is one of the most important adverse events known to lead to dose reductions therefore failure to achieve SVR and withdrawal of therapy [8, 9].

Hemolytic anemia induced by ribavirin has been explained by the oxidative stress induced to erythrocytes by triphosphate [10, 11]. The ITPA gene encodes inosine triphosphatase. Mutations in the ITPA gene lead to inosine triphosphatase deficiency, a benign enzymopathy resulting in accumulation of inosine triphosphatase in the erythrocytes thus increasing hematoxocity [12, 13]. Variations in the gene (rs1127354) lead to ITPase deficiency and thus protects against hemolytic anemia that occurs in the early stages of treatment, in patients infected with HCV [7].

In the present study, we also observed that rs1127354 minor allele protect against hemolytic anemia, and found that CC major allele can predict anemia in the first four weeks of treatment and also throughout the course of therapy. Although ribavirin dose reduction was necessary in our study, we could not correlate the lack of SVR with necessity of dose modification, and also ITPA single nucleotide polymorphisms could not correlate in any way with treatment outcome. Previous reports show that the effect of ribavirin dose reduction is not as important on treatment outcome as long as RBV treatment is not discontinued, which is reconfirmed in our study [14, 15].

The rs6051702 SNP showed considerable linkage with hemoglobin reduction in the first weeks of treatment [7]. We could not find similar correlations in our cohort, but major allele AA individuals were more prone to developing anemia over the course of treatment but not but not necessarily in the first weeks of therapy. We again confirmed the utility of these genetic markers in predicting anemia, in patients treated for HCV infection with combination therapy. Moreover, we observed in our population a high number of patients who were protected for thrombocytopenia being predominantly homozygous for the major allele AA. For the rs1127354 SNP, no such connection could be demonstrated as it has previously been shown [16]. Further studies are needed to investigate whether this information can be used in the future as a predictor for thrombocytopenia in patients undergoing HCV treatment. To our knowledge, it is the first time C20orf194 SNP variants were ever correlated with sustained virological response. We cannot find an explanation for why this occurred. A hypothesis was that patients who developed anemia are more likely to obtain SVR, although, as a separate hypothesis, this could not be demonstrated. Intensive statistical analysis is needed further, in order to determine a link between these two variables.

Since PegIFN and RBV treatment is so burdened by adverse effects, the need to involve some predictors of response, has increased considerably. This has motivated many studies to identify factors related to virus/host involved in a higher response rate to treatment. Patients with genotype 1 HCV infection, can be included in a “difficult to treat” category of patients, having a low rate of SVR. The issue is of high interest in Romania, given that a national multicenter study showed that genotype 1b is almost exclusively present in patients with chronic HCV infection [17].

In 2009, a correlated set of polymorphisms near the gene IL28B were associated with clearance of HCV genotype 1, in patients undergoing treatment with IFN and RBV. The same genotypes were later associated with a spontaneous HCV clearance in untreated patients [2, 18–20]. The single nucleotide polymorphism was located on chromosome 19, 3 kb upstream of the IL28B gene that encodes IFNL3 [21–23]. Interferon lambda has antiviral activity, inhibiting HCV replication, through stimulation of expression of the ISGs (interferon-stimulated genes) and increasing immunity of natural killer (NK) cells and cytotoxicity of antigen-specific CD8+ cell [24–26].

The way IL28B SNPs exerts its antiviral activity, remains questionable. Studies on IFNL expression in peripheral blood mononuclear cells are inconclusive. Most research, points out that the liver tissue mRNA expression
of IL28B is different depending on the genotype IL28B, but there is a different expression of ISG [27].

The second polymorphism, rs8099917, associated with SVR was described in three studies [2, 18, 19]. The polymorphism rs8099917 is located 7.5 kb upstream of the start codon of IL28B, in the intergenic region of IL28A and IL28B. In these studies, the T-allele was favorable and allele G was unfavorable, yielding three possibilities: homozygosity T/T and G/G and heterozygosity G/T.

In our study, the same correspondence was maintained, as 69.23% of the patients that successfully completed treatment were CC genotype carriers for IL28B gene, and 63.29% of the responders were TT genotype carriers for rs8099917 SNP.

The link between IL28B gene and HCV clearance may influence the decision to initiate antiviral therapy, design and interpretation of clinical trials, treatment economy and the regulatory approval process for new anti-HCV therapeutic agents.

So far, in our country there is no similar study on all four-gene polymorphisms, in terms of response to treatment with IFN and RBV, and impact on hematotoxicity. Our paper tried to draw attention to the importance of determining genetic variations, in order to pinpoint genetic therapy in the future.

Conclusions

We confirmed through our study, that both SNP variants for rs12979860 and rs8099917 are important genetic predictors for sustained virological response. Although they do not provide strong, categorical evidence that certain genotypes are associated with attainment of SVR, they can successfully be utilized as clinical tools in choosing the appropriate treatment. Genetic polymorphisms of ITPA gene were associated with higher rates of Hb level drop at week 4 and throughout the course of therapy. Some variants proved to be protective against ribavirin-induced hemolytic anemia. Similar findings were available for C20orf194 gene polymorphisms. In addition, rs6051702 genotyping showed that AA allele carriers achieved SVR in higher proportions and protected against thrombocytopenia. Since our study was conducted on a small number of cases and all patients included in the study were Romanian, we cannot consider these findings as representative, but extended studies on different ethnicities are needed in order to confirm representative, but extended studies on different ethnicities were Romanian, we cannot consider these findings as small number of cases and all patients included in the study achieved SVR in higher proportions and protected against pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet, 2009, 41(10):1105–1109.


References


Grigorescu M; Romanian Society of Gastroenterology and Hepatology. HCV genotype 1 is almost exclusively present in Romanian patients with chronic hepatitis C. J Gastrointestin Liver Dis, 2009, 18(1):45–50.


Corresponding author
Kamal Constantin Kamal, MD, Department of Family Medicine, University of Medicine and Pharmacy of Craiova, 2 Petru Rareș Street, 200349 Craiova, Romania; Phone +40748–117 735, e-mail: kamalconstantin@gmail.com

Received: February 8, 2016
Accepted: August 9, 2016