UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
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SUMMARY
PHD THESIS

CHRONIC VIRAL HEPATITIS C.
IMMUNOLOGICAL PROFILE AND CORRELATIONS WITH TREATMENT RESPONSE

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SUMMARY

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CURRENT KNOWLEDGE

Hepatitis C virus (HCV) is one of the most common causes of progressive liver disease, with significant impact on human health worldwide. Recent studies estimate that more than 185 million people worldwide have been infected with HCV, of which 350,000 die each year (1) (2).

The impact of the viral infection on the liver tissue range from minimal histological lesions to intense fibrosis and cirrhosis with hepatocellular carcinoma.

HCV has a high genetic variability because the virus has a high replication rate and RNA polymerase is prone to errors. There are at least seven genotypes, in Europe genotype 1b is prevalent in 47% of cases, followed by 1a to 17% and genotype 3 in 6% of the (3). Recent research showed that 1b genotype achieved high levels of viremia and is associated with increased risk of chronic evolution, responds poorly to IFN therapy and progresses to liver cirrhosis and hepatocellular carcinoma. Chronic infection is due to: inadequate response of innate immunity, the adaptive immune mechanisms deficiencies, the production of viral quasispecies and immunotolerance of the host.

The transmission routes are blood transfusions, surgery, dental treatments, intravenous drug use, unprotected sex, perinatal.

The humoral immune response is weak and inconstant, antibodies develop 5-6 weeks post-infection with a protective activity difficult to assess. Cellular immune response is achieved by the intervention of CD8 cytotoxic T lymphocytes that can recognize HCV under the stimulation of CD4 T clones. A large percentage of people infected with HCV have a chronic evolution, because the immune response is unable to achieve the clearance of the virus.

Some cytokines' SNPs (Single Nucleotide Polymorphism) affects their synthesis and secretion in HCV infection and modulate the immune response leading to chronic liver infection (3).

The diagnosis of viral Hepatitis C Diagnosis is made by using biochemical, serological, hematological and molecular tests (HCV RNA). Additionally are used the imaging investigations - abdominal ultrasound and Fibroscan® or other noninvasive methods for measurement of the degree of liver fibrosis.

The goal of antiviral therapy is to cure HCV infection by viral clearance, achieving a sustained virological response (SVR) which means negative HCV-RNA at 6 months after therapy.

Standard therapy is a combination of Pegylated interferon (PEG-IFN) and Ribavirin (RBV). A sustained virological response is defined as undetectable HCV-RNA at either 12 weeks (SVR 12) or 24 weeks (SVR 24) after the end of therapy. Currently, 40-50% of patients infected with HCV genotype 1 treated with PEG IFN and RBV regimen have the chance to achieve a sustained virological response to treatment.

Protease inhibitors (PIs) are a class of direct acting antivirals (DAA). Inhibitors of NS3/4A protease, *Boceprevir* and *Telaprevir* in combination with PEG-interferon and Ribavirin, increase the SVR chance by approximately 30% compared to double therapy.

Protease inhibitors of second generation, *Simeprevir*, *Daclatasvir* acting on nonstructural protein NS 3 / 4A, 5A along with *sofosbuvir* (an inhibitor of RNA polymerase NS5B) have a strong efficacy and achieve SVR at 12 weeks in most cases (1).

There is a combination of *Sofosbuvir* (SOF) and *Ledipasvir* (LDP), which in a 12-week regimen with or without RBV increases the chance of SVR to more than 99% in patients without cirrhosis (4).

Other new generation molecules, recently approved by the European Commission, include *Paritaprevir* (inhibitor of NS3/4A protease), *Ritonavir* (protease inhibitors enhancer) and *Ombitasvir* (NS5A inhibitor). This is used with another antiviral drug called *Dasabuvir* (non-nucleoside inhibitor of polymerase NS5B), with or without addition of Ribavirin according to genotype (1a/1b) and the type of patient (decompensated, compensated or without liver cirrhosis). In clinical trials the combination therapy had a success rate of 95-100% in patients infected with HCV genotype 1.

In Romania it was recently approved (Order MS / CNAS no. 1379/1023/2015) a therapeutic protocol based on Ombitasvir , Paritaprevir, Ritonavir and Dasabuvir, which is settled by the National Health Insurance House for patients with severe fibrosis/cirrhosis (F4), patients who relapse after liver transplantation, patients with genotype 1 HCV with advanced liver fibrosis (F3) and contraindications to interferon therapy.

1. OBJECTIVES OF THE STUDY

The main objective was to evaluate sustained virological response rate in patients with HCV chronic infection treated with Peginterferon and Ribavirin in conjunction with clinical, biological and genetic factors. Another objective was to assess liver fibrosis in patients with chronic hepatitis C by various methods (Fibroscan®, APRI) and the impact of the degree of fibrosis on virological response to antiviral standard therapy.

2. MATERIAL AND METHODS

2.1. Patient lots

I included in this study a group of 188 patients with chronic hepatitis C, genotype 1b that consists of two subgroups, one from Medical Clinic I of Emergency Hospital, Craiova, Romania and another subset of patients from Hospital Santa Maria, Lisbon, Portugal.

The study was conducted over a period of 3 years (2012-2015) and involved the collection of informative data about patients, collection and storage of blood samples in order to determine the genetic polymorphisms of interleukins involved in the response to treatment and in liver fibrosis.

I also had a control group of 168 healthy people without infections with hepatitis viruses.

All patients were treated with PEG interferon and Ribavirin for 48 weeks and then were monitored clinically and biologically for 24 weeks after the end of therapy.

2.2. Methods

The patients were evaluated both clinically and biologically before, during and 24 weeks after antiviral therapy.

Patients were registered in a database containing age, sex, route of infection, associated diseases and the side effects of therapy. Also I included laboratory test results in dynamic:

- **Diagnostic tests for detecting the HCV infection**:
  - Serological tests for HCV antibodies by Enzyme Linked Immuno Sorbent Assay (ELISA).
  - Molecular tests for the detection of viral genotype and viral load quantification in patient blood: PCR (Cobas 6800, Roche Molecular Diagnostics).
- **Monitoring the viral load**:

- HCV-RNA was tested**:**
  - *at the start* of therapy;
    - *after 4 weeks* of therapy;
    - *after 12 weeks* of therapy if HCV-RNA was detectable at 4 weeks;
    - *after 24 weeks* of therapy if at 12 weeks if the clearance was not achieved but the viral load decreased by more than 2 log10;
    - *at the end* of therapy (48/72 weeks depending on the type of virological response);
    - *24 weeks after completion* of therapy (72 weeks after the start).

- **Biochemical tests**: GOT (ALT), GPT (AST), GGT, FA (ALP), urea, creatine. These were measured by spectrophotometric method.
- **Hematological tests**: the full blood count was performed by automated method.
- **Genetic tests**:
  - interleukins IL 28 B and IL10 genotyping (using kits Custom Taqman SNP Genotyping Assays (Applied Biosystems, Life Technologies, Foster, CA, USA).
- **Ultrasound of the liver**: chronic liver disease, liver lobes dimensions, liver tissue description: echogenicity, the presence of regenerative nodules, liver edges, caliber of portal vein (signs of portal hypertension), signs of liver cirrhosis.

To assess **liver fibrosis** I used FibroScan® method also called transient unidimensional elastography (TE) as a noninvasive form of measuring the degree of liver fibrosis. This non-invasive procedure successfully replaced liver biopsy in patients infected with HCV because it has high positive predictive value for advanced/severe fibrosis.

APRI score (AST related to platelet count) was calculated as the ratio of AST / (upper limit of normal platelet range / platelet count of the patient) x 100.

For data processing I used Microsoft Excel (Microsoft Corp., Redmond, WA, USA) with XLSTAT suite for MS Excel (Addinsoft SARL, Paris, France) and IBM SPSS Statistics 20.0 software (IBM Corporation, Armonk, NY , DOOR).

3. RESULTS

The study group consisted of 123 women (65.43%) and 65 men (34.57%), 127 patients (67.55%) aged over 50 years. A total of 136 patients (72.73%) came from urban areas and the rest from rural areas.

Following evaluation by Fibroscan® 109 patients (52.98%) had advanced/severe fibrosis. 18 patients (9.57%) did not have fibrosis (stage F0), 14 patients (7.45%) had mild fibrosis (F1), 47

(25.00%) moderate fibrosis (F2), 67 (35 64%) advanced fibrosis and 42 patients (22.34%) had severe fibrosis (F4).

Most patients had normal hemoglobin values (average 13.39 g/dL), anemia prevalence in the studied group was 30.85% and thrombocytopenia prevalence was 41.71% (average platelet count was 189,000 media / mL). White blood cells count was normal, with an average of 5,598/mL.

Regarding the medical history, 41 patients (21.81%) had hepatic steatosis, peripheral neuropathy was present in 16 patients (8.51%), hypothyroidism in 9 patients (4.79%) and diabetes at 24 patients (12.77%).

Viral load had an average of 2,668,385 IU/mL, 118 patients (62.77%) had a load over 400,000 IU/mL.

Biochemical parameters of liver function showed cytolysis. Thus, ALT had an average of 76.39 IU/L, AST 94.12 IU/L, GGT 98.64 IU/L and ALP 84.50 IU/L.

Renal function was relatively well preserved as urea had an average value of 38.90 g/L and creatinine 0.84 g/L.

IL 28 B SNP rs12979860 CC genotype appeared less frequently in patients infected with HCV (OR: 0.553, p = 0.008), suggesting a protective effect against the infection. Also, CT genotype was more frequent in patients (53.41%) compared with controls (36.31%) (OR: 2.011, p <0.001), and T allele had a higher frequency in patients with HCV (67.05%) than in controls (52.98%) (OR = 1.806, p = 0.008), both results suggesting that the T allele increases the risk of HCV infection.

None of IL 10 R genotypes frequencies did not differ significantly between patients group and control group. However, IL 10 R GG genotype had a higher frequency in patients (15.79%) than in the control group (12.50%) (OR: 1.312, p = 0.4134). Prevalence of IL 10 R G allele was 63.16% in patients and 57.74% in healthy individuals (OR: 1.255, p = 0.340). These results suggest that G allele might increase the risk of infection with HCV, however, extensive studies are necessary to confirm this.

Virological response analysis showed that 98 out of 176 patients (55.68%) had a sustained virological response, 59 (33.52%) were non-responders and 19 (10.80%) were relapsers. Greater reduction was observed in the mean HCV-RNA both at 4 weeks and at 12 weeks. We observed a marked HCV RNA decrease in responders in the first 12 weeks of therapy, this being a positive prognostic factor for SVR.

By comparing clinical and biological parameters among responders and non-responders we observed statistically significant differences regarding age over 50 years ($p = 0.007$), number of patients with liver fibrosis degree F1 ($p = 0.038$), viral load before treatment ($p = 0.014$) AST ($p = 0.003$) and ALT ($p = 0.006$) levels.

Patients under age 50 had an increased likelihood of treatment response. Also, patients with a baseline viral load less than 400,000 IU / mL responded better to treatment. Hepatic cytolysis was a negative predictor of the response to antiviral therapy. Advanced/severe liver fibrosis (F3-F4) was associated with decreased chance of therapy response (OR: 0.492, $p = 0.0257$). We observed different dynamics of platelet counts in responders and non-responders. Thus, the responder platelet count falls within the first week of treatment, but increases at the end of therapy slightly above the normal value of 200,000/mL. In non-responders thrombocytopenia is maintained after treatment.

AST and ALT were increased in non-responders and declined moderately under the therapy, but remained above the normal; in responders the levels decreased significantly by the end of therapy and were normal afterwards.

There was a strong link between IL 28 B SNP rs12979860 genotypes link and response to therapy. Patients who received the combination therapy of PEG-IFN and RBV with the CC genotype had higher SVR rates (78.95%, OR: 4.56, 95% confidence interval: 2.152 to 9.666, $p <0.001$) than subjects who had CT (51.61%) or TT genotypes (23.08%, OR: 0.17, 95% confidence interval: 0.062 to 0.471, $p = 0.0003$).

IL 28 B C allele was strongly associated with SVR (OR: 5.867, 95% CI (2.124 to 16.106), $p = 0.0003$). Virological response rate was 61.97% for the C allele compared to 21.74% for those without this allele (genotype TT).

Patients receiving dual therapy with PEG-IFN and RBV the response rate was higher in patients with IL 10 R GA (62.82%) and AA (56.45%) genotypes compared to GG (47.22%) (OR: 2.200, 95% confidence interval: 0,701- 6.889, $p = 0.1251$). This difference did not reach statistical significance probably because there were only 17 responders with genotype GG. SVR rate in patients with genotype GG of IL 10 R was 47.22%, in those with GA 62.82% and 56.45% in AA. HCV patients with A allele (non-GG genotype) achieved SVR in 64.36% cases.

It can be seen as the combination of genotypes IL 28 B CC plus IL 10 R AA was present with higher frequency among patients who achieved SVR (12.00%) than non-responders (6.38%) and also the combination IL 28 B CC plus IL 10 R GA (OR: 3.151).

The combination of GG genotype of the IL 10 R with C allele of IL 28 B (OR: 0.750) and especially with the T allele (OR: 0.275) had a negative effect on SVR and the T allele of IL 28 B combined with the G allele of IL 10 it also has a negative effect (OR: 0.522). The C allele of IL 28 B plus A allele of IL 10 R greatly increased the chance of SVR (OR: 3.227).

We observed significant differences between patients with fibrosis F0-F2 and F3-F4 on the following parameters: patients age over 50 years (53.16% vs. 68.81%, OR: 1.39, p=0.0207), response to treatment (64.56% vs. 49.54%, OR: 0.49, p=0.0236), HCV RNA (4,079,865 vs. 1,722,831 IU/L, Student's t: 2.00, p=0.0471), serum glucose (93.69 versus 102.96 g/L).

GOT had higher values in patients with advanced/severe fibrosis (85.18 IU/L) than in those with mild/moderate fibrosis (64.31 IU/L) (p = 0.0235). Thrombocytopenia was more common in patients with fibrosis F3-F4 (44.04%) than in those with fibrosis F0-F2 (29.11%). APRI score has differentiated patients with mild/moderate fibrosis (average APRI: 0.72) from those with advanced/severe fibrosis (average APRI: 1.28). Thrombocytopenia under 150,000/mL was a positive predictor of advanced/severe fibrosis. A total of 56 patients (70.89%) from those with mild/moderate fibrosis had platelet counts above 150,000/mL, compared with only 61 (55.96%) patients with advanced/severe fibrosis.

If we used APRI score to exclude severe fibrosis (F4), for a cut-off value of 0.5 we have achieved a sensitivity of 83.33% and a specificity of 53.33%, values that are acceptable for clinical use, and the area under the ROC curve in this case was 0.7541.

In conclusion APRI score can be used to determine patients with a high probability of having advanced/severe fibrosis (cirrhosis), or exclude those who do not have fibrosis, which can be confirmed by Fibroscan® or liver biopsy.

There were no significant differences between IL 28 B genotype distribution in patients with F0-F2 fibrosis and those with F3-F4, as Chi square test result was p=0.808, > 0.05.

Patients with F3-F4 fibrosis had much lower percentage for genotype AA of IL 10 R. The genotype GA was a risk factor for advanced/severe fibrosis (OR: 2.151, 95% confidence interval: 1.042 to 4.434, p=0.038) as was the G allele of IL 10 R (OR: 2.400, 95% confidence interval: 1.159 to 4.974, p = 0.018). The A allele of IL 10 R SNP was more frequent in patients with mild to moderate fibrosis (OR: 1.13). IL 28 B CC genotype showed a protective effect in combination with IL 10 R allele (OR: 0.698) and AA genotype (OR: 0.526), but the association was not statistically significant (p=0.3610). GG genotype had an increased risk of hepatic fibrosis, both in combination with the C allele of the IL 28 B (OR: 1,348) and the T allele (OR: 1.091).

Advanced/severe fibrosis was significantly associated with the presence of the G allele of the IL 10 R, both in combination with the C allele of the IL 28 B (OR: 2,744) and the T allele (OR: 2.402).

We identified by multivariate logistic analysis, we identified the following significant positive predictors for **the chance of SVR**: mild/moderate fibrosis (OR: 2.84, p=0.0897), viral load <400,000 IU/L (OR: 3.39, p=0.0169), the CC genotype of IL 28 B (OR=7.62, p=0.0827) and CT genotype of IL 28 B (OR=7.77, p=0.0221). Age under 50 was a positive predictor, without reaching statistical significance (OR: 2.26; p = 0.1117).

For **advanced/severe fibrosis** we identified the following positive predictors: platelet count <150,000/mL (OR: 15.81; p = 0.0075), GA genotype of IL 10 R (OR = 26.84; p = 0.0482) and GG genotype of IL 10 R (OR: 4.732, p=0.2880). Male gender was a positive predictor, but without reaching statistical significance (OR: 5.19, p=0.1622). Age under 50 was a protective factor for advanced/severe fibrosis (OR = 0.07, p = 0.0329).

4. CONCLUSIONS

1. We identified as positive predictors for SVR: age under 50 years, a reduced degree of liver fibrosis (mild/moderate), absence of hepatic steatosis and other comorbidities that make the IFN and RBV treatment more difficult. Low viral load less than 400,000 IU/L was a positive predictive factor for the favorable evolution under treatment.
2. The negative predictors for SVR were: increased AST, ALT and GGT before treatment.
3. The C/T polymorphism rs12979860 of IL 28 B significantly affected the chance of SVR. CC genotype was associated with increased likelihood of SVR, followed by the CT genotype. The C allele was strongly associated with the chance of response to treatment.
4. IL 10 R polymorphism -1082 A/G had no statistically significant effect on SVR (p=0.1251), although the response rate was lower in patients with genotype GG, which supports the hypothesis that these patients produce a greater quantity of IL 10, which in turn inhibit the antiviral activity of interferon.
5. We identified the following as positive predictors for liver fibrosis: male gender, age over 50 years, thrombocytopenia and elevated serum glucose.
6. The negative predictors for liver fibrosis included achievement of SVR.
7. The C/T polymorphism rs12979860 of IL 28 B had no significant influence on liver fibrosis.
8. The -1082 polymorphism A/G of IL 10 R had a significant influence on liver fibrosis. We observed that the G allele and GG genotype were positive predictors for advanced/severe fibrosis.
9. The positive effect of the G allele on hepatic fibrogenesis manifested in whatever combination of C or T allele of SNP rs12979860 of IL 28 B and any genotype.
10. Further studies are needed to clarify the role of clinical-biological factors in combination with IL 10 R polymorphisms on liver fibrogenesis in chronic viral hepatitis C.
11. A genetic determinism of HCV infection can have impact on the evolution of the liver disease and the quality of patient’s life, and influence the therapeutic decision based on the real and actual possibilities.
12. The therapeuthic decision to treat the patients that are already in the stage of liver cirrhosis cannot exclude the ones that have a high risk to develop advanced fibrosis in a short period of time, or those that already tried an IFN based treatment and failed to acieve SVR or didn’t tolerate the therapy due to severe adverse effects.

5. REFERENCES

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