"OVIDIUS" UNIVERSITY OF CONSTANȚA 2016

DOCTORAL SCHOOL OF MEDICINE

FIELD OF MEDICINE PhD

DOCTORAL THESIS

- SUMMARY -

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IMMUNOLOGICAL AND THERAPEUTIC CORRELATES BETWEEN THE GENETIC HOST SPECTRUM VARIATION AND DEVELOPMENT OF HEPATITIS C VIRUS INFECTION

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Motto:

“Somewhere, something incredible is waiting to be known.
Science is a way of thinking much more than it is a body of knowledge.”
— *Carl Sagan*

Keywords: Human Leukocyte Antigen-HLA; HLA-DRB1 alleles; HLA-DQB1 alleles; hepatitis C; Interferon-alpha therapy with Peg / Ribavirin- PegIFN-α / RBV

TABLE OF CONTENTS

Introduction:

Although extensively studied the mechanism of Hepatitis C virus (HCV) pathogenesis remains elusive. The host genetic factors along with the viral factors and environmental factors are incriminated to governing the disease pathology in worsening or regression the disease. Subject to the restriction of T lymphocytes by human leukocyte antigens HLA (human leukocytic antigen), the antibodies secreted by B lymphocytes, natural killer cells (NK) and cytokines (IL28B) are under the conditions of the immune response to infection by the hepatitis C virus. HLA class II and I presentation of the viral antigen by the CD4 + and TCD8 + cells is the key to optimal immune response and all of this depends on viral clearance or persistence. It has also been observed a mismatch in the response to the vaccine against the hepatitis B virus (HBV), and the patient undergoing treatment with interferon following infection with hepatitis B or C. The response varied vaccine is due to the specific combinations of HLA class II polymorphisms that are influencing the ability of HLA class II molecules to bind and present a great diversity of antigens to the CD4 + lymphocytes which determines in turn the production of antibodies and lymphocyte viral clearence

activation. Many clinical and laboratory investigations have identified a number of immunogenic factors involved in conditioning the evolution of hepatitis C.

Genetic polymorphisms and the heterozygosity of HLA loci enable HLA molecules to present a wide variety of viral epitopes and diversify the properties of HLA molecules in agreement with binding and antigen presentation.

The association of HLA susceptibility to infection with hepatitis C, protection, disease severity, response to treatment with interferon and to vaccine have been extensively studied in the overall population.

The thesis consists of one general part which provides a synthesis of specialties published literature and a special part, intended for research and personal results. The information gathered at the end finally come to a conclusion to an overview of the frequency of HLA class II DRB1 / DQB1 marker alleles of protection and susceptibility in patients infected with hepatitis C in Romanian population and a research overview on the association of HLA alleles with important factors in prognosis (viremia, degree of liver fibrosis, genotype IL28B) and the response to standard treatment with interferon and ribavirin that patients are follow, this in order to define a genetic profile of the patient that can guide the clinician on the disease progression.

There is possible that some other future results will be joining from other studies in view of the global population and in the future can be designed new strategies for developing a combined vaccine against hepatitis C and better management of therapy with interferon and ribavirin in these patients. Elucidation of viral and immunological processes leading to persistent viremia or viral clearance in infected patients can go to new antiviral therapeutic strategies that lead to stopping chronic infection, reducing the risk of developing life-threatening complications and treat patients more effectively customized.

Motivation and the importance of research

By determining the HLA-DRB1 and HLA-DQB1 alleles in subjects enrolled in this study, I proposed to determine which of these alleles, genotypes and haplotypes generated by them, act as risk factors for susceptibility or protective, viral persistence and respond in treatment.

Observeing the results of previously published studies emerged that is a great diversity in association with HLA susceptibility, protection, response and evolution of hepatitis C in different regions and ethnic

groups in the overall population (Annex 3). It is noted that HBV and HCV infections tend to be repressors one to each other. HBV infection and infection with hepatitis D virus (HDV) can suppress the HCV infection and the other viruses HCV and HDV may have a negative effect on the hepatitis B virus.
Interferon can activate antigen presentation by training several cytotoxic T lymphocytes and thus having a protective response against viral assault. T lymphocyte responses restricted class I and II are effective against the virus, are polyclonal and multi-specific in patients with acute infection that heal and successfully eliminate the virus or can be relatively weak and ineffective infected patients who go to chronicity. The main cause of viral persistence during infection with hepatitis C virus develop a weak immune response against viral antigens. Aggression cellular processes along with chronic liver inflammatory and regenerative response is mutagenic and mitogenic stimuli for the development of lesions in the DNA can cause hepatocellular carcinoma.

The HLA system is very polymorphic and the phenotype of an individual largely determine the response to various viral antigens and the disease. Recently, it was demonstrated the protective effect of HLA alleles in the HCV viral infection could be explained by a specific response of CD4 + T cells directed against viral epitopes. This effect may be due more proficient presentation of antigens via HLA molecules or a polymorphism linked a gene immunomodulatory neighbor.

There are many reasons to study the association between polymorphisms of MHC and consequences of HCV infection. In terms of pathology is sought explanation for the phenomenon that some patients recovered without sequelae after infection while other patients develop end-stage liver disease and hepatocellular carcinoma. It is also important immunological mechanism that confers protection against infection immunological reaction. The clinical significance of the research is to highlight new prognostic markers to guide therapeutic conduct for each patient individually.

PERSONAL CONTRIBUTION

The study goals

Through this study I wanted to accomplish an analytical case-control study about the importance of the genetic markers of the host organism, gene alleles represented by major histocompatibility complex (HLA) class II HLA-DRB1 and HLA DQB1, in a group of patients infected with the hepatitis C virus and one group of controls, uninfected individuals, represented by unrelated, uninfected randomized subjects from the same population.

For this purpose we performed:

- The developing of an experimental model based on the documentation previously published in the literature;
- selection the tests of molecular biology and optimization of working protocols used for genotyping of analyzed subjects;
- establishing the criteria for setting up lots of patients included in the study;
- establishment of the control group;
- HLA-DRB1 alleles and determination of the HLA-DQB1 in all subjects studied, the specific procedures of molecular biology, in the Laboratory of Molecular Genetics of the Faculty of Medicine Constanta;
- setting up the data and information on the subjects included in the study;
- determining the existence of association between HLA-DRB1 and HLA-DQB1 loci and the developments of hepatitis C and establish the gene susceptibility and protection present in patients with hepatitis C through the statistical study compared the frequency of HLA-DRB1 and HLA-DQB1 in patients and control group ;
- determining HLA-DRB1 loci existence of association and HLA-DQB1 with certain risk factors related to the host (viral levels, degree of fibrosis) which may be present in patients before treatment;
- association of HLA-DRB / DQB1 and IL28B genotypes with clearence and viral persistence after double PegIFN and RBV therapy to define the patient profile that are more likely to achieve sustained virological response (SVR).

General methodology

The composition of the study

In the present study I aimed to conduct a study in order to associate HLA-DRB1 and HLA-DQB1 as genetic markers for clearence/persistent viral infected patients with hepatitis C to guide the prognosis favorable or less favorable terms disease progression and response to treatment with PegIFN / RBV.

The study focuses mainly on comparing the frequencies of the different alleles of HLA in the presence of certain risk factors in patients infected with hepatitis C with variable response to treatment and the subjects in the control group who could guide the clinically by some prognostic evolution disease.

To highlight genetic markers in patients with hepatitis C, I hypothesized that certain HLA-DRB1 / DQB1 will be present in significant numbers increased in the group of patients with increased viremic values and high degree of liver fibrosis, non-responders to treatment, representing alleles that confer risk for chronic and persistent viral, while other alleles of the same gene will be better represented in subjects in the control group / patients who achieved SVR 6 months after treatment, representing alleles protection for people infected with hepatitis C.

Subjects in the study

The study group included a group of 153 subjects of which 51 patients enrolled in the The Infectious Diseases Hospital Constanta, and a control group comprised healthy subjects unrelated from the same population, enrolled in the register of marrow donors in theClinical Institute Fundeni, Bucharest.

To enroll the subjects in the study I have participated in the periodic assessments of patients diagnosed with hepatitis C, between June 2012 - September 2014. Enrollment of subjects in the group of patients and control group was not difficult because the studied disease casuistry was rich in the mentioned Infectious Diseases Clinic, I met a relatively high number of patients and willing to participate in the study but nevertheless the material considerations have selected and enrolled in a study no. 51 cases from which I obtained consent for participation and blood samples for molecular determinations. Healthy subjects enrolled in the control group who participated in the survey were to be screened for enrollment in the National Marrow Donor Registry at the Center of immunogenetics and virology of Fundeni Clinical Institute, Bucharest.

Inclusion criteria:

- subjects diagnosed with chronic hepatitis C virus genotype 1b;
- Stable clinical status, including hematocrit stable
- A positive HCV antibodies using a laboratory test of the third generation;
- detection of HCV RNA by RT-PCR method positive
- liver biopsy in the past 18 months, Metavir score ≥ ≥ A1 and F1 or A1 ≥ and> F2
- ALT> 1.5 time normal range within 24 weeks before inclusion;
- patients who have not been treated with ribavirin or PEG-IFNalpha IFNalpha;
- albumin and bilirubin within normal limits;
- alpha-fetal protein-less than or equal to 3 times the normal range for the laboratory;

Exclusion criteria:

- significant clinical infections other than HCV, defined as any viral, bacterial, that requires specific therapy (anti-infective therapies must have completed at least 14 days prior to therapy with PegIFN / RBV)
- Co-infection with hepatitis B virus or human immunodeficiency virus (HIV);
- hemochromatosis, liver disease related to alcoholism, heart disease, hepatocellular carcinoma;
- pacient with increased liver cirrhosis;

Material and method

Genetic analysis itself was performed between June 2013 - December 2014 in the Laboratory of Molecular Genetics of the Faculty of Medicine, University "Ovidius".

All HLA-DRB1 and DQB1 alleles in subjects that I have studied were determined HLA typing technique adapted in order to apply this method in our laboratory. The techniques listed below are part of the protocol HLA typing of immunogenetics and Virology Center, Fundeni. This center is accredited EFI (European Federation of immunogenetics), accounting reference center for transplantation immunology in Romania, including HLA typing.

Laboratory methods

Identification of the type of histocompatibility antigens present on an individual cells is called HLA typing. Identification data of patients and witnesses and appropriate HLA haplotypes designated patient and witnesses are presented in Annex 5.

For all 153 subjects in the study we performed HLA-DRB1 and DQB1 alleles typing. For each subject were collected for HLA 2 ml whole blood collected in EDTA. Of this amount, 200-600 ml was used for genomic DNA isolation and the remaining blood was stored at -20 ° C.

From the collected blood was extracted genomic DNA, which was then analyzed by any of the methods of typing SSO (sequence-specific oligonucleotide probes) or SSP (sequence specific primers) for HLA typing. Protocols presented are standard operating HLA Laboratory of Molecular Genetics in the Faculty of Medicine finds.

The lab method chosen for HLA typing

For HLA-DRB1 DQB1 gene typing for the subjects in the study we chose initial use for all samples SSO method, which has lower costs. This method enables medium-high resolution typing at a locus allele HLA-DQB1 in most situations; Instead HLA-DRB1 locus, which is polymorphic, allelic group to obtain a resolution of (two digits - low) and high (4 digits) based on the combination of genes present. Another advantage of the method is that it can perform more than one determination being made as for the typing of many loci and for several patients at the same time. For when we got ambiguous results from the SSO typing method, or we could assign a resolution allelic result, I used the method with SSP kits for high resolution. This method, although it takes less than SSO method not only allows typing a sample once, and is less useful for large groups of patients. In addition, high-resolution typing method SSP has a high cost and therefore could not be used as a method of choice.

Extracting the genomic DNA from samples

To have used DNA isolation kits of reagents from the company Qiagen - QIAmp DNA Blood Mini Kit which are based on a quick method based on repeated centrifugation of filtering columns, with minimal sample manipulation - reduced contamination.

The concentration of DNA extracted from samples was determined using a photometer Nano, to compare the ratio of the concentration of nucleic acid in the sample (OD260) ratio contaminants, proteins,

and organic substances (OD280); salts and alcohol (OD230). Moreover the DNA isolated from blood samples (3µl) was loaded onto a 1% agarose gel and visualized by staining with ethidium bromide.

HLA polymorphism detection methods (HLA)

HLA typing method SSO HLA-DRB1 gene amplification and HLA-DQB1 PCR with sequence-specific DNA oligonucleotide probes (SSO)

The method principle

Polymorphic regions in genes HLA-class II were amplified from genomic DNA samples obtained from patients by a PCR reaction (Polymerase Chain) using primers complementary to sequences from the 5 'and 3' contiguous; sequences used to design primers are conserved between individuals, thus ensuring the amplification of the allele regardless of the type and gain of the two alleles present equal to heterozygous individuals (mostly).

After the PCR reaction, the resulting DNA was further used in hybridization reaction with sequence-specific oligonucleotide probes (SSO) chosen so as to detect polymorphisms regions. Stringency solutions used lashing and specific detection of single nucleotide even between different alleles. Alleles are identified by hybridization analysis of positive and negative reactions with oligonucleotide probes, each specific to a particular sequence.

SSO method chosen for further study allowed determination at the same time, and the analysis of the amplification products by hybridization to their specific SSO probes immobilized on a nitrocellulose membrane and developing a color reactions (Fig.13) to give low resolution -Media which meant potential for ambiguous results in some cases.

Detection kit is being used RELI SSO HLA typing systems from Invitrogen.

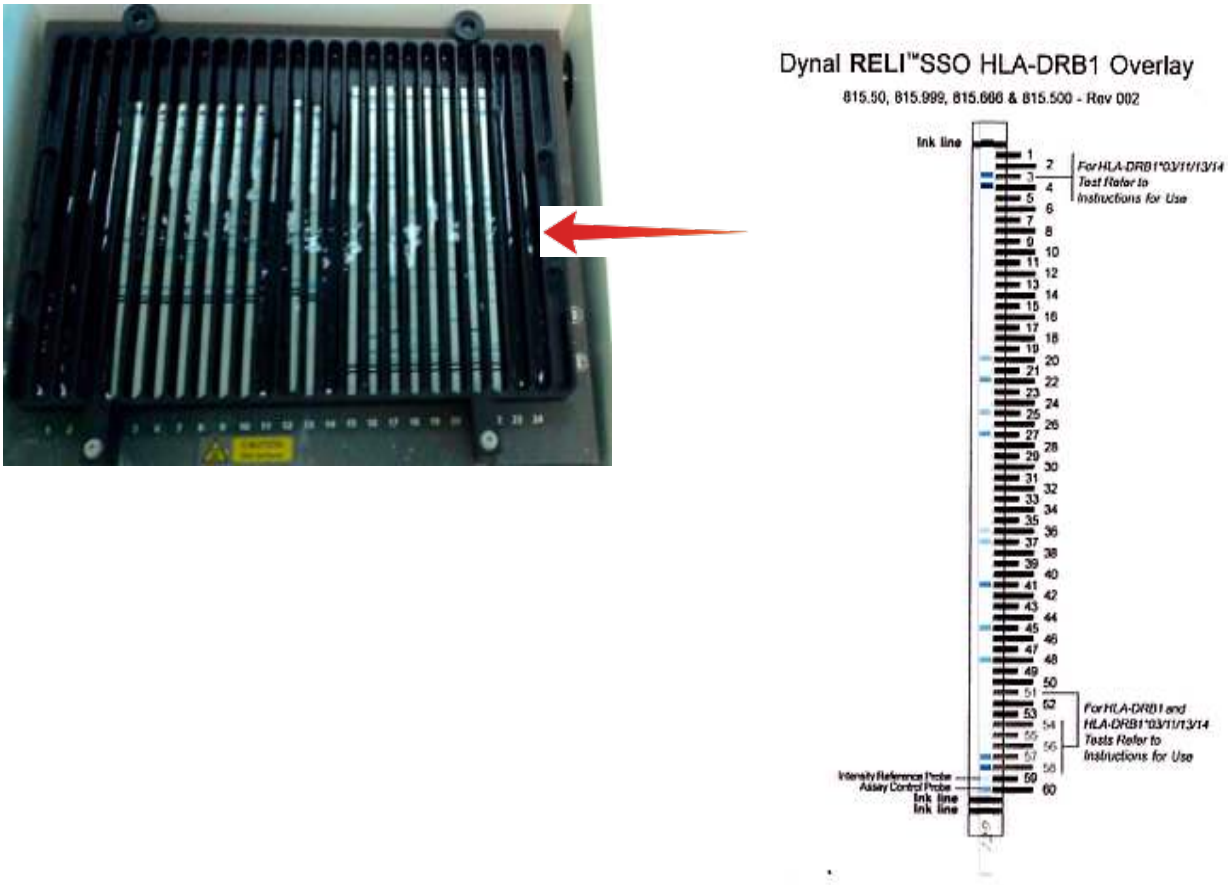


Fig.12 *Imagine a unei serii de stripuri, după finalizarea determinării RELI SSO HLA-DRB1*

Fig . 13 *Interpretarea rezultatului RELI SSO HLA-DRB1*

Cardul de citire HLA-DQB1 utilizat pentru identificarea liniilor pozitive de pe strip. 38 de poziţii corespund liniilor specifice, poziţiile 5 şi 41 reprezintă controlul de intensitate al probei, iar poziţia 40 reprezintă controlul pozitiv al reacţiei. Specificităţi pozitive: 3, 4, 20, 22, 25, 27, 36, 37, 41, 45, 48, 59, 60

SSP HLA typing method - HLA-DRB1 gene amplification and HLA-DQB1 with sequence-specific primers

Principiul metodei

Determination of HLA alleles by the method SSP was performed by amplifying a gene region of interest by PCR with primer pairs specific to different polymorphisms known, the analysis of the amplification reaction products by agarose gel electrophoresis and interpretation of the results. The method

is based on the fact that only primers whose sequences are perfectly complementary to the DNA in the sample will bind to it and will produce an amplicon in the PCR reaction. Non-complementary primers, on the other hand, does not bind to DNA, and the amplification does not occur.

The kits used were prepared containing different sets of primers in PCR with 20-96-well plates. The wells of the plate-dint aliquot is added a reaction mixture containing the reaction buffer, the genomic DNA; and Taq DNA polymerase. The plate was then sealed and placed in a thermal cycler for conducting the PCR amplification. At the end of the PCR amplification products was loaded onto an agarose gel, and stained with migrate ethyl bromide. The gel is then photographed and interpret the results. We analyzed the genes of class II HLA-DRB1 and HLA-DQB1 using two types of kits for commercial high-resolution typing: AllSet + ™ Gold SSP from Invitrogen ™ HLA SSP Kits and Protrans.

Statistical methods used in the study

Statistical data processing methods used in the case in combination studies were considered useful for determining the contribution of genes to susceptibility to a particular disease or phenotype that are commonly used in families or populations.

Although HLA genes are known for many years and all the major histocompatibility complex was sequenced, combination studies HLA are still used to identify genes predisposing and protective specific to certain conditions, to specify the profile immunogenetics of patients for risk assessment and sometimes for establishing therapeutic measures.

In study 1 and 2 I planed a case-control association study by determining the distribution of HLA DRB1 / DQB1 in a patient infected group with hepatitis C genotype 1b, between a group of patients diagnosed with HCV and a control group consisting of uninfected subjects clinically healthy persons recruited from the marrow donor registry.

I chose a case-control study that enables us to compare healthy people with diseased to identify a possible link between disease and risk factor thought to be involved in the production or its development (in this case, the presence of certain HLA -DRB1 and / or DQB1). The frequency of allele or haplotype frequency were calculated based on the total chromosomes, not the number of subjects in the study (number of chromosomes is twice the number of people), and the frequency of genotypes was calculated based on the number of subjects.

The experiment began with a null hypothesis which postulates that there is no difference between the two groups, drawn from the same population, in terms of the parameter tested. Then the null hypothesis was rejected by a statistical test, the alternative hypothesis (that there is a difference between the two groups) were successful. I used to test the statistical significance test $\chi 2$ (hi square) hypothesis was rejected when $\chi 2$ test provided a p-value <0.05, and to compare the two groups we used a 2×2 contingency table. To study the risk we conducted a statistical analysis using tests of association of HLA alleles in the two groups of patients responders and non-responders to interferon therapy and calculated the relative risk between the variable "Response to treatment" (non responder-Non-R / responder-SVR) and HLA DRB1 alleles variables / DQB1 (presence / absence)

The existence of a relationship of dependency (the association of relationship) was made by determining $\chi 2$, and the ratio of probabilities (odds) calculation. Exposure to patients and probability (odds) Exposure to witnesses, OR (odds ratio or the ratio of risk / chance), the ratio between the risk of having a positive (DRB1 / DQB1 - Present) in the group of patients Non-R and the risk of having a positive (DRB1 / DQB1 - Present) in the group of patients SVR, statistically significant value when confidence interval 95% CI for OR contains value 1 (equal risk). It was considered the following meanings of the RR value obtained:

RR = 1 risk allele does not change the test for response to treatment with interferon;

RR <1 in the presence of low risk allele tested allele modifies the risk for onset of response to treatment and acts as a protective allele conferring viral clearance - provides protection for illness;

RR> 1 risk allele in the presence of allele modifies the risk for developing non-tested treatment response and act as disease susceptibility allele leading to viral persistence and chronic disease associated with the occurrence of complications.

The program used in processing experimental data was statistical IBM SPSS Statistics software version 20, and the procedures used were: Descriptive statistics (characterization variables defined in the database), graphics, non-parametric statistical tests.

Study 1 The determination of distribution of HLA DRB1 and DQB1 group of patients infected with hepatitis C genotype 1b

3.4. Resoults

General analysis of the study group

The 153 patients in the study group had an average age at baseline of 40.01 years (range 13-75 years). The group included 76 female subjects (49.7%) with mean age 40.3 years (range 18-68 years) and 77 male subjects (50.3%) with mean age 39.6 years (range 13-75 years).

The number of patients who responded to therapy is higher (32) with little difference between the sexes, which is slightly higher for female patients in the No. 17 with an average age of 47.8 (range 26-68 years) compared with males who achieved SVR (15) to which the average age is 41.1 years lower (between 14 and 65 years).

Vârsta și sexul în raport cu răspunsul la tratament

Raspuns la tratament	Sex		nr.	Minimum	Maximum	Media	Deviația Std.
Non-R	Masculin	Varsta (ani)	13	25.00	75.00	43.0000	16.17096
	Feminin	Varsta (ani)	6	40.00	63.00	53.8333	9.36839
SVR	Masculin	Varsta (ani)	15	14.00	65.00	41.1333	16.30016
	Feminin	Varsta (ani)	17	26.00	68.00	47.8235	11.02971
Martor	Masculin	Varsta (ani)	49	13.00	63.00	38.3673	12.17527
	Feminin	Varsta (ani)	53	18.00	65.00	36.4151	11.63455

Table 5. The distribution by sex and age of the study group compared to treatment response

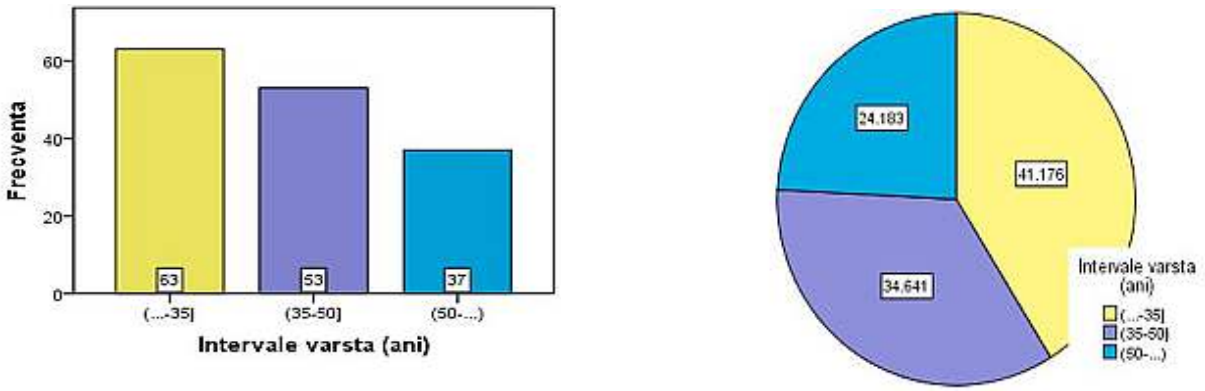


Figure 3. Distribution by age ranges of the studied group

HLA genotyping results obtained from subjects in the study group

The HLA-DRB1 and HLA-DQB1 individual alleles, genotypes and haplotypes DRB1 / DQB1 of the 51 patients and 102 control subjects are shown in thesis in Annex 5.

HLA-DRB1 * allele distribution in the patient and control group

By analysis of HLA-DRB1 alleles in the 51 patients with hepatitis C I have identified 102 HLA-DRB1 alleles with the following distribution shown in Chart. 4

DRB1 * 01 allele group with two alleles * 0101 (13.72%) * 0102 (2.94%), DRB1 * 07 (9.80%), and DRB1 * 11 * 1104 (6.86%) * 1101 (14.71%) which had the highest frequency among patients.

DRB1 * 04 allelic group exhibited the greatest variability of alleles (* 0401 * 0404 * 0405 * 0407) and have not found any allele from DRB1 * 12 and * 08 groups.

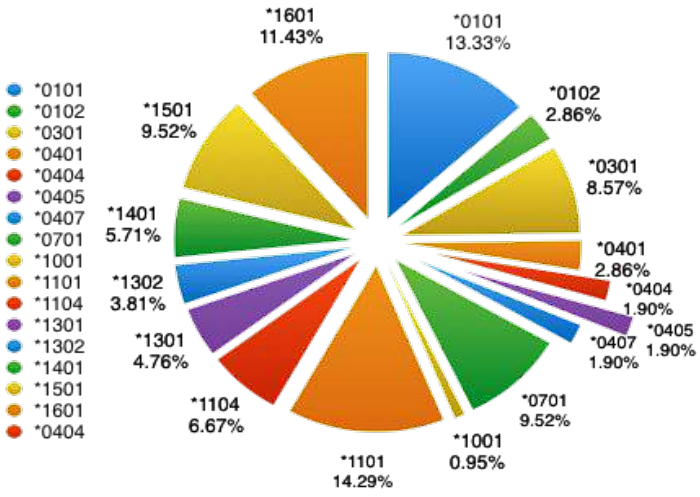


Figure 4. The frequency of HLA-DRB1 alleles in the group of patients

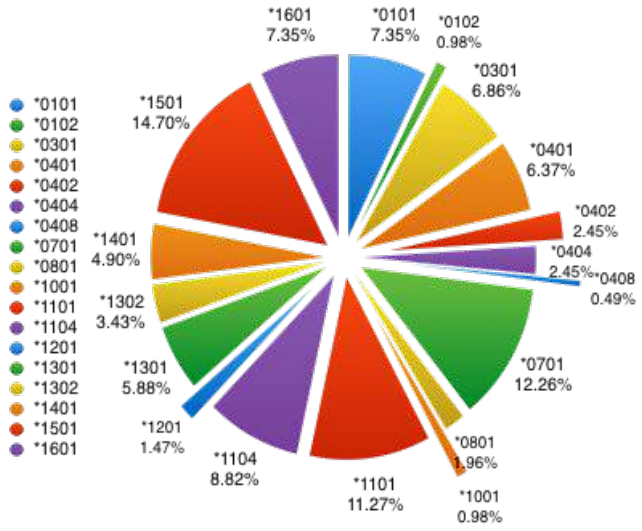


Figure.5. HLA-DRB1 alleles Frequency in the control group

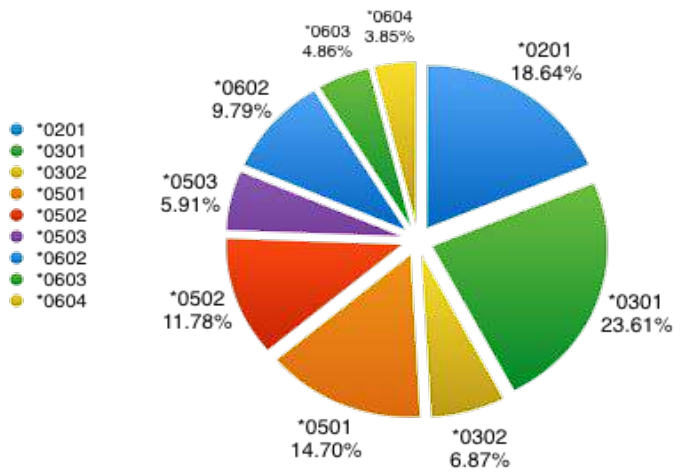
In the control group the 102 subjects have identified HLA-DRB1 alleles 204, with the following distribution shown in Figure. 5.DRB1 * 03 allelic group (6.86%), DRB1 * 04, DRB1 * 07 (12.25%) and DRB1 * 13 allele * 1301 (5.88%) and 1302 * (3.43%) were the most frequent alleles in group Control.

HLA-DRB1 * allele distribution of the patient population and the control group

HLA-DQB1 alleles were determined in 153 subjects with SSO method. Because DQB1 locus polymorphic and there is less information on linkage disequilibrium with these genes are transmitted, I could designate the 306 allele, unambiguous method by typing SSO.

DQB1 allele distribution of the patient population

By analysis of the HLA-DQB1 alleles in patients with hepatitis C in the study were identified for HLA-DQB1 102, with the following distribution shown in Figure No.7.



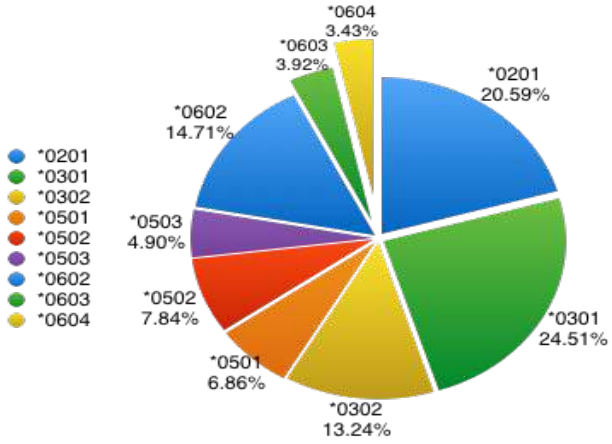


Chart 7. *DQB1 allele frequency of the patient population* **Chart no.8**. *DQB1 allele frequency in controls*

- alleles from the group DQB1 * 02 (18.63%), DQ7 allele * 0301 (23.59%), DQB1 * 05 three allele * 0501 (14.69%), allele * 0502 (11.76%) * 0503 (5.90%) were most commonly in the patient.

- By analyzing HLA-DQB1 in control group we have identified 204, HLA-DQB1 alleles with the following distribution shown in Chart no.8.
- Alleles of DQB1 * 02 * 0201 group (20.59%), DQ7 * 0301 (24.51%) are significantly better represented than other DQB1 alleles in the patient group.
- ***Distribution haplotype DRB1 / DQB1***
- Haplotypes HLA DRB1 / DQB1 gene region is transmitted to progeny through stronger linkage disequilibrium that exists between the two loci. After designating HLA-DRB1 and DQB1, haplotypes were designated in turn using the database for major histocompatibility complex (dbMHC) of NCBI, electronic resource created with the Medical University of Gratz, Austria. (234) The most common

haplotype DRB1 / DQB1 which may occur in populations in Europe are presented in thesis in Annex 3.

Distribution haplotype DRB1 / DQB1 the patient population

I designated the 102 haplotypes DRB1 / DQB1 in all patients, represented by a number of 17 combinations with the following distribution (Chart 8) and the control group have designated the 204 haplotypes DRB1 / DQB1 alleles in 18 combinations. (Chart No. 11)

Haplotypes with the same allele group DR11, *1101 and *1102 were the most common haplotypes (21.56%) in the combination *1101 / *0301 (14.70%) and * 1102 / *0301 (6.86%) and haplotype *1601 / *0502 was the second frequency in the group of patients (11.70%)

haplotype * 1501/ *0602 was the most common haplotype (18.62%) and haplotype * 0701/*0201, was the second in frequency in the control group (12.25%) in contrast the poorly represented haplotypes were * 0801/0302 * 4 cases (1.96%), haplotype * 1201 / * 0301 found in 3 cases (1.47%) and haplotype * 1001 / * 0501 in only one case (0.49%);0501 in only one case (0.49%);

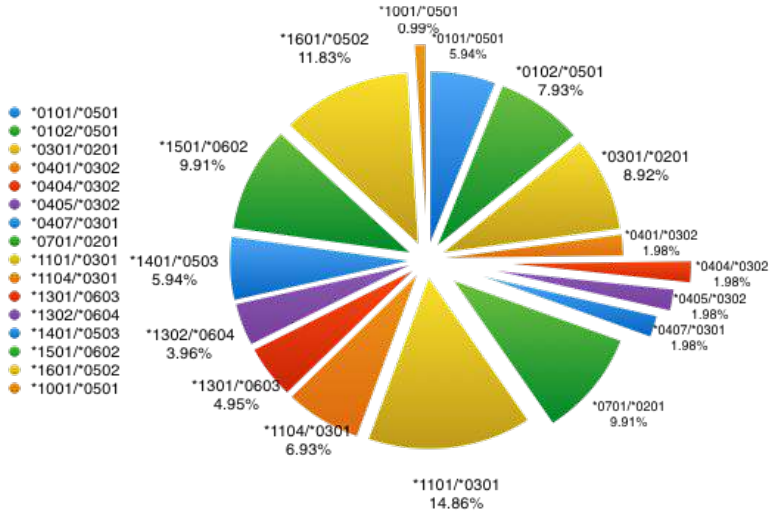


Chart 10.. HLA-DRB1 / DQB1 haplotype frequency in the group of patients

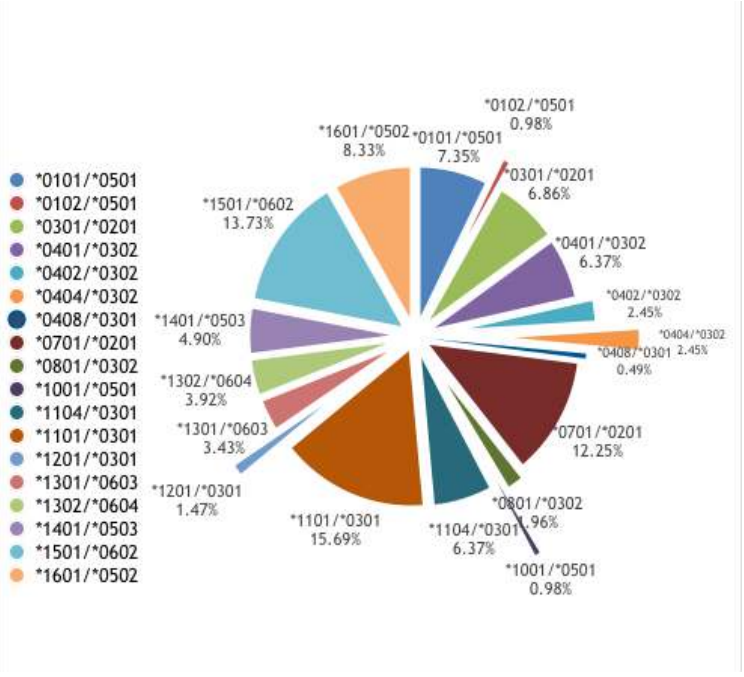


Chart no.11. HLA-DRB1/DQB1 haplotype frequency in the control group

Conclusions:

Alleles of the DRB1 group were present in a proportion significantly increased in the group of patients were * 0101 (16.66%) * 0301 (8.82%), * 1601 (11.76%) and allele group that prevailed in the

control group was represented by 0701 (12.25%) * 1104 (8.82%) and * 1501 (14.70%).

When were compared all frequencies of alleles group DQ in the group of patients and control group, distribution polymorphisms allelic prevailed in the control group by alleles group DQB1 * 02 were more frequent in the control group (20.59%) as the group serological DQ7 (24.51%) and DQB1 * 0302 allele (13.24%) and DQB1 * 05 allele group were significantly higher in patients (15.69%).

Study 2 - Correlation between evaluating different possible genetic risk factors present in patients before treatment and the presence of HLA-DR-DQ

Material and method

In the present study, we investigated the association of the onset of viremia and the degree of hepatic fibrosis with respect to HLA genotypes in patients infected with the hepatitis C virus.

Depending on the degree of fibrosis and corresponding score necro-inflammatory 'activities METAVIR patients were divided: patient-minimal fibrosis (F0-F1, F1: portal fibrosis without septa HSCs; A0, A1, A1: minimum activity), patients with grade moderate fibrosis (F1-F2, 2- portal fibrosis with rare septa A1-A2 A2 moderate activity) and patients who experienced a relatively severe degree of fibrosis (F3, F3 F4-fibrosis with many septa, without cirrhosis, A2 -A3- activity necroinflammatory moderately marked).

Depending on the load values before treatment viremic patients were divided into two groups as follows: patient-than-average viremia with 1,000,000 copies / ml - 5,000,000 copies / ml and patients with high viral load greater -very 5000 .000 - 25,000,000 copies / ml and above.

Results:

Of all patients in the study 29 patients had elevated basal levels of viremia (HCV RNA) correlated with higher levels of hepatic transaminases before starting standard treatment with PegIFN / RBV. Of the 102 alleles in patients with HCV present in a number increased alleles were present in 58 patients who presented high vs cargo viremic 22 cases of patients who presented viremia small-medium (a total of 44 alleles). Regarding the degree of fibrosis presented by patients at study group has a distribution generally stable, with a slight difference is observed between the group of patients who developed fibrosis minimal 17 cases (34 alleles studied, approximately 33.3% of study group), 20 cases (40 alleles studied, 39.2% of group total) representing a degree of fibrosis patients with moderate to severe fibrosis patients with 14 cases (28 alleles studied, 27.4% of the study group).

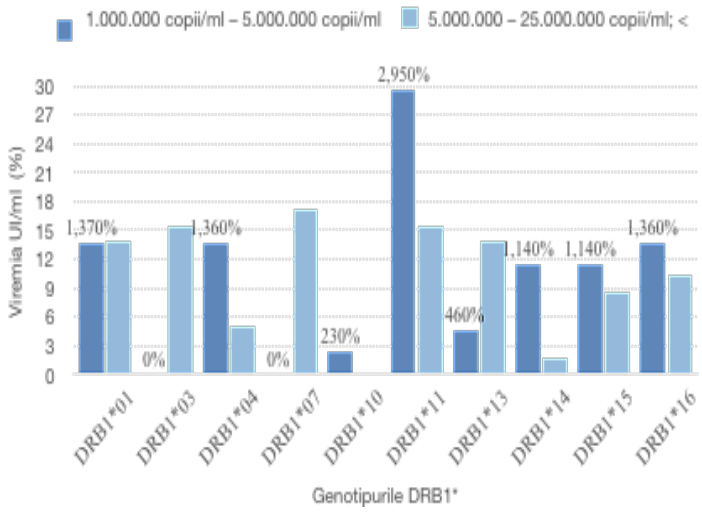
HLA-DRB1 alleles distribution -DQB1în compared with viremia at onset in patients with HCV

DR alleles groups * 03 and * 07 were present only in patients with high and very high viremia in the thesis presented in Table 10:

- In the group of patients with severe viremia *0301 allele (15.5%) *0701 allele (17.2%) were well represented and grupulrile DR * 11 allele (43.1%) and * 04 allele *0401, *0404, *0405, * 0407 were better represented in patients with low-moderate viremia. (Chart No. 13)

By analyzing HLA-DQB1 in patients with hepatitis C in the study were identified HLA-DQB1, with the following distribution in the thesis presented in Table No.11

DQ*02 group alleles were present in all 19 cases in patients with high viral load * 0201 allele having represented the largest share in this category viremia alleles compared with 32.8% of others groups.



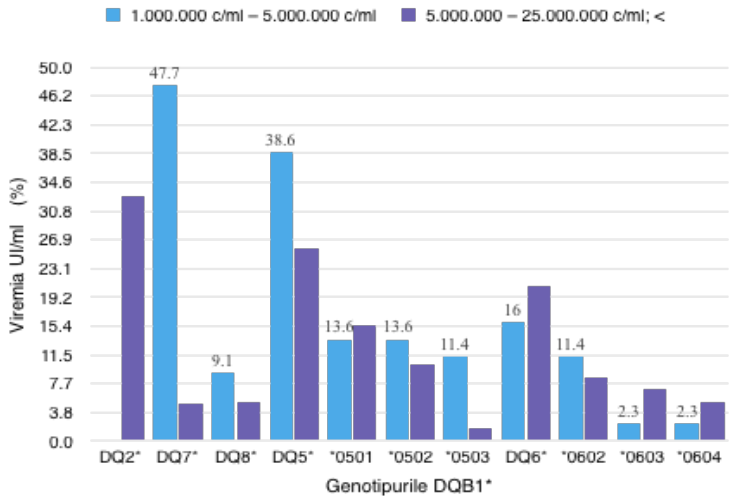


Chart No.13. The association of HLA DRB1 *genotypes with the onset of viremia

Chart No.14. The association of HLA -DQB1 * with the onset of viremia viremia

- DQB1 *03 allelic heterogeneous group accounted for most of the alleles in the group of patients with low-moderate viremia (44, 43.2%) and group alleles DQ2 were better represented in the group of patients with viremia high and very high 38.2% are absent in the group of patients who have small or medium viremia. (Chart 14)

The association of HLA-DRB1, DQB1 with the degree of fibrosis in patients with HCV

HLA-DRB1 allele distributions and their association with the degree of liver injury are presented in Table No sentence. 13 Chart no. 16.

Group allelic DR * 03 represented by allele 0301 was not present in patients with fibrosis minimal, being distributed fairly evenly in the other two groups of patients with fibrosis moderate (10%) and severe (17.8%) also group DR * 07 * 0701 allele has the highest share among patients with severe fibrosis (15%).

DR * 11 allelic group was by far the best represented in the group of patients with minimal fibrosis and moderate represented by two alleles: - * 1101 allele (37.6%) * 1104 allele (17.2%

Analysis of HLA-DQB1 in patients with hepatitis C in the study identified alleles with the following distribution graph No.17:

DQ * 02 alleles were more frequent in the non-R group in patients with very severe fibrosis 11 cases, 39.2% compared with the group of patients with minimal fibrosis where we found one case with this allele.

DQ * 03 allelic heterogeneous group accounted for the alleles in patients group is minimal fibrosis 25.4% moderate 38.1% (Chart no. 17)

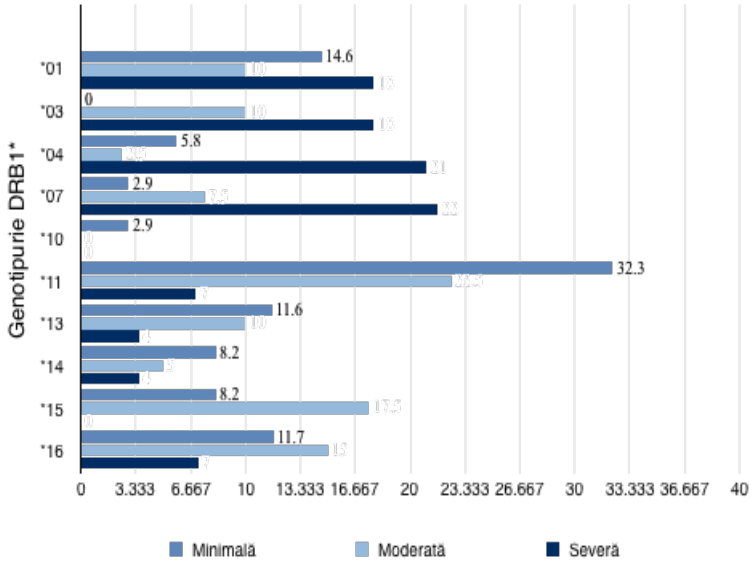


Chart No.16. T*he frequency of DRB1 genotypes depending on the degree of liver fibrosis (score Metavir)*

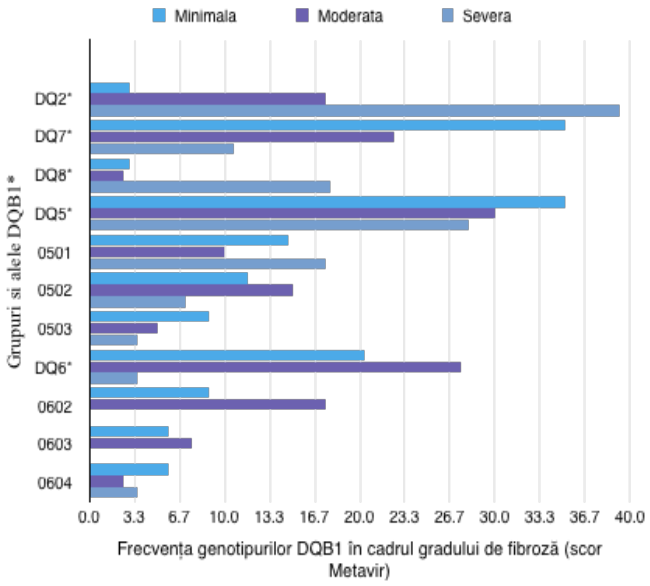


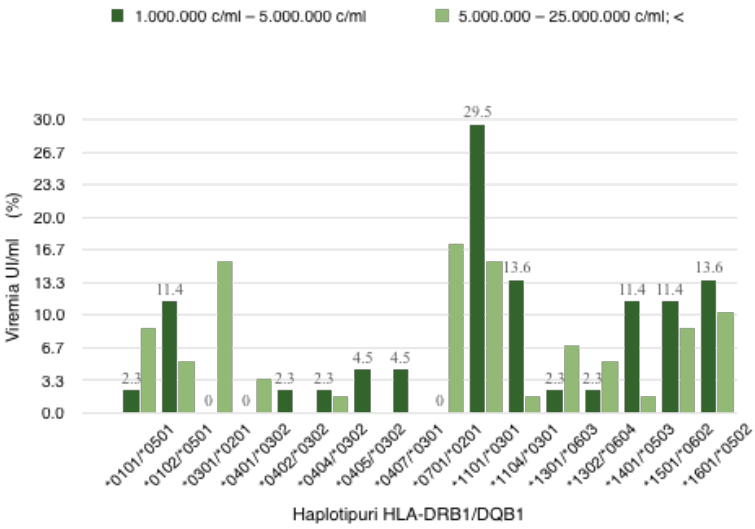
Chart No.17. *HLA DQB1 distribution of alleles* depending on the fibrosis degree

HLA haplotype DRB1-DQB1 association with viremia at onset and degree of fibrosis in the case patients with HCV

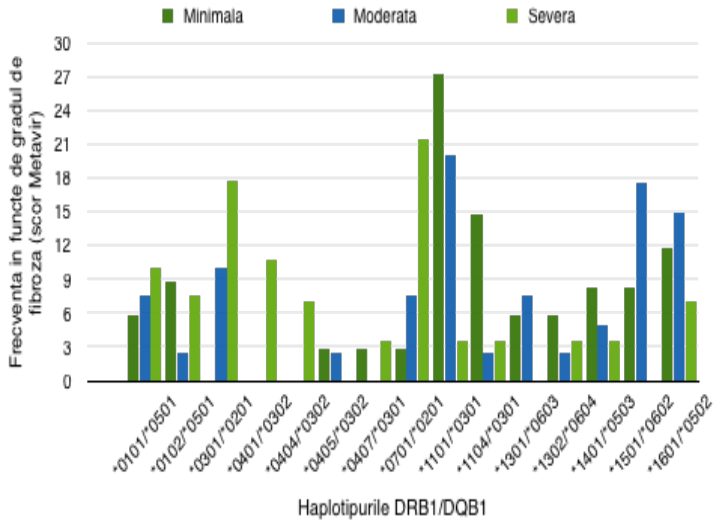
The biggest share is represented by haplotype * 0701 / * 0201 in viremic patients with high values before treatment (Graph.15) and patients who had low viral load averages and presented their most common haplotype * 1101 / * 0301.

The biggest share is represented by haplotype * 1101 / * 0301 in patients who had minimal fibrosis before treatment (graph. No. 18), and patients with moderate fibrosis presented their most common haplotype * 1101 / * 0301, * 1501/0602 or * 1601 / * 0502. In contrast haplotype * 0701 / * 0201 haplotype is more frequently present in patients with marked fibrosis.

HLA haplotype DRB1



Graph nr.15. *HLA DRB1/DQB1 haplotype depending on the viral load of patients*



Graph nr.18.*HLADRB1/ DQB1 depending on the degree of* fibrosis

Conclusions:

DRB1 locus, alleles observed to confer predisposition to viral persistence and chronic group were from DR * 03 and * 07 which were present only in patients with severe viremia, * 0301 (15.5%) * 0701 (17.2 %), the same allelic groups were equally well represented in cases with severe fibrosis * 0701 (15%), moderate fibrosis * 0301 (10%) and severe (17.8%). We found a higher frequency of allele * 11 of the DR group well represented in viremic patients with low and moderate values (43.1%) also was by far the best represented in the group of patients with mild fibrosis, * 1101 (37.6%). Alleles DQ2 group is best represented by * 0201 allele in the group of patients with high HCV-RNA (38.2%) and moderate-marked

necroinflamatorie activity.

Study 3 - Correlation between change in response to treatment with Peg IFN / RBV and the presence of HLA-DRB1-DQB1 and IL28B genotypes in patients with hepatitis C

Target of antiviral therapy with IFN / RBV was sustained virological response ("virological sustained response," SVR) defined as undetectable HCV RNA 6 months after completion of treatment that characterized group of patients SVR and the lack of virological response ("non- response ") defined by a decrease in viral load of <2 log10 from baseline to 12 weeks after start of treatment defined group of patients non responders (non-R) who stopped the treatment of antiviral later in week 12.

Patients who remained negative for HCV RNA during this period were grouped as SVR while others were grouped as Non-R. A lower overall average age was observed in all patients achieved SVR regardless of gender and patients in the control group (average for males 13 years 38.367 min - max 63 years and 36 years 41.5 in women (min 18 years -max 65 years). (Table.16)

Răspunsul la tratamentul cu RBV/IFN	nr.	Frecv (%)	Sex (%)		Media de vârstă		Deviația standard	
			Masculin	Feminin	Masculin	Feminin	Masculin	Feminin
	(n=153)		n=77 (50,3%)	n=76 (49,7%)	min/max	min/max		
SVR	*32*	*20,9*	*15*	*17*	*41.133(25-75)*	*47.823(26-68)*	*16.300*	*11.029*
Non-R	*19*	*12,4*	*13*	*6*	*45.000 (24-65)*	*53.833(40-63)*	*16.170*	*9.368*
Lot control	*102*	*66,7*	*49*	*53*	*38.367(13-63)*	*36.415(18-65)*	*12.175*	*11.634*

Table.16. The frequency of cases studied patient groups SVR, Non-R and the control group by gender and age average, n = no. of subjects.

HLA-DRB1 alleles DQB1 association with response to treatment with PegIFN / RBV

When the frequency of alleles for 32 patients (P) SVR (2n = 64) and 19 patients non-R (2n = 38) at each locus was compared with that obtained for the 102 Control (C) (2n = 204), a statistically significant difference was found for the HLA-DRB1 * 0101 allele in both comparisons made between groups vs. SVR C (p = 0.003) and non-R vs.C (p = 0.06), showing an association with the group of patients being found at a higher frequency (7.8%) than in the control group (1%). Another statistically significant association was found for DRB1 * 0701 (P = 0.012), when the groups were compared SVR vs. C, with a higher frequency

in the control group 12.3%. * 07 allele also reached statistical significance when comparing the groups and non-R vs.C (p = 0.043), being one of common alleles in patients non-R 23.7%. Other alleles that have statistical significance after comparisons between groups were DRB1 * 0301 patients with non-R group (p = 0.005) and DRB1 * 1101 (p = 0.001) of patients with SVR group.

Following comparisons made between DQB1 allele frequency between patient groups vs. SVR C and non-A group of patients vs.C one allele DQB1 * 0201 was found with statistical significance. p = 0.002, OR 0.136 95% CI 0.03-0.58 it with greater frequency in group C, and when comparing groups vs. non-R C (23.1%), the significant association with group C was observed throughout DQB1 * 0201 allele where, p = 0.01, OR 3.42, 95% CI 1.65-7.09.

The risk on the response to hepatitis C treatment in patients with HLA-DRB1 alleles conferred by-DQB1 in the group of patients studied

A highly significant association was found for HLA-DRB1 *0701 (P <0.001), the risk of having patients present in the group of Non-R is 19.55 times greater, 95% CI pt. OR = (2.36 to 161.63) and the risk of not achieving SVR is 15.15 times higher than the Others The patients. IC 95% pt. RR = (1.99 to 115.02). (Table 19) Another significant association in this group compared to the allele DRB1 * 0301 was a non-responder patients (P <0.001), the risk of patients having with DRB1 *0301 Non-R group present is 16.80 times the IC 95% pt. OR = (2.00 to 140.50) and the risk of non-response to treatment is 13.47 times greater than Others The 95% CI for patients. RR = (1.75 to 103.59). (Table 19) The statistical analysis revealed * 1101 allele that is associated with the response to treatment in this case p <0.001, the risk of having DRB1 * 1101 patients with Non-R group present is very low 95% CI 0.30 pt. OR = (0.09 to 0.96) and the risk of not responding to treatment is also low in these patients 95% CI 0.37 pt. RR = (0.13 to 1.02).

To determine the relative risk in the response to treatment in patients with hepatitis C frequency of HLA DQB1 in the patient non-SVR was compared with the group of patients who achieved SVR and a highly significant association was found for HLA-DQB1*0201 (p <0.001) in patients with the risk of having DQB1*0201 present in the non-R group is 9.25 times the 95% CI for. OR = (5.34, 117.81), and the risk of not responding is 14.31 times higher compared with 95% for patients SVR IC. RR = (3.49, 58.58). (Table no.21). Another significant association in this comparison group was the DQB1 * 0301 allele patients SVR responders to therapy (P <0.01), OR 0.25, 95% CI .08-.82), they risk very low 0.33 times of not responding

	non-R vs. SVR							
Alela/ locus	nr.	frecv.	χ^2	p val.	OR	95% C.I	RR	95% C.I
DRB1	nonR/ SVR	non-R/SVR (%)	non-R +SVR (2n=102)					
0101	1/5	2,6/7,8	0,156	0,282	0,31	0,03-2,83	0,33	0,04-2,77
0102	3/5	7,9/7,8	0,001	0,988	1,01	0,22-4,49	1,01	0,25-3,99
0301	8/1	21,1/1,6	11,258	**<0,001**	16,80	2,00-140,50	**13,47**	1,75-103,59
0401	1/2	2,6/3,1	0,020	0,887	0,83	0,07-9,56	0,84	0,07-8,97
0404	1/1	2,6/1,6	0,142	0,707	1,70	0,10-28,03	1,68	0,10-26,15
0701	9/1	23,7/1,6	13,195	**<0,001**	19,55	2,36-161,63	**15,15**	1,99-115,02
1101	4/18	10,5/28,1	4,365	**<0,01**	0,30	0,09-0,96	**0,37**	0,13-1,02
1301	2/3	5,3/4,7	0,017	0,896	1,13	1,18-7,08	1,12	0,19-6,42
1302	1/3	2,6/4,7	0,267	0,605	0,55	0,05-5,48	0,56	0,06-5,20
1501	2/8	5,3/12,5	1,412	0,235	0,38	0,07-1,93	0,42	0,09-1,88
1601	6/6	15,8/9,4	0,945	0,331	1,813	0,54-6,08	1,684	0,585-4,852

Tabelul. 19. *Asocierea alelelor HLA -DRB1 cu răspunsul la răspunsului tratamentul cu FN/RBV studiu comparativ între grupul pacienților non-responderi (non-R)și pacienții responderi (SVR). Alele cu semnificație statistică sunt marcate cu caractere în bold, unde “- ” reprezintă alela lipsă, n= nr. de cromozomi.*

	non-R vs. SVR							
Alela/ locus	nr.	frecv.	χ^2	p val.	OR	95% C.I	RR	95% C.I
DQB1	nonR/ SVR	non-R/SVR (%)	non-R +SVR (2n=102)					
DQ2*								
0201	17/2	44,7/3,1	27,237	**<0,001**	25,09	5,34-117,8	14,31	3,49-58,58
DQ7*								
0301	4/20	10,5/31,2	5,691	**<0,01**	0,25	0,081-0,82	0,33	0,12-0,91
DQ8*								
0302	2/5	5,3/7,8	0,242	0,622	0,65	0,12-3,58	0,67	0,13-3,30
DQ5*								
0501	4/11	10,5/17,2	0,843	0,358	0,56	0,16-1,92	0,61	0,21-1,78
0502	1/5	2,6/7,8	0,156	0,282	0,31	0,03-2,83	0,33	0,04-2,77
DQ6*								
0602	2/8	5,3/12,5	1,412	0,235	0,389	0,07-1,93	0,421	0,09-1,88
0603	2/3	5,3/4,7	**0,017**	0,896	1,130	1,18-7,08	1,123	0,19-6,42
0604	1/3	2,6/4,7	0,267	0,605	0,550	0,05-5,48	0,561	0,06-5,20

Tabelul.21. *Riscul alelelor HLA –DQB1 în cadrul la terapia cu PegIFN/RBV prezentată în comparație între grupul de pacienți SVR și grupul de pacienți non-R: SVR vs. grupul non-R. Asociatia cu boala (fie pozitivă sau negativă) în cadrul grupului alelic DQB1 este evidențiată cu caractere bold. Alelele individuale sunt prezentate cu caractere italice. Frecvența alelică a fost calculată ținând cont de numărul diploid de cromozomi în fiecare grup (2n). Unde n=numărul de cromozomi.*

IL28B interleukin gene polymorphism association with response to treatment with PegIFN / RBV in HCV patients group

In the analyzed group there is a low percentage of patients homozygous C / C gene IL28B (15.1%), profile confirmed as predictor of favorable evolution, being associated with obtaining rapid virological response (RVR) and sustained virological response (SVR).

Rs12979860 C allele frequency is observed in a proportion of 62.5% in patients with genotype C/C

achieved an SVR compared with 48.3% in SVR patients who were heterozygous C/T. In this study we found that although C alleles are more common among patients with SVR, paradoxically were associated with higher levels of HCV RNA (viremic high loads 5000000-25000000 copies / ml; < 25.000.000 copies / mL) as compared to patients carriers of the allele T

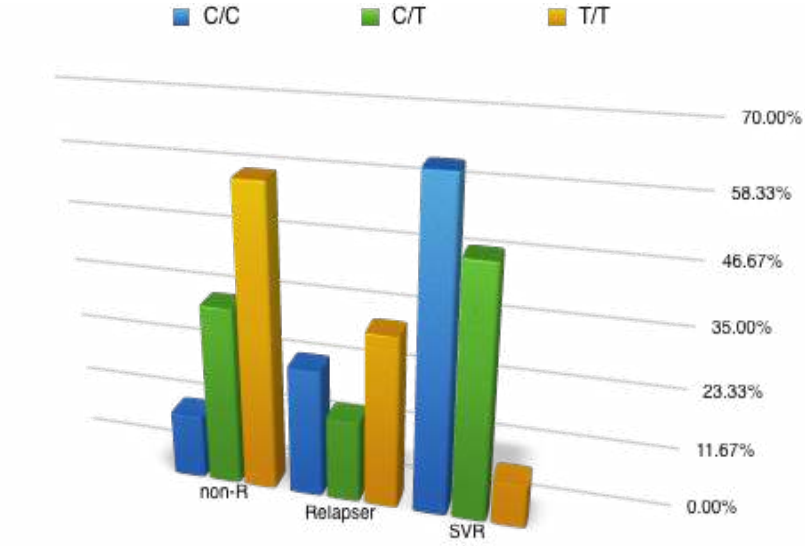


Chart no.21. IL28B gene polymorphism association with interleukin
Response to treatment with PegIFN / RBV in HCV patients group

Higher levels of HCV RNA were observed in some patients before treatment and patients were homozygous carriers of the allele C allele carriers T/T were highlighted in a high percentage in the patient non-R 58.3% in the group of patients who relapsed (33.3%), being present in a small percentage of patients who achieved SVR case during therapy (8.4%).

Conclusions:

In the study the correlations between variation in response treatment with PegIFN / RBV and the presence of genotype HLA-DRB1-DQB1 and IL28B patients with hepatitis C have observed both positive associations (allele/haplotype confer risk for disease) and negative (allele/haplotype It provides protection for illness). Analysis of relative risk (RR) given locus alleles HLA-DRB1 of the relative risk values were between 25.09 (the predisposing allele) and 0.03 (allele that confers the greatest protection). In the study presented was shown a relatively high number of patients with genotype homozygous C/C who had relapsed at different time intervals after treatment and there may be the possibility that patients with genotype favorable C / C to provide an adequate rate SVR after treatment standard for a shorter period (24

weeks vs. 48 weeks or longer). Another explanation is given by a recent study which showed that the risk of relapse after standard antiviral Usually treatment may also be dependent on minimal residual viremia and not IL28B genotype.

General conclusions and research perspectives

- In univariate analysis present patients with sustained virological response have aged significantly lower (mean 42.8 years), comes mainly from urban areas, have polymorphism CC of the IL28B and values relatively high viral load baseline (HCV RNA) at the time initiating therapy with IFN. Also, these patients had low values of significance; Metavir score for evaluating the degree of liver fibrosis and necroinflammatory activity and favorable virological response at 4 and 12 weeks of treatment (RVR or EVR rapid viral response).
- When comparing the two groups of patients when the response to treatment, significant associations were observed at different loci, especially when the group of non-R was compared with the group SVR, a highly significant association was found for DQB1 HLA-DRB1 * 0201 and HLA-patients in 0701 * Non-R, DRB1 * 0701 allele with a frequency significantly higher in this group compared to SVR. As one of common alleles in patients non-R but also in the control group it can be argued that the study population is one risk for viral persistence.
- Another association with viral clearance significant was found also in the case of HLA-DQB1 * 0301 where the group of Non-R was compared with the group of SVR, suggesting that there may be a trend in the association of this alleles with liver disease "less severe".
- These observations are interesting because they indicate a possible mechanism by which HLA-DQB1 * 0301 is somehow involved in viral clearance in patients with spontaneous natural immunity or an immune system supplemented by treatment with IFN and we can assume that the studied patients who had HLA DQB1 * 0301 could not be achieved spontaneously eliminate the virus, but had SVR after IFN therapy.
- Similarly, comparisons of groups were performed for haplotypes, where only two haplotypes of HLA-DRB1 * 0701 / DQB1 * 0201 and HLA-DRB1 * 1101 / DQB1 * 0301 showed significant association when the group of non-R was group compared with SVR.
- Because allele DRB1 * 1101 was found at a lower frequency compared to patients non-R, I

assumed that this is probably responsible for viral clearance, in agreement with previous studies in other European Caucasian population.

- Higher levels of viral load (HCV RNA) in patients carriers of the C allele which is considered to be associated with a higher rate of SVR in the present study could be explained by the fact that although differences values viremic are not very high, higher levels of HCV RNA in patients with genotype C / C might facilitate the activation of the innate immune system and the infection control during treatment. You might assume that in addition to the baseline HCV RNA level, IL28B genotype may influence the kinetics and response to treatment with PegIFN / RBV.
- Available data in the study may guide on patients having genotype unfavorable (C / T or T / T) could be treated as "difficult to treat" in the same way as they are considered other prognostic factors negative (eg, advanced fibrosis) however further studies are needed on a larger group of patients to confirm this effect, it is known that genotype IL28B has a positive predictive value of 100% SVR, so should not be used as the sole predictor in determining therapeutic decisions.
- In the group of subjects analyzed strong association between DQB1 * 0201 response unfavorable treatment suggest that no haplotype but rather an allele is involved in non response to treatment which is in disequilibrium linkage is present both in the composition haplotype DRB1 * 0701 / DQB1 * 0201 and DRB1 * 0301 as / DQB1 * 0201 * 0701 and both alleles DR * 0301 is present in patients non-responder.
- So far I have not found any other study that demonstrated the association of a haplotype or allele HLA-DRB1 / DQB1 with the evolution of HCV patients in Romania, which is proved by other similar studies conducted in patients from other populations, indicating again the need for to repeat such studies
- Information presented in the study could have important implications for the management of patients with HCV genotype 1 in Romania, unlike other studies reported in various European and non-European populations, they found a higher rate of response to treatment in patients with HLA DRB1 * 0701, we found that these patients in our population could not achieve viral clearance (SVR) considering it a risk allele in the present study population for the date.
- This is important if he is confirmed by other studies in our population, because in normal clinical

practice in Romania patients treated with standard therapy IFN / RBV (due to the problems of high cost) and if HLA typing in these patients could be achieved before treatment and one allele or haplotype associated with viral persistence was found, these patients could receive more aggressive treatment, instead of being treated with standard therapy.

- Important implications of the current study are only relevant for patients receiving standard therapy IFN / RBV and may not be for patients who received other therapies
- The study is available for less developed countries where patients can not afford other standard therapies and the treatment is largely first choice.
- Estimating the evolution of HCV infection currently based solely and only on genotype HLA is very unlikely to use in clinical practice because it was established the relative risk conferred by the possession of the particular alleles specific to not be all too high (generally between 2 and 4), especially when it calculated in the general population and usually take in considerer low frequency alleles in the general population, it is as a result of the high degree of polymorphisms at MHC loci.

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