„CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST, ROMANIA

DOCTORAL SCHOOL

FACULTATY OF MEDICINE

DISSERTATION

SUMMARY

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Ph.D. Student:

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BUCHAREST

2022

„CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST, ROMANIA

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The impact of the interferon-free treatment in hepatitis C virus. Biochemical, imagistic and epigenetic perspectives

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1. Isac T, Isac S, Rababoc R, Cotorogea M and Iliescu L: Epigenetics in inflammatory liver diseases: A clinical perspective (Review). Exp Ther Med 23: 366, 2022 https://doi.org/10.3892/etm.2022.11293
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5. Elena L. Iliescu, Mihaela Grumeza, Adriana Mercan, Letitia Toma, Mihai Dodot, Teodora Isac. Sa1526 – The Impact of Ombitasvir/Paritaprevir/Ritonavir with Dasabuvir in Patients with Hcv Associated Cryoglobulinemia- Experience of a Single Center. Gastroenterology, Volume 156, Issue 6, Supplement 1, 2019, Page S-1232, ISSN 0016-5085. doi: 10.1016/S0016-5085(19)40074-7.
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INTRODUCTION

Hepatitis C infection is a major global health problem, with a significant socio-economic impact, not solely due the impaired quality of life and complications but also due the significant pressure on the health systems worldwide, being a chronically progressive disease with the need for repeated hospitalizations. The prevalence of HCV infection is globally 2.2%. Chronic hepatitis HCV increases the risk of complications such liver cirrhosis or hepatocellular carcinoma.

The main objective of the doctoral thesis is to evaluate patients with hepatitis C virus (HCV) infection in acute phase, to monitor them , highlighting new elements in assessing the progression of liver disease and the response to treatment. In addition, this thesis aims to describe an epigenetic approach to the disease, assessing miRNA (miR) species, as modern diagnostic and treatment markers in HCV infected patients.

Therefore, the thesis aims to monitor the dynamics of alpha-fetoprotein (AFP) in HCV infected patients before and after direct antiviral agents therapy (DAA), as well as the degree of liver fibrosis measured by FibroScan and the incidence of mixed cryoglobulinemia (MC).

The thesis purposes an innovative epigenetic approach, by assessing various miR species in plasma: miR 7-1, miR 21, miR 122 and miR 885 of HCV infected patients in acute phase and after achieving sustained viral response (SVR) and to establish a correlation with the degree of cytolysis, cholestasis, inflammation, viral load and hepatic fibrosis.

The scientific research methodology included the following steps:

The study was performed on 63 patients: of these 36 patients, healthy volunteers, constituted the control group (C) and 27 patients constituted the study group (VHC and RVS groups). The 27 patients included in the study group were evaluated at the time of diagnosis of HCV infection (VHC) and 3 months later (RVS).

The first stage of the doctoral dissertation focused on the comparative analysis of demographic data. The following parameters were considered: age, patient gender, social status, smoking status, body mass index (BMI).

Further, plasma level of albumin (g/dl), AST (U/l), ALT (U/l), GGT (U/l), AP (U/l), DB (mg/dl) and TB (mg/dl) were determined spectrophotometrically. PCR (mg/l), triglycerides (mg/dl), total cholesterol (mg/dl), plasma levels of AFP (ng/ml) and the presence of MC were assessed by chemiluminescence. The blood count was determined automatically using a dedicated flow cytometry kit.

The coaguloation parameters were assessed from blood samples collected on EDTA vacutainers. The following parameters were determined: aPTT (sec), PT/ INR (sec) and plasma fibrinogen. FibroScan® technology was used for the non-invasive assessment of liver fibrosis. The viral load was determined using RT-PCR.

For epigenetic markers, we assessed plasmatic miR 7-1, miR 21, miR 122 and miR 885 in healthy voluntiers and in HCV infected patients in acute phase and after sustained viral response. The main steps included: total RNA collection, reverse transcription and amplification of the gene of interest, according to the total protocol.

The doctoral thesis is structured in two parts: a general part and a special part.

The general part of the doctoral thesis includes 13 chapters. The first few chapters refer in an illustrative way to HCV infection. These chapters provide information on the epidemiology, etiology of HCV transmission, pathophysiology of the disease, clinical picture, paraclinical diagnosis, imaging aspects of chronic liver disease, staging according to severity, characteristic histopathological characteristicsaspects, differential diagnosis, complications of infection with HCV as well as the prognosis and evolution of the disease.

Chapter 12 focuses on treatment principles including DAA treatment.

The special part includes four original chapters that refer to the working hypothesis, general objectives, the general methodology of scientific research and the experimental including three above-mentioned research perspectives : biochemistry, imaging and epigenetics.

The experimental part consists of a brief introduction, specific materials and methods, results, discussions and conclusions.

Finally, the conclusions of the doctoral thesis are structured. The confirmed objectives and the refuted hypotheses were specified. The own contributions were highlighted but also the results in accordance with the specialized literature

SPECIAL PART

General objectives of the study

The dissertation focuses on the biochemical, imaging and epigenetic diagnostic strategies in HCV-infected patients as well as their variation secondary to DAA

The aim of the dissertation is to analyze a new potential diagnostic strategy and to monitor the therapeutic efficacy from an epigenetic perspective.

The main objectives of the study are:

• To analyze the demographic data from the cohort included in the study taking into account the main anthropometric and social characteristics: patient gender, environment of origin, smoker / non-smoker status, BMI, average age

• To compare the plasma level of AFP in patients with HCV infection, in the acute, replicative phase but also at 3 months after diagnosis following antiviral treatment in patients with SVR, in the absence of HCC

• To assess the variability of the liver fibrosis (FibroScan®) in patients with acute HCV infection before and after DAA therapy and to compare them with healthy volunteers.

• To compare the incidence of CM in HCV infected patients before and modern antiviral therapy, in the absence of specific glomerulopathy.

• To identify novel diagnostic tools and to correlate the plasmatic AFP with the following biological markers: AST, ALT, GGT, AP, DB, TB, CRP, triglycerides, total cholesterol, plasma albumin, coagulation parameters, viral load in the acute phase of HCV hepatitis.

• To validate new innovative epigenetic markers in HCV infection: miR 7-1, miR 21, miR 122, miR 885

• To emphasize the role of the validated epigenetic markers in the therapy response

• To correlate the level of miR 7-1 in plasma with the following markers: AST, ALT, CRP, viral load, AFP, GGT, AP, erythrocytes sedimentation speed (ESS), fibrinogen, and the degree of the liver fibrosis with regard to the stages of the HCV infection.

• To analyze the variability of the miR 21 in HCV infected patients and to correlate them with the plasma level of standard biomarkers: AST, ALT, CRP, viral load, AFP, GGT, AP, ESS, fibrinogen, and the degree of the liver fibrosis.

• To correlate the miR 122 expression to plasmatic AST, ALT, PCR, viral load, AFP, GGT, AP, ESS, fibrinogen and liver fibrosis in the replicative phase

• To correlate the miR 885 expression with the following standard markers: AST, ALT, CRP, viral load, AFP, GGT, AP, ESS, fibrinogen and the degree of the liver fibrosis, with regard to the stages of the HCV infection (acute vs. sustained viral response)

General methodology of the scientific research

The present study is an interventional, prospective, randomized, double-blind clinical trial that includs an initial cohort of 104 patients. The patients were enrolled between February 2020 and December 2020. Healthy volunteers and HCV-infected patients in acute phase with no previous antiviral treatment.

All clinical procedures were performed following the approval of the Ethics Council of the Fundeni Clinical Institute (48358/01/10/2019) in accordance with the European Council Directive 2001/20 / EC and respecting the rules of confidentiality of personal data according to the directive European 95/46 / EC. Informed consent was obtained from all subjects included in the study.

The exclusion criteria were: patients with decompensated cirrhosis, chronic kidney disease, life expectancy below 12 months, decompensated heart failure, extrahepatic manifestation of the HCV infection, alcohol or drug use, HBV or HIV co-infection. Twenty-five patients had at least one exclusion criterion. Another 16 patients refused the enrollment in the study and 9 patients did not achieved the SVR after DAA.

Of the remaining 63 patients, 36 patients, healthy volunteers constituted the control group and 27 patients constituted the study group. The 27 patients included in the study group were evaluated at the time of diagnosis of HCV infection and 3 months after achieving SVR secondary to antiviral therapy with direct agents.

Therefore, the study groups were:

• Control group (C) (n = 36) consisting of healthy volunteers

• HCV group (VHC) (n = 27) consisting of HCV-infected patients at the time of diagnosis, in the replicative phase

• SVR group (RVS) (n = 27) consisting of patients with sustained viral response 3 months after initiation of DAA.

The analyse of AFP dinamics in HCV-infected patients

The first part of the dissertation focuses on the comparative analysis of demographic data. The following parameters were considered: age, patient gender, social status, smoking status, body mass index.

Biochemical markers were assessed according to a local protocol. Whole blood from each patient was collected in dedicated vacutainers and maintained at 4 ° C until the sample coagulated. After coagulation, the samples were centrifuged for 20 minutes at 1000xg and processed immediately for the markers listed below.

We assessed, spectrophotometrically, the plasma levels of albumin (g / dl), AST (U / l), ALT (U / l), GGT (U / l), AP (U / l), DB (mg / dl), TB (mg / dl), CRP (mg / l), triglycerides (mg / dl) and total cholesterol (mg / dl), using compatible kits for Siemens Advia 1800 Reader (Siemens, Erlangen-Germany). AFP (ng / ml) and MC were determined using Advia Centaur XPT Reader (Siemens, Erlangen-Germany) by chemiluminescence. The blood count was determined automatically using a dedicated flow cytometry kit (Sysmex XN-1000 Reader) (Sysmex, Kobe-Japan) and the results were expressed per ml of blood.

The coagulation parameters were assessed in blood samples collected on EDTA vacutainers according to the manufacturer's recommendations using StaR Max 3 Reader (Stago, France). The following parameters were determined: aPTT (sec), PT / INR (sec) and plasma fibrinogen.

The level of viral load was determined using RT-PCR (Montania 4896 Real Time PCR Reader, Anatolia, Istanbul-Turkey), using the Bosphore HCV quantification kit (Anatolia, Istanbul-Turkey), according to the manufacturer's recommendations. The main steps included: total RNA collection, reverse transcription and amplification of the gene of interest, according to the local protocol. The assessments were performed in triplicate and viral load was expressed in virion / ml blood.

The analyse of epigenetic markers in HCV-infected patients without hepatocelular carcinoma

For miR assessment, the total RNA was isolated from 200 µl plasma using the miRNeasy Serum / Plasma kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. An amount of 3 µl of RNA was reverse transcribed using the miRCURY LNA RT kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's recommendations.

For each sample, six amplification reactions of 1 µl of cDNA per reaction were performed using miRCURY miRNA Assays (Qiagen, Hilden, Germany). The miR species considered were: miR-7 1-3p, miR 21-3p, miR122-5p, miR-885-5p and miR-16-5p (endogenous control). Reactions were performed according to the manufacturer's recommendations using the PCR 7500 Fast (Applied Biosystems, Foster City, CA, USA).

The Ct values obtained were normalized to control using double delta Ct method, considering miR-16-5p endogenous control, according to Qiagen recommandation and after checking the stability. Plasma levels of miR were expressed as fold-change for comparative analysis between study groups and as $2^{-\Delta\Delta Ct}$ for correlation analysis.

Results

Demographic data

Gender distribution revealed 47.2% men in control group vs. 30.7% in the study group (p=NS).

We identified 22.2% of the patients in control group as smoker vs. 34.6% in the study group (p=NS). The data are revealed in Fig. 17 and 18.

Regarding the distribution of the patients according to their residency (urban/rural), 75% of the patients from the control group lived in cities vs. 61.5% in the study group. The data are revealed in Fig. 19 and 20.

The mean age of the patients in the control group was 50±2.3 vs. 61.8±2.3 in the study group (p=NS). The data revealing the patients age included in the study are revealed in the Fig. 21 și 22.

Patient's distribution according to their BMI in the control group revealed a mean BMI of 25.6±3.8 kg/m^2 vs. 27.06±5.6 kg/m^2 (p=NS) (in the study group). The results are revealed in Fig. 23 and 24.

The analyze of AFP dynamics in HCV-infected patients

The comparative analysis of the plasmatic levels of AFP among groups is illustrated in the Fig. 1. The results revealed a statistically significant increased AFP in acute phase (VHC group) as well as after achieving the sustained viral response (RVS group) compared with control: 5.79±0.7 vs. 2.4±0.3, P=0.0009, and 5.9±2.5 vs. 2.4±0.27, P=0.042, respectively. We didn't observe any changes in plasmatic AFP between VHC and RVS groups: 5.79±0.73 vs. 5.9±2.5, P=NS.

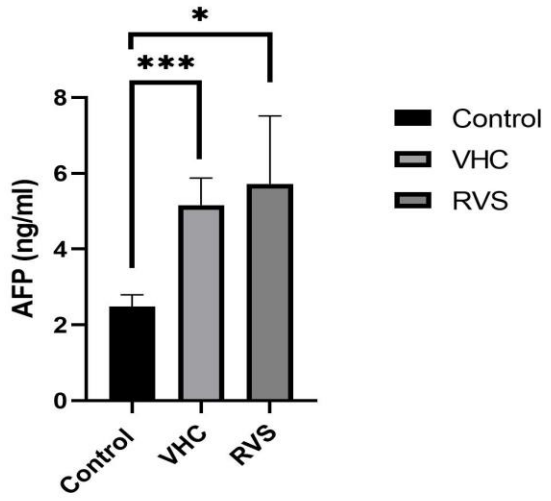

Fig. 1 Comparative analysis of plasmatic AFP among groups. The data is expressed as means± SEM. * represents p=0.05-0.01 and *** represents p=0.001-0.0001.

The presence of the mixed cryoglobulinemia is revealed in Fig. 2. We observed a statistically significant increase of the prevalence of the mixed cryoglobulinemia in VHC group compared to control (46% vs. 14%, $p<0.0001$). Additionally, the prevalence of the mixed cryoglobulinemia was reduced in RVS group compared to VHC group (25% vs. 46%, $p<0.0001$).

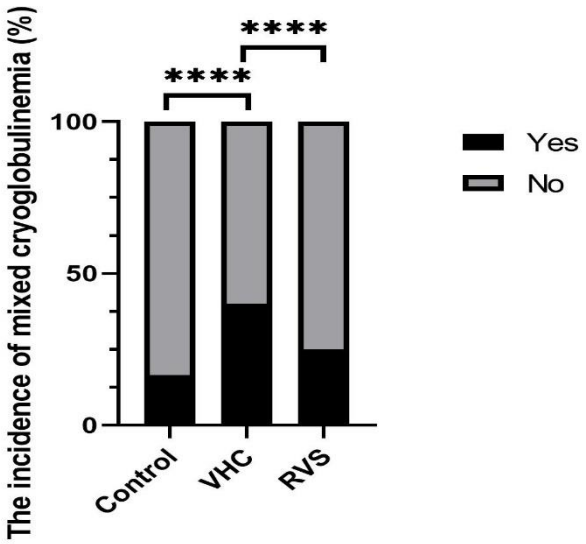

Fig. 2 The incidence of mixed cryoglobulinemia among groups. **** represents p<0.0001

The comparative analysis of the hepatic fibrosis using FibroScan is illustrated in Fig. 3. The data revealed an increased fibrosis levels in both, VHC and RVS groups compared to control: 9.8±0.9 vs. 4.5±0.37, p=0.001 and 13±3.2 vs. 4.5±0.37, p=0.0006, respectively. No differences were observed between VHC and RVS groups: 9.8±1 vs. 13±3.2, p=NS.

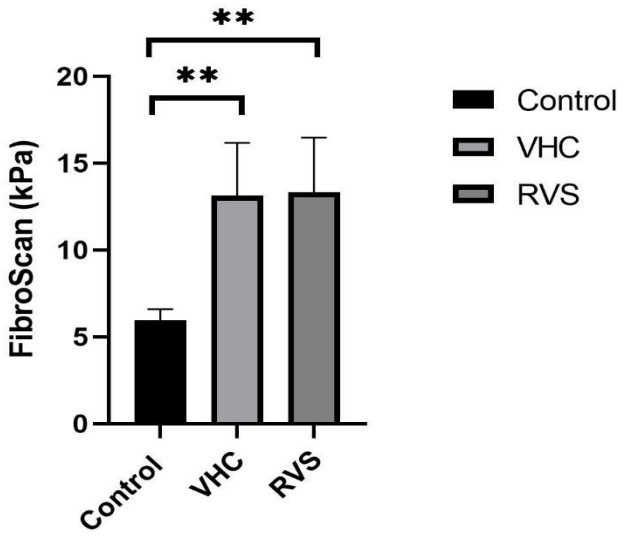

Fig. 3 The mean fibrosis levels among groups. The data are expressed in kPa as means ± SEM. ** represents p=0.01-0.001

The correlative analysis between AFP and biochemical markers and imagistic findings are revealed in Fig. 4-9. We observed a statistically significant positive correlation between AFP and the values of the following markers: AST (R^2=0.37, p = 0.0001), ALT (R^2 = 0.37, p = 0.0002), BT (R^2 = 0.3, p = 0.0009), GGT (R^2 = 0.38, p = 0.0001), FA (R^2 = 0.45, p<0.0001) and a negative correlation between AFP and plasmatic fibrinogen (R^2 = 0.15, p = 0.002).

No statistically significant correlations were observed between the plasma level of AFP and FibroScan® (R^2 = 0.0007, p = 0.88), the viremia (R^2 = 0.0009, p = 0.86), platelets count (R^2 = 0.9, p = 0.08) or CRP (R^2 = 0.01, p = 0.64)

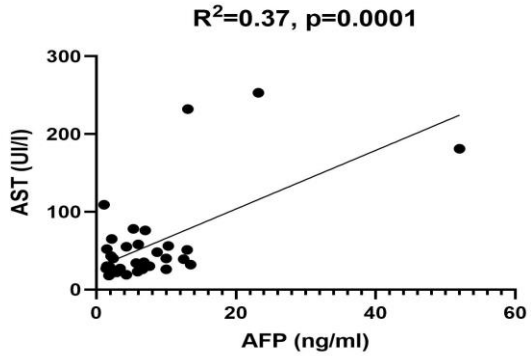

Fig. 4 Correlative analysis of AFP and AST. R^2- correlation coefficient. p- statistical significance.

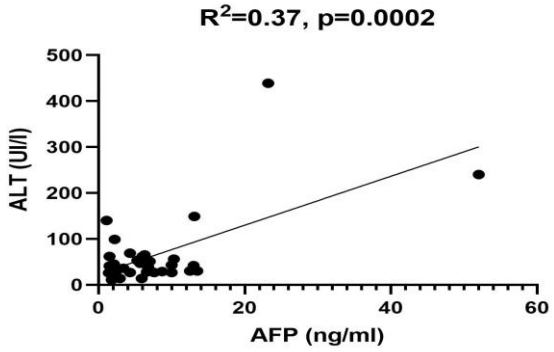

Fig. 5 Correlative analysis of AFP and ALT. R^2- correlation coefficient; p-statistical significance

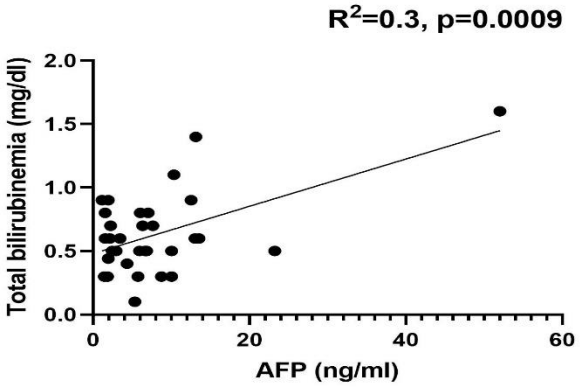

Fig. 6 Correlative analysis of AFP and total bilirubinemia (TB). R^2- correlation coefficient; p-statistical significance

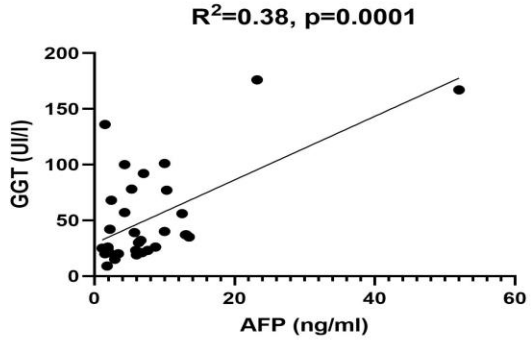

Fig. 7 Correlative analysis of AFP and GGT. R^2- correlation coefficient; p-statistical significance

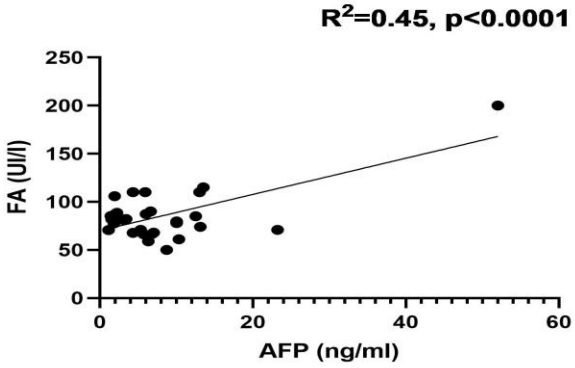

Fig. 8 Correlative analysis of AFP and alkaline phosphate (AP). R^2- correlation coefficient; p-statistical significance

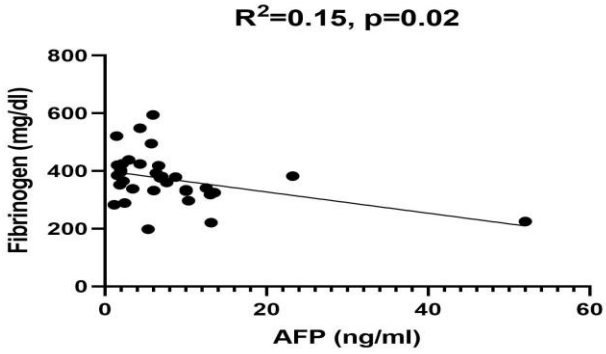

Fig. 9 Correlative analysis of AFP and plasmatic fibrinogen. R^2- correlation coefficient; p-statistical significance

The analyse of epigenetic markers in HCV-infected patients without hepatocelular carcinoma

The comparative analysis of miR 7-1-3p expression between the studied groups is shown in Fig. 10. No difference was observed between the Control and HCV groups (1 ± 0.2 vs. 0.65 ± 0.08, p = NS). The miR 7-1-3p was up-regulated in the RVS group compared to the VHC group: 1.03 ± 0.11 vs. 0.65 ± 0.08 ($p<0.006$).

Additionally, the level of miR 7-1-3p was not correlated with any marker considered (Fig. 11-19): AST (R2 = 0.007, p = 0.67), ALT (R2 = 0.08, p = 0.16), PCR (R2 = 0.32 , p = 0.23), viral load (R2 = 0.07, p = 0.18), AFP (R2 = 0.01, p = 0.54), GGT (R2 = 0.03, p = 0.47), AP (R2 = 0.01, p = 0.75), ESS (R2 = 0.15, p = 0.09), FibroScan® (R2 = 0.01, p = 0.54).

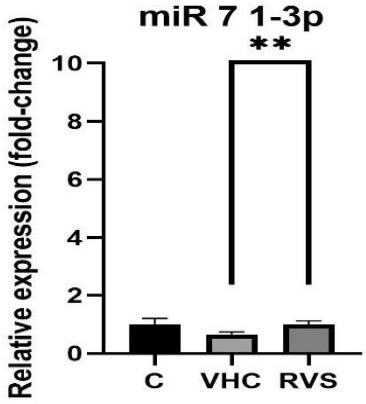

Fig. 10 Relative expression of miR 7 1-3p among groups. The results are expressed as fold-change± SEM. ** represents p=0.01-0.001

The MiR 21-3p was up-regulated in RVS compared to Control: 2.76±0.52 vs. 1±0.29, p=0.17 (Fig. 11).

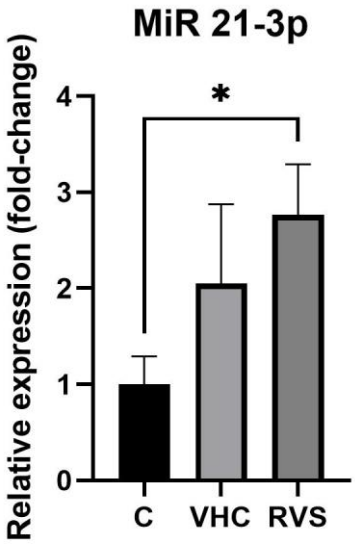

Fig. 11 Relative expression of miR 21-3p among groups. The results are expressed as fold-change± SEM. ** represents p=0.01-0.001

The correlative analysis of miR 21-3p expression with the following standard markers did not identify any statistically significant relationship: AST (R^2 = 0.0001, p = 0.95), ALT (R^2 = 0.001, p = 0.87), PCR (R^2 = 0.21, p = 0.35), viremia (R^2 = 0.03, p = 0.75), AFP (R^2 = 0.03, p = 0.41), GGT (R^2 = 0.1, p = 0.19), FA (R^2 = 0.0001, p = 0.98), ESR (R^2 = 0.11, p = 0.15), FibroScan® (R^2 = 0.01, p = 0.57).

MiR 122 was upregulated in both, VHC and RVS groups compared with control group: 6.24±1.5 vs. 1±0.3 ($p<0.001$), and 3.96±0.72 vs. 1±0.3 ($p<0.001$), respectively (Fig. 12).

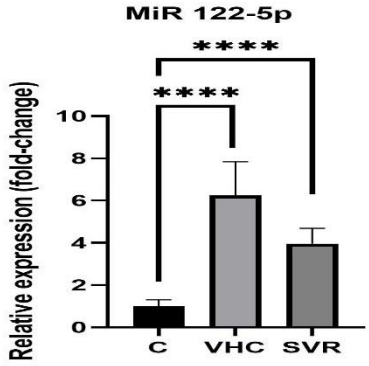

Fig. 12 Relative expression of miR 122-5p among groups. The results are expressed as fold-change± SEM. **** represents $p<0.0001$

Plasma levels of miR 122-5p were positively correlated with AST (R^2= 0.35, p = 0.0018), ALT (R^2 = 0.3, p = 0.0045), and CRP (R^2 = 0.76, p = 0.022) (Fig. 13-15). The other markers did not suggest statistically significant correlations in association with miR 122-5p: viral load (R^2 = 0.03, p = 0.37), AFP (R^2 = 0.009, p = 0.65), GGT (R^2 = 0.11, p = 0.15), FA (R^2 = 0.0006, p = 0.93), VSH (R^2 = 0.001, p = 0.85), FibroScan® (R^2 = 0.008, p = 0.67)

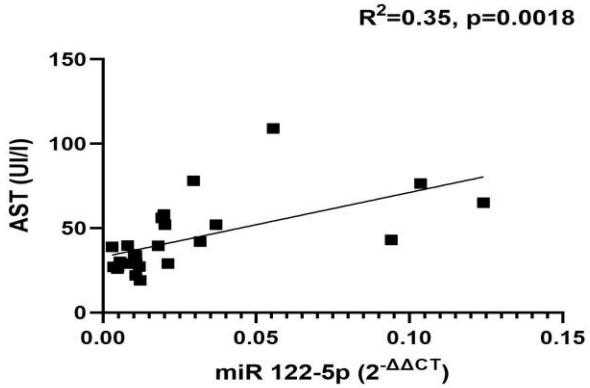

Fig.13 Correlative analysis of miR 122-5p and AST. R^2- correlation coefficient. p statistical significance

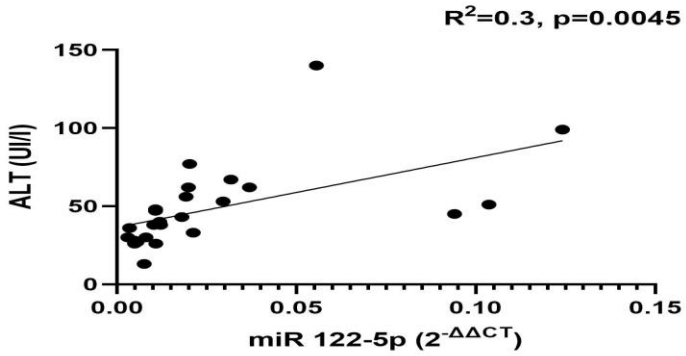

Fig.14 Correlative analysis of miR 122-5p and ALT. R^2- correlation coefficient. p statistical significance

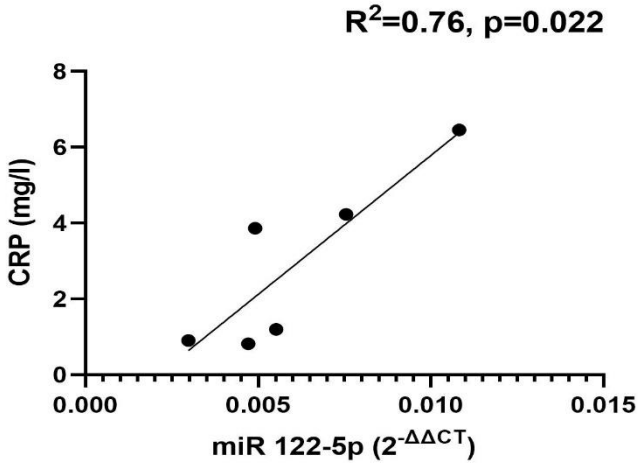

Fig. 15 Correlative analysis of miR 122-5p and CRP. R^2- correlation coefficient. p statistical significance

MiR-885-5p was up-regulated in the HCV group compared to the Control group: 7.57 ± 1.7 vs. 1 ± 0.31, p<0.001 and a down-regulated in the RVS group compared to the HCV group: 2.96 ± 0.44 vs. 7.57 ± 1.7, p = 0.007. In addition, overexpression of miR 885-5p was observed in the RVS group compared to the Control group: 2.96 ± 0.44 vs. 1 ± 0.31, p = 0.001 (Fig. 16).

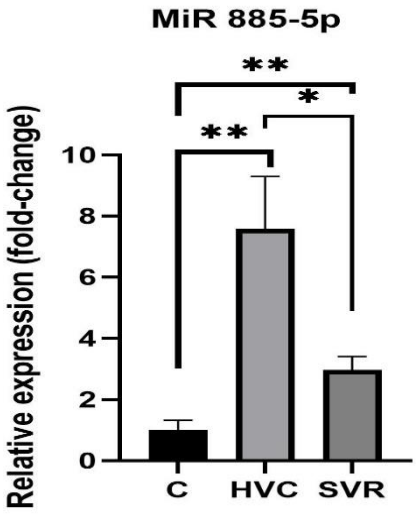

Fig. 16 Relative expression of miR 885-5p among groups. The results are expressed as fold-change± SEM. * represents p=0.01-0.05, ** represents p=0.001-0.01

Moreover, miR 885-5p was found to be positively correlated with: AST (R^2= 0.16, p = 0.04) and CRP (R2 = 0.9, p = 0.03) (Fig. 17-18). The other hypotheses were not confirmed. We found no linkage between miR 885-5p and: ALT (R^2=0.06, p = 0.22), viral load (R^2 = 0.03, p = 0.39), AFP (R^2 = 0.0002, p = 0.93), GGT (R^2 = 0.2, p = 0.057) , AP (R^2 = 0.005, p = 0.82), ESS (R^2 = 0.0001, p = 0.96), FibroScan® (R^2 = 0.01, p = 0.52)

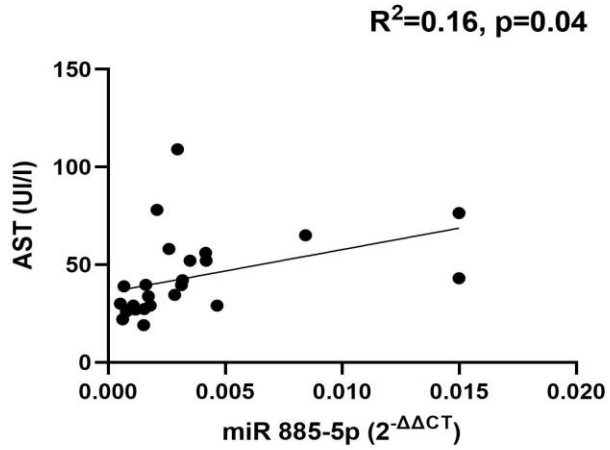

Fig. 17 Correlative analysis of miR 885-5p and AST. R^2- correlation coefficient. p statistical significance

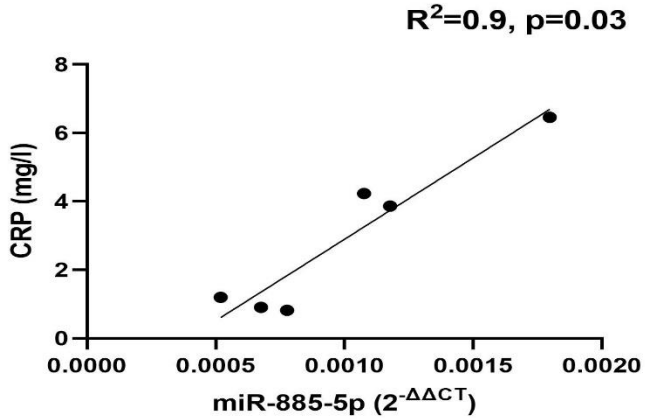

Fig. 18 Correlative analysis of miR 885-5p and CRP. R^2- correlation coefficient. p statistical significance

CONCLUSIONS AND PERSONAL CONTRIBUTION

The conclusions of the doctoral thesis are:

• The plasma level of AFP, even if under the cut-off levels for the diagnosis of a HCC, was elevated in HCV infected patients, regardless of their response to DAA

• MC increases in patients with HCV and then decreases after sustained viral response secondary to DAA

• HCV can cause liver fibrosis in the early stages of the infection, which persists at 3 months, despite the sustained viral response

• The plasma level of AFP in HCV infected patients is positively correlated with the hepatic cytolysis syndrome as revealed by plasmatic AST and ALT

• The plasma level of AFP in HCV infected patients is positively correlated with the cholestasis syndrome as revealed by the level of AP, GGT and TB

• In the replicative phase of HCV infection, fibrinogenemia is negative related to plasmatic AFP.

• The plasmatic down-regulation of miR 7-1-3p could represent a marker of sustained viral response after DAA in HCV infected patients.

• MiR 21-3p increases in HCV infected patients after successful DAA compared to control

• Acute HCV infection causes up-regulation of miR 122-5p, which will decrease after DAA: Therefore, this epigenetic marker could have a predictive role in therapy response. Plasma levels of miR 122-5p positively correlate with the hepatic cytolysis syndrome in the replicative HCV infection.

• MiR 885-5p is up-regulated in acute, replicative phase, of HCV infection and decreases, subsequently, after DAA. This marker could help in predicting the progress of chronic HCV infection, as well as the therapeutic response.

• There are minor direct correlations between miR 885-5p and ALT in the acute phase of HCV infected patients.

• Inflammatory status, as revealed by PCR levels, positively correlates with plasma levels of miR 122-5p and miR 885-5p

Although future clinical trials are needed to confirm the hypotheses and results discussed in this thesis, we have highlighted an innovative epigenetic panel in the early diagnosis of HCV-infected patients, which could prove their utility in monitoring the response to DAA.

In addition, we revealed potential correlations of these biomarkers with the standard biochemical and sonographic diagnostic strategies.

We also stated, through comparative and correlative analyzes, the role of AFP in HCV-infected patients in the absence of HCC as well as its dynamics secondary to DAA.

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