AIM OF THE WORK

Aim 1: To evaluate the prevalence of insulin resistance in Egyptian patients infected with chronic hepatitis C virus genotype 4.

Aim 2: To assess factors associated with insulin resistance in those patients (viral, metabolic, histopathologic including steatosis, fibrosis and necroinflammatory changes).

Aim 3: To test the impact of insulin resistance on treatment outcomes in patients chronically infected with hepatitis C virus genotype 4 receiving pegylated IFN-α and ribavirin.

STUDY SUBJECTS

The prevalence study assessed one hundred non-diabetic chronic hepatitis C patients (CHC) with genotype 4 infection. Patients were recruited from those attending the 'Centre for Treatment of Hepatitis Virus' in Sharq El Madina Hospital (being one of the 23 centers established by the Ministry of Health for treatment of CHC patients) after testing for eligibility to be included in the study, for a period of 6 months for the prevalence study. Chronic hepatitis was defined as presence of HCV-Ab for at least 6 months and positive viremia. Diabetes was diagnosed using the1997 American Association Criteria (fasting glucose >126 mg/dl).[147, 148]

The following patients were excluded from the study by appropriate virological, serological, biochemical and ultrasound data and by a clinical history: those with clinical evidence of hepatic decompensation or liver cirrhosis, concomitant hepatitis B infection (defined as HBsAg positive), patients with CHC by a genotype other than 4, autoimmune hepatitis, hemochromatosis, primary biliary cirrhosis, Wilson's disease, drug-induced liver disease and laboratory values serum creatinine > 1.5 mg/dl, an absolute neutrophil count <1,000/ml, a platelet count < 50,000/ml or hemoglobin <11 g/dl.

All patients were asked to give their informed consent before being included in the study, for withdrawal of blood samples and obtaining medical data from the filing system in the hospital. The study was approved by Local Research Committee of Alexandria Main University Hospital.

Based on the results of the 100 patients included in the prevalence study, 71 consecutive non-diabetic treatment-naïve CHC patients were enrolled for testing treatment outcome associated with various degrees of insulin resistance. All patients were given a combination of pegylated interferon and ribavirin for an intended duration of 48 weeks. The optimal dose of peginterferon alpha-2b (Peg-Intron, Schering Plough Corp., USA) is 1.5 µg/kg/week, given subcutaneously once/week or peginterferon alpha-2a (PEGASYS, Hoffman La Roche, Switzerland) at a dose of 180 µg once a week subcutaneously together with oral Ribavirin 10.6 mg/kg per day (1,000 mg for those who weigh <75 kg and 1200 mg>75 kg).

After 12 weeks of treatment, the early virological response was assessed by measuring the viral load. Patients who did not demonstrate a 2 $\log_{10}$ or more decrease in viral load as compared to baseline, were considered early non-responders. Therapy was discontinued for those patients according to the protocol approved by the Ministry of Health. Patients showing > 2 $\log_{10}$ reduction in viral load as compared to baseline after 12 weeks of therapy, were considered as partial early virological responders (pEVRs) while those showing HCV RNA level below limit of detection at 12 weeks of therapy were considered as complete early virological responders (cEVRs). Those patients (pEVRs , cEVRs) continued the antiviral treatment regimen until 48

weeks. Viral load was re-assessed after 48 weeks of therapy. End-of-treatment response (ETR) was defined as serum HCV-RNA below the limit of detection at the end of treatment.

As for the assessment of insulin resistance, it was done by Homeostatic Model Assessment for Insulin Resistance or the HOMA-IR test, being a more practical and easier test as compared to the gold standard which is the hyperinsulinemic–euglycemic clamp test.[149, 150] Subjects with HOMA-IR > 3 were considered insulin resistant.[151, 152] HOMA-IR test was done to the studied 71 genotype 4 chronic HCV patients before the start of therapy, after 12 weeks of therapy and after 48 weeks of therapy to those who continued treatment.

A control group comprised of 60 healthy non diabetic HCV-negative individuals was included in the study. Their insulin resistance was also measured by HOMA-IR and compared to pretreatment HOMA-IR of the CHC patients included in the study.

METHODS

Clinical and Laboratory assessment:

Data base and filing system included personal data (age, residence, alcohol intake and smoking), medical history (diabetes mellitus, autoimmune disorders and metabolic liver diseases, prior antiviral or immunosuppressive therapy), known risk factors (disease duration, contact with hepatitis patients, drug abuse, blood transfusion, schistosomiasis and parenteral antischistosomal therapy, haemodialysis, major surgery, occupational exposure and interferon therapy) and laboratory results (liver function tests, hematological tests and HBV state). Clinical assessment data was recorded. Antiviral treatment data and all laboratory results and follow up of patients was recorded as well.

The following data was collected: sex, age, weight (kilograms), height (meters), waist circumference (centimeters), blood pressure, and concomitant medications. Body mass index (BMI) was calculated as weight divided by the square of the height (kg/m2). Overweight was defined as a BMI 25-30 and obesity as a BMI >30. The metabolic syndrome was diagnosed according to the recently revised WHO definition as the presence of 3 or more of the following criteria: central obesity (waist circumference >102 cm, [male] and > 88 cm, [female}, hypertension (blood pressure> 135/85 mm Hg), fasting plasma glucose > 110 mg/dl, triglycerides > 150 mg/dl, high density lipoprotein (HDL) cholesterol < 40 mg/dl (male) or < 50 mg/dl (female). [153]

Virological assessment:

Venous blood samples were collected from chronic hepatitis C patients. Determination of the genotype of the virus was done using a real-time PCR kit (AmpliSens®/*HCV*-FRT PCR kit, InterLabService Ltd, Moscow, Russia). Assessment of the HCV viral load by quantitative measurement of RNA was carried out using real-time PCR (COBAS Ampliprep^{TM}/COBAS TaqMan^{TM}, Roche Molecular Systems, Pleasonton, CA, USA). Level of viremia was classified as high, intermediate and low according to viral load being $>10^6$, 10^5 -10^6 or $<10^5$ IU/ml respectively. Quantitative HCV RNA estimation was done before the start of therapy, at 12 weeks of therapy and at the end of treatment.

HCV Genotyping using RT-PCR kit AmpliSens®/*HCV*-FRT PCR kit: [154]

Principle of PCR Detection:

AmpliSens® *HCV*-genotype-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection and differentiation of *hepatitis C virus* (*HCV*) genotypes 1a, 1b, 2, 3, and 4 in the clinical materials (peripheral blood plasma) by means of real-time hybridization-fluorescence detection (figure 11).

HCV genotypes 1a, 1b, 2, 3, and 4 detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using special primers. In real-time PCR the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. The real-time PCR monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run. AmpliSens *HCV*-genotype-FRT PCR kit uses "hot-start", which greatly reduces frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by separation of nucleotides and Taq-polymerase by application of chemically modified polymerase (TaqF) that is activated by heating at 95ºC for 15 min.

HCV genotypes 1a, 1b, 2, 3, and 4 detection includes:

(a) Total RNA extraction from blood plasma simultaneously with the Internal Control sample.

(b) Reverse transcription of cDNA on RNA matrix.

(c) Real-time PCR of *HCV* genotypes 1a, 1b, 2, 3, and 4 cDNA

Figure 11: AmpliSens®/*HCV*-FRT PCR genotyping kit

Sampling:

Peripheral blood plasma was taken in a vacutainer with 3% EDTA solution (1 : 20) after overnight fasting. Plasma samples were stored at -80 C.

Protocol of use according to the manufacturer's recommendations:

I.RNA Extraction:

The volume of sample for RNA extraction was 0.1 ml.
1. Lysis Solution and Washing Solution 1 were warmed up (if stored at 2 – 8 ºC) at 56 ºC until the ice crystals disappeared.

2.The required number of 1.5 ml tubes including the tube for Negative Control of Extraction (C) were prepared.

3. Four hundred and fifty µl of Lysis Solution and 10 µl of internal control **(IC)** were added per each tube. The tubes were labelled.
4. One hundred µl of plasma sample per each tube containing Lysis Solution and IC were added. The tubes were closed and vortexed. The tubes were centrifuged briefly to make sure there are no drops on the walls of the tubes.

5. One hundred µl of Negative Control were added to the eppendorfs intended for the Negative Control of Extraction (C-). The tubes were closed and vortexed. The tubes were centrifuged briefly to make sure there are no drops on the walls of the tubes.

6. Sorbent was thoroughly resuspended with the vortex, 25 µl of resuspended sorbent were added to each test tube.

7. The tubes were vortexed and incubated at room temperature for 10 min with stirring every 2 min.

8. The tubes were centrifuged at 10,000 g for 1 min.

9. The supernatant was removed and discarded from the tubes.

10. Five hundred µl of Washing Solution 1 were added to each tube then vortexed thoroughly (until the sorbent was fully resuspended) then centrifuged at 10,000 g for 1 min. The supernatant was removed and discarded.

11. Five hundred µl of Washing Solution 3 were added to each tube then vortexed thoroughly (until sorbent was fully resuspended) followed by centrifugation at 10,000 g for 1 min. The supernatant was removed and discarded.

12. Step 11 was repeated.

13. Five hundred µl of Washing Solution 4 were added to each tube , vortexed thoroughly (until sorbent is fully resuspended) then centrifuged at 10,000 g for 1 min. The supernatant was removed and discarded .

14. The tubes were incubated at 56 °C for 15 min to dry the sorbent.

15. Fifty µl of RNA-buffer were added per each tube. The sorbent was resuspended in RNA-buffer, incubated at 56 °C for 5 min, and then vortexed. To sediment the sorbent, the tubes were centrifuged at 10,000 g for 2 min. RNA was processed once extracted and was never stored.

II.Reverse Transcription:

The total reaction volume was 20 µl, the volume of RNA sample was 10 µl.
1. Reaction mixture was prepared for 12 reactions at a time:
5 µl of RT-G-mix-1 were added to the tube with RT-mix and thoroughly mixed by vortexing. 6 µl of Revertase (MMlv) were added to the tube with the reaction mixture, pipetted 5 times, and vortexed.

2. Ten µl of the prepared mixture were transferred per each tube.

3. Ten µl of RNA-sample were added per each tube with the reaction mixture and carefully mixed.

4. The tubes were placed in a thermal cycler and incubated at 37 °C for 30 min.

5. cDNA sample obtained during reverse transcription was diluted for further PCR test by adding 20 µl of DNA-buffer to the tube with 20 µl of cDNA sample and carefully mixing by pipetting (10 times). cDNA samples were stored at -40 ◌C.

III.Preparing the PCR

The total reaction volume was 25 µl, the volume of cDNA sample was 12.5 µl.
1. The following reaction mixtures were prepared: “1b/3”, “1a/2”, and “4/IC”. To do this, 65 µl of RT-PCR-mix-2-FEP/FRT and 6 µl of polymerase (TaqF) were added per each tube with PCR-mix-1-FRT *HCV* genotypes 1b/3, PCR-mix-1-FRT *HCV* genotypes 1a/2, PCR-mix-1-FRT *HCV* genotype 4/IC and thoroughly vortexed.

2. Twelve and half µl of prepared mixture were transferred to the PCR tubes.

3. Twelve and half µl of cDNA samples obtained from clinical or control samples at the stage of RNA extraction and reverse transcription were added to the tubes. Each sample was tested in 3 separate reaction tubes carrying the 3 different reaction mixtures.

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3. Control amplification reactions were carried out:
    - NCA (Negative Control of Amplification): 12.5 µl of TE-buffer were added to the tube labeled NCA.
    - C+1b/3 (Positive Control of Amplification): 12.5 µl of Positive Control cDNA *HCV* genotypes 1b/3 were added to the tube with "1b/3" reaction mixture labeled C+1b/3.
    - C+1a/2 (Positive Control of Amplification): 12.5 µl of Positive Control cDNA *HCV* genotypes 1a/2 were added to the tube with "1a/2" reaction mixture labeled C+1a/2.
    - C+4 (Positive Control of Amplification): 12.5 µl of Positive Control cDNA HCV genotype 4 were added to the tube with "4/IC" reaction mixture labeled C+4.

IV.Amplification:

Amplification temperature profile was created on the Real-Time PCR Applied Biosystems StepOne Instrument as follows (table 6, figure 12):

Table 6: Protocol of HCV genotype amplification

Step	***Temperature °C***	***Time***	***Fluorescence detection***	***Cycle repeats***
Hold	95	15 min	_	1
Cycling	95	20 sec	_	45
	60	40 sec	FAM/Green JOE/Yellow	

Figure 12: Real-Time PCR Applied Biosystems StepOne Instrument

V.Data Analysis and Interpretation:

Amplification products of the Internal Control and the *HCV* RNA fragments were analyzed within the test. Matching of the fluorescence channels with the *HCV* genotypes are specified in table 7:

Table 7: Flourescence channels in RT-PCR HCV genotyping

Channel	Reaction mixture		
	1b/3	1a/2	4/IC
FAM/Green	1b	1a	IC
JOE/Yellow/HEX/Cy3	3	2	4

The *HCV* genotype found in a sample was confirmed by the results of amplification obtained from three test tubes (with PCR-mix-1-FRT *HCV* genotypes 1b/3, PCR-mix-1-FRT *HCV* genotypes 1a/2, and PCR-mix-1-FRT *HCV* genotype 4/IC). The results were interpreted by the software of the used instrument by the crossing (or no crossing) of the fluorescence curve with the threshold line that corresponds to the presence (or absence) of Ct value in the result grid (table 8, figure 13).

Table 8: Amplification of control samples.

Control	Stage for controls	Reaction mixture						Interpretation
		1b/3		1a/2		4/IC		
		FAM/ Green	JOE/ Yellow	FAM/ Green	JOE/ Yellow	FAM/ Green	JOE/ Yellow	
C-	RNA extraction	–	–	–	–	<Ct*	–	OK
NCA	PCR	–	–	–	–	–	–	OK
$C+_{1b/3}$	PCR	<Ct*	<Ct*					OK
$C+_{1a/2}$	PCR			<Ct*	<Ct*			OK
$C+_{4}$	PCR					–	<Ct*	OK

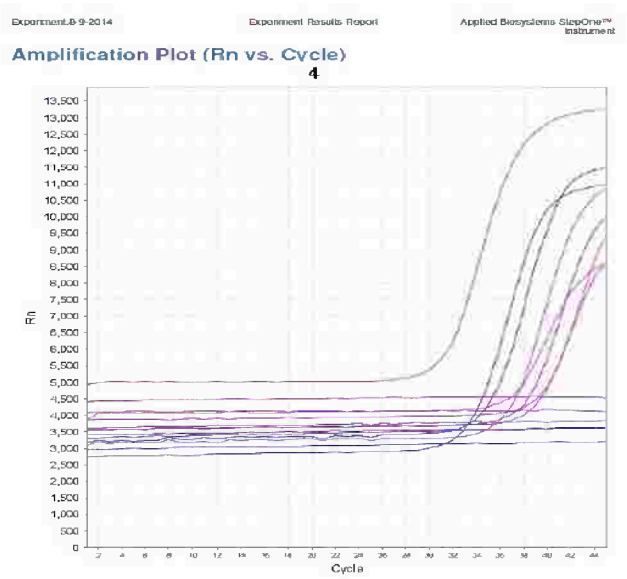

Figure 13: The amplification plot of HCV isolates of genotype 4

Quantitative estimation of viral load COBAS Ampliprep™/COBAS TaqMan™, "CAP/CTM": (155, 156)

I.CAP based extraction of nucleic acid

1- HCV RNA was isolated from 0.85-ml aliquots of serum using the automated COBAS Ampliprep instrument (figure 14).
2- HCV quantification standard which is a non-infectious RNA construct containing fragments of HCV sequences with primer binding regions identical to those of the HCV 5' non-translated region (NTR),was added to each sample as an internal control.
3- After a protease incubation step, the lysis reagent, together with the magnetic glass particles, were introduced into each specimen. HCV RNA and HCV quantification standard RNA were bound to the surfaces of the magnetic glass particles.
4- After completion of several washing steps, the adsorbed nucleic acids were eluted at elevated temperature with an aqueous solution.

Figure 14: RT-PCR COBAS Ampliprep instrument.

II.Amplification and detection using the COBAS TaqMan 48 Analyzer

After CAP-based extraction of nucleic acids, samples and controls were processed for amplification and detection using the COBAS TaqMan 48 Analyzer according to the instructions of the manufacturer.

1- Reverse transcription and amplification were carried out using primers that bind within the highly conserved 5' NTR of HCV; deoxynucleoside triphosphates, including deoxyuridine and *Thermus* species strain Z05 polymerase (a single-tube, single-enzyme, single-primer set process).

2- In the presence of manganese, Z05 has both reverse transcription and DNA polymerase activities.
3- For destruction of potential contaminating DNA from previous amplifications, the AmpErase system (AmpErase) was used.
4- For detection of amplification products, the assays utilize real-time PCR technology with two different dual-labeled fluorescent oligonucleotide probes, which are able to bind HCV target amplicon and quantification standard amplicon, respectively, within the regions spanned by the primers. The two different probes for the HCV target and the quantification standard are labeled with two different fluorescent reporter dyes. The reporter fluorescence is suppressed in the intact probe by the proximity of the quencher dye due to inductive-resonance-based energy transfer (Forster-type energy transfer).

During elongation, the hybridized dual-labeled oligonucleotide probe is cleaved by the 5'-3' exonuclease activity of Z05 polymerase, leading to the separation of reporter and quencher dyes. Within each cycle during the annealing and elongation phase of PCR, the increasing emission of fluorescence light from such cleaved dual-labeled oligonucleotides is collected independently for the HCV target and quantification standard at different wavelengths. The larger the original HCV RNA amount of a specimen, the earlier the fluorescence of the reporter dye rises above certain assigned fluorescence levels (the critical-threshold value), whereas for the constant titer of quantification standard RNA, the fluorescence of the reporter dye should appear at the same cycle for all specimens.

Due to the large dynamic range of the CAP/CTM assay, none of the specimens, had to be diluted. The CAP/CTM assay is standardized against the WHO HCV international standard (96/790) and results are automatically reported in international units per milliliter (IU/ml).

Interpretation

By comparison of critical-threshold values obtained for the target HCV RNA and the quantification standard RNA, the original HCV RNA concentration of the specimen is calculated. Four different results are possible for a sample analysed by the CAP-CTM assay:

(1) undetectable, (2) detectable but unquantifiable (<15 IU/ml), (3) detectable and quantifiable (>15 IU/ml), reported as an exact HCV-RNA concentration in IU/ml, or (4) detectable, quantifiable but above the upper limit of quantification (>6.9 x 10^7 IU/ml).

As indicated in the test package insert, linear quantification of HCV RNA with the CAP/CTM assay ranges from 43 IU/ml up to 6.9×10^7 IU/ml. However, lower HCV RNA concentrations can be detected and reported as positive, but below 15 IU/ml (the assay lower detection limit) results are highly variable and cannot be considered accurate.

Measurement of insulin resistance by HOMA-IR:

After an overnight fast of 12 hours, venous blood samples were collected to test the lipid profile and to determine serum levels of glucose and insulin. Insulin resistance was assessed using homeostasis model assessment for insulin resistance (HOMA-IR) method, using the following equation: HOMA-IR = fasting insulin (μU/mL) X fasting glucose (mmo/L)/22.5 (using Insulin kit™, Cobas e analyzer™, Roche diagnostics, Germany).[137, 138] A HOMA –IR score of more than 3.0 was considered as the criterion of insulin resistance. This was done before start of therapy, after 12 weeks of therapy and at the end of treatment.

Estimation of HOMA-IR score Insulin kit™, Cobas e analyzer™, Roche diagnostic: [157]

Venous blood samples were drawn from patients and control subjects after 12 hours fasting for measurement of homeostatic model of insulin resistance HOMA-IR. Fasting blood glucose was measured. Insulin level in blood was estimated using Cobas e analyzer Insulin kit. The kit is an immunoassay kit for the in vitro quantitative determination of human insulin in human serum and plasma. The electrochemiluminescence immunoassay "ECLIA" is intended for use on Elecsys and cobas e immunoassay analyzers (figure 15).

Figure 15: Cobas e immunoassay analyzers

Liver Histopathology and Immunohistochemistry:

Core liver biopsies were performed on mandatory basis according to MOH protocol for chronic hepatitis C patients before starting the combined Peg INF/ Ribavirin therapy in Sharq Elmadina hospital. They were done to monitor disease progression, to exclude other possible causes of chronic hepatitis as well as to determine the patients that will mostly benefit from the combined therapy. An ultrasound guided percutaneous liver biopsy was analyzed by our experienced pathologist, who was blinded to clinical and biologic data. The biopsies were immediately transferred to Histopathology lab. Specimens were kept in formalin for 24 hours, then, were processed for paraffin embedding. Sections of 5μm thickness were cut from each specimen on 3 separate glass slides.

- The first slide was stained with Hematoxylin and Eosin for routine examination and grading.
- The second slide was stained with Reticulin for evaluation of liver architecture.
- The third slide was stained with Trichrome for estimation of fibrosis.

All specimens were staged and graded according to the Metavir system.[158] Necroinflammation activity (A) was graded as A0 (absent), A1 (mild), A2 (moderate), A3 (severe). Fibrosis stage (F) was scored as F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis), and F4 (cirrhosis). In accordance with the American Association for the Study of Liver Diseases (AASLD) guidelines, fibrosis was considered to be clinically significant for F2-F4 stages.[159, 160] Hepatic steatosis was scored as the percentage of hepatocytes containing macrovesicular fat droplets and was graded from 0 to 3 (grade 0, <5% steatosis; grade 1, 5-33% hepatocytes affected; grade 2, 34-66% of hepatocytes affected; grade 3, 67% or more of hepatocytes affected).[161]

As for the immunohistochemical study, paraffin-embedded liver sections from CHC patients were deparaffinized and subjected to immunohistochemical staining using an anti-human IRS1 (Insulin receptor substrate-1) polyclonal antibodies to examine the protein expression levels of IRS1 in liver samples from randomly selected patients (Ultravision Detection System Antipolyvalent, HRP/DAB kit™, Thermo Fischer Scientific, UK).

Immunohistochemistry for estimation of Insulin receptor substrate -1(IRS-1)Thermo Scientific UltraVision Detection System, Anti-polyvalent, HRP/DAB [162]

The reagents in this universal kit constitute a labeled streptavidin-biotin immunoenzymatic antigen detection system. This technique involves the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen, a biotinylated

secondary antibody that reacts with the primary antibody, enzyme-labeled streptavidin, and substrate-chromogen.

The primary antibody insulin receptor substrate 1 (IRS-1), the major intracellular substrate of the insulin receptor, is an adaptor protein that transmits signals from the IR to downstream effectors that are important for the biological effect of insulin. After insulin stimulation, IRS proteins are rapidly phosphorylated on multiple tyrosine residues. IRS-1 functions as one of the key regulators of IR and disruption of IRS-1 causes insulin resistance associated with hypertension and hypertriglyceridemia.

Principle of the procedure:

This UltraVision detection system detects a specific antibody bound to an antigen in tissue sections. The specific antibody is located by a biotin-conjugated secondary antibody. This step is followed by the addition of a streptavidin-enzyme conjugate that binds to the biotin present on the secondary antibody. The specific antibody, secondary antibody, and streptavidinenzyme complex is then visualized with an appropriate substrate/chromogen.

Procedure

STAINING PROTOCOL:

1.Tissue sections were deparaffinized and rehydrated.
2. To reduce nonspecific background staining due to endogenous peroxidase,slides were incubated in Hydrogen Peroxide Block for 10-15 minutes.
3. Slides were washed 2 times in buffer.
4. Tissues were incubated in digestive enzyme.
5. Slides were washed 4 times in buffer.
6. Ultra V Block was applied and incubated for 5 minutes at room temperature to block non specific background staining (not exceeding 10 minutes for fear of a reduction in desired stain)
7. Slides were then rinsed.
8. Primary antibody was applied and incubated according to manufacturer's protocol.
9. Slides were washed 4 times in buffer.
10.Biotinylated Goat Anti-Polyvalent was applied and incubated for 10 minutes at room temperature.
11. Slides were washed 4 times in buffer.
12. Streptavidin Peroxidase was applied and incubated for 10 minutes at room temperature.
13. Slides were rinsed 4 times in buffer.
14. One drop (40 µl) DAB Plus Chromogen was added to 2 ml of DAB Plus Substrate, mixed by swirling and applied to tissue. Slides were incubated for 5-15 minutes, depending on the desired stain intensity.
15.Slides were counterstained and covered by a coverslip using a permanent mounting media.

Tissue sections of breast adenocarcinoma were used as control for immunostaining as recommended by the kit manufacturer.

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM *SPSS software package version 20.0.* Qualitative data were described using number and percent. Quantitative data were described using Range (minimum and maximum), mean, standard deviation and median. Comparison between cases and control was done using Chi-square test. The distributions of quantitative variables were tested for normality using *Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test.* If data reveals normal distribution, parametric tests were applied. If the data were abnormally distributed, non-parametric tests were used. For abnormally distributed data, comparison between cases and controls was done using Mann Whitney test while Kruskal Wallis test was used to compare between HOMA-IR categories. Correlations between pretreatment HOMA and different parameters were assessed using Spearman coefficient. Significance test results were quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS

I. Demographic characteristics of the study subjects

Among the hundred genotype 4 chronic hepatitis C patients included in the study, 40 were males and 60 were females. The median age of the studied group was 45 years with **a** range of 19-59. The mean ± SD of their ages was 42.82 ± 10.23. (table 9)

Table (9): Demographic characteristics of the study subjects (n=100)

	No.	%
Sex		
Male	40	40.0
Female	60	60.0
Age (years)		
Min. – Max.	19.0 – 59.0	
Mean ± SD.	42.82 ± 10.23	
Median	45.0	

II. Body Mass Index, Lipid profile and metabolic syndrome of the studied genotype 4 CHC patients:

The median of the body mass index (BMI) of the 100 CHC patients included in the study was 27.60 with mean ± SD 27.06 ± 3.44. As regards their lipid profile; the mean ± SD of their cholesterol, triglycerides, HDL and LDL was 149.22 ± 43.76, 102.20 ± 44.35, 40.24 ± 43.76 and 95.26 ± 36.42 respectively with a median of 147.0, 94.50, 33.90 and 92.50 respectively. As for the total lipids, the mean ± SD was 420.90 ± 121.37 and median 426.50. (table 10)

Metabolic syndrome was diagnosed as mentioned before in the methodology section. Fifteen patients (15%) met the criteria of the metabolic syndrome.

Table (10): Body Mass Index and Lipid profile of the studied genotype 4 CHC patients (n=100)

Variable	Min. – Max.	Mean ± SD.	Median
BMI	17.50 – 35.0	27.06 ± 3.44	27.60
Cholesterol	63.0 – 276.0	149.22 ± 43.76	147.0
TG	27.0 – 285.0	102.20 ± 44.35	94.50
HDL	4.0 – 375.0	40.24 ± 43.76	33.90
LDL	11.0 – 195.0	95.26 ± 36.42	92.50
Total Lipids	2.75 -- 862.0	420.90 ± 121.37	426.50

*BMI: Body mass index,TG: Triglycerides, HDL: High density lipoproteins, LDL: Low density lipoproteins.

III. Histopathological examination of liver biopsies of the studied genotype 4 CHC patients

The liver biopsies of the 100 CHC patients included in the study were examined and assessed according to the Metavir system. Necroinflammatory activity in liver biopsies of these patients was as follows: 55% A1, 44% A2, 1% A3 and none were graded as A0. As for fibrosis staging, 51% were F1, 45% were F2, 4% were F3 and none were F0 or F4. As for steatosis: mild and moderate steatosis was equally distributed among the studied cases (20.7% each), severe steatosis was detected in 10.3%, whereas steatosis was absent in 42% of cases (table 11, figure 16-17).

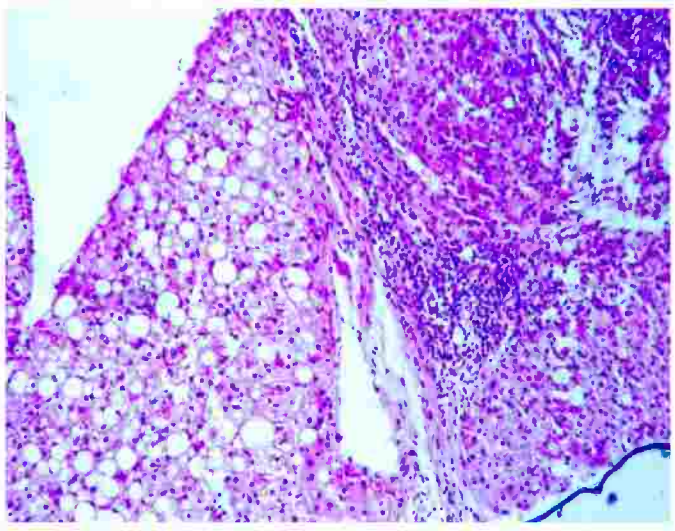

Figure (16): Liver section showing severe degree of macrosteatosis and lymphocytic infiltration.

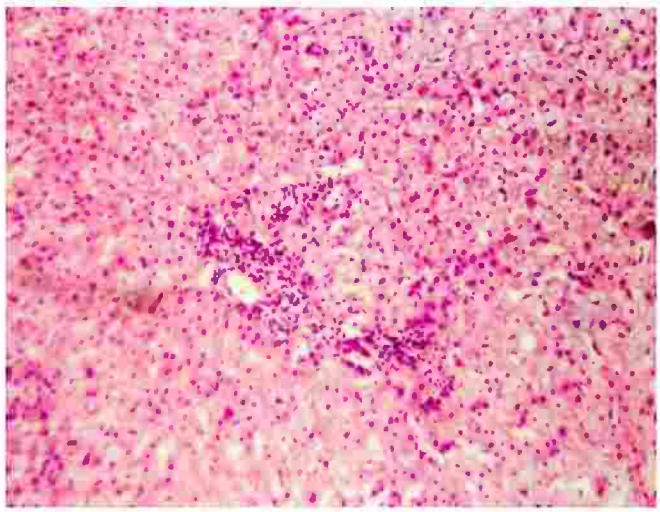

Figure (17): Liver section showing absent steatosis with minimal degree of lymphocytic infiltration.

Table (11): Distribution of the studied cases according to Histopathological findings

Histology	No.	%
Necroinflammatory Activity (n=100)		
A0	0	0.0
A1	55	55.0
A2	44	44.0
A3	1	1.0
Fibrosis (n=100)		
F0	0	0.0
F1	51	51.0
F2	45	45.0
F3	4	4.0
F4	0	0.0
Steatosis (n=29)		
No	14	48.3
Mild	6	20.7
Moderate	6	20.7
Severe	3	10.3

IV. Basal Viral load among the genotype 4 Chronic Hepatitis C group of patients:

The distribution of the 100 genotype 4 chronic HCV patients included in the study with respect to their baseline viral load was as follows: 22% had low level viremia, 43% had intermediate level viremia and 35% had high level viremia. The median was 487.3 $\times 10^3$ IU/ml with mean and standard deviation of $2400.89 \pm 6854.84 \times 10^3$ IU/ml. Level of viremia was classified as high, intermediate and low according to viral load being $>10^6$, 10^5 -10^6 or $<10^5$ IU/ml respectively. (table 12, figure 18)

Table (12): Basal Viral load among the studied genotype 4 Chronic Hepatitis C group of patients (n=100)

Basal Viral load ($\times 10^3$)	No.	%
Low level viremia	22	22.0
Intermediate viremia	43	43.0
High level viremia	35	35.0
Min. – Max.	1.3 - 56000.0	
Mean ± SD.	2400.89±6854.84	
Median	487.23	

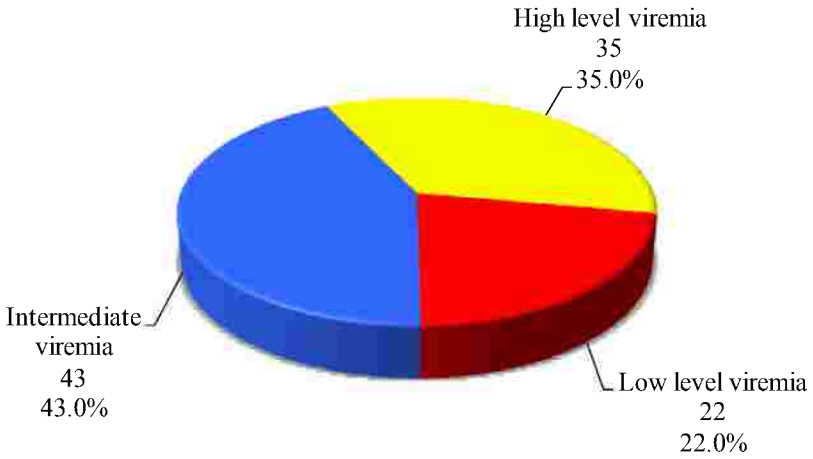

Figure (18): Basal Viral load among the studied genotype 4 Chronic Hepatitis C group of patients (n=100)

V. HOMA-IR values of the studied genotype 4 CHC patients and control subjects:

Insulin resistance was present in 31 of the 100 non diabetic genotype 4 chronic hepatitis C patients (31%). For statistical purposes, HOMA scores were categorized into three groups (<2, 2-4, >4). Comparing between the studied genotype 4 chronic hepatitis C patients before onset of therapy and the control subjects regarding HOMA-IR showed that among the chronic hepatitis C patients, 49% had HOMA–IR of <2, 40% had HOMA-IR of 2-4 and 11% had a HOMA-IR value >4 while that of the 60 HCV negative subjects (the control group) was as follows: 73.3% had <2 HOMA-IR, 21.7% had a 2-4 score and 5% had a >4 HOMA-IR score, the difference being statistically significant (p= 0.009). Likewise the median of HOMA-IR of controls was significantly lower than that of the chronic HCV patients (1.61 ± 1.29 and 2.55 ± 2.36 respectively, p= 0.001). (table13, figure 19)

Table (13): HOMA-IR values of the studied genotype 4 CHC patients and control subjects

Pre-treatment HOMA-IR	Control subjects (n=60)		Patients (n=100)		Test of sig.	P
	No.	%	No.	%		
<2	44	73.3	49	49.0		
2 – 4	13	21.7	40	40.0	χ^2=9.168*	0.009*
>4	3	5.0	11	11.0		
Min. – Max.	0.03 – 6.81		0.23 – 15.17			
Mean ± SD.	1.61 ± 1.29		2.55 ± 2.36		Z=3.322*	0.001*
Median	1.41		2.05			

χ^2: Chi square test
Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$
HOMA-IR: Homeostatic model for assessment-insulin resistance

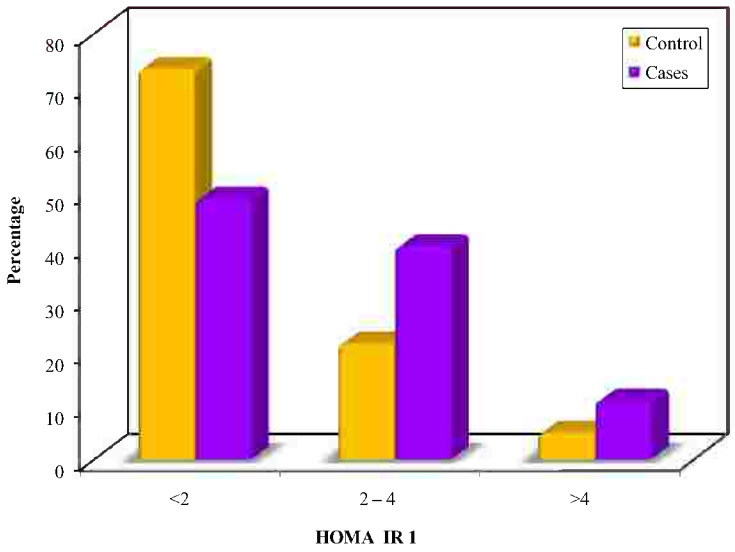

Figure (19): HOMA-IR values of the studied genotype 4 CHC patients and control subjects

VI. Correlation between Pre-treatment HOMA-IR and different variables in the studied genotype 4 CHC patients

Data on the relationship between pretreatment HOMA-IR and clinical and biological variables are presented in table 14. In the univariate analysis, HOMA-IR was positively correlated with age, basal viral load, BMI, triglycerides, fibrosis and steatosis, resulting regarding basal viral load and incidence of fibrosis mounting to a statistical significance (p=0.029 and p=0.026 respectively). Conversely, it was negatively correlated with cholesterol, HDL, LDL and total lipids, results falling short of statistical significance. (table 14, figure 20)

When the data were analyzed by multivariate linear regression to determine variables independently associated with high HOMA-IR, results suggested that viral load remained the only independent factor associated with elevated HOMA-IR levels (p=0.001). (table 15)

Table (14): Univariate analysis of correlations between Pre-treatment HOMA-IR and clinical and biological variables in the studied genotype 4 CHC patients (n=100)

Variable	Pre-treatment HOMA-IR	
	r_s	P
Age	0.101	0.315
Basal viral load	0.218*	0.029
BMI	0.151	0.133
Cholesterol	-0.098	0.338
TG	0.118	0.246
HDL	-0.081	0.430
LDL	-0.120	0.238
Total Lipids	-0.037	0.716
Fibrosis	0.223*	0.026
Steatosis	0.336	0.075

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$
BMI: Body mass index, TG: Triglycerides, HDL: High density lipoproteins, LDL: Low density lipoproteins.

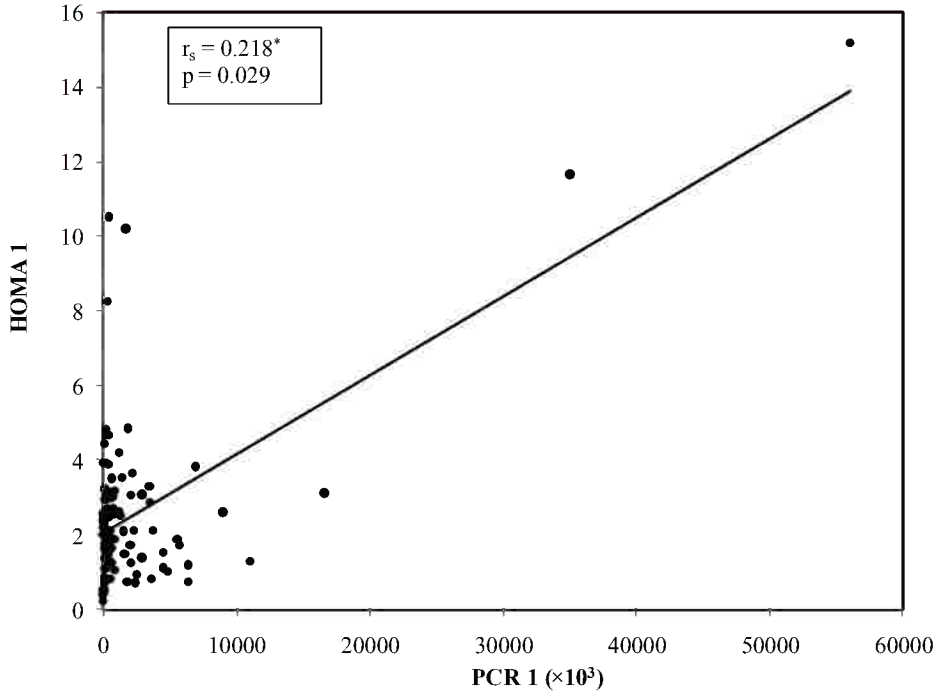

Figure (20): Correlation between pre-treatment HOMA-IR and basal viral load

Table (15): Linear regression analysis for factors associated with high pre-treatment HOMA-IR in patients with genotype 4 CHC (n=100)

	B	S.E	t	p	95% CI	
					Lower	Upper
Basal viral load	0.201	0.051	3.967*	0.001	0.097	0.306
Fibrosis	1.407	1.137	1.237	0.228	-0.935	3.748
$F = 7.803^*$, $p = 0.001^*$, $R = 0.695$, $R^2 = 0.484$						

B: Unstandardized coefficient, SE: Standard error, t= t value, p: Significance of the predictor, OR: Odds Ratio, 95% CI: 95% confidence interval

For testing the treatment outcomes of chronic hepatitis C patients infected with genotype 4 and their association with various degrees of insulin resistance, a total of 71 patients, among those chosen for the prevalence study, were assessed for treatment response after starting dual therapy with peg-interferon and ribavirin. The treatment response in those patients was as follows: ten patients were early non-responders (with $\leq 2\log_{10}$ decrease in HCV RNA in serum 12 weeks after therapy as compared with baseline). For those, therapy was discontinued. Seven patients were partial early virological responders (pEVRs) showing a $\geq 2\log_{10}$ decrease in HCV RNA in serum 12 weeks after start of therapy as compared with baseline and 54 patients were complete early virological responders (cEVRs) with undectectable HCV RNA in serum 12 weeks after start of therapy. Of the 61 patients demonstrating an early virological response and allowed to continue therapy, 59 patients were followed till end of treatment; 2 of the cEVRs being missed during the follow up. All 59 patients achieved end-of-treatment response (ETR) in which serum HCV RNA was undetectable 48 weeks after start of therapy (figure 21).

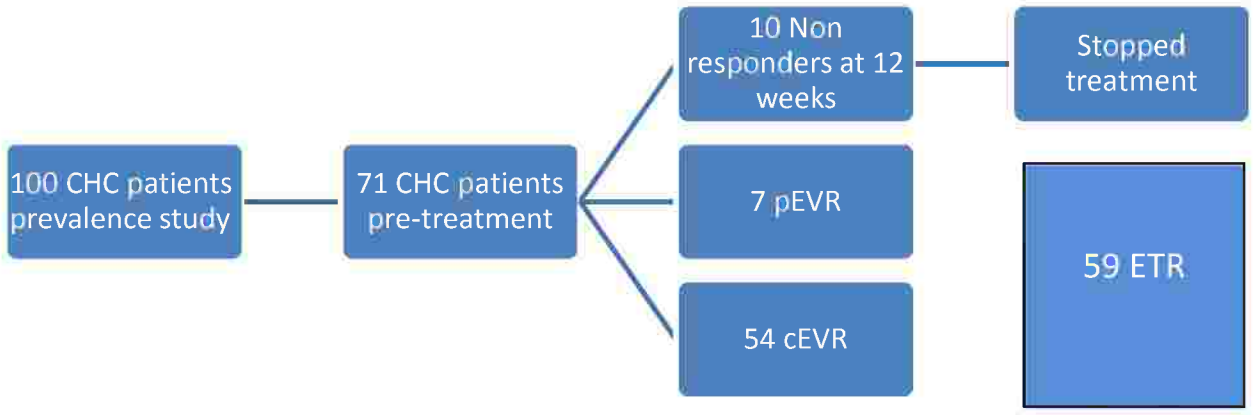

Figure (21): Work flow chart of genotype 4 CHC patients included in the study
pEVR: partial early virological responders, cEVR: complete early virological responders, ETR: end of treatment responders

VII. Pretreatment variables associated with End of Treatment Response

Overall, 59 patients of those followed for assessing treatment outcomes achieved an ETR (59/71, 83 %). We examined the baseline disease and demographic characteristics of the patients influencing the ETR A univariate analysis showed that patients who achieved an ETR were significantly younger, had lower HOMA-IR scores, less viral load and less steatosis compared with non-responders. (Table 16) There was no association between BMI, cholesterol, triglycerides, high density lipoproteins, low density lipoproteins, total lipids or fibrosis in relation to ETR. When the data were analyzed by multivariate logistic regression, results suggested that baseline viral load (odds ratio=0.098,p=0.006) remained the only independent factor associated with ETR in Egyptian patients with genotype 4 chronic hepatitis C infection treated with Peg-IFN plus RBV (Table 17).

Table (16): Pretreatment variables associated with end of treatment response in genotype 4 CHC patients receiving pegylated interferon and ribavirin

Variable	Early Non-Responders (n=10)	ETRs (n=59)	Test of sig.	P value
Age	35.70 ± 5.91	43.97 ± 11.05	t = 3.527*	0.002*
HOMA-IR	2.73 ± 1.08	2.32 ± 2.57	Z = 2.157*	0.031*
Viral load($\times 10^3$)	74.83 ± 123.05	0.83 ± 3.75	t = 3.527*	0.002*
BMI	25.98 ± 5.40	27.41 ± 3.07	t = 0.814	0.435
Cholesterol	141.30 ± 45.83	146.25 ± 46.24	t = 0.314	0.755
Triglycerides	102.80 ± 43.57	101.37 ± 48.09	Z = 0.218	0.827
HDL	40.57 ± 18.73	43.17 ± 54.67	Z = 0.688	0.491
LDL	80.30 ± 36.81	93.35 ± 38.23	t = 1.004	0.319
Total lipids	410.40 ± 109.78	415.97 ± 126.75	Z = 0.050	0.960
Fibrosis				
F1	60.0%	52.5%		
F2	40.0%	42.6%	0.291	1.000
F3	0.0%	4.9%		
Steatosis				
No	0.0	54.5%		
Mild	25.0%	22.7%	6.749*	0.042*
Moderate	75.0%	13.6%		
Severe	0.0	9.1%		

χ^2: Chi square test, FE: Fisher Exact test, t: Student t-test, Z: Z for Mann Whitney test, *: Statistically significant at $p \leq 0.05$. Values of variables of early non-responders and ETRs are in *mean ± SD*. BMI: Body mass index, TG: Triglycerides, HDL: High density lipoproteins, LDL: Low density lipoproteins, HOMA-IR: Homeostatic model for assessment-Insulin resistance.

Table (17): Multivariate logistic regression analysis for factors affecting end-of-treatment response in genotype 4 CHC patients receiving pegylated interferon and ribavirin

	B	S.E	p	OR	95% CI	
					Lower	Upper
Age	0.108	0.073	0.140	1.114	0.965	1.285
HOMA -IR	-0.267	0.233	0.252	0.766	0.485	1.209
Viral load	-2.326	0.854	0.006*	0.098	0.018	0.521

B: Unstandardized coefficient, SE: Standard error, p: Significance of the predictor, OR: Odds Ratio, 95% CI: 95% confidence interval

VIII. Relation between Insulin Resistance and Response to Therapy

Seventy one genotype 4 CHC patients were followed after receiving pegylated interferon and ribavirin and had different treatment outcomes. Their HOMA–IR scores were recorded before initiation of therapy. Ten of those patients were early non-responders as they did not show ≥2 log_{10} decrease in serum HCV viral load by RT-PCR as compared to baseline after 12 weeks of therapy, and further treatment was stopped for them. The mean± SD of their HOMA-IR was 2.95 ± 1.14. Seven patients showed ≥2 log_{10} decrease in viral load compared to baseline after 12 weeks of therapy (partial early virological responders), whereas 54 patients showed complete early virological response, with undetectable HCV RNA after 12 weeks of therapy. The mean± SD of HOMA-IR for the partial and complete early responders was 3.98 ± 2.13 and 2.32 ± 2.35 respectively.

The relation between the baseline HOMA-IR of the three groups of patients showed statistically significant difference (p=0.006). The relation was also statistically significant between the non-responders and the cEVR group of patients (p=0.037), and that between the pEVR and the cEVR group of patients (p=0.007). The relation was not statistically significant between the non-responders and the pEVR patients (p=0.435). (table 18)

Table (18): Relationship between pretreatment values of HOMA-IR and response to therapy in patients with genotype 4 chronic hepatitis C infection

HOMA-IR	Response to therapy			$^{KW}\chi^2$	P
	Early Non responders (n=10)	pEVRs (n=7)	cEVRs (n=54)		
Min. – Max.	1.10 – 4.67	1.90 – 8.24	0.37 – 11.66		
Mean ± SD.	2.95 ± 1.14	3.98 ± 2.13	2.32 ± 2.35	10.303*	0.006*
Median	3.07	3.66	1.64		
p_1		0.435	0.037*		
p_2		0.007*			

χ^2: Chi square for Kruskal Wallis test
p_1 : p value for Mann Whitney test for comparing between Non responder with pEVR and cEVR
p_2 : p value for Mann Whitney test for comparing between pEVR and cEVR
*: Statistically significant at $p \leq 0.05$
pEVR: partial early virological responders, cEVR: complete early virological responders, ETR: end of treatment responders

IX. HOMA-IR before start of therapy, after 12 and 48 weeks of therapy in genotype 4 CHC patients achieving complete early virological response:

Table 19 displays the HOMA -IR scores of the 54 genotype 4 CHC patients that achieved cEVR. Values were determined before commencement of therapy, then after at weeks 12 and 48. The median of the pre-treatment HOMA-IR, after 12 weeks from therapy and at the end of treatment was 1.64, 1.39 and 1.2 respectively with mean± SD 2.32 ± 2.35, 2.20 ± 2.63 and 1.66 ± 1.61 respectively. A highly statistical significant decline in the HOMA score values was noted on comparing the three measurements ($p<0.001$). (table 19)

Table (19): Comparison between HOMA-IR values at the start of therapy, after 12 weeks and after 48 weeks of therapy in cEVR group of patients:

	HOMA-IR of cEVR patients			χ^2	P
	Pre-treatment (n = 54)	After 12 weeks therapy (n = 54)	After 48 weeks therapy (n = 52)		
Min. – Max.	0.37 – 11.66	0.20 – 15.05	0.13 – 10.50		
Mean ± SD.	2.32 ± 2.35	2.20 ± 2.63	1.66 ± 1.61	27.038*	<0.001*
Median	1.64	1.39	1.20		
p_1		0.081	<0.001*		
p_2		<0.001*			

χ^2: Chi square for Friedman test
p_1 : p value for Wilcoxon signed ranks test for comparing between 1st with each stage
p_2 : p value for Wilcoxon signed ranks test for comparing between 2nd and 3rd
*: Statistically significant at $p \leq 0.05$
cEVR: complete early virological response

X. Relation between Immunohistochemistry and Pre-treatment HOMA-IR:

The expression of insulin receptor substrate -1 (IRS-1) was estimated by immunohistochemical staining of randomly selected 29 liver tissue sections from patients with genotype 4 chronic hepatitis C among those included in the study . Results were as follows: 9 cases were grade 0 (10% positive cells), 6 cases 1+ (10–50% positive cells with weak staining), 10 cases 2+ (10–50% positive cells with strong staining or 50% positive cells with weak staining) and 4 cases 3+ (50% positive cells with strong staining) (Figure 22).[163] Pretreatment HOMA-IR values in patients having different grades of immunohistochemical staining for IRS-1 were compared. No statistically significant difference was found between any grades of immunohistochemical staining and pre-treatment HOMA-IR (p= 0.942). (table 20)

Table (20): Relation between IHC and Pre-treatment HOMA-IR

	IHC				$^{KW}\chi^2$	P
	-ve (n = 9)	+ (n = 6)	++ (n = 10)	+++ (n = 4)		
HOMA-IR						
Min. – Max.	0.40 – 10.52	0.37 – 15.17	0.72 – 10.20	0.82 – 4.85		
Mean ± SD.	2.82 ± 3.08	4.33 ± 5.50	2.54 ± 2.77	2.54 ± 1.84	0.393	0.942
Median	1.90	2.39	1.51	2.25		
r_s (p)	0.001 (0.995)					

χ^2: Chi square for Kruskal Wallis test
r_s: Spearman coefficient

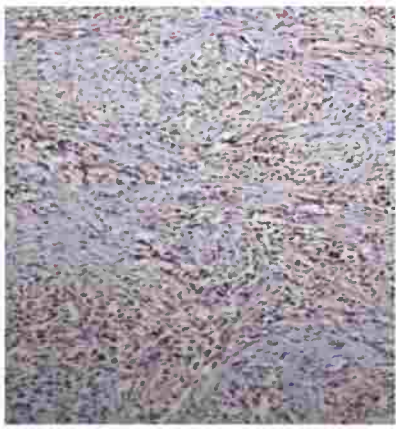

Figure 22 (a) shows the breast cancer tissue sections used as control for immunostaining

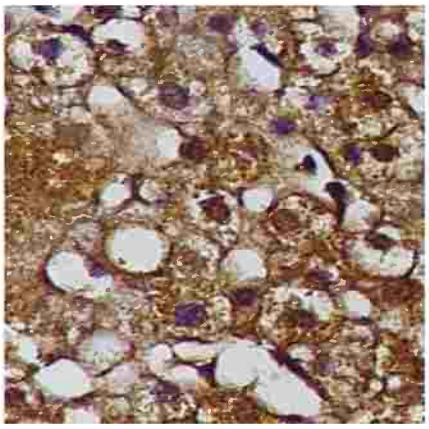

Figure 22 (b) shows grade 3+ immunostaining

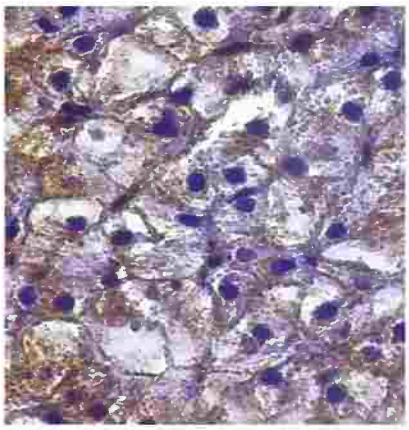

Figure 22 (c) shows grade 1+ immunostaining of the selected liver sections of our patients