AIM OF THE WORK

The present study is devoted to evaluate the role of IL28B single nucleotide polymorphism (SNP) rs12979860 and some host related factors in the responsiveness of chronic hepatitis C Egyptian patients to pegylated interferon plus ribavirin given as the standard of care (SOC) treatment. Host factors will include sex, age, obesity, insulin resistance, ethnicity (race) and degree of liver fibrosis.

SUBJECTS AND METHODS

The present study was carried out after the approval of the Ethical Committee Medical Research Institute. A signed formal consent was obtained from all subjects enrolled in the study that is divided into:

Group I: Consists of 30 apparently healthy volunteers of comparable age, sex and body mass index and served as a control group.

Group II: Consists of 88 CHC infected patients who were positive HCV antibody and HCV RNA (HCV- Ribonucleic acid). Patients of this group were under treatment with pegylated interferon (PEG-IFN) plus ribavirin therapy for 24 weeks. According to the response to the standard of care (SOC) therapy patient group was divided into the following groups:

Group II.1: Responders: those are patients whose Polymerase chain reaction (PCR) results showed EVR.

Group II.2: Non-responders to the (SOC) therapy: those are patients whose PCR results didn't show EVR.

Criteria of Exclusion:

None of the patient's positive for neither hepatitis B surface antigen (HBs Ag) nor positive for Anti-Nuclear Antibody (ANA).

Methods:

All subjects and patients were subjected to:-

I. Full history taking with special emphasis on the onset of diabetes mellitus and HCV infection

II. Thorough clinical examination including:

a. Anthropometric measurements including height, weight, body mass index (BMI) which was calculated as body weight in kilograms divided by the square of height in meter (kg/m^2) [(198)].

b. Liver biopsies were done to assess the stage of liver fibrosis [(108)].

c. Abdominal ultrasonography [(199)].

Sampling:

I. Following twelve hours fasting venous blood were withdrawn from each subject and were taken into four tubes:

- **First**: Tripotassium citrate tube for prothrombin time and activity.
- **Second**: K3- EDTA (tri-potassium ethylene diamine tetraacetic acid) for complete blood count and DNA extraction, the later was done daily and stored at -80 Degree centigrade (-80^oC) before IL-28B genotyping.

- **The last**: A plain tube without anticoagulant. Sera were immediately separated divided into aliquots which were utilized in routine biochemical and virological investigations and aliquots stored at -20°C till analysis serum for Homeostasis Model for Assessment of insulin resistance (HOMA-IR) calculation.

II. Two hours following meal, a venous blood sample was obtained from each individual for the determination of serum postprandial glucose level (PP2h).

The subjected to the following investigations:

I. Biochemical investigations:

- Liver function tests:

  Alanine aminotransferase (ALT), aspartate transferase (AST) , serum Gamma Glutamylatransferase (GGT), alkaline phosphatase (ALP), total and direct bilirubin BIL(T&D), serum albumin (S.ALB).

- Lipid profile: Total cholesterol (T.Ch), Triglycerides (TG), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL).
- Fasting and postprandial blood Glucose.
- Serum creatinine.
- Anti-Nuclear Antibody (ANA), Thyroid Stimulating Hormone (TSH).
- The assay of serum insulin.
- Interleukin-28B (IL-28B) genotyping.

II. Virological investigation:

- Enzyme Linked Immuno Sorbent Assay (ELISA) was carried to confirm the presence of HCV antibodies (HCV Ab), Anti-HCV in these patients and exclude of positive hepatitis B virus antigen (HBV Ag) from patients [200].
- Detection of HCV-RNA by Real Time Polymerase PCR (ABI: Applied Biosystem) baseline, at weak 12 and 24 [201].

Methods:

Chemical analysis was performed on the automated chemistry instrument The Olympus AU400 analyzer (Olympus, Tokyo, Japan) for turbidmetric, spectrophotometric and ion selective electrode measurements, using reconstituted freeze dried forms of multi-analyte calibrators for the serum samples:

1- Determination of serum glucose concentration: [202]

Glucose was determined without deproteinization using an enzymatic method based on the following reaction:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{Glucose\ oxidase} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{Peroxidase} \text{Quinoneimine} + 4H_2O$$

The oxidized rose coloured product was proportionate to the concentration of glucose in the sample (T). It was read spectrophotometrically at wave length (λ) 546nm, and compared to a standard glucose solution (S) of a known concentration (Cs) similarly treated. Serum Glucose (C_T) was calculated as follows:

$$\text{mg/dL glucose } (C_T) = \frac{A_T}{A_S} \times Cs \text{ (mg/dL)}$$

mmol glucose/L = mg/dL × 0.0555

2- Determination of serum creatinine concentration: [203]

Creatinine was determined without deproteinization using Jaffé reaction in a kinetic manner. After a 20 seconds delay, the rate of increase in absorbance(ΔA/min) due to complex formation between creatinine in the sample (T) and alkaline picrate reagent was monitored kinetically over a period of 1 minute at λ 500 nm, and compared to a standard creatinine solution (S) of a known concentration (Cs) similarly treated. Creatinine concentration in the sample (C_T) was determined as follows:

$$\text{mg/dL creatinine } (C_T) = \frac{\Delta A_T}{\Delta A_s} \times Cs \text{ (mg/dL)}$$

mmol creatinine/L = mg/dL x 0.0884

3- Determination of serum alanine aminotransferase (ALT) activity: [204]

ALT activity was determined as follows:

$$\alpha\text{- oxoglutarate + L-alanine} \xrightarrow{ALT} \text{L-glutamate + Pyruvate}$$

$$\text{Pyruvate + NADH + H}^+ \xrightarrow{Lactate\ dehydrogen\ ase} \text{Lactate + NAD}$$

The decrease in absorbance per minute (ΔA/min) at 340 nm (due to NADH + H+ oxidation) was monitored kinetically for 3 minutes. The enzyme activity – expressed in units/L – was calculated as follows:

ALT Activity (U/L) = ΔA/min x -1481.

NAD (Nicotinamide adenine dinucleotide), NADH (Nicotinamide adenine dinucleotide hydrogenates).

4-Determination of serum Aspartate aminotransferase (AST) activity: [205]

AST activity was determined as follows:

$$\alpha\text{-oxoglutarate + L-aspartate} \xrightarrow{AST} \text{L-glutamate + oxaloacetate}$$

$$\text{Oxaloacetate + NADH + H}^+ \xrightarrow{Malate\ dehydrogenase} \text{L-malate + NAD}$$

The decrease that occurs in absorbance at 340 nm (due to NADH + H+ oxidation) was monitored kinetically for 3 minutes. The enzyme activity expressed in units/L was calculated as follows: AST Activity (U/L) = ΔA/min x -1481.

5- Determination of serum alkaline phosphatase activity (ALP): [206]

This was determined kinetically according to the following reaction:

$$\text{P-nitrophenylphosphate} + H_2O \xrightarrow{\text{ALP}} \text{Phosphate} + \text{P-nitrophenol}$$

The resulting increase in absorbance at λ405nm due to the yellow p-nitrophenol product was monitored kinetically at 37°C for 3 minutes Absorbance rate per minute (ΔA/min) and it was directly proportional to the activity of ALP in the sample. The ALP activity was determined from the following equation:

$$\text{ALP Activity (IU/L)} = (\Delta A/\text{min}) \times 2757$$

6- Determination of total serum cholesterol (TC): [207]

Total serum cholesterol was determined enzymatically without deproteinization according to the following reactions:

$$\text{Cholesterol ester} \xrightarrow{\textit{cholesterol esterase}} \text{cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} \xrightarrow{\textit{cholesterol oxidase}} \text{Cholest-4-ene-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-aminoantipyrene} \xrightarrow{\textit{peroxidase}} \text{quinonemine} + 4\ H_2O$$

The rose coloured chromogen produced was proportionate to cholesterol concentration in the sample (T). It was measured at λ 546 nm, and compared to the colour of a standard of a known cholesterol concentration milligram per deciliter (mg/dL)) similarly treated (S). The concentration of cholesterol was obtained by the following equation:

$$\text{mg / dL cholesterol } (C_T) = {}^{A_T}\!/_{A_S} \times Cs \ \text{(mg/dL)}$$

Millimole per liter (mmol / L) cholesterol = mg / dL x 0.026

7- Determination of High density lipoprotein cholesterol (HDL-C): [208]

HDL-C was determined by precipitating Apoprotein-B (Apo-B) 100 containing lipoproteins (LDL and very low density lipoproteins (VLDL)) with phosphotungestic acid in the presence of magnesium ions. Following centrifugation, the cholesterol concentration in the supernatant (HDL-C fraction) was determined enzymatically in the same manner as total cholesterol. The final result was multiplied by a factor of 3 to correct for the sample (1 part) to precipitating mixture (2 parts) ratio.

8- Determination of Low density lipoprotein cholesterol (LDL-C): [209]

An estimated LDL-C concentration was obtained using the Friedewald equation:

$$\text{LDL-C} = \text{Total-C} - (\text{HDL-C} + \text{VLDL-C})$$

Where VLDL-C concentration was estimated using TG/5, provided that TG is < 400 mg/dl.

9- Determination of serum triglycerides (TG): [210]

Triglycerides were determined enzymatically without deproteinization according to the following reactions:

a) Enzymatic hydrolysis: $TG \xrightarrow{Lipase} Glycerol + Fatty\ acids$

b) $Glycerol + ATP \xrightarrow{glycerol\ kinase} Glycerol\text{–}3\text{-}PO_4 + ADP$

c) $Glycerol\text{-}3\text{-}PO_4 \xrightarrow{glycerolphosphate\ oxidase} Dihydroxyacetone\ PO_4 + H_2O_2$

d) Colour development **:**

$2H_2O_2$ + Parachlorophenol + 4 aminoantipyrene $\xrightarrow{peroxidase}$ quinoneimine (red coloured) + $4H_2O$

The rose coloured chromogen, proportionate to TG concentration in the sample (T), was measured spectrophotometrically at λ 546 nm and compared to a standard of known TG concentration (mg/dL) similarly treated (S). TG level was obtained by the following equation:

$$mg / dL\ triglycerides\ (C_T) = {}^{A_T}\!/_{A_S} \times Cs\ \ (mg/dL)$$

mmol / L triglycerides = mg / dL x 0.01126

10- Determination of serum total bilirubin: [211]

It was determined using dimethylsulfoxide as a solvent for total bilirubin assay. Bilirubin in this solvent reacted with diazotized sulfanilic acid and produced an intensely coloured diazo-dye. The intensity of the color of the dye was proportional to the concentration of total bilirubin measured.

Absorbance of sample was read at 546nm and the results were obtained after comparison with a standard bilirubin of known concentration similarly treated using the following equation:

$$Bilirubin\ mg/dl = {}^{A_T}\!/_{A_S} \times Cs$$

Bilirubin µmol/L= (mg/dl) /17.1

11- Determination of serum direct bilirubin: [212]

Direct bilirubin was determined by the reaction with diazotized sulfanilic acid to produce a diazo-dye. The intensity of the color of the dye was proportional to the concentration of direct bilirubin. Absorbance of sample was read at 546nm and the results

were obtained after comparison with a standard bilirubin of known concentration similarly treated using the following equation:

$$\text{Bilirubin mg/dl} = {}^{A_T}\!/_{A_S} \times Cs$$

12- Determination of serum albumin levels: [213]

Test principle:

Albumin measurements were widely simplified by the introduction of dye binding methods. Bromocresol purple (BCP) dye is capable of forming a coloured complex with albumin, providing a greater specificity to albumin determination.

The reaction employed in this assay is:

$$\text{Albumin} + \text{BCP} \xrightarrow{\text{Acid}} \text{Coloured complex.}$$

Formation of the complex is proportional to the albumin concentration in the specimen. The amount of complex formed is determined bichromatically at A 600/800nm.

Results expressed as gram per liter (g/L) or gram per deciliter (g/dL) is automatically calculated. SI Conversion g/L x 0.1 = g/dL.

13- Determination of serum Gamma Glutamylatransferase (GGT): [214]

The International Federation of Clinical Chemistry (IFCC) recommended the standardized method for determining GGT including optimization of substrate concentrations, employment of NaOH glycylglycine buffer and sample start. The GGT liquid reagent followed the formulation recommendation according to Szasz, but was optimized for performance and stability. The assay is optionally standardized against the original IFCC and Szasz methods.

Test principle:

Enzymatic colorimetric assay γ-glutamylatransferase transfers the γ -glutamyl group of L- γ -glutamyl-3-carboxy-4-nitroanilide to glycylglycine.

L- γ -glutamyl-3-carboxy-4-nitroanilide+glycylglycine $\xrightarrow{GGT}$ L- γ -glutamyl glycylglycine + 5-amino-2-nitrobenzoate

The amount of 5-amino-2-nitrobenzoate liberated is proportional to GGT activity in the sample. It is determined by measuring the increase in absorbance photomterically.

➢ Determination of Prothrombin Time (PT) and activity by (Sysmex CA -1500): [215]

Prothrombin time was determined in plasma by adding a thromboplastin reagent (Thromborel® S, Dade Behring, Marburg GmbH, Germany) and the clotting time was recorded in seconds. To obtain the prothrombin index, a curve was made with dilutions of

normal pool plasma corresponding to 100% activity. In each patient, results were expressed as the percentage of normal plasma yielding the same prothrombin time in seconds.

➢ Determination of Complete blood picture (CBC) by (Sysmex 1800 i) (Roche Diagnostics) cell counter [216].

➢ Determination of Thyroid-Stimulating hormone (TSH): [217]

TSH was performed on the auto-analyzer Cobas 411 Roche Hitachi electrochemolysis, using the serum samples: [217]

Thyroid-Stimulating hormone (TSH; Thyrotropin) is a glycoprotein.The Elecsys TSH-assay employs monoclonal antibodies specifically directed against Human TSH. Antibodies labeled with ruthenium complex consist of a chimeric contrast of human and mouse-specific components.

Test principle

Sandwich principle: Total duration of assay; 18 minutes.

- 1st incubation: 50 microliter (µL) of sample, abiotinylated monoclonal TSH-specific antibody and a monoclonal TSH-specific antibody labeled with a ruthenium complex react to form a sandwich complex.
- 2nd incubation: After addition of streptavidin-coated microparticles the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticales are magnetically captured onto the surface of the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Result are determined via a calibration curve which is instrument –specifically generated by 2-point calibration and master curve provided via reagent barcode.

Analyzer automatically calculates the analyte concentration of each sample either in µIU/ml or mlU/L.

➢ Serodetection of anti-hepatitis C virus antibodies by sandwich enzyme linked immunosorbent assay (ELISA) technique [218] (Murex Diagnostic Limited. Dartford, England):

The diluted samples were incubated in micro-wells coated with highly purified HCV antigen (HCV-Ag) that contained sequences from the putative C, NS3, NS4, and NS5 regions of HCV that were bound to any anti-HCV antibody in the sample during the first incubation. Unbound material was removed by washing.

The formed Antigen-Antibody (Ag-Ab) complexes were then incubated with peroxidase conjugated monoclonal anti-human IgG. An antibody-antigen-antibody-enzyme complex was developed in those wells containing samples with anti-HCV antibodies.

Excess unbound enzyme conjugate was removed by washing. Wells that contained anti-HCV antibodies and hence the bound enzyme conjugate developed a purple color on addition of a suitable substrate, 3,3',5,5' tetramethyl benzidine (TMB) which was converted to orange when the enzyme reaction was terminated with sulphuric acid (2M). The color developed was read photo metrically at λ450.

The amount of conjugate bound, and hence the color developed in the wells was directly related to the concentration of the antibodies in the sample. All readings above the cutoff value of the positive control were considered positive.

➢ Serodetection of hepatitis B virus surface antigen (HBs-Ag) by sandwich enzyme linked immuno-sorbent assay (ELISA) technique [(219)] (Murex Diagnostic Limited. Dartford, England)

The samples were pre-incubated in micro wells coated with a mixture of mouse monoclonal antibodies specific for different epitopes on HBsAg. Then, affinity purified goat antibody to HBsAg conjugated to horse-reddish peroxidase was added to the sample in the well.

During the two incubation steps, any HBs-Ag in the sample would bind to the well in an antibody-antigen-antibody-enzyme complex. Excess unbound enzyme conjugate was removed by washing. Wells that contained HBs-Ag and hence the bound enzyme conjugate developed a purple color on addition of a suitable substrate, 3,3',5,5' tetramethyl benzidine (TMB) which was converted to orange when the enzyme reaction was terminated with sulphuric acid (2M). The color developed was read photometrically atλ450. All readings above the cutoff value of the positive control were considered positive.

➢ Determination of serum insulin: [(220)]

Principle of the Procedure:

Serum insulin was measured using a two site, solid phase chemiluminescent enzyme immunometric assay (CLIA) by the Immulite 1000 Automated Analyzer (Diagnostic Products Corporation). The solid phase consisted of a polystyrene bead, coated with a monoclonal murine anti-insulin antibody. Both the bead and the sample (or calibrator) were dispensed into a specially designed reaction tube. (N.B: The reaction tube served as a vessel for incubation, wash, and signal development).

The sample (or calibrator) was incubated at 37°C with both the alkaline phosphatase conjugated to polyclonal sheep anti-insulin antibody and the monoclonal murine anti-

insulin antibody in the reaction tube. The insulin molecules present in the sample (or calibrator) bound to both anti-insulin antibodies forming a sandwich complex.

The reaction mixture was separated from the bead by spinning the reaction tube at a high speed along its vertical axis, followed by transferring the fluid (reaction mixture) to a coaxial sump chamber, which is integral to the bead / tube wash station.

Unbound conjugate in the reaction tube was removed using four discrete wash cycles within seconds, leaving the beads in the reaction tubes with no residual unbound label. A luminogenic substrate was then added to the reaction tube. The reaction tube was then transferred to the luminometer chain, where the light generated by the luminometric reaction was detected by the photomultiplier tube (PMT).

In the luminogenic reaction, the substrate (adamantly dioxetane phosphate) was dephosphorylated into an unstable anion (unstable intermediate dioxetane) by the alkaline phosphatase conjugate captured on the bead. The unstable intermediary emitted photons upon decomposition, is directly proportional to the amount of bound enzyme, and therefore directly proportional to the concentration of insulin in the serum sample.

Reagents

Insulin Test Units:

Each barcoded test unit contained one bead coated with monoclonal murine anti-insulin. The kit contained 100 test units. Test unit bags were left to come to room temperature before opening.

Insulin Reagent Wedge:

Reagent with barcode consisted of 18.5ml alkaline phosphatase conjugated to polyclonal sheep anti-insulin in buffer, with preservative.

Insulin Adjustors:

Two vials (Low and High), lyophilized insulin in a nonhuman serum matrix, with preservative. Each vial was reconstituted by adding 4.0 mL distilled water.

Sample:

10 µl serum were aspirated from sample cup.

Expected Values*:* Up to 28.4 micro international unit per millimeter (µIU/mL).

- A simple mathematical model named **Homeostasis Model for Assessment of insulin resistance** (HOMA-IR = fasting insulin (µIU/ml) × fasting glucose (mmol/L)/22.5) has proved to be useful in the measurement of insulin sensitivity in euglycemic patients. It is a model of interactions between glucose and insulin dynamics that is then used to predict fasting steady-state glucose and insulin concentrations for a wide range of possible combinations of insulin resistance and β-cell function [(221)].

➢ Detection of HCV-RNA by Real Time PCR:

The Real-time PCR system is based on the detection and quantitation of fluorescent reporter. [222]

HCV RNA extraction

RNA extraction was performed using Qiagen QIAamp viral RNA mini spin protocol**.**

Reagents used for RNA extraction, QIAamp viral RNA mini kits.

QIAamp viral RNA mini kits used for viral RNA preparation.

Reagents:

QIAamp spin columns

Collection tubes (2 ml)

RNA Carrier (poly A) 310 µg

Buffer AVL is a viral lysis buffer used for purifying viral nucleic acids. It is supplied as 155 ml with 5 x 310 µg Carrier RNA. QiaAmp Viral Lysis (AVL) buffer (Qiagen), which has a high chaotropic salt concentration.

Buffer washing nucleic acid (AW) 1 contains Guanidinium Chloride (guanidine hydrochloride). This is used to denature proteins in your sample. They will then flow through the column and will be discarded with the wash.

Buffer AW2 is essentially 70% Ethanol (EtOH). 30 ml of 100% EtOH is added to the 13 mls of "concentrate "included in the bottle. 70% EtOH is used to remove salts from your column and aid in purifying your DNA.

Buffer Eluted viral nucleic acid (AVE) is RNase-free water that contains 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNases.

I-Procedure:

HCV RNA extraction, QIAamp viral RNA mini spin protocol

1- Five hundred and sixty µl of AVL buffer containing lyophilized carrier RNA were pipetted into a 1.5 ml micro centrifuge tube.

2- One hundred and forty µl serum was added, mixed by pulse-vortexing for 15 second. And incubated at room temperature for 10 minutes, and then briefly centrifuged to remove drops from the inside of the lid.

3- Five hundred and sixty µl of ethanol (96-100%) were added to the sample, and mixed by pulse-overtaxing for 15 second.

4- Six hundred and thirty µl of the solution from step 3 were carefully applied to the QIAamp spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and centrifuged at 6000 g (relative centrifugal force) (8000 revolutions

per minute (rpm)) for 1 minute. The QIAamp spin column was placed into a clean 2 ml collection tube containing the filtrate was discarded.

5- The QIAamp spin column was carefully opened, and step 4 was repeated.

6- The QIAamp spin column was carefully opened and 500 µl of AW1 buffer were added. The cap was closed and centrifuged at 8000 rpm for 1 minute. The QIAamp spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

7- The QIAamp spin column was carefully opened and 500 µl of buffer AW2 were added. The cap was closed and centrifuged at full speed 14000 rpm for 3 minutes.

8- The QIAamp spin column was placed in a new 2 ml collection tube, centrifuged at 14000 rpm for 10 minutes.

9- The QIAamp spin column was placed in a new 2 ml collection tube and the old collection tube with filtrate was discarded. The QIAamp spin column was carefully opened and 60 µl of AVE were added equilibrated to room temperature for 1 mint, then centrifuged at 8000 rpm for 1 minute.

II- The amplification reaction was performed as follows:

HCV-RNA was detected by using (ABI; applied biosystem) RNA protocol with TaqMan assay reagents

- 0.5 µl of HCV forward primer for Hepatitis C Virus genotype 4 (HCV4).
- 0.5 µl of HCV reverse primer (HCV4).
- 0.5 µl of HCV TaqMan probe (HCV4).
- 0.5 µl of HCV IC forward primer.
- 0.5 µl of HCV IC reverse primer.
- 0.5 µl of HCV IC probe.
- 0.25 µl reverse transcriptase enzyme (RT).
- 0.5 µl RNAase inhibitor.
- 12.5 µl TaqMan universal PCR master mix 2 fold (ABI; applied biosystem) were added to the reaction mixture bringing the reaction volume to 15.5 µl.
- 9.5 µl of Qiagen extracted RNA were added to bring the reaction to a final volume of 25 µl

Figure (12). Step One Applied Biosystem**.**

Thermal profile:

The reaction took place under the following thermal profile:

Incubation at 48°C for 30 minutes to transcribe viral RNA to cDNA by RT. This was followed by AmpliTaq gold activation for 95° C for 10 minutes, followed by 45 cycles of two PCR-step amplification, denaturation for 95° C for 15 second, followed by annealing and extension at 60° C for 1 minute with end point fluorescence detection [222].

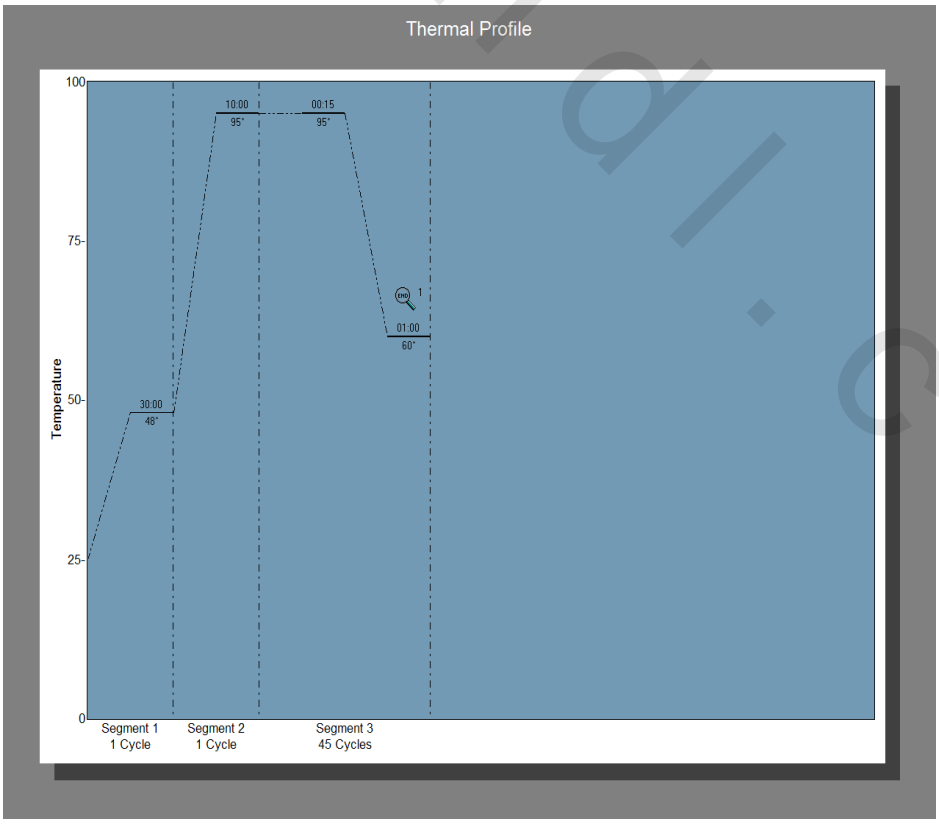

Figure (13): Thermal profile setup of HCV RT-PCR

➢ Single nucleotide polymorphisms (SNPs) of the IL28B rs1297980 genotyping:

I. Genomic DNA extraction:

A freshly withdrawn whole blood sample was taken on a sterile K3-EDTA (tri-potassium ethylene diamine tetraacetic acid) vacutainer tube. The genomic DNA Extraction from peripheral blood leukocytes was carried out using QIAamp® DNA blood Mini kit (Qiagen Hilden, Germany) according to the manufacturer's instructions.

Principles:

The QIAamp® DNA blood Mini kit is basically an ion-exchange column chromatography kit and it comprised of four main steps (Lysis, Binding, Washing and Elution).

Reagents:

1. **QIAamp DNA Mini Kit Buffer AL:** Cell lysis buffer containing chaotropic salt guanidine hydrochloride to disrupt the cell and nuclear membranes and to denature proteins.
2. **Protease**: prepared by resuspending the provided lyophilized Qiagen protease with the provided protease resuspension (nuclease-free H2O) containing 0.04% sodium azide, the ready to-use protease is then divided into aliquots and store at -20 °C until first use.
3. **Ethanol**: 96-100%
4. Q**IA amp Spin column:** silica-gel membrane spin columns that selectively bind DNA based on Ph and salt concentrations.
5. **QIAamp Buffer AW1:** Wash buffer 1
6. **QIAamp Buffer AW2:** Wash buffer 2
7. Q**IAamp Buffer AE:** Elution buffer (10 mM Tris HCL;0.5 mM EDTA; PH 9.0).

Procedure:

1) Step 1(Lysis):

a. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.

b. Add 200 µl sample to the microcentrifuge tube. Use up to 200 µl whole blood, or up to 5 x 10^6 lymphocytes in 200 µl peripheral blood sample (PBS).

Note: It is possible to add QIAGEN Protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.

c. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s. To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

d. Incubate at 56°C for 10 minutes. DNA yield reaches a maximum after lysis for 10 minutes at 56°C. Longer incubation times have no effect on yield or quality of the purified DNA.

e. Briefly centrifuge the 1.5 ml micro-centrifuge tube to remove drops from the inside of the lid.

f. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 second. After mixing, briefly centrifuge the 1.5 ml micro-centrifuge tube to remove drops from the inside of the lid.

2. Step 2: Adsorption to the QIAamp membrane (binding):

a. Carefully apply the mixture (the lysate) from the last step to the QIAamp Mini spin column (packed with silica membrane) placed into a 2 ml collection tube without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min.

b. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Notes:

Close each spin column to avoid aerosol formation during centrifugation.

Centrifugation is performed at 6000 x *g* (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty.

3. Step 3 Removal of residual contaminants (Washing)

a. Washing Step I:

(1) Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1minute.

(**2**) Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

b. Washing Step II:

(1) Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 minutes.

(2) Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 minute.

This step helps to eliminate the chance of possible Buffer AW2 carryover.

4. Step 4 Elution of genomic DNA (Elution):

a. Place the QIAamp Mini spin column in a clean 1.5 ml micro-centrifuge tube (not provided), and discard the collection tube containing the filtrate.

b. Carefully open the QIAamp Mini spin column and add 100 µl Buffer AE or distilled water.

c. Incubate at room temperature (15–25°C) for 1 minute, and

d. Then centrifuge at 6000 x *g* (8000 rpm) for 1 minute.

Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

A second elution step with a further 200 microliter (µl) Buffer AE will increase yields by up to 15%.

For long-term storage of DNA, eluting in Buffer AE and storing at –20°C is recommended, since DNA stored in water is subject to acid hydrolysis.

A 200 µl sample of whole human blood (approximately $5x10^6$ leukocytes/ml) typically yields 6 micrograms (µg) of DNA in 200 microliter (µl) water 30 Nanograms per microliter (ng/µl) with an A260/A280 ratio of 1.7–1.9.

II. Assessing the integrity of the extracted genomic DNA [223]

The integrity of the extracted genomic DNA was assessed by agarose gel electrophoresis (AGE) in four main steps:

The integrity of the extracted genomic DNA was assessed by agarose gel electrophoresis (AGE) in four main steps:

1) Agarose gel preparation :

a. 1% agarose powder was dissolved in 100 mL Tris Borate EDTA (TBE) 1x buffer using a hot plate with a magnetic stirrer.

b. Ethidium Bromide: 1µL for every 10 mL of the gel was added after the dissolved hot gel was allowed to cool under running tap water.

c. Gel casting was done by pouring the warm gel into a plastic plate with plastic combs placed inside it to form the wells.

d. As soon as the gel cooled and solidified, the plastic combs were removed leaving the formed application wells in which the DNA ladder and samples were dispensed.

2) Sample preparation:

5 µL autoclaved sterile water + 2 µL gel loading dye (bromophenol blue, EDTA and glycerol) + 5 µL extracted DNA.

3) AGE run condition:

After submerging the cast gel into the electrophoresis tank filled with TBE 1x buffer and samples were applied inside the wells, electrophoresis was done for 30 – 40 minutes at 90 – 100 volts.

4) Visualization:

Discrete intense bands were observed using an ultraviolet (UV) *trans* illuminator scanner and a Polaroid gel documentation camera was used to take a shot of the gel as shown in (figure 14).

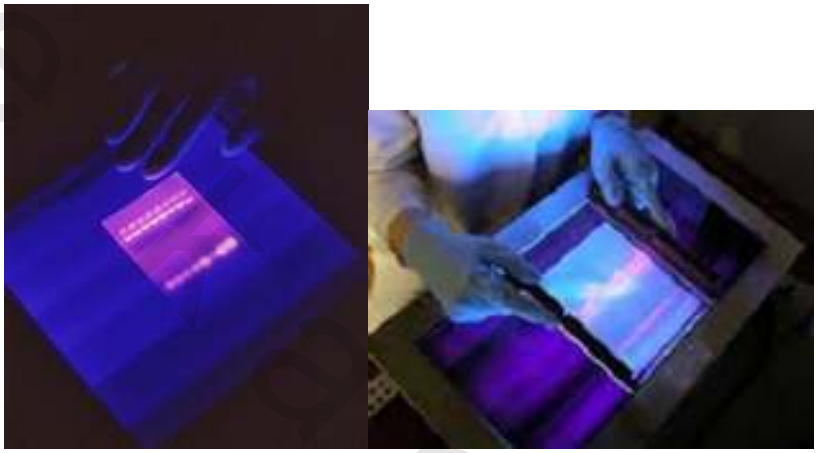

Figure (14). Digital photo shot of the gel showing the bands of the genomic DNA

III. Quantification and uniformity of stored DNA:

Just before performing the Real-Time PCR SNP genotyping, the purity and concentration of stored extracted genomic DNA was determined using the Thermo Scientific Nano Drop™ 1000 Spectrophotometer *(Thermo Fisher Scientific, Wilmington, Delaware USA)*.

1. Since DNA absorbs strongly at or near 260 nm, while other contaminants especially proteins absorb strongly at or near 280 nm. The ratio of absorbance at 260 nm and 280 nm (260/280) was used to assess the purity of DNA. Samples with ratio of ~1.8 was accepted as "pure" for DNA while the extraction of samples with appreciably lower ratio (indicating the presence of other contaminants especially proteins) was repeated.

2. 260/230 ratio was also used as a secondary measure of nucleic acid purity. Since the 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Samples with 260/230 values in the range of 2.0-2.2 was accepted as "pure" for DNA, while the extraction of samples with appreciably lower ratio (indicating the presence of other contaminants especially EDTA, Guanidine HCL used for DNA isolations and phenol all have absorbance near 230 nm) was repeated. Accordingly variable volume of DNAase free water was used to dilute all the samples with accepted purity and bring them all to a uniform final concentration of 1ng/µl Nano gram per micro liter.

IV. Real Time Polymerase Chain Reaction SNP genotyping:

Genotyping of single nucleotide polymorphisms (SNPs) of the IL28B rs1297980 was carried out by the allelic discrimination Real-Time Polymerase Chain Reaction SNP genotyping technology using dual labeled fluorogenic TaqMan® minor groove binder (MGB) probes: [224]

I. Reagents:

1. **TaqMan® SNP Genotyping Assay 20✕**

(*Applied Biosystems-Life Technologies, Carlsbad*, California, USA) it contains:

a. **Sequence-specific forward and reverse primers** to amplify the polymorphic sequence of interest (NB: their sequences are not revealed by the company).

b. **Two allele specific TaqMan® MGB* probes** for distinguishing between the two Alleles, each TaqMan MGB probe contains:

1) A reporter dye at the 5' end of each probe:

a. VIC® fluorescent dye is linked to the 5' end of the Allele C probe. i.e. Allele "C" (wild allele) would associate with TaqMan® MGB VIC labeled probe

b. FAM™ (6-carboxyfluorescein) fluorescent dye is linked to the 5' end of the Allele C probe .i.e. Allele "T" (mutant allele) would associate with TaqMan® MGB FAM labeled probe

2) A Non-fluorescent quencher (NFQ) at the 3′ end of each probe

3) A minor groove binder (MGB) at the 3' end of each probe: This modification increases the melting temperature (Tm) for a given probe length [225] which allows the design of shorter probes. Shorter probes result in greater differences in Tm values between matched and mismatched probes, producing robust allelic discrimination. Even single nucleotide mismatches between a probe and the target sequence reduce the efficiency of probe hybridization, which in turn reduces the amount of reporter dye cleaved from a quenched probe. Furthermore, AmpliTaq Gold DNA polymerase used is more likely to displace a mismatched MGB probe without cleaving it. Each of these factors minimizes the production of nonspecific fluorescence signals

2. **Double-concentrated (X2), TaqMan® Universal PCR Master Mix** (*Applied Biosystems-Life Technologies, Carlsbad*, California, USA) which contains :-

a - AmpliTaq DNA Polymerase: It is an enzyme with a DNA-directed DNA polymerase activity that catalysis the synthesis of DNA from deoxyribonucleotide triphosphates in the presence of a DNA template and primer. It also has a 5′ to 3′ exonuclease activity[226] .This thermally stable DNA polymerase enabled applying a hot start PCR, a technique that reduces non-specific amplification during the initial set up stages of the PCR, where the AmpliTaq DNA polymerase's activity is

inhibited at ambient temperature by the presence of bound inhibitors which dissociate only after a high-temperature activation step that was done. i.e holding temperature at 95 °C for 10 mint[227] .

b - Deoxynucleoside, 5´triphosphates (dNTPs) 10 mM each: These PCR-Grade Nucleotides constituted the building blocks of new DNA used by the AmpliTaq DNA polymerase. They included, 2'-deoxyadenosine triphosphate (dATP), 2'-deoxycytidine 5'-triphosphate (dCTP), 2'-deoxyguanosine 5'-triphosphate (dGTP), and 2'-deoxythymidine 5'-triphosphate (dTTP).

c - ROX™ dye: This is a Passive Reference dye that did not participate in the 5' nuclease PCR yet it provides an internal reference to which the reporter-dye signal can be normalized during data analysis.

d - Optimized buffer.

3. **DNase-free, deionized sterile-filtered water.**

II. Principals

1. Polymerase Chain Reaction

The PCR process is a simple and powerful method, [228, 229] which allows amplification of DNA segments in vitro through a succession of incubation steps at different temperatures. Typically, the double stranded DNA is heat-denatured, two pre-designed primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature by the heat stable Taq DNA polymerase enzymatic activity. One set of these three consecutive steps is referred to as a cycle (Figure 15). The PCR process is based on the repetition of this cycle and can amplify DNA segments by at least 10^5 fold.[230-232]

2. TaqMan® SNP Genotyping:

The TaqMan SNP Genotyping assay is a 5'-nuclease assay that uses 5'-nuclease activity of the Taq DNA polymerase for SNP genotyping and it is performed concurrently with the PCR reaction and during PCR, the following steps occur:

1. The forward and reverse PCR primers within our TaqMan® SNP Genotyping Assay, which are pre-designed to amplify the sequence containing the SNP, anneals specifically to their complementary sites that includes the SNP polymorphic site in between.

2. Each one of the two allele specific TaqMan MGB probes, anneals specifically to its complementary sequence between the forward and reverse primer sites. As long as the TaqMan MGB probe is intact, the proximity of the reporter dyes (FAM or VIC) to their non-fluorescence quencher dyes results in quenching of the reporter fluorescence primarily by fluorescence (or Förster) resonance energy transfer (FRET) [233]

3. During the PCR amplification step, AmpliTaq Gold® DNA polymerase in the TaqMan® Universal PCR Master Mix with its DNA 5'→3' polymerase enzymatic

activity uses the nucleotides within the Master Mix to extend the primers bound to the template DNA.

4. AmpliTaq® Gold DNA polymerase within the TaqMan Universal PCR Master Mix, with its 5'→3' exonuclease activity, displaces then cleaves the matching allele specific TaqMan MGB probe that hybridized perfectly to its specific complementary target, cleavage separates the reporter dye from its quencher dye, which results in an increase in specific fluorescence by this reporter [234]. (Figure.16 on the left).

5. The mismatching allele specific TaqMan MGB probe which is not perfectly complementary, will consequently has a lower melting temperature and will not bind efficiently. This prevents the polymerase from acting on the probe and consequently no fluorescence signal would be detected from this probe.(Figure 16 on the right).

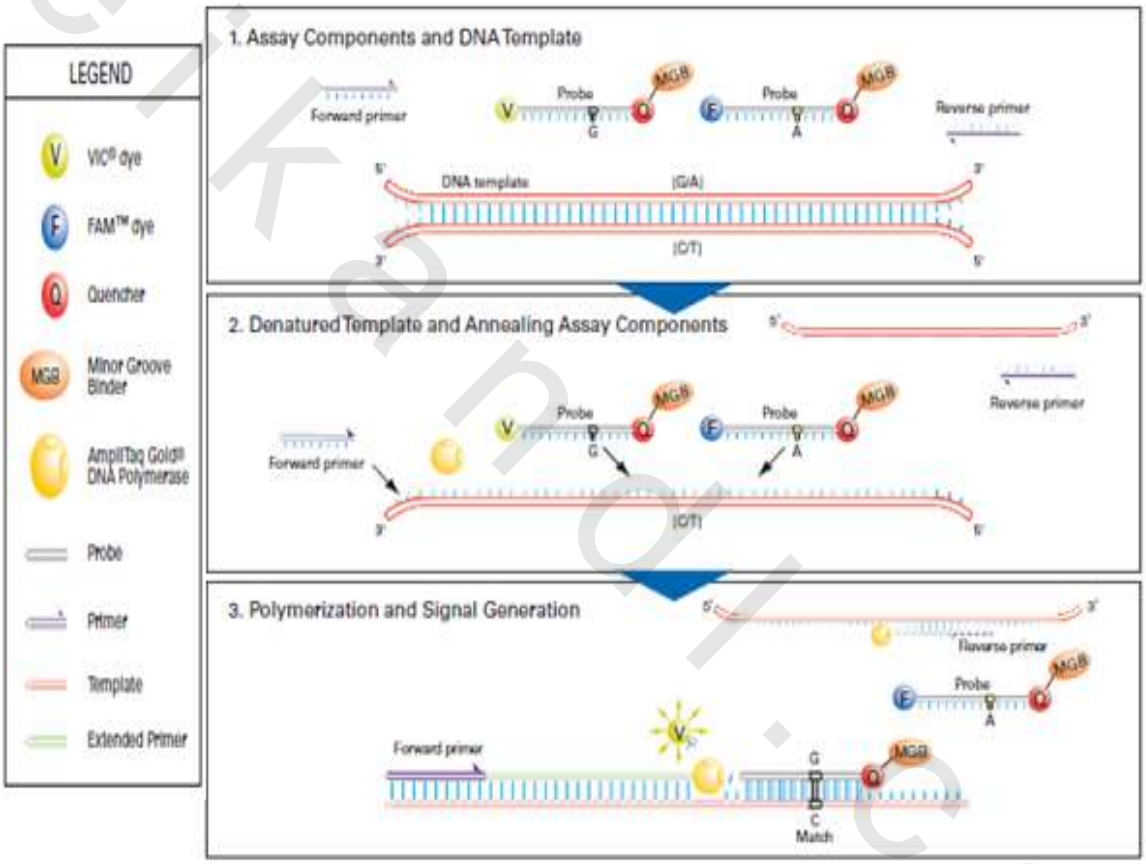

Figure (15): 5'-nuclease assay process (TaqMan® SNP Genotyping Assay (Applied Biosystems) (ABI) User Protocol) [234]

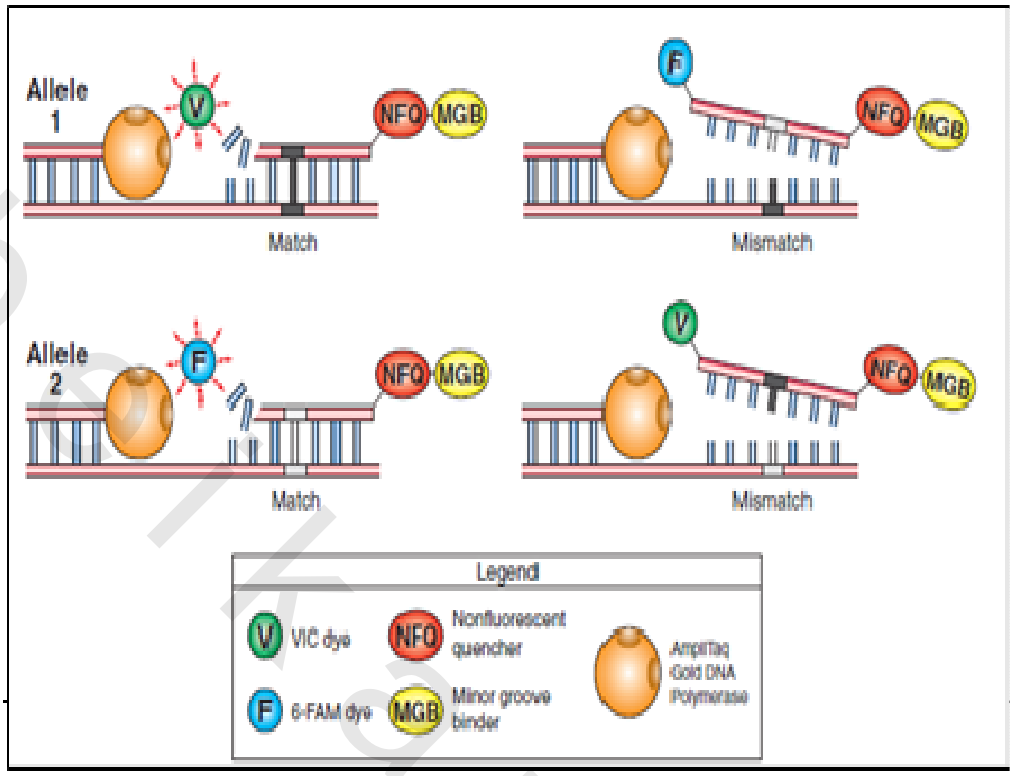

Figure (16): Results from matches and mismatches between target and probe sequences in TaqMan SNP Genotyping Assays. [234]

III. Procedure Steps:

1. Preparing the reaction mixture:

All reagents were brought to room temperature (20–25°C) prior to use. The TaqMan Universal PCR Master Mix bottle was thoroughly mixed by swirling. After gradual thawing of the frozen genomic DNA samples and the TaqMan SNP Genotyping Assay, a pulse vortexing was done followed by a brief spinning.

2. Calculations were done to prepare the total amount of reaction mix so that each was as follows:

in a 20 µl total volume; 5 µl total DNA; 0.5 µl IL28B Rs60(rs12979860) forward primer; 0.5 µl IL28B Rs60 reverse primer; 0.5 µl IL28B Rs60 prob T allele; 0.5 µl IL28B Rs60 Prob C allele ; 10 µl Universal TaqMan PCR master mix(2X) and 3 µl nuclease free water.

QRT-PCR Procedure: Table 4

1- Vortex each vial before use and spin briefly as in table (4):

Reagent	Volume/reaction	Description
Universal TaqMan PCR Master Mix	10 µl	Universal TaqMan PCR master mix(2X)
IL28BRs60 Forward primer	0.5 µl	HPLC purified, ready to use
IL28BRs60 reverse primer	0.5 µl	HPLC purified, ready to use
IL28BRs60 Probe T allele	0.5 µl	HPLC purified, ready to use,(FAM^{TM} ~dye labeled probe)
IL28BRs60 C allele	0.5 µl	HPLC purified, ready to use,(VIC^{TM} ~dye labeled probe)
PCR Grade H_2O	3 µl	RNase , DNase-free water
DNA Extract	5 µl	(~ 20ng)

HPLC **(**High performed liquid gas chromatography).

2- Centrifuge the reaction tubes in a centrifuge for 30 sec. in order to collect prepared reaction volume in the bottom of the well.

3- Programming the thermal cycling conditions:

The thermal cycling conditions of the StepOne real-time PCR system (*Applied Biosystems-Life Technologies, Carlsbad*, California, USA) were programmed as follows: 30 second at 60^0 C, 10 minutes at 95^0C, 40 cycles consisting of 15 second at 95^0C and 1 minute at 60^0C and 30 second at 60^0C.

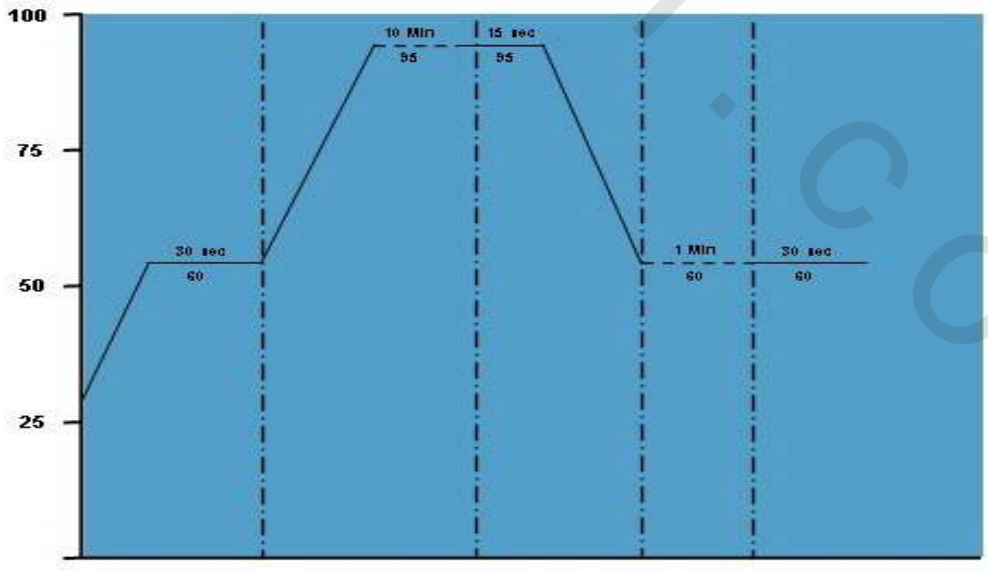

Figure (**17).**IL-28B genotyping Thermal cycling.

3. Loading the reaction tubes:

The reaction tubes were then loaded onto the StepOne real-time PCR system and the run was started.

1. PCR tube reading:

After PCR amplification, an end point tube reading was done using the StepOne real-time PCR system. The real-time readings all through the run were also used to plot a multicomponent plot (

Figure). The growth of the three fluorescent signals FAM, VIC and ROX (the Passive Reference dye used), It is clear that VIC fluorescent signal (indicating the presence of the wild C-allele) and FAM fluorescent signal (indicating the presence of the mutant T-allele), both start to increase exponentially about the same cycle. This indicates the uniformity of the concentration among the extracted genomic DNA samples.

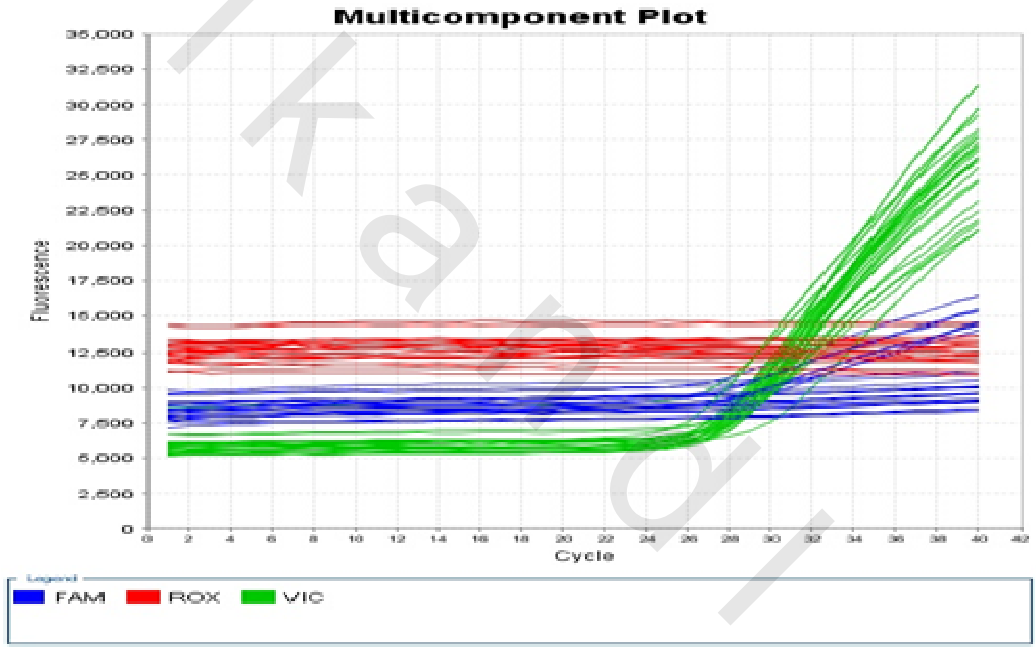

Figure (18): A multicomponent plot showing the growth of the three fluorescent signals FAM, VIC and ROX vs Cycles .The growth of the three fluorescent signals FAM, VIC and ROX (the Passive Reference dye used), It is clear that VIC fluorescent signal (indicating the presence of the wild C-allele) and FAM fluorescent signal (indicating the presence of the minor T-allele), both start to increase exponentially about the same cycle. This indicates the uniformity of the concentration among our extracted genomic DNA samples.

Data analysis and calculations:

Data analysis was done using StepOne v2.2.1 Sequence Detection System (SDS) Software (Applied Biosystems-Life Technologies, Carlsbad, California, USA), which uses the three different types of fluorescence measurements made during the tube read i.e. FAM, VIC and ROX (the Passive Reference dye used) to calculate Reporter normalized (Rn) ratios for each reporter dye in each reaction tube . This normalization was necessary

to correct for fluorescent fluctuations due to changes in concentration or volume. Normalization was accomplished by dividing the emission intensity of the reporter dye (FAM or VIC) by the emission intensity of the Passive Reference (ROX dye) to obtain a ratio defined as the Rn (Reporter normalized) (Figure 19) so that:

a. **Rn+**: was the Rn ratio of a reaction containing all components including the template.

b. **Rn–:** was the Rn ratio of a sample without a template i.e. the Non Template Control (NTC) wells.

c. **ΔRn**: was the difference between the Rn+ and the Rn–. It reliably indicated the magnitude of the fluorescence signal generated by the given set of PCR conditions. The following equation expressed the relationship of these terms: ΔRn = (Rn+) – (Rn–) where:

$$R_n^+ = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}} \quad \text{PCR with template}$$

$$R_n^- = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}} \quad \text{PCR without template or early cycles of a real-time reaction}$$

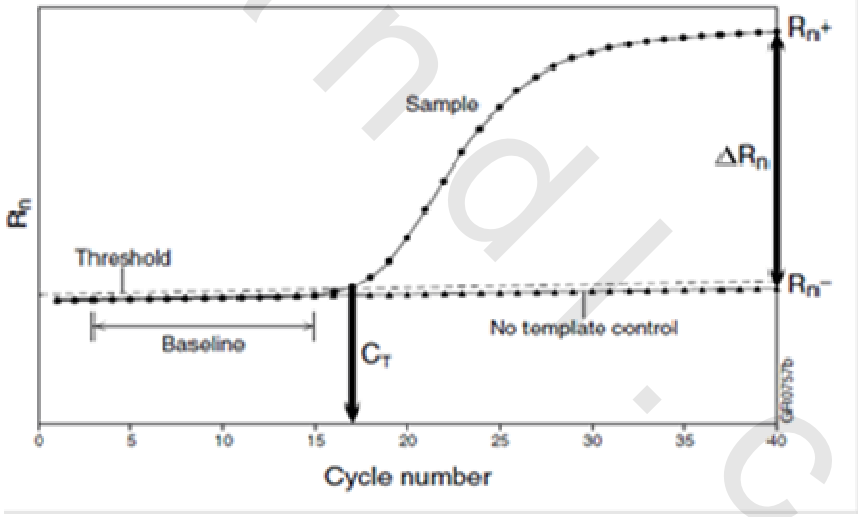

Figure (19): The graph of Rn versus cycle number, the threshold cycle occurs when the Sequence Detection System begins to detect the increase in signal associated with an exponential growth of PCR product

Simple workflow for quick results

TaqMan® SNP Genotyping Assays constitute the simplest SNP genotyping technology available. We deliver your ready-to-use SNP genotyping assay at ambient temperature in a convenient, single-tube format. The rest is easy. Just combine the assay with TaqMan® Genotyping Master Mix or TaqMan® Universal PCR Master Mix and your

purified DNA sample. There is no need to optimize probe, primer, salt concentrations, or temperature because all assays use universal reagent concentrations and thermal cycling conditions.

After generating an endpoint read using a thermal cycler or real-time PCR instrument, no transfers, washes, or additional reagents are required, and the plate remains sealed; just read the plate and analyze the genotypes (Figure 20).The analysis software allows you to auto-call genotypes, minimizing manual intervention.

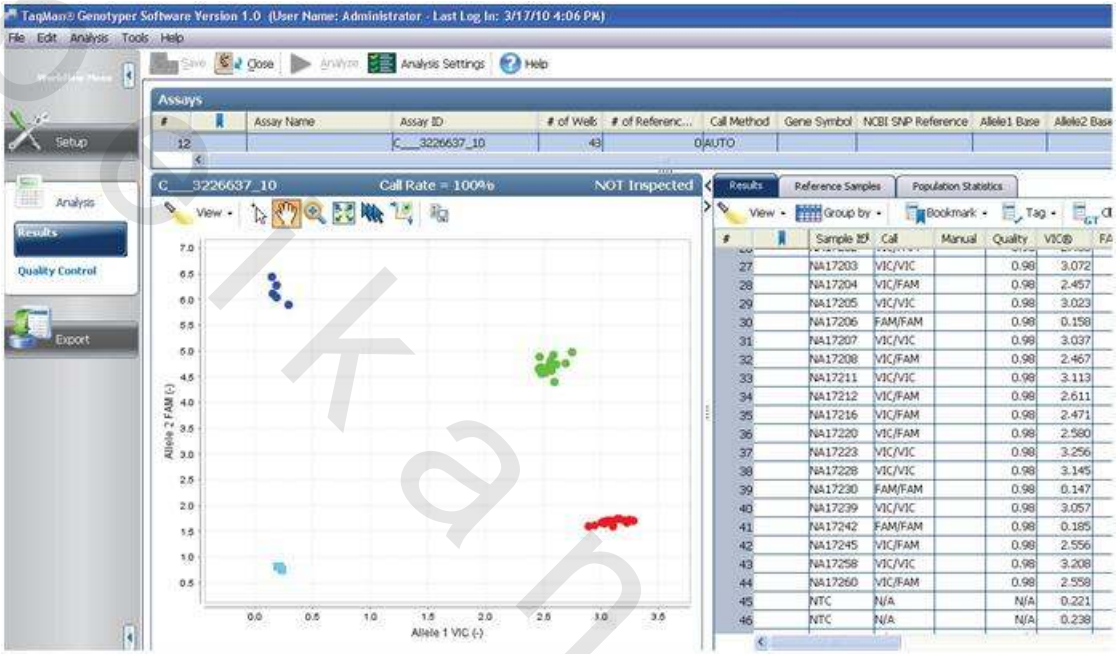

Figure (20): TaqMan® Genotyper Software automatically determines sample genotypes and displays data.

2. Allele calls:

Accordingly, the substantial increase in Rn ratio of each reporter dye in each reaction tube was then correlated to the specific allele(s) present in this sample and accordingly its genotype could be interpreted (Table 5).

Table 5: The allele calls and the IL28B rs12979860 genotyping according to the fluorescence signals of the sample

A substantial increase in…	Indicates….	Genotyping
VIC-dye fluorescence only (VIC / VIC)	Homozygosity for T allele (Homo-mutant genotype)	TT
FAM-dye fluorescence only (FAM/FAM)	Homozygosity for C allele (Homo-wild genotype)	CC
Both VIC- and FAM-dye fluorescence (FAM/ VIC)	Allele T-Allele C (heterozygosity)	TC

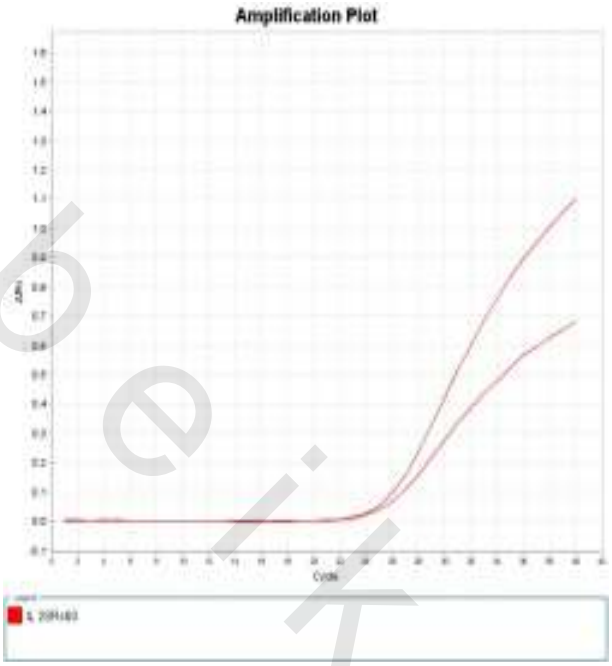

Figure (21). Heterozygote

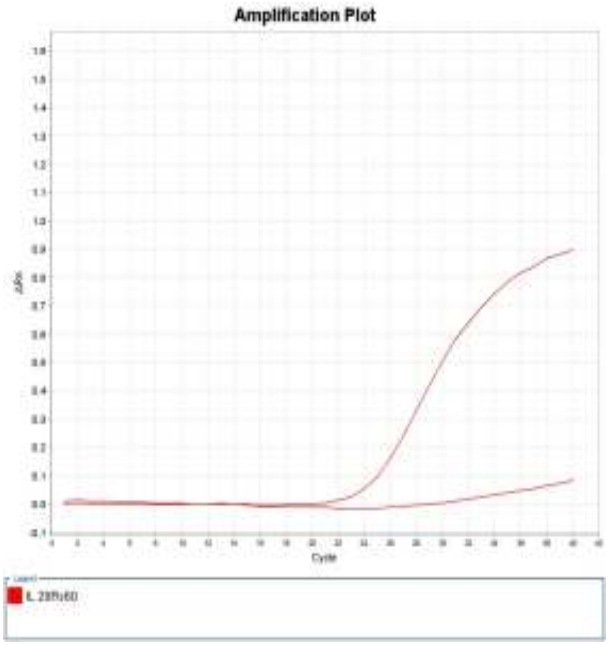

Figure (22). Homozygote

➢ Histopathology:

Liver biopsy specimens were analyzed by a single experienced pathologist who was blinded to the clinical and biological data. Liver biopsy specimens not less than 15 mm in length or the presence of at least 10 complete portal tracts were required [(235)].

For each liver biopsy, a stage of fibrosis and a grade of activity were established. Liver fibrosis was staged on a scale of 0 to 4 (0 = no fibrosis, 1 = portal fibrosis without septa, 2 = few septa, 3 = numerous septa without cirrhosis, 4 = cirrhosis) [(236)] .These features have been shown to be highly reproducible between pathologists [(236)]. The grading of activity which evaluates the intensity of necro inflammatory lesions was indicated as follows: A0 = no histological activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity [(237)].

Determination of the Fibrosis Progression Rate:

The fibrosis progression rate per year was defined as the ratio between the fibrosis stage (expressed according to the METAVIR scoring system) and the estimated duration of infection in years.

Histological grading & staging:

All specimens were staged and graded according to the METAVIR [237,238] system.

Algorithm for Evaluation of Histological Activity*				
Piecemeal Necrosis	+	Lobular Necrosis	=	Histological Activity Score
0 (none)		0 (none or mild)		0 (none)
0		1 (moderate)		1 (mild)
0		2 (severe)		2 (moderate)
1 (mild)		0, 1		1
1		2		2
2 (moderate)		0, 1		2
2		2		3 (severe)
3 (severe)		0, 1, 2		3

Fibrosis Scoring†	
Score	Description
0	No fibrosis
1	Stellate enlargement of portal tract but without septa formation
2	Enlargement of portal tract with rare septa formation
3	Numerous septa without cirrhosis
4	Cirrhosis

Figure (23): Liver fibrosis and histological activity according to METAVIR

➢ Statistical analysis :

Statistical calculations were performed using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 20 for Microsoft Windows.

Mean ± standard error (X ± SE), frequencies (number of cases) and relative frequencies (percentages) were used where appropriate. Mann–Whitney U test and Kruskal–Wallis test were used to analyze continuous variables where appropriate. We used the Spearman rank correlations to evaluate the relationship between pairs of markers. For comparing categorical data, Chi square ($\chi 2$) test was performed. Fisher exact test was used instead when the expected frequency is less than 5. A probability value (p value) less than 0.05 was considered statistically significant. Hardy-Weinberg Equilibrium: G. H. Hardy and W. Weinberg noted that with some well-defined assumptions, the population allele frequencies could be used to calculate the equilibrium-expected genotypic proportions.

RESULTS

Individual data of all subjects and patients enrolled in the present study are shown in appendix (1- 11).

HCV patients baseline characteristics and their statistical analysis are shown in (Table 6).HCV patients with genotype-4 group number=88 (n=88) the male/female ratio is 63.6%/36.4%. The average age is 43.5 ± 1.1 years (X ± S.E). Their mean body mass index is (BMI) 27.7 ± 0.2 Kg/m^2 which is significantly higher than that in apparently healthy subjects group, $p = 0.0001$ (Table 6). The mean values of liver function tests including ALT, AST, ALP and GGT are significantly higher than those in apparently healthy subjects group, $p = 0.0001$ (Table 6).Albumin are significantly lower than those in apparently healthy subjects group, $p = 0.0001$(Table 6). Bilirubin (total and direct) are significantly higher than those in apparently healthy subjects group, $p = 0.001$, 0.009 respectively (Table 6). Also, serum creatinine concentration level in HCV patients group is significantly lower when compared to that in apparently healthy subjects group, $p = 0.008$ (Table 6). Moreover, in HCV patient group, the mean concentration levels of fasting blood sugar and fasting insulin; 6 ± 3 mmol and 9.9 ± 0.9µIU/ml, respectively, are significantly higher than that in apparently healthy subjects group, $p = 0.043$, $p = 0.019$ respectively (Table 6). In addition, the mean level of homeostasis model assessment – insulin resistance (HOMA-IR) in HCV patients group (3.2 ± 0.7, X ± S.E) is significantly elevated when compared to that in apparently healthy subjects group (1.49 ± 0.1, X ± S.E), $p = 0.005$ (Table 6).

Table (6): Statistical Analysis of Baseline Demographic, and Biochemical Characteristics of Apparently Healthy Subjects and HCV Patients

Parameter	Healthy Subjects (n = 30)	HCV Patients (n = 88)	p^*
Age (yrs)	27.4 ± 1.2	43.5 ± 1.1*	0.0001[a]
Sex M/F	19(63.3%)/11(36.7%)	56(63.6%)/32(36.4%)	
Body Mass Index (Kg/m^2)	23.8 ± 0.2	27.7 ± 0.2*	0.0001[a]
ALT (IU/L)	16.9 ± 1.2	50.5 ± 3.5*	0.0001[a]
AST (IU/L	19.8 ± 1.1	47.8 ± 3.2*	0.0001[a]
ALP (IU/L	35.1 ± 4	90.1 ± 3.6*	0.0001[a]
GGT (IU/L	19.7 ± 2	60.6 ± 7.3*	0.0001[a]
Albumin (g/dL)	4.4 ± 0.07	4.1 ± 0.05*	0.0001[a]
Total Bilirubin (mg/dL)	0.55 ± 0.04	0.78 ± 0.42*	0.001[a]
Direct Bilirubin (mg/dL)	0.15 ± 0.01	0.26 ± 0.02*	0.009[a]
Creatinine (mg/dL)	0.97 ± 0.03	0.86 ± 0.02*	0.008[a]
Fasting Blood Sugar (mmol)	4.9 ± 0.1	6 ± 0.3*	0.043[a]
Fasting Insulin (µIU/ml)	6.7 ± 0.5	9.9 ± 0.9*	0.019[a]
Postprandial Blood Sugar (mg/dL)	99.2 ± 1.8	131.7 ± 5.7*	0.0001[a]
TSH (µIU/ml)	2 ± 0.2	2.3 ± 0.1	0.280[a]
HOMA-IR	1.49 ± 0.1	3.2 ± 0.7*[a]	0.005[a]
Triglycerides (mg/dL)	81 ± 6.1	108 ± 5.4*	0.008[a]
Total Cholesterol (mg/dL)	181 ± 8.6	152 ± 3.7*	0.003
HDL Cholesterol(mg/dL)	52 ± 2.2	52 ± 2	0.557[a]
LDL Cholesterol(mg/dL)	112 ± 7.6	78 ± 3.1*	0.0001
White Blood Cells (WBCs) x 10^3/µl	7.9 ± 0.5	4.83 ± 0.2*	0.0001[a]
Hemoglobin (mg/dL)	13.8 ± 0.2	12.6 ± 0.1*	0.0001[a]
Platelets x 10^3/µl	273.8 ± 14	185.4 ± 5.9*	0.0001[a]
International Normalized Ratio (INR)	1.00 ± 0.0003	1.06 ± 0.01*	0.0001[a]
Prothrombin concentration %	100 ± 0.000	94.48 ± 0.85*	0.0001[a]
Alpha-Feto protein(mg/ml)		8.40 ± 1.73	
Baseline Viral Load $\log_{10}$(IU/ml)		1445813 ± 237860	

Student "t" test applied for all except for [a] Mann Whitny U test was applied
p^* value was considered significant at $p < 0.055$

It should be noted that all responded cases is noticed in 64 % (n=57) of HCV patients while non-responders are 36 % (n=31), (Figure 24)

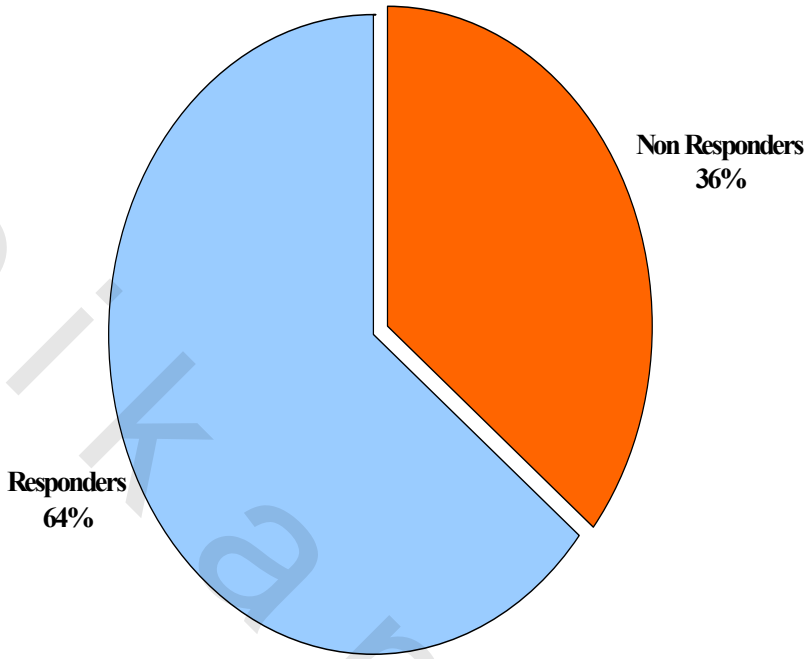

Figure.(24): The Percentage Distribution of HCV Patients According to the Response to Standard Treatment

Statistical analysis of baseline demographic and biochemical characteristics of non-responders and responders HCV Patients are shown in (Table 7). HCV responded patients with genotype-4, group (n=57) the male/female ratio is (59.6%)/(40.4%) but in non-responder patients with genotype-4, group(n=31) the ratio is (71%)/(29%). The mean body mass index is (BMI) of HCV responder patients is 27.7 ± 0.2 Kg/m^2 which is significantly lower than that in non-responded group, $p = 0.001$ (Table 7). In HCV responder patients, the serum activity level of GGT was significantly lower than that in apparently non-responded group, $p = 0.0001$ (Table 7). The mean concentration level of serum albumin in HCV responder patients was significantly higher than that in apparently non-responded group, $p = 0.043$ (Table 7). The concentration levels of total and direct bilirubin in HCV responder patients were significantly lower than those in non-responded group, $p = 0.005$, 0.0001, respectively (Table 7). Also, the mean fasting serum insulin concentration level in HCV responder patients group is significantly lower when compared to that in non-responded group, $p= 0.048$ (Table 7). Moreover, in responded patient group, the mean concentration levels of thyroid stimulating hormone (TSH) and alpha-feto protein (AFP); 2.07 ± 0.15 μIU/ml and 5.36 ± 0.92 mg/ml, respectively, are significantly lower than that in non-responded group, $p = 0.018$, $p = 0.004$ respectively (Table 7). In addition, the mean level of HDL-cholesterol in HCV responder patients group (54.9 ± 2.7, X ± S.E) is significantly elevated when compared to that in HCV non-responder patients group (46.6 ± 2.3, X ± S.E), $p = 0.021$ (Table 7). Furthermore, in HCV responder patients group, the mean value of international normalized ratio (INR) was significantly lower than that in apparently non-responder patients group, $p = 0.0001$ (Table 7).

Table (7): Statistical Analysis of Baseline Demographic and Biochemical Characteristics of Non Responders and Responders HCV Patients

Parameter	HCV Patients (n = 88)		p^*
	Non Responders (n= 31)	Responders (n = 57)	
Age (yrs)	44 ± 1.7	43.2 ± 1.4	0.705[b]
Sex M/F	22(71%)/9(29%)	34(59.6%)/23(40.4%)	
Body Mass Index (Kg/m^2)	28.7 ± 0.4	27.1 ± 0.2*	0.001[a]
< 30 Kg/m^2	25 (80.6 %)	55 (96.5 %)	
≥ 30 Kg/m^2	6 (19.4 %)	2 (3.5 %)	
ALT (IU/L)	51.3 ± 6.1	50.1 ± 4.2	0.529[a]
AST (IU/L)	48.5 ± 4.2	47.4 ± 4.4	0.196[a]
ALP (IU/L)	88.2 ± 7	91.1 ± 4.2	0.701[a]
GGT (IU/L)	73.8 ± 8.1	53.4 ± 10.4	0.0001[a]
Albumin (g/dL)	3.97 ± 0.08	4.17 ± 0.05	0.043[b]
Total Bilirubin (mg/dL)	0.93 ± 0.1	0.70 ± 0.05	0.005[a]
Direct Bilirubin (mg/dL)	0.36 ± 0.05	0.20 ± 0.02	0.0001[a]
Creatinine (mg/dL)	0.88 ± 0.03	0.85 ± 0.02	0.653[a]
Fasting Blood Sugar (mmol)	6.68 ± 0.81	5.66 ± 0.28	0.389[a]
Fasting Insulin (µIU/ml)	12.41 ± 2.17	8.56 ± 0.61	0.048[a]
Postprandial Blood Sugar (mg/dL)	136.7 ± 12.6	128.9 ± 5.7	0.286[a]
TSH (µIU/ml)	2.71 ± 0.2	2.07 ± 0.15	0.018[a]
HOMA-IR	4.8 ± 1.8	2.34 ± 0.34	0.110[a]
Triglycerides (mg/dL)	94.2 ± 6.8	114.9 ± 7.4	0.170[a]
Total Cholesterol (mg/dL)	142.9 ± 6.5	156.6 ± 4.3	0.109[a]
HDL Cholesterol(mg/dL)	46.6 ± 2.3	54.9 ± 2.7	0.021[b]
LDL Cholesterol(mg/dL)	77.6 ± 5.4	78.3 ± 3.8	0.916[b]
White Blood Cells (WBCs) x 10^3	4.68 ± 0.23	4.91 ± 0.22	0.875[a]
Hemoglobin (mg/dL)	12.7 ± 0.3	12.6 ± 0.17	0.780[a]
Platelets(x 10^3µl)	189.61 ± 10.9	183.12 ± 6.97	0.653[a]
International Normalized Ratio (INR)	1.11 ± 0.02	1.03 ± 0.01	0.0001[a]
Prothrombin concentration %	90.1± 1.9	96.8 ± 0.7	0.0001[a]
Alpha-Feto protein(mg/ml)	14.00 ± 4.48	5.36 ± 0.92*	0.004[a]
Baseline Viral Load($\log_{10}$ IU/ml)	986598 ± 142855	1695561 ± 355799*	0.564[a]

Student "t" test applied for all except for [a] Mann Whitny U test was applied

p^* value was considered significant at $p < 0.05$

Clinico-pathological Characteristics

Pretreatment liver biopsy samples were assessed for 88 patients. The liver biopsy data showed that the majority of HCV patients were staging F1 (46.6 %) with total count 41 case of patients divided to 9 non responder (NR) (29.03% of non-responder) and 32 responder (R) (56.14% of responder), F2 (29.54 %) divided to 8 NR (25.8% of NR) and 18 NR (31.58% of NR), and F3(22.72%) with a total count 20 patients divided to 13 (41.93% NR) and 7(12.28% R) as well as grading of histological Activity A1 23 (26.13 %) of patients, A2 (47.72 %) and A3 (14.77%) in table (8).

Table (8): Baseline Clinico-pathological characteristics of HCV Patients as well as Non-responders and Responders HCV patients

	HCV Patients (n = 88)	Non responders (n = 31)	Responder (n = 57)
Fibrosis stage			
F_1	41 (46.59 %)	9 (29.03%)	32 (56.14 %)
F_2	26 (29.55 %)	8 (25.81%)	18 (31.58 %)
F_3	20 (22.72 %)	13 (41.94%)	7 (12.28 %)
F_4	1 (1.14 %)	1 (3.22%)	----------
Histological Activity			
A_1	23 (26.14%)	8 (25.81 %)	24 (42.11 %)
A_2	42 (47.72%)	9 (29.03 %)	33 (57.89 %)
A_3	13 (14.77%)	13 (41.94 %)	0
A_4	1 (1.14%)	1 (3.22 %)	0
Ultra Sound			
Fatty liver	33 (37.5%)	10 (32.3 %)	23 (40.4%)
Non Fatty liver	55 (62.5%)	21(67.7 %)	34 (59.6%)

On the other hand, univariate analysis identified GGT (OR: 0.219, CI 95%:0.068 – 0.710, *p* = 0.006), body mass index (OR: 0.152, CI 95%: 0.029 – 0.804, *p* = 0.021), HOMA-IR (OR: 0.215, CI 95%: 0.084 – 0.547, p = 0.001) and fibrosis stage (OR: 0.170, CI 95%: 0.059 – 0.491, *p* = 0.001) as independent predictors for virlogical response, Table (9).

Table (9): Logistic Regression of Some Factors Associated with Response in Patients with HCV

Factor	OR	CI 95%	*p*
AFP (mg/ml)	0.302	0.081 – 1.120	0.065
GGT ((IU/L)	0.219	0.068 – 0.710	0.006*
Albumin (mg/dL)	2.758	0.721 – 10.55	0.106
BMI (> 30 Kg/m^2)	0.152	0.029 – 0.804	0.021*
HOMA-IR	0.215	0.084 - 0.547	0.001*
Virus load, log_{10} IU/mL	1.538	0.636 – 3.719	0.231
Fibrosis (F1+F2 *vs.* F3 + F4)	0.170	0.059 – 0.491	0.001*
Fatty Liver	0.704	0.280 – 1.767	0.304

OR = Odds Ratio, CI = Confidence Interval
* *p* value was considered significant at p < 0.05

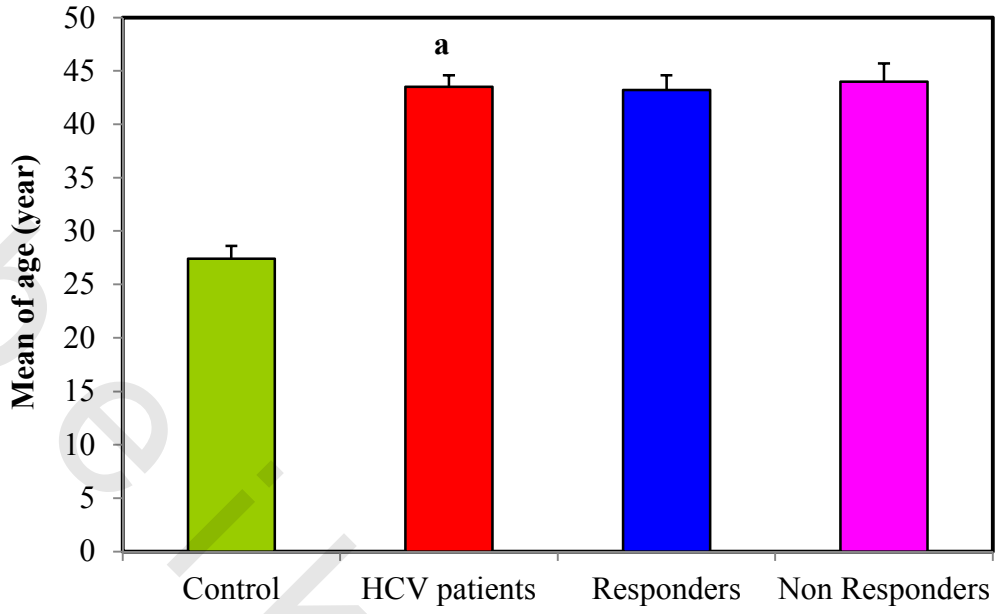

Figure (25): Average Age (year) of the different Studied Groups

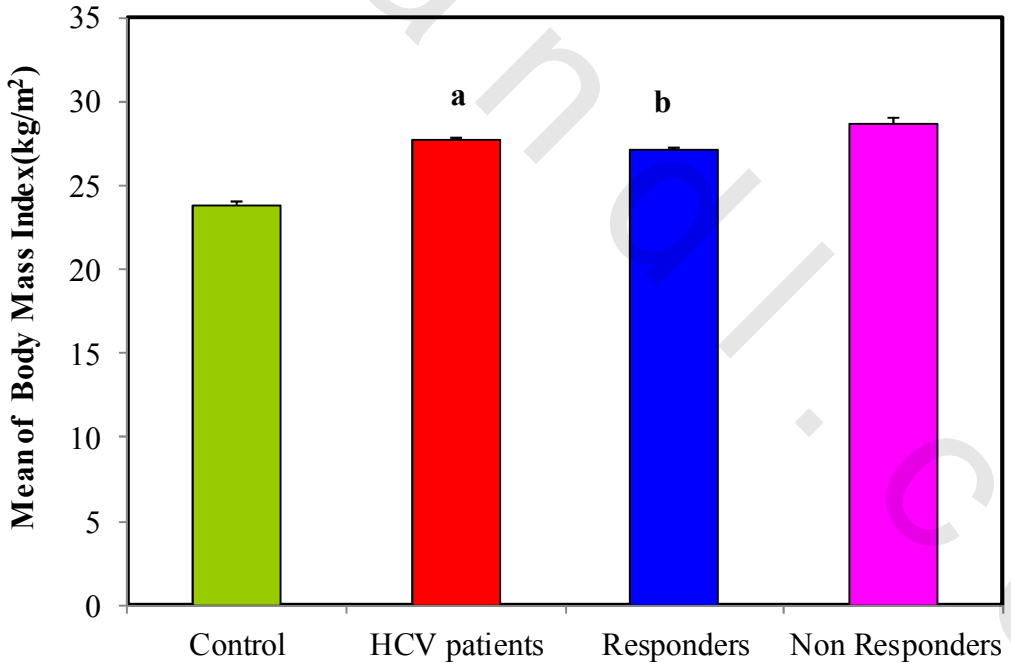

Figure (26): Mean Body mass index (kg/m2) in the Different Studied Groups
a: Statistically significant when compared to healthy subjects group $p < 0.05$.
b: Statistically significant when compared to non-responder subjects group $p < 0.05$.

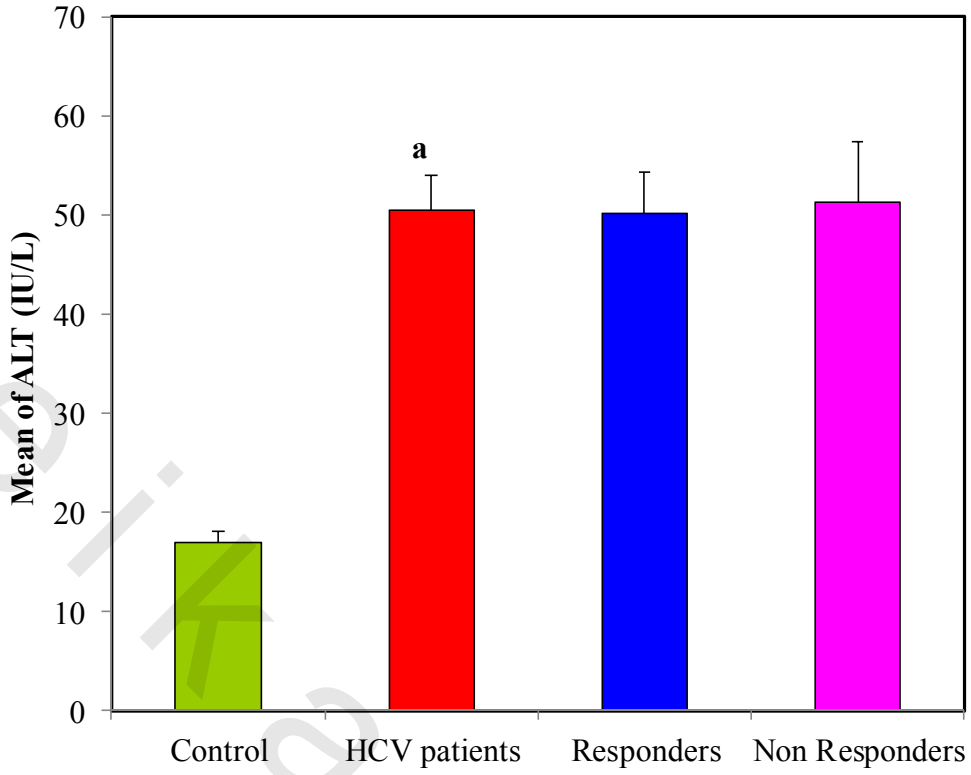

Figure (27): Mean Serum Activity Level of ALT (IU/L) in the Different Studied Groups

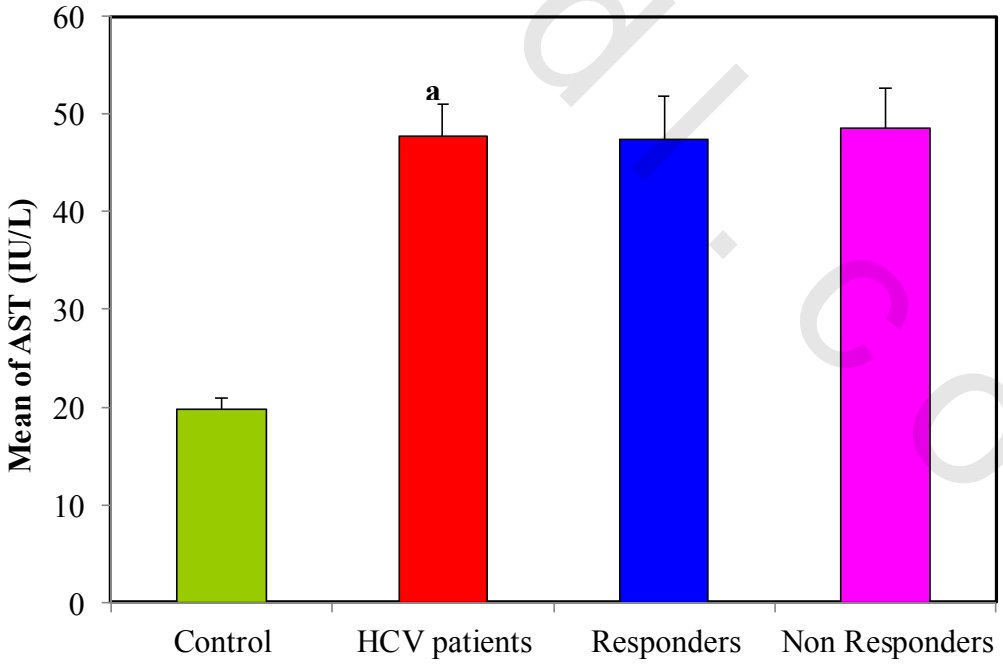

Figure (28): Mean Serum Activity Level of AST (IU/L) in the Different Studied Groups

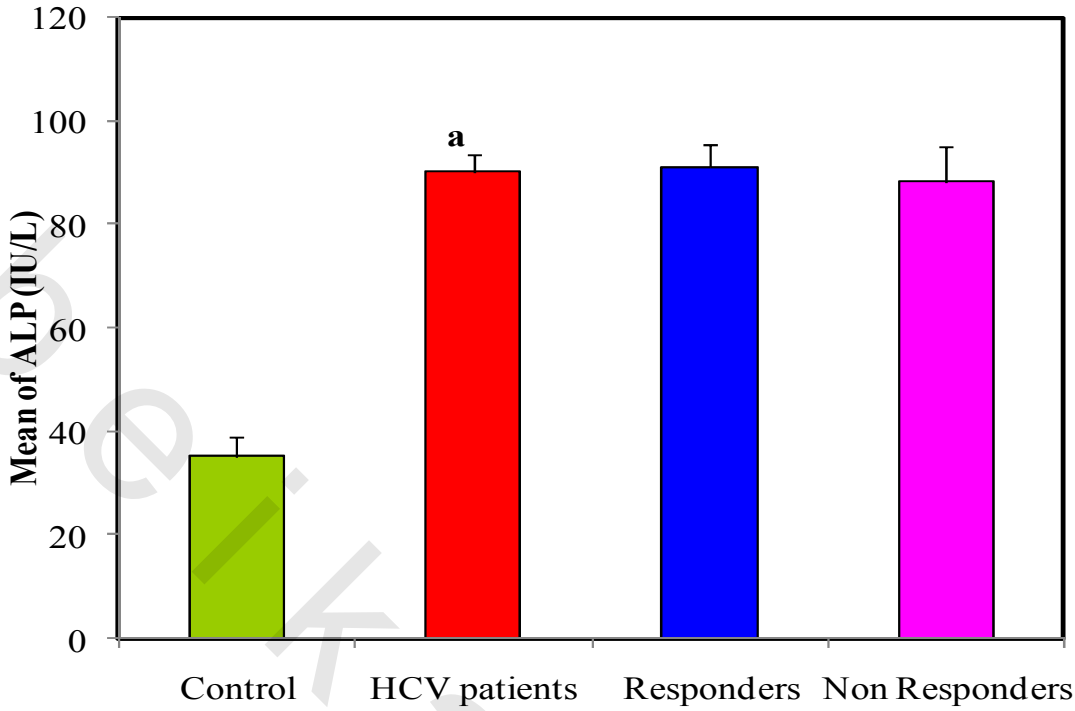

Figure (29): Mean Serum Activity Level of ALP (IU/L) in the Different Studied Groups.

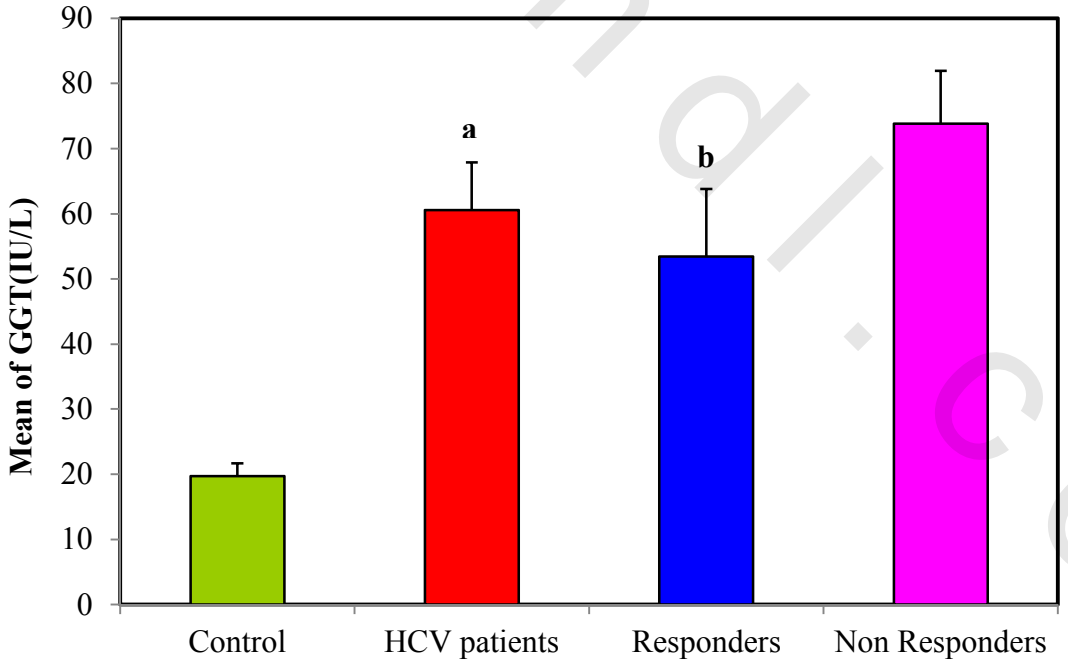

Figure (30): Mean Serum Activity Level of GGT (IU/L) in the Different Studied Groups

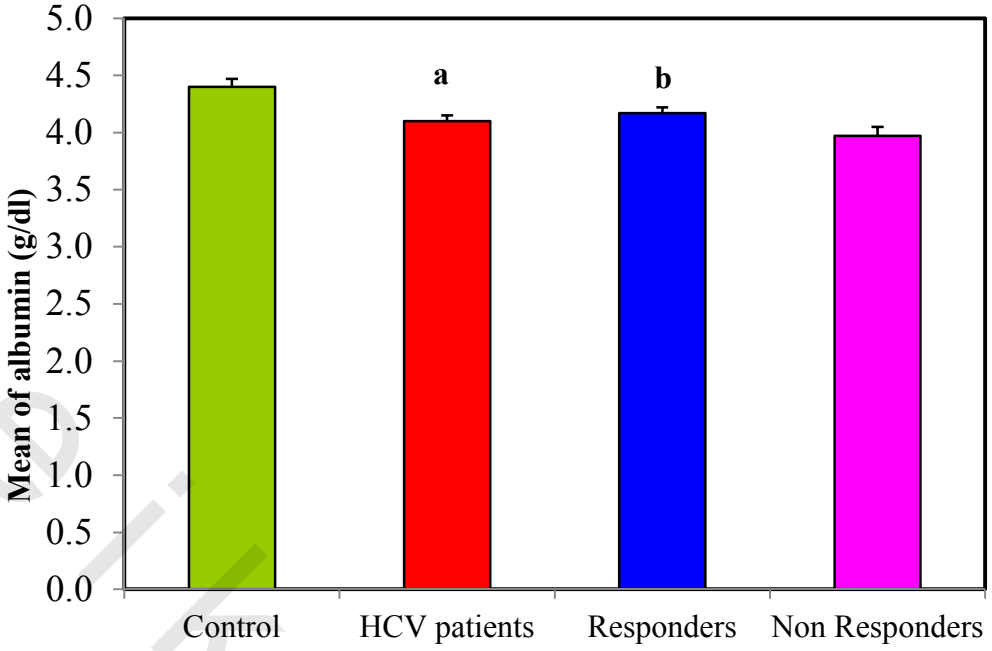

Figure (31): Mean Serum Concentration Level of Albumin (g/dl) in the Different Studied Groups

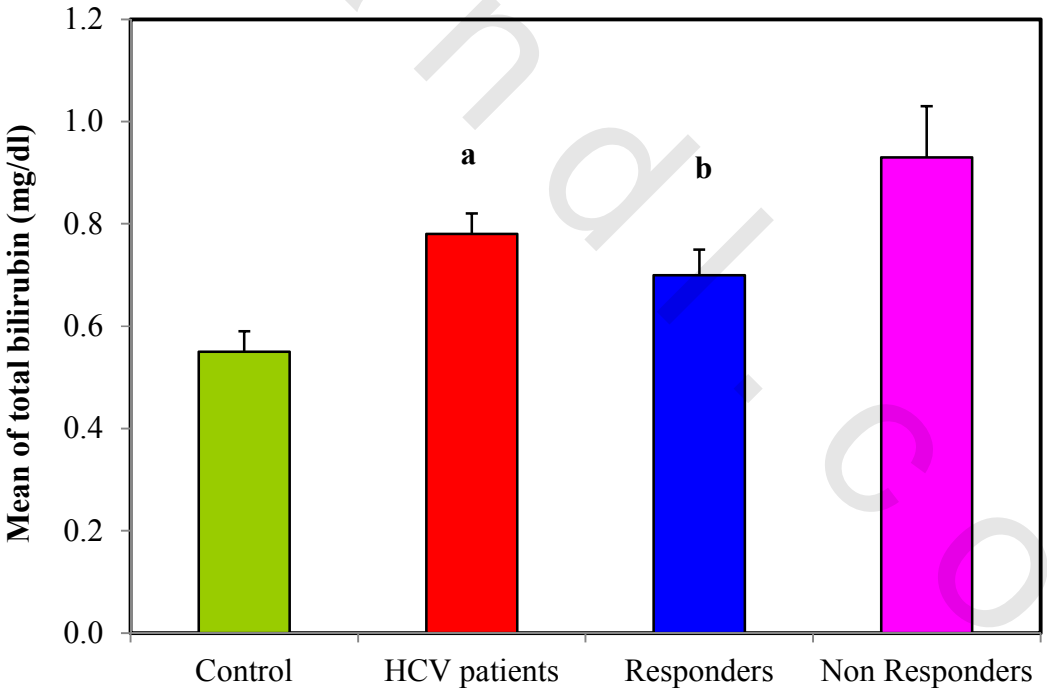

Figure (32): Mean Serum Concentration Level of Total bilirubin (mg/dl) in the Different Studied Groups

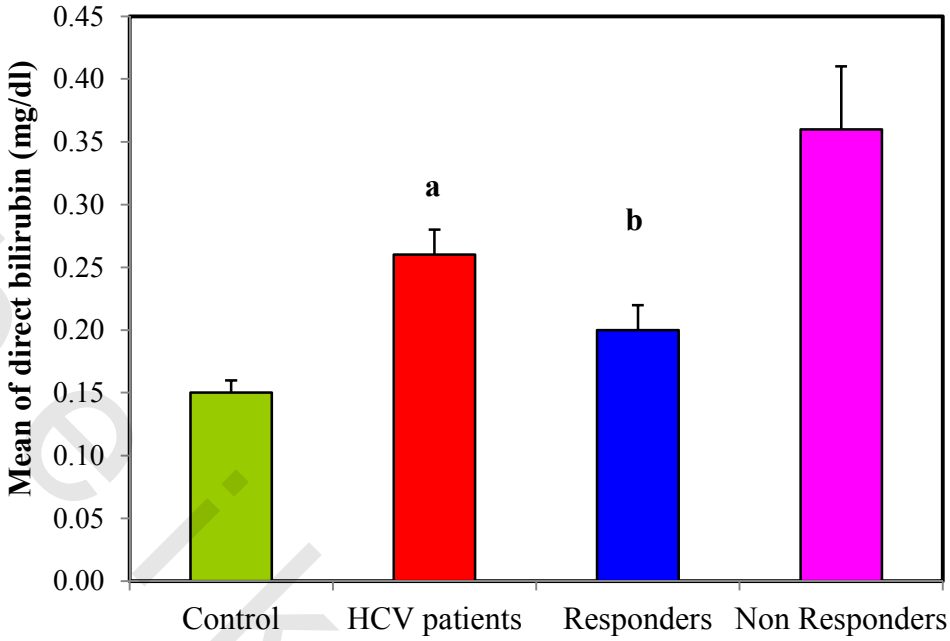

Figure (33): Mean Serum Concentration Level of Direct bilirubin (mg/dl) in the Different Studied Groups

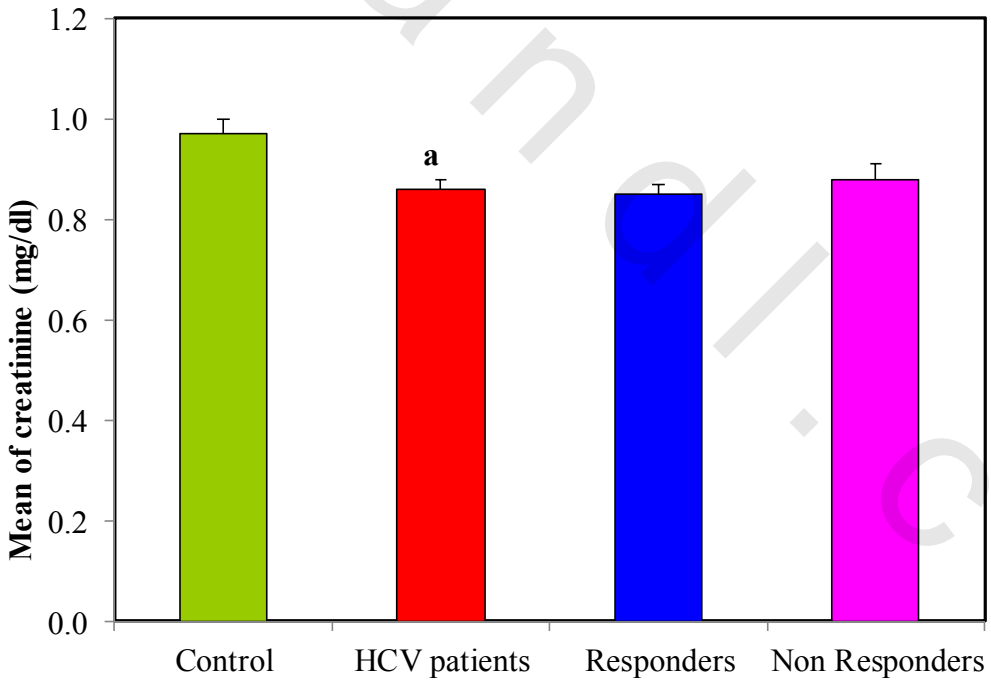

Figure (34): Mean Serum Concentration Level of Creatinine (mg/dl) in the Different Studied Groups

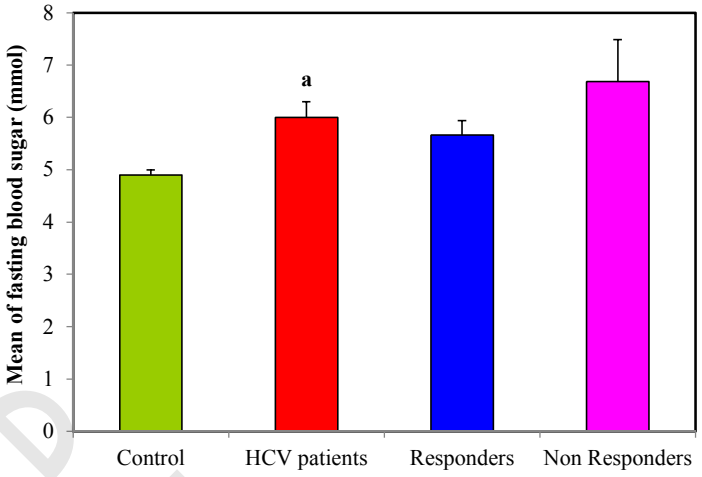

Figure (35): Mean Serum Concentration Level of Fasting blood glucose (mmol) in the Different Studied Groups

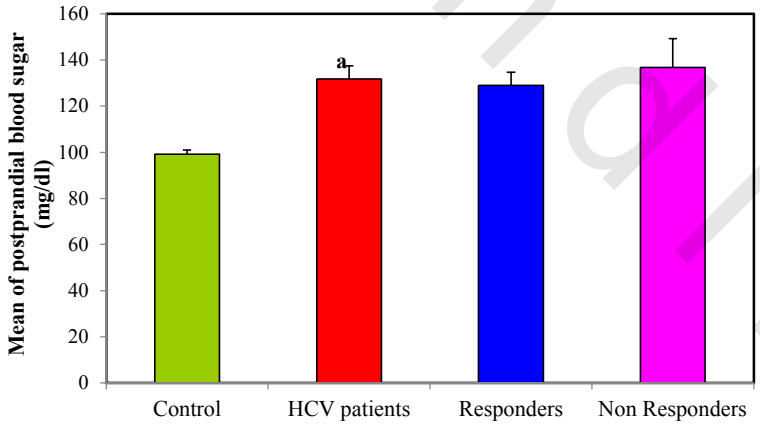

Figure (36): Mean Serum Concentration Level of Postprandial Blood glucose (mg/dl) in the Different Studied Groups

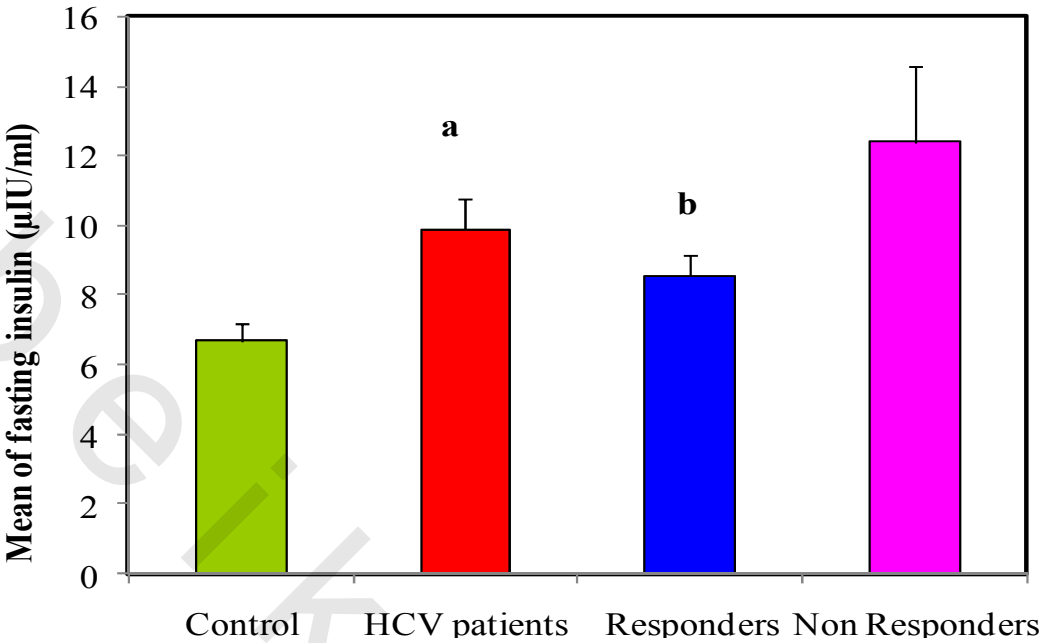

Figure (37): Mean Serum Concentration Level of Fasting Insulin (µIU/ml) in the Different Studied Groups

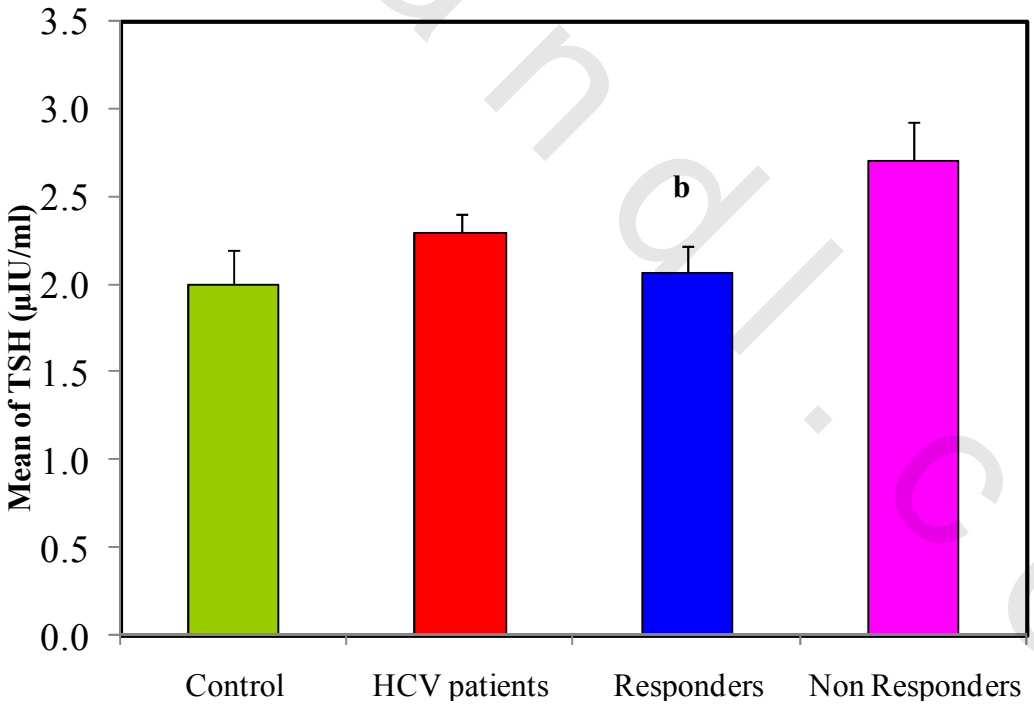

Figure (38): Mean Serum Concentration Level of TSH (µIU/ml) in the Different Studied Groups

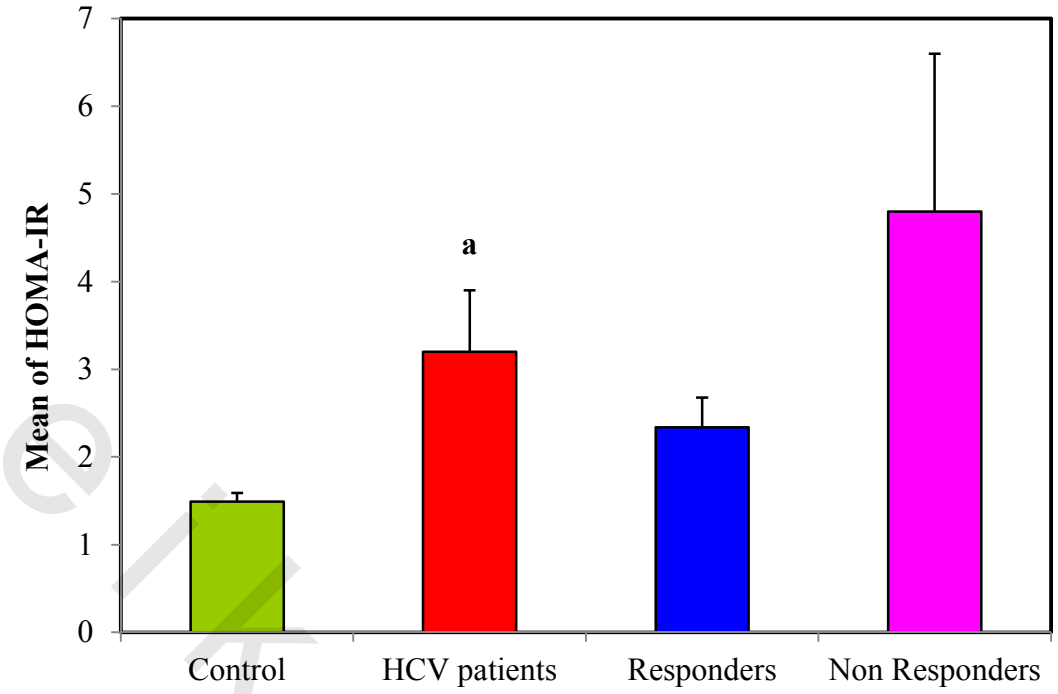

Figure (39): Mean Level of HOMA-IR in the Different Studied Groups

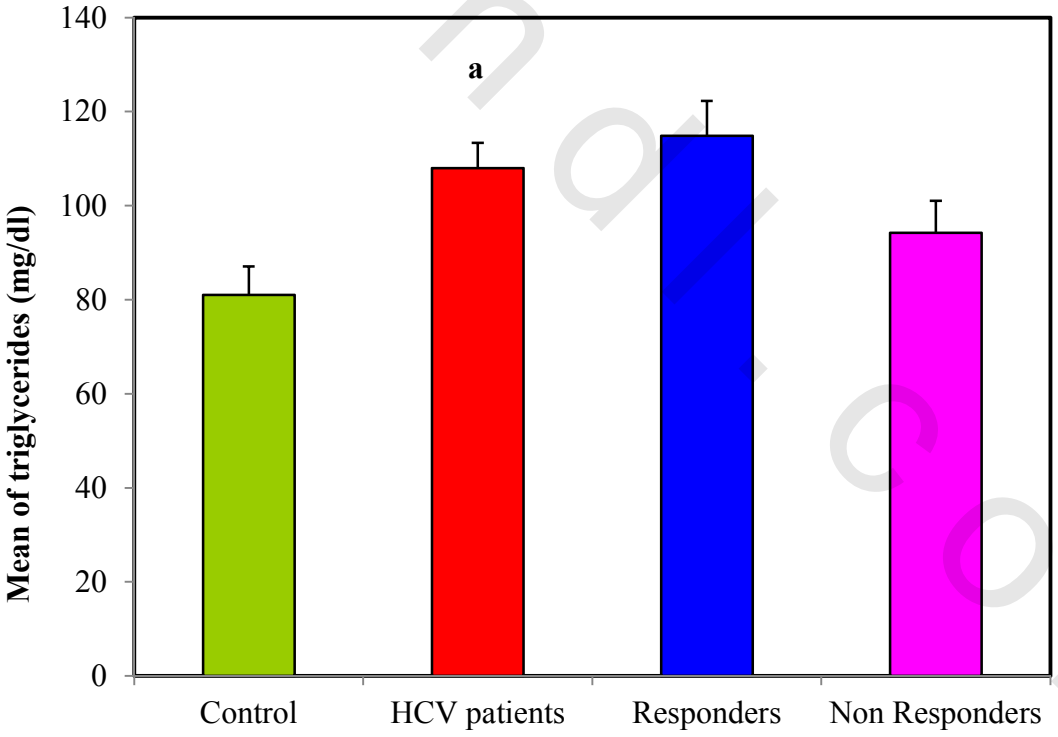

Figure (40): Mean Serum Concentration Level of Triglycerides (mg/dl) in the Different Studied Groups

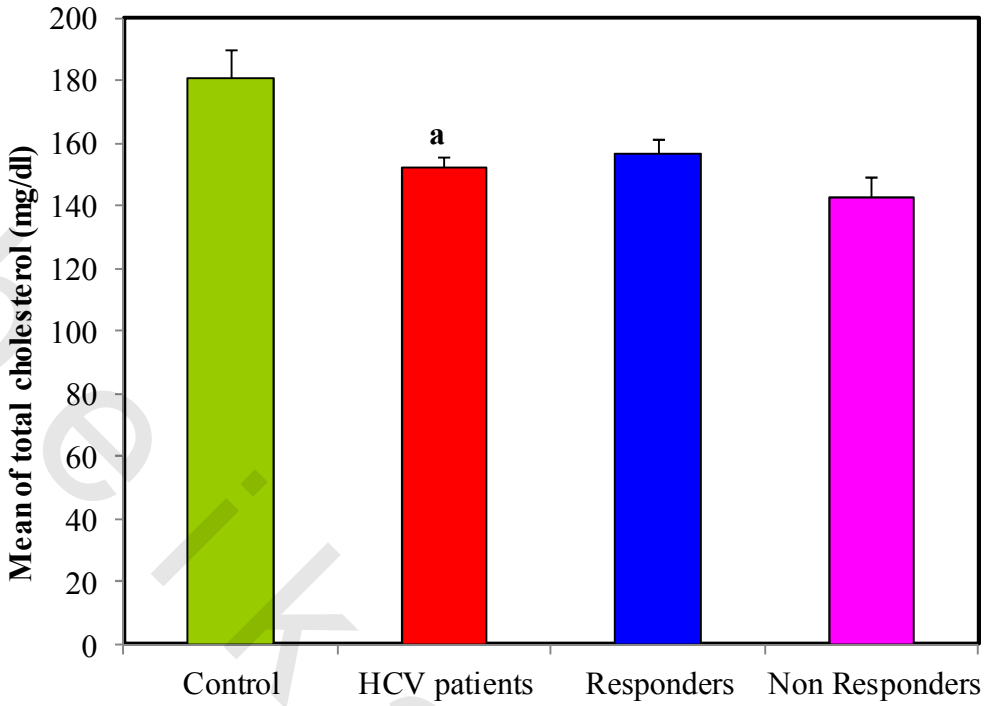

Figure (41): Mean Serum Concentration Level of Total Cholesterol (mg/dl) in the Different Studied Groups

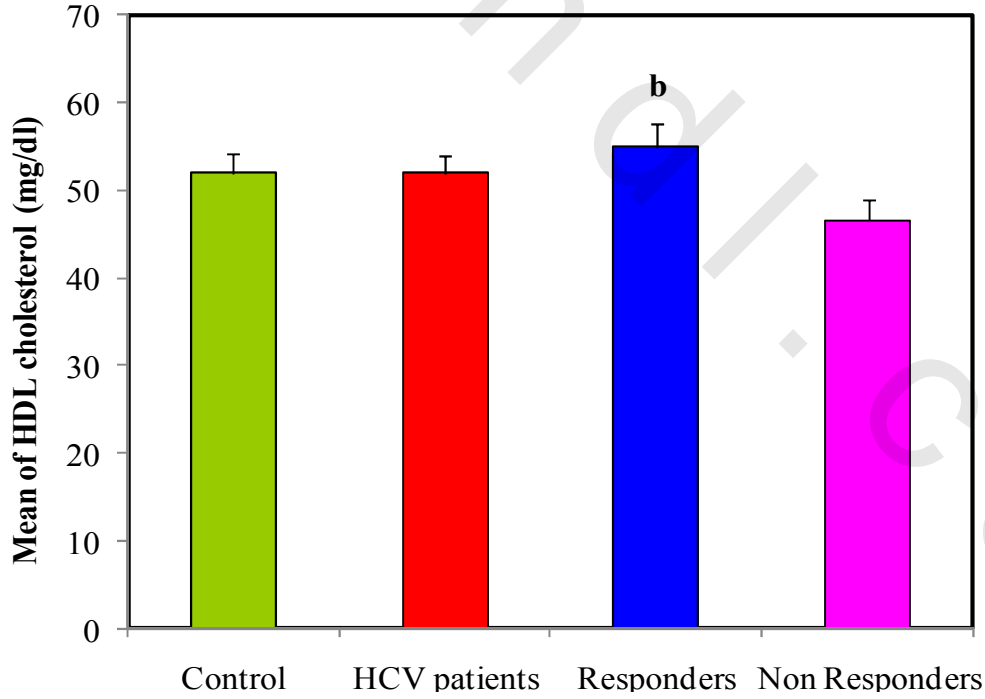

Figure (42): Mean Serum Concentration Level of HDL Cholesterol in the Different Studied Groups (mg/dl)

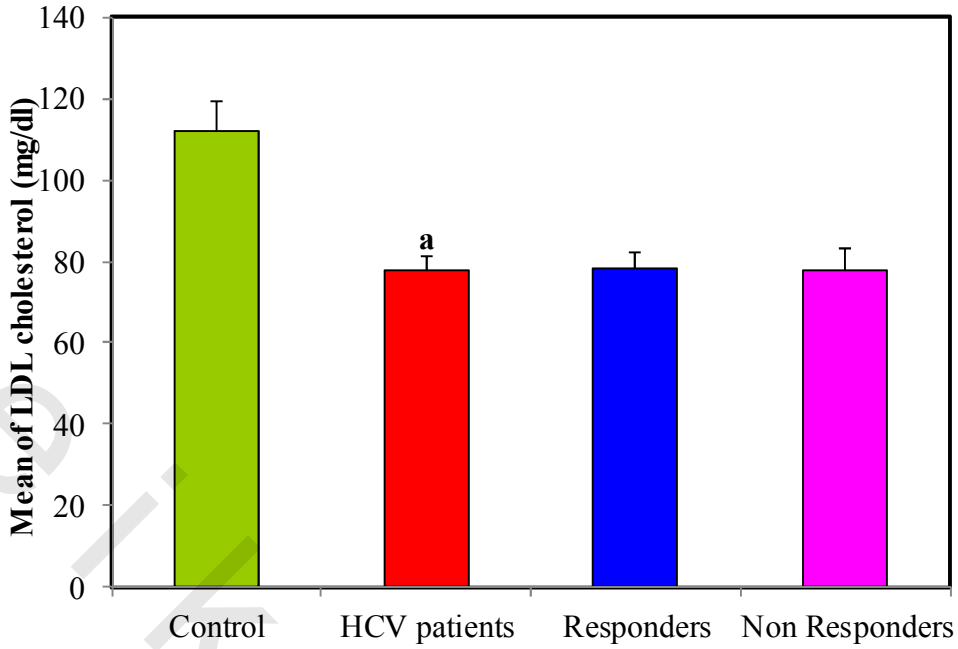

Figure (43): Mean Serum Concentration Level of LDL cholesterol (mg/dl) in the Different Studied Groups

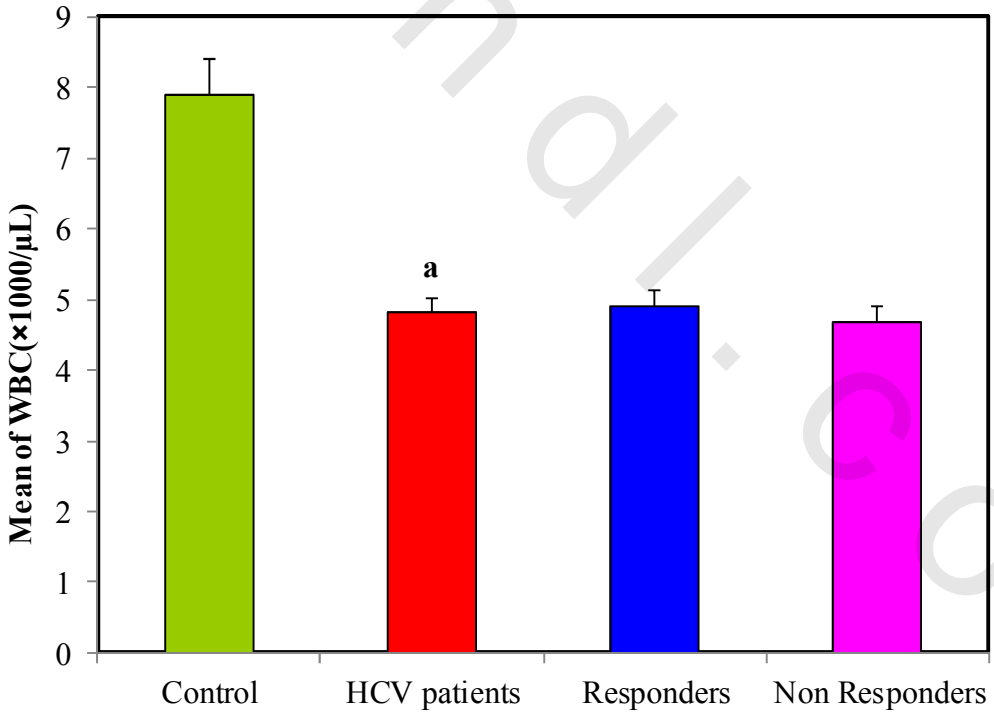

Figure (44): Mean Count of WBC ($\times 10^3/\mu L$) in the Different Studied Groups

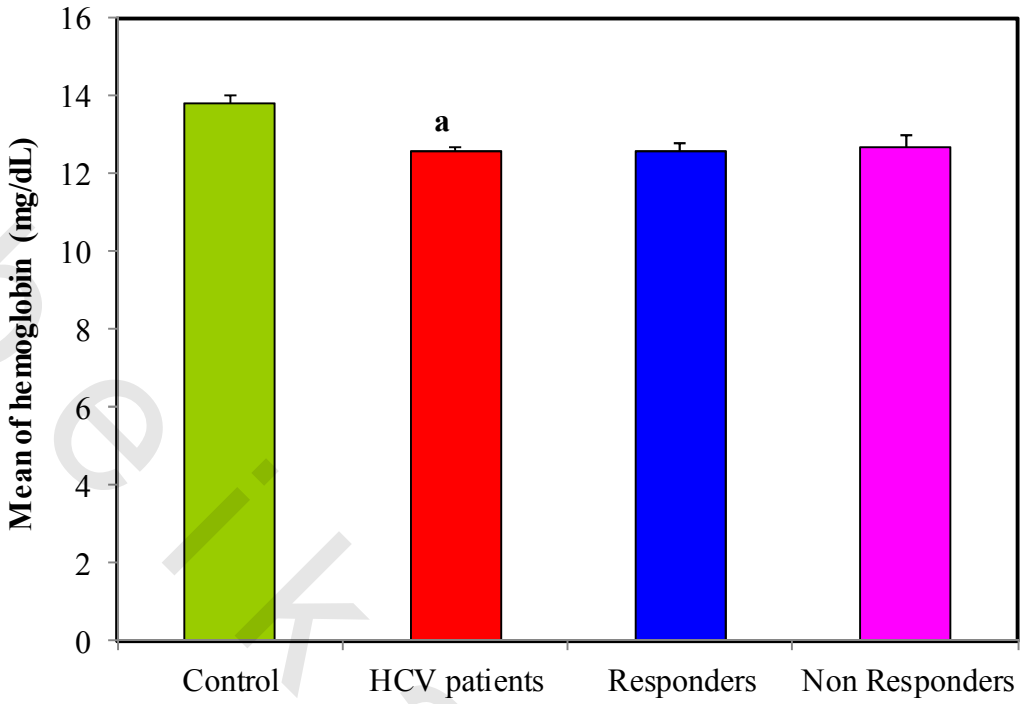

Figure (45): Mean Concentration Level of Hemoglobin (mg/dL) in the Different Studied Groups

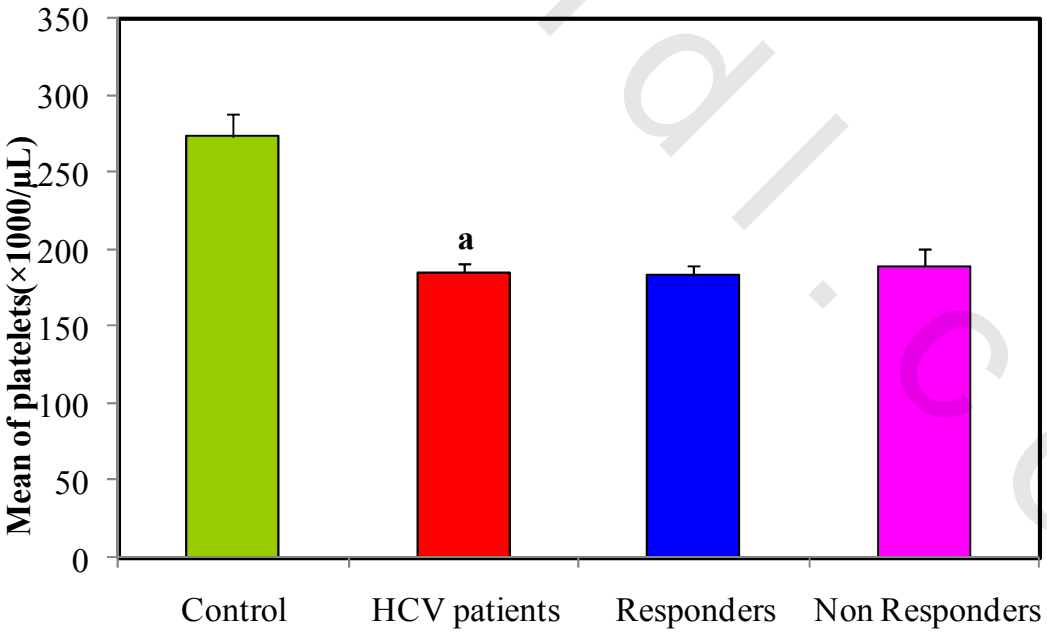

Figure (46): Mean Count of Platelets ($\times 10^3/\mu L$) in the Different Studied Groups

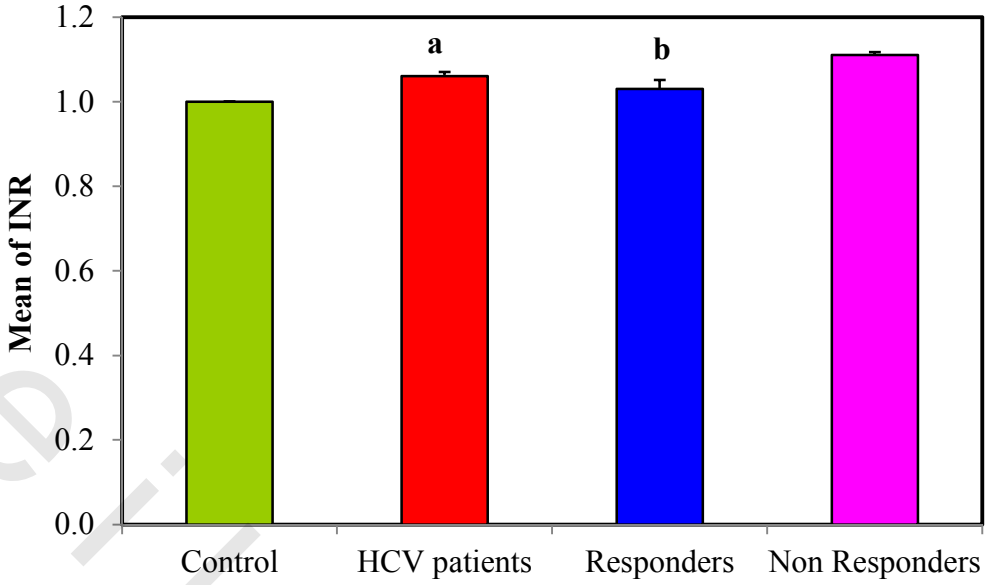

Figure (47): Mean Level of INR in the Different Studied Groups.

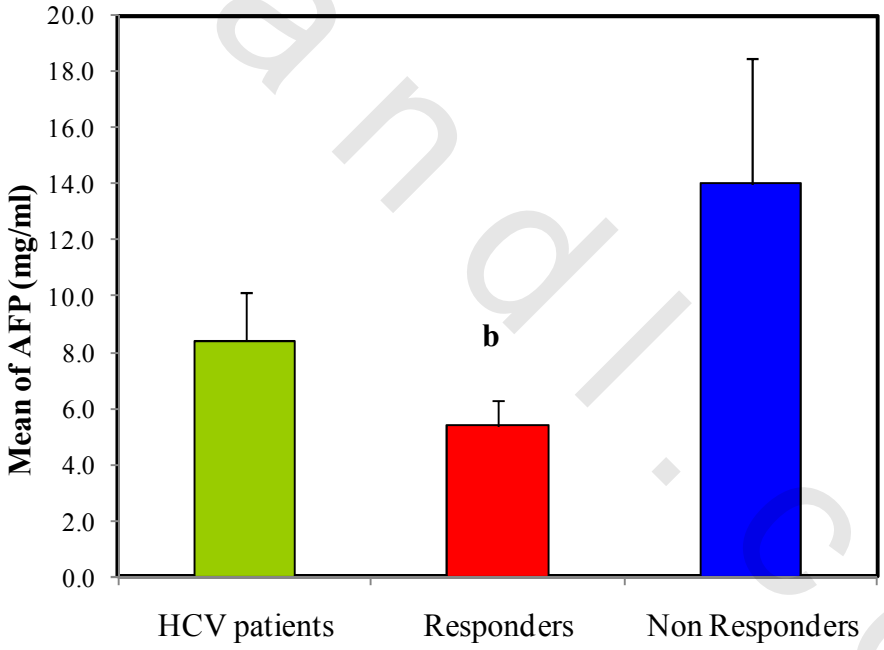

Figure (48): Mean Level of AFP (mg/ml) in the Different Studied Groups

IL-28 rs12799860 Genotype Distribution

The IL-28 rs12799860 genotypes (CC, CT and TT) distribution in apparently healthy subjects and in HCV patients is shown in Table (10). (Figure 49) shows the IL-28 rs12799860 genotype distribution in HCV patients. The frequencies of the IL-28 rs12799860 different genotype in healthy subject are as follows; CC 43.3 % (n = 13), CT 53.3 % (n = 16) and TT 3.3 % (n = 1). In HCV patients (n = 88), the frequencies of the IL-28 rs12799860 different genotype are as follows; CC 26.1 % (n = 23), CT 58.0 % (n = 51) and TT 15.9 % (n = 14). It should be noted that 91.3 % of HCV patients with CC alleles (21/23), 56.7 % of HCV patients with heterozygous CT (29/51) and 50.0 % HCV patients with recessive alleles TT (7/14) were responders, respectively. The distribution of these different IL-28 rs12799860 genotypes among all studied groups are in agreement with Hardy-Weinberg equilibrium, $p > 0.05$, except for non-responders HCV patients is not consistent with Hardy-Weinberg equilibrium, $p = 0.011$, Table (11), (figure 50). The frequency of C allele was higher in all groups except for non responder group where the frequency of T allele was higher. The results of the present study showed that the C allele of rs12979860 was significantly associated with virological response, Table (12)

Table (10): Distribution of IL-28 rs12799860 genotypes (CC, CT and TT) in apparently healthy subjects and in HCV patients

	Healthy Subjects	HCV Patients	HCV Patients (NR)	HCV Patients (R)
CC	13 (43.3%)	23 (26.1%)	2 (08.7%)	21 (91.3%)
CT	16 (53.3%)	51 (58.0%)	22 (43.1 %)	29 (56.7%)
TT	1 (3.3%)	14 (15.9%)	7 (50.0%)	7 (50.0%)
Total	**30**	**88**	**31**	**57**

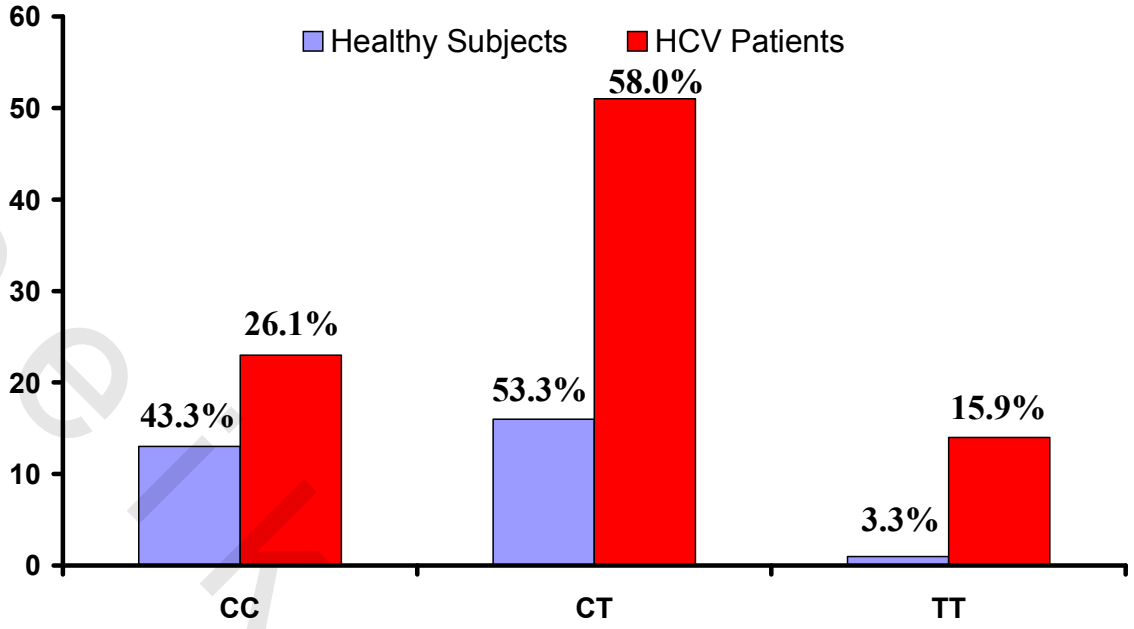

Figure (49). Distribution of IL-28 rs12799860 genotypes (CC, CT and TT) in apparently healthy subjects and in HCV patients

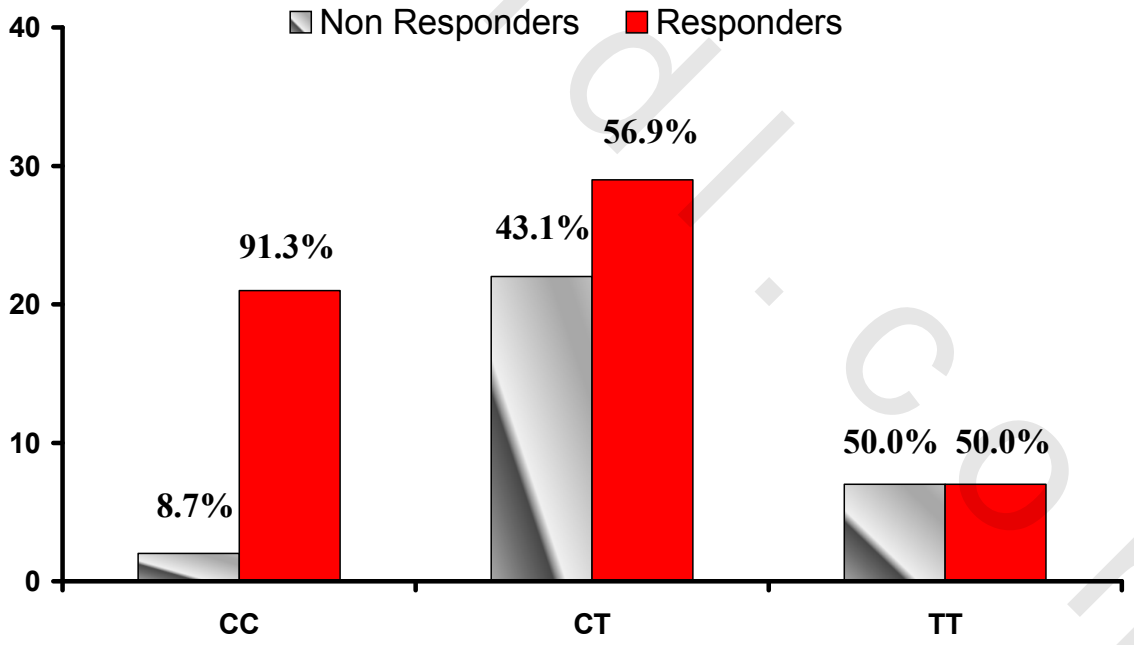

Figure (50) Distribution of IL-28 rs12799860 genotypes (CC, CT and TT) in HCV patients.

Table (11): Exploring the Consistence of IL-28 rs12799860 SNP genotypes distribution with Hardy-Weinberg Equilibrium in Apparently Healthy subjects and HCV Patients Groups

	Observed		HW Expected		Observed vs. HW Expected		IL-28 rs12799860	
	Count	%	Count	%	X^2	p - Value	Allele	Frequency
Apparently Healthy Subjects (n = 30)								
CC	13	43.3	14.7	49	**2.184**	**0.139**	**Wild (p)**	0.70
CT	16	53.3	12.6	42			**Mutant (*q*)**	0.30
TT	1	03.4	02.7	09				
HCV Patients (n = 88)								
CC	23	26.1	26.73	30.4	**2.584**	**0.108**	**Wild (p)**	0.55
CT	51	58.0	43.54	49.5			**Mutant (*q*)**	0.45
TT	14	15.9	17.73	20.1				
Non-Responder HCV Patients (n =31)								
CC	2	06.4	05.5	17.7	**6.481**	**0.011***	**Wild (p)**	0.42
CT	22	71.0	15.1	48.7			**Mutant (*q*)**	0.58
TT	7	22.6	10.5	33.9				
Responder HCV Patients (n =57)								
CC	21	36.8	22.1	38.8	**0.391**	**0.532**	**Wild (p)**	0.62
CT	29	50.9	26.8	47.0			**Mutant (*q*)**	0.38
TT	7	12.3	08.1	14.2				

* *p*-value was considered significant at *(p)* value < 0.05

Table (12): Allele Frequencies of rs12979860 Polymorphism Among Apparently Healthy Subjects and HCV Patients Groups and its Relation to HCV

	C		T		OR	CI 95%	*p*
	No.	%	No.	%			
Apparently Healthy Subjects	42	70	18	30			
HCV Patients	97	55.1	79	44.9			
Non Responders Patients	26	41.9	36	58.1		0.233-0.822	0.007*
Responders Patients	71	62.3	43	37.7	0.737		

The results of the present study showed that the CC alleles of rs12979860 were significantly associated with virological response, Table (13).

Table (13): IL-28B Polymorphism in Relation to Response of HCV Patients

SNP	OR	CI 95%	*p*
CC *vs*. TT	0.095	0.016 – 0.570	0.014*
CC *vs*. TT/CT	0.118	0.026 – 0.546	0.002*

OR = Odds Ratio, CI = Confidence Interval
* *p* value was considered significant at $p < 0.05$

Triglycerides, total-cholesterol and LDL-cholesterol were the only parameters that showed significant differences on examining the mean significant differences of baseline biochemical characteristics between HCV with CC genotype and HCV with non CC genotype (CT and TT), (Table 14). In HCV patients with genotype CC, the mean concentration serum levels of triglycerides and total cholesterol were significantly higher than those in HCV patients with non CC genotype (CT and TT), $p = 0.012$ and $p = 0.010$, respectively. In contrast, the mean concentration level of LDL-cholesterol was significantly lower in HCV patients with genotype CC when compared to that in HCV patients with non CC genotype (CT and TT), $p = 0.012$ (Table 14).

Table (14): Statistical Analysis of Baseline of Some Biochemical Characteristics in All HCV Patients According to IL-28B rs12979860 Genotypes

Biochemical Parameter	rs12979860 genotypes		*p*
	CC (n= 23)	CT + TT (n = 65)	
Triglycerides (mg/dL)	128.7 ± 11.6	100.1 ± 5.9*	0.012
Total Cholesterol (mg/dL)	168.7 ± 7.4	145.8 ± 3.9*	0.010
LDL Cholesterol (mg/dL)	89.9 ± 5.9	73.8 ± 3.5*	0.012

Mann Whitny U test was applied
p^* value was considered significant at $p < 0.05$

The results of the present study showed the frequencies of high degree of fibrosis were observed in Non-responders HCV patients who are with non CC genotype (Table 15). Also, the results revealed that almost 89% of non-responder HCV patient's non CC genotype has high serum GGT activity.

Table (15): Distribution of Degree of in Non- Responders and Responders HCV Patients According to IL-28B rs12979860 Genotypes

	Non Responders (n= 31)		Responders (n = 57)	
	CC (2)	CT/TT (29)	CC (21)	CT/TT (36)
Stage of Fibrosis				
F1 + F2	0 (00.0 %)	17(58.6 %)	18 (85.7 %)	32 (88.9 %)
F3 + F4	2 (100 %)	19 (65.5 %)	3 (14.3 %)	4 (11.1 %)
Serum GGT Activity (IU/L)				
≥ 23.7 (cut off value**)**	2 (100 %)	26 (89.7 %)	9 (42.9 %)	25 (69.4 %)
< 23.7 (cut off value**)**	0 (00.0 %)	3 (10.3 %)	12 (57.1 %)	11 (30.6 %)