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RESEARCH ARTICLE

PEROXISOME PROLIFERATORS-ACTIVATED RECEPTOR- GAMMA GENETIC POLYMORPHISM AND NONALCOHOLIC STEATOHEPATITIS, CAN IT PREDICT DISEASE PROGRESSION!

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Abstract

Background and study aims:- nonalcoholic fatty liver disease (NAFLD) is now the most common liver diseases. It progresses to liver cirrhosis with the incidence of hepatocellular carcinoma. Multiple hits theory in NAFLD progression indicates that genetic factors are important. Peroxisome proliferators-activated receptor-gamma (PPAR-gamma) has an essential role in the regulation of adipocyte-specific genes and lipid and glucose metabolism. We aimed at evaluating the role of PPAR-gamma single nucleotide polymorphisms (SNPs) in NAFLD and predicting its progression to nonalcoholic steatohepatitis (NASH).

Material and methods:- Fifty obese patients with NAFLD preliminary diagnosed by liver ultra-sound were recruited. Full medical history, anthropometric measurements, biochemical studies, liver biopsy for the NAS score to identify NASH patients and the presence of fibrosis and assessment of SNPs of C681G, C689T and C34G of PPAR-gamma by Polymerase chain reaction-restriction fragmented length polymorphism (PCR-RFLP), were conducted for each patient. Thirty ages matched average weight healthy adult had been chosen as controls.

Results:- The GG genotype of C681G was higher in cases (18%) than in control (10%) and also of C34G was higher in cases (12%) than in control (3.3%). The TT genotype of C689T was higher in cases (6%) than in control (0%).

The GG genotype of C681G was higher in NASH group (19.4%) than in non NASH group (14.3%).

Conclusion:- PPAR–gamma genetic polymorphism (C681G, C689T and C34G) was raised in patients with NAFLD and also of (C681G and C34G) was raised in cases with NASH but didn't reach statistical significance..

Introduction:-

Nonalcoholic fatty liver disease (NAFLD) is now the most common liver diseases. It progresses to liver cirrhosis and organ failure **(Hardy et al., 2016)**.

The incidence of NAFLD is increasing all over the world, representing 30% in developed countries and about 10% in developing countries **(smith and Adams, 2011)**, and greater than 30% of patients with NAFLD have NASH may progress to end stage liver disease (cirrhosis) **(Rinella, 2015)**, liver cell failure and hepatocellular carcinoma **(Torres and Harrison, 2008).**

The majority of the cases with NAFLD is associated with features of metabolic syndrome (MS) involving obesity, diabetes, hypertension and dyslipidemia **(McCullough, 2011).** Recently NAFLD is considered the hepatic component of MS **(Torres and Harrison, 2008)**.

There are multiple causes have been attributed for occurrence of hepatic insulin resistance in NAFLD such as [inflammation, endoplasmic reticulum (ER) stress and the accumulation of hepatocellular lipids] **(Kumashire et al., 2011).**

Peroxisome proliferators-activated receptor-gamma (PPAR-gamma), which is a member of the nuclear hormone receptor superfamily, it has an essential role in the control of adipocyte-specific genes, lipid and glucose metabolism. PPAR-gamma is produced by adipose tissue **(Semple et al., 2006)**, it involves two protein isoform, PPAR-gamma 1 and PPAR-gamma 2. PPAR-gamma 2 is presented primarily in adipose tissue and the large intestine **(Fajas et al., 1997).**

The PPAR-gamma gene is recognized as a potential candidate gene to detect insulin sensitivity **(Day, 2004).** PPAR-gamma has been involved in the etiology of type 2 DM and its role has been investigated in several epidemiological studies **(Gouda et al., 2010).**

In MS, Reports documented a huge number of SNPs of the gene belong to insulin resistance (IR) and energy metabolism, which might be associated with the risk to NAFLD **(Meirhaeghe et al., 2005 and Ranjith et al., 2008)**.

Previous studies don't provide adequate proof for the great role of PPAR-alpha and PPAR-gamma single nucleotide polymorphism (SNP) and susceptibility for NAFLD **(Dongiovanni and Valenti, 2013),** also its role in the pathogenesis and progression of fatty liver disease is still undetermined **(Domenici et al., 2013).** Aim of work in this study was to investigate the role of PPAR-gamma SNPs polymorphism in the diagnosis and progression of NAFLD to NASH by liver biopsy.

Materials and methods:-

The current study is a prospective case-control study, performed in Kasr Al-eini hospital, Internal Medicine out-patient clinic (Liver and gastroenterology clinic), faculty of medicine, Cairo University, over a 7 month period (From January-July 2014). NAFLD patients' preliminary diagnosed by liver ultra-sound scan showing a picture of fatty liver ± elevated liver enzymes.

The selection of patients in this study was based on the following inclusion criteria; both sexes, aged between 18-60 years, with body mass index (BMI) over 30Kg/m² in cases, having a bright liver on liver ultra-sound scan (picture suggestive of hepatic steatosis) and no history of alcoholic intake. While participants with hepatitis C or B infection, patients with known causes of liver disease (autoimmune, genetic, or drug induced), patients with major systemic conditions and pregnant women were excluded from the study. Fifty male and female participants (46 female and 4 male) had a complete work-up including history, general physical examination, anthropometric measurements, serum biochemistry profiles; including hepatitis markers [hepatitis C virus (HCV) antibody and hepatitis B virus surface (HBVs) antigen], liver ultra-sound scan and True-cut ultrasound guided liver biopsy.

Thirty healthy age matched average weight (BMI<25kg/m²) participants (20 female and 10 male), with the normal appearance of liver during scanning and normal liver enzymes had been chosen as a control for 3 SNPs of C681G, C689T and C34G of PPAR-gamma. Liver biopsy was not taken for control for ethical consideration.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by Cairo University Hospital Research Ethics Committee (REC) (No. n-17-2014). A written informed consents were obtained from all participants in the study, including consent for genetic study.

Methods:-

Clinical and biochemical assay:-

All participants were interviewed for their medical history and clinical examination. The blood samples were obtained from each participant after a fasting period of at least 12 hours. The blood glucose level was measured using the glucose oxidase method. Serum total cholesterol, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol and triglyceride levels were measured on an autoanalyser using enzymatic calorimelery. Serum levels of liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyle transferase (GGT)] were also measured.

Anthropometric measurements:-

The weight and height of each participant were measured and the BMI was calculated by the formula: body weight in kg divided by height square in meters (Kg/m^2). The waist circumference was measured midway between the rib margin and the iliac crest in a standing position by the same examiner.

Abdominal ultrasound:-

Was performed by the same operator using a Toshiba Apilo XV scanner equipped with a broad band 3.5 MHz curved array probe to assess the presence of liver steatosis (bright liver),which was defined and graded as **(Ricci et el., 1997).** Steatosis was graded using this semi-quantitative scale from 1-4.

Liver biopsy:-

All patients had undergone ultrasound guided liver biopsy (according to guidelines of liver biopsy for patients with NAFLD in 2002 by the American Association for the study of Liver Diseases, **AASLD**). Controls did not undergo liver biopsy for ethical reasons.

Liver biopsy was fixed in ten percent neutral buffered formalin, then embedded in paraffin blocks. Five micrometer thick sections were cut and stained with hematoxylin and eosin and examined under light microscope for histopathological diagnosis and scoring using the NAS scoring system according to Histological Scoring System for Nonalcoholic Fatty Liver Disease **(Kleiner et al., 2005).** This scoring system addresses the full spectrum of lesions of NAFLD and allows a diagnostic categorization into NASH, borderline NASH or not NASH. Fibrosis staging was evaluated (separately from NASH) from 0 to 4 scales **(Brunt, 2009).**

According to results of liver biopsy, NAFLD patients subdivided into two groups: Group I includes patients had NASH (NASH and borderline NASH) and group II which includes patients didn't have NASH (simple steatosis).

Genetic study:-

Genomic DNA was isolated from whole blood samples using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instruction. The analysis of 3 SNPs of PPAR-gamma polymorphism was determined by PCR based restriction fragment length polymorphism (PCR-RFLP) according to **(Liao et al., 2006).** The sequences of PCR primers were listed in table I. PCR conditions were as follows: an initial denaturation at 94 º c for 5 minutes, followed by 35 cycles of denaturing at 94 ºc for 30 seconds, annealing at 51 º c for 40 seconds and extension at 72 º c for 40 seconds. The final extension was continued at 72 º c for 10 minutes and cooling to 4 º c. After amplification, the amplified PCR products were subjected to restriction digestion by restriction endonuclease (Fermentas, Vilnius, Lithuania) for 8 hours at 60 º c. The DNA fragments were then separated on 2% agarose gel electrophoresis to identify the genotypes.

Table 1: Oligonucleotide primer sequence of the 3 SNPs at PPAR-γ gene.

PPAR-γ & 3 SNPs		**Primers**
PPAR-γ	**Forward**	5'-GCCAATTCAAGCCCAGTC-3'
	Reverse	5'-GATATGTTTGCAGACAGTGTATCA-3'
rs10865710(C681G)	**Forward**	5'-TGTCGGGTCTCGATGTTG-3'
	Reverse	5'-TGGTTATTAAGCCTAAGGTG-3'
rs7649970 (C689T)	**Forward**	5'-TAGAGAACTCCATTTTTTCATTATGACATAGCACTGAT-3' †
	Reverse	5'-ACTGACTGCTATCTAAATTCTG-3'
rs1801282 (C34G or Pro12A1a)	**Forward**	5'-ACTCTGGGAGATTCTCCTATTGGC-3' †
	Reverse	5'-CTGGAAGACAAACTACAAGAG-3'

Forward, forward primer; Reverse, reverse primer

† The underlined mismatched base introducing a restriction endonuclease recognition site (T →G for rs7649970 and C →G for rs1801282).

The genotype CC is wild type and others are polymorphism either homozygous or heterozygous.

Statistical methods:-

Data were coded and entered using the statistical package SPSS version 21. Data was summarized using mean, standard deviation, median, minimum and maximum for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparison of quantitative variables was done using the nonparametric Kruskal-Wallis when comparing more than 2 groups and using the nonparametric Mann-Whitney U test when comparing 2 groups. For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. Genotype frequencies were compared between the different study groups using chi-square tests. Odds ratio (OR) with 95% confidence intervals was calculated. P value <0.05 was taken as statistically significant **(Chan, 2003).**

Results:-

The current study was conducted on eighty participants. The participants were divided into two groups:
Group A (NAFLD cases), 92 % were female and 8% were male, their mean age was 43.54±7.28.
Group B (control), 66.66% were female and 33.33% were male, their mean age was 40.67±6.9.
The demographic data of the study groups were shown in Table 2 &3.

Table 2: The demographic data of the study groups.

	Cases (n. 50)	**Controls (n. 30)**	**P value**
	Mean± SD	**Mean± SD**	
BMI (kg/m^2)	34.87±3.70	22.47±1.81	<0.001*
Waist Circumference (cm)	105.78±14.64	89.00±2.70	<0.001*
ALT (IU/L)	33.20±19.09	17.17±3.44	<0.001*
AST (IU/L)	33.32±20.60	20.73±2.60	<0.001*
GGT (IU/L)	44.02±25.31	38.27±5.45	0.382
Creatinine (mg/dl)	0.89±0.30	0.90±0.21	0.67
FBS (mg/dl)	92.85±9.472	82.40±4.84	< 0.001*
T-CHOL (mg/dl)	201.54±28.69	141.27±4.27	< 0.001*
LDL-CHOL (mg/dl)	104.36±21.62	61.85±5.38	< 0.001*
HDL-CHOL (mg/dl)	47.58±14.41	55.67±3.70	< 0.001*
TG (mg/dl)	173.32±40.53	118.7±5.99	< 0.001*

* Significant difference.

ALT alanine aminotransferase; AST aspartate aminotransferase; GGT γ glutamyle transferase; FBS fasting blood sugar; T-CHOL total cholesterol; LDL-CHOL low density lipoprotein cholesterol; HDL-CHOL high density lipoprotein cholesterol; TG triglyceride.

Table 3: The demographic data of the study groups.

	NAS scores by liver biopsy		
	NASH (n. 36)	**Non NASH (n. 14)**	**P value**
	Mean± SD	**Mean± SD**	
BMI (kg/m^2)	34.96±3.98	34.64±3.02	0. 721
Waist Circumference (cm)	105.39±15.99	106.79±10.89	0.581
ALT (IU/L)	37.06±20.59	23.29±9.25	0.02*
AST (IU/L)	35.67±23.43	27.29±8.18	0.430
GGT (mg/dl)	47.50±27.43	35.07±16.39	0.187
Creatinine (mg/dl)	0.88±0.32	0.90±0.23	0.680
FBS (mg/dl)	119.89±34.18	123.79±20.12	0.261
T-CHOL (mg/dl)	196.33±26.88	214.93±29.80	0.084
LDL-CHOL (mg/dl)	101.89±17.81	110.71±29.11	0.375
HDL-CHO (mg/dl)	46.36±15.76	50.71±9.93	0.060
TG (mg/dl)	170.44±44.98	180.71±25.74	0.531

* Significant difference.

NASH Non alcoholic steatohepatitis; Non NASH non non alcoholic steatohepatitis
BMI and waist circumference were significantly higher in NAFLD patients.

ALT, AST, fasting blood sugar (FBS) and lipid profile [total cholesterol, HDL, LDL cholesterol and triglycerides] were significantly higher in NAFLD patients.

NASH was found in 72% of cases (Group A I), the NASH group includes patients with NASH (38%) and borderline NASH (34%), and (Group A II), 28% of cases didn't have NASH.

In the NASH group, there were higher levels of ALT, AST and GGT, which was significant as regards ALT but other parameters didn't reach significance as shown in Table 3.

Genetic study; GG genotype of the C681G gene was higher in cases (18%) than in the control group (10%), also the G allele frequency of C681G genes in NAFLD (41%) was higher than that (31.6%) in controls (p value =0. 23) with no significance difference. While CC and C allele of the C681G genes was lower in NAFLD cases than that in controls, as shown in Fig. 1 and Table 4.

TT genotype of the C689T genes and T allele was higher in NAFLD cases than that in controls but didn't reach level of significance, as shown in Fig. 2 and Table 4.

GG genotype of C34G gene was higher in cases (12%) than in the control group (3.3%) (p value = 0.23). The G allele frequency of C34G genes in NAFLD (22%) was higher than that (11.7%) in controls (p value = 0.100) as shown in Fig. 3 and Table 4.

Table 4: Comparison of gene distribution and allele frequencies in NAFLD patients and controls

		Cases (n. 50)		**Control (n. 30)**		**P-value**	**OR (95% CI)**
		Count	**%**	**Count**	**%**		
C681G	GG	9	18%	3	10.0%	0.315	2.333 (0.53-10.267)
	CG	23	46%	13	43.3%	0.520	1.376 (0.519-3.648)
	GG+ CG	32	64.0%	16	53.3%	0.346	1.556 (0.619-3.907)
	CC	18	36%	14	46.7%	Reference	
	Allele G	41	41%	19	31.6%	0.238	1.5 (0.764-2.943)
	Allele C	59	59%	41	68.3%	Reference	
C689T	TT	3	6%	0	0%	0.285	----------
	CT	4	8%	2	6.7%	1	1.302 (0.223-7.591)
	TT+CT	7	14.0%	2	6.7%	0.471	2.279 (0.441-11.772)
	CC	43	86%	28	93.3%	Reference	
	Allele T	10	10%	2	3.3%	0.213	3.222(0.681- 15.237)
	Allele C	90	90%	58	96.7%	Reference	
C34G	GG	6	12%	1	3.3%	0.235	4.235 (0.479-37.487)
	CG	10	20%	5	16.7%	0.570	1.41(0.24-6.59)
	GG+CG	16	32.0%	6	20.0%	0.245	1.882(0.643-5.51)
	CC	34	68%	24	80.0%	Reference	
	Allele G	22	22%	7	11.7%	0.100	2.136(0.852-5.355)
	Allele C	78	78%	53	88.3%	Reference	

Distribution of the GG genotype of the C681G gene was higher in NASH group (19.4%) than non NASH group (14.3%) (p value =1. 00). The G allele frequency of C681G genes was higher in NASH group (41.7%) than in non NASH group (39.3%) (p value = 0.82) but didn't reach significance as shown in Fig. 4 and Table 5.
The G allele frequency of C34G genes it was higher in NASH group (22.2%) than in non NASH group (21.4%) as shown in Fig. 5 and Table 5.

Table (5): Comparison between NASH and non NASH groups regarding genetic results.

		NASH scores by liver biopsy				P value	OR (95% CI)
		NASH (n. 36)		Non NASH (n. 14)			
		Count	%	Count	%		
C681G	GG	7	19.4%	2	14.3%	1.00	1.346 (0.205-8.819)
	CG	16	44.4%	7	50.0%	0.853	0.879 (0.225-3.429)
	GG+ CG	23	63.9%	9	64.3%	0.979	0.983 (0.271-3.562)
	CC	13	36.1%	5	35.7%	Reference	
	Allele G	30	41.7%	11	39.3%	0.828	1.104 (0.453-2.692)
	Allele C	42	58.3%	17	60.7%	Reference	
C 689 T	TT	2	5.6%	1	7.1%	1.00	0.688 (0.057-8.344)
	CT	2	5.6%	2	14.3%	0.304	0.344 (0.043-2.741)
	TT+CT	4	11.1%	3	21.4%	0.384	0.458 (0.088-2.378)
	CC	32	88.9%	11	78.6%	Reference	
	Allele T	6	8.3%	4	14.3%	0.460	0.545 (0.142-2.101)
	Allele C	66	91.7%	24	85.7%	Reference	
C 34 G	GG	4	11.1%	2	14.3%	1.00	0.833 (0.131-5.304)
	CG	8	22.2%	2	14.3%	0.702	1.667 (0.3-9.272)
	GG+CG	12	33.3%	4	28.6%	1.00	1.25 (0.324-4.826)
	CC	24	66.7%	10	71.4%	Reference	
	Allele G	16	22.2%	6	21.4%	0.931	1.048 (0.363-3.024)
	Allele C	56	77.8%	22	78.6%	Reference	

Discussion:-

In Egypt the incidence of NAFLD in patients with metabolic syndrome (57.9%) and (62.7%) in general adult **(Hegazy et al., 2006)**, the national institute of health in Egypt termed NAFLD "a silent disease" **(NCEP)**, because it develops over a prolonged period of time and most of the people complaining of few, if any symptoms until it progress to NASH or cirrhosis.

Two factors are important for the developments of NAFLD are genetic and environmental factors **(Giby and Ajith, 2014).**

Recently PPAR considered attractive drug targets for the treatment of NAFLD, NASH and related disorders as (type 2 diabetes and the metabolic syndrome) **(Dongiovanni and Valenti, 2013).**

In a study done by **Merihaeghe et al., 2003 and 2005** demonstrated that the G allele of rs10865710 and the T allele of rs7649970 were accompanied with reduced PPAR–gamma promoter activity.

In the present study GG genotype of the C681G gene was higher in cases (18%) than in the control group (10%) but didn't reach significance. The G allele frequency of C681G genes in NAFLD (41%) was higher than that (31.6%) in controls with no significance difference.

Our results, going on with **Cao et al., 2012**, reported that the GG genotype of the C681G gene was higher in cases (17.2%) than in the control group (11.2%) and the G allele frequency of C681G genes in NAFLD (41.1%) was higher than that (34.8 %) in controls.

In our study, we found that the TT genotype of C689T gene was higher in cases (6%) than in the control group (0%) but didn't reach level of significance. The T allele frequency of C689T genes in NAFLD (10%) was higher than that (3.3%) in controls.

In contrary to **Cao et al., 2012,** reported that the TT genotype of C689T gene was not detected in both cases and control (0%) and there is no influence for C689T as precipitating factors for NAFLD.

The results of the current study showed that GG genotype of C34G gene was higher in cases (12%) than in the control group (3.3%) (p value = 0.23). The G allele frequency of C34G genes in NAFLD (22%) was higher than that (11.7%) in controls (p value = 0.100).

Our results were in agreement with a previous study **(Rey et al., 2010 and Chen et al., 2013)** stated that the incidence of the A1a12 mutant was higher in NAFLD patients from that in the controls but with no significant difference.

A study done by **Gupta et al., 2010** and **Yang et al., 2012** also reported that genotype of Pro12A1a in NAFLD group has been significantly more frequently than that in controls and it was an independent risk factor for NAFLD.

In difference to our results, a previous study done by **Cao et al., 2012** observed that GG genotype of C34G gene was not detected in both cases or control (0%) and The G allele frequency of C34G genes in NAFLD (3%) and in control (3.1%).

Moreover, results from previous work showed controversy, these could be partially explained by the difference of the ethnicity in the studying populations **(Cao et al., 2012)**, which might suspect complex interaction between PPAR– gamma SNP, environmental factors and SNP of other genes **(Domenici et al., 2013)** and reduced incidence of the A1a allele in Asian populations **(Cao et al., 2012).**

In the present study, BMI, waist circumference, ALT, AST, LDL, HDL and TGs were significantly higher in cases than in control. This was in agreement with several studies **(Marchesini et al., 2003; Fracanzani et al., 2011; Ong et al., 2005 and Kashyap et al., 2009).**

To our know knowledge, there is only one study **(Cao et al., 2012)** which evaluates the role of several SNPs (C681G, C689T and C34G) in PPAR–gamma gene and susceptibility to NAFLD.

Previous work was done on NAFLD but to our know knowledge, this is the first study which detect relation between 3 SNPs (C681G, C689T and C34G) in PPAR –gamma gene and liver biopsy proved NASH.

In the current study, we observed that GG genotype of the C681G gene was higher in NASH group (19.4%) than non NASH group (14.3%) and the G allele frequency of C681G genes was higher in NASH group (41.7%) than in non NASH group (39.3%) but didn't reach significance.

In our study, we found that the TT genotype of C689T gene was lower in NASH group (5.6%) than in non NASH group (7.1%). The distribution of the T allele of C689T genes accounts for 8.3% in NASH group and 14.3% in a non NASH group.

The results of the present study showed that GG genotype of C34G genes in NASH group (11.1%) and in non NASH group (14.3%) but levels not reach significance. The G allele frequency of C34G genes it was higher in NASH group (22.2%) than in non NASH group (21.4%).

Kotronen et al., (2009) observed that the SNP at the Pro12A1a was significantly associated with hepatic fat content which, measured by proton magnetic resonance spectroscopy in study involved 302 patients.

Dongiovanni et al., (2010) reported that the A1a to Pro substitution in PPAR–gamma gene was not associated with the grades of steatosis, necroinflammation or liver fibrosis.

We should mention some limitation of the present study. It is a pilot study; was done on a small number of patients because many patients suspect to have NAFLD by abdominal ultrasound had normal liver enzyme and according to recent guidelines these patients are not eligible to perform liver biopsy and also many indicated patients refused to do a liver biopsy. Also in the present study, we had an unequal distribution of the sex of the participants.

Our study stated that the possibility of NAFLD in patients with SNP of C681G and C689T and C34G in PPAR–gamma and also SNP of C681G and C34G associated with susceptibility for progression to NASH. Further studies are required on large scale of patients to detect the role of SNP of C681G, C689T and C34G in PPAR–gamma in pathogenesis and progression of NAFLD. We conclude that PPAR–gamma genetic polymorphism (C681G, C689T and C34G) was raised in patients with NAFLD and also of (C681G and C34G) was raised in cases with NASH but didn't reach statistical significance.

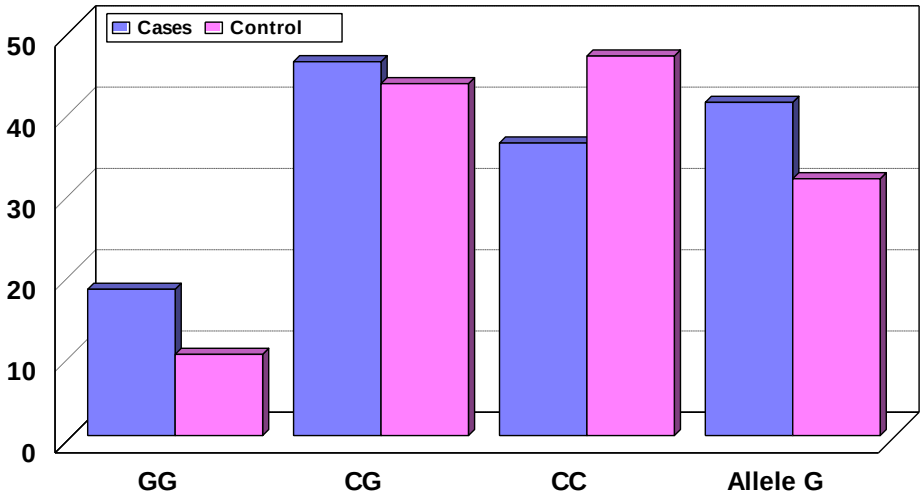

Fig. 1: Distribution of the genotype of C681G genes in cases and in control group.

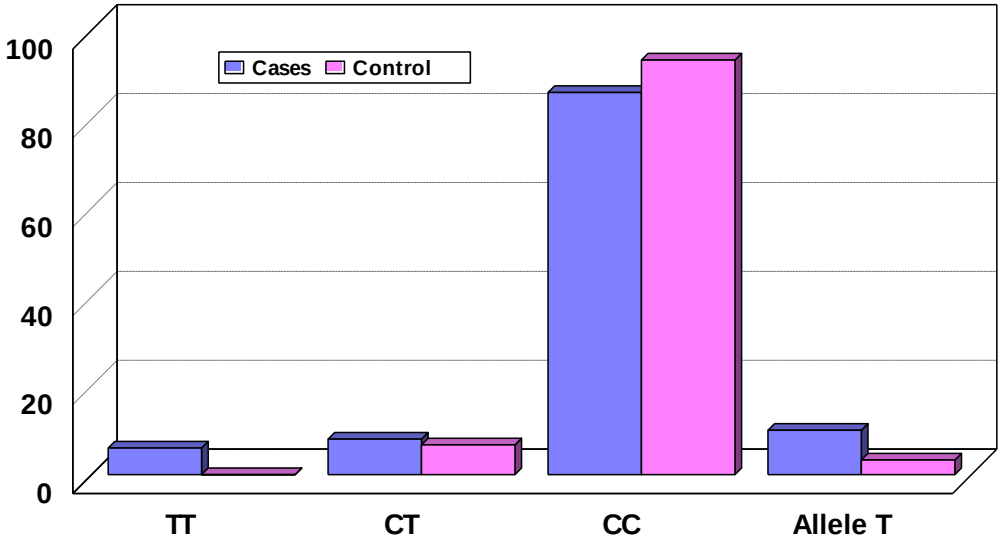

Fig. 2: Distribution of the genotype of C689T genes in cases and in control group.

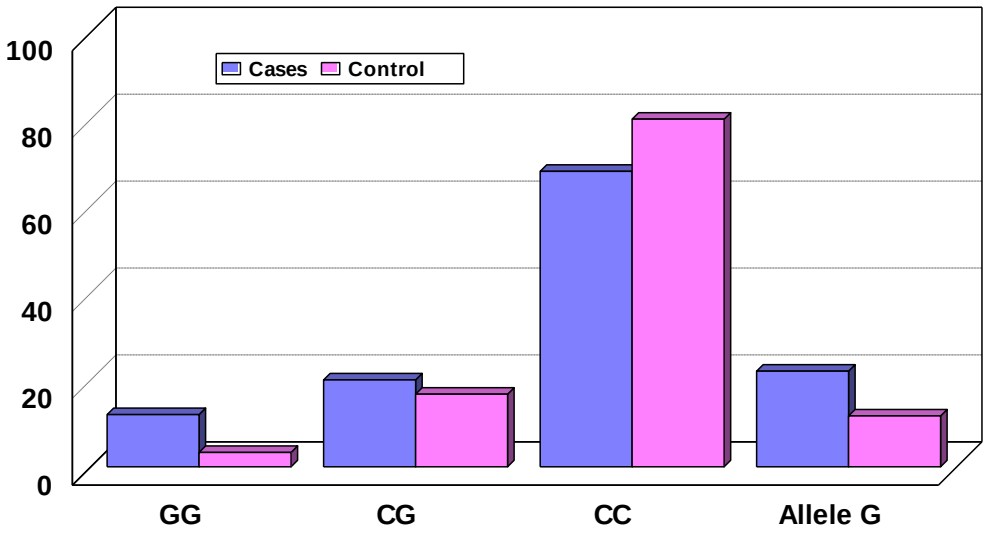

Fig. 3: Distribution of the genotype of C34G genes in cases and in control group.

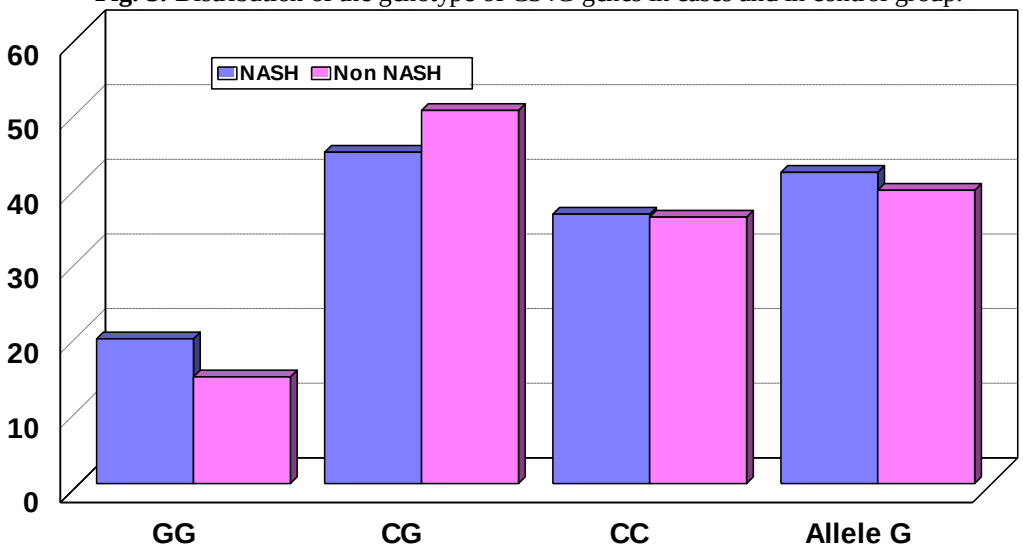

Fig. 4: Distribution of the genotype of C681G genes in NASH group and in non NASH group.

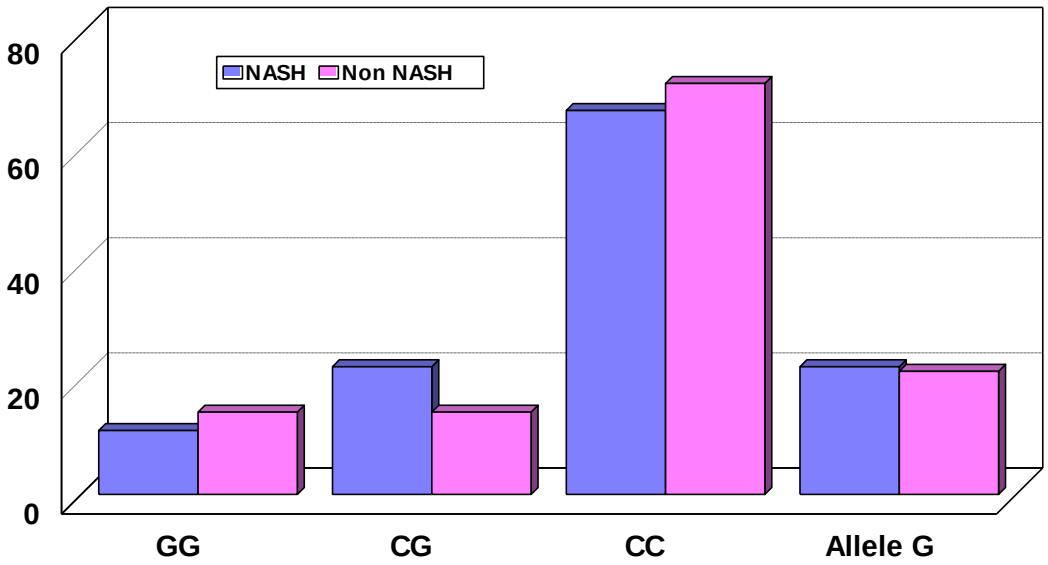

Fig. 5: Distribution of the genotype of C34G genes in NASH group and in non NASH group.

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