Study of the Relation between Endothelial Nitric Oxide Synthase G894T Gene Variants and Occurrence of Type II Hepatorenal Syndrome

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Running title: Nitric Oxide Synthase G894T gene in type II HRS

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Background and study aim: The progress of hepatorenal syndrome (HRS) in hepatic patients is not yet understood. Nitric oxide level, through its impact on haemodynamic circulation, may be engaged in the progress of the disease. Our study expected to clarify the possible role of eNOS G894T in decompensated liver cirrhosis and HRS and its relationship with nitrite level.

Methods: Study included 80 cirrhotic patients (40 decompensated cirrhotic and 40 HRS) and 40 healthy participants as controls. Renal and liver function tests, CBC, serum electrolytes, plasma nitrite and eNOS G894T by real-time PCR were surveyed.

Results: There were higher frequencies of TT and GT genotypes of eNOS G894T versus GG in both cirrhotic (22.5% and 50% respectively) and HRS (30% and 45% respectively) than in controls (10% and 30%) (P=0.007). T allele was more prevalent than G allele in cirrhotic (47.5%) and HRS (52.5%) patients compared to controls (25.0%) (P=0.001). TT and GT genotypes increase risk of cirrhosis by OR 4.909 [95% CI: 1.24 – 19.46] and OR 3.636 [95% CI: 1.32 – 9.99] and HRS OR7.200 [95% CI: 1.86 – 27.77] and 3.600[95% CI: 1.27 – 10.7] respectively. T allele can increase risk of cirrhosis by OR 2.714 [95% CI: 1.39 – 5.30] and HRS OR 3.316 [95% CI: 1.70 – 6.48]. Clear connections were observed between TT genotype and lower plasma nitrite level (P<0.001).

Conclusion: Mutant variants of eNOS G894T gene in cirrhotic and HRS patients from controls could partially explain the progress to decompensation and HRS in liver cirrhosis.

INTRODUCTION

Hepatorenal syndrome (HRS) is defined as the development of renal failure in patients with advanced chronic liver disease that is complicated by portal hypertension and ascites **[1]**. The incidence and the prevalence of HRS in advanced liver disease are 7.6% and 13%, respectively **[2].**

There are two kinds of HRS: Type 1 HRS is recognized by quick and dynamic renal impairment and is hastened by spontaneous bacterial peritonitis. Without treatment, the median survival is not more than 2 weeks. Type 2 HRS is described by a moderate and stable decline in the glomerular filtration rate and generally takes place in patients with preserved functions of the liver. Type 2 HRS patients are frequently resistant to diuretic therapy and they have a median survival about three to six months **[3]**.

Renal vasoconstriction is considered the hallmark of HRS; however, its pathogenesis is not entirely recognized. Several mechanisms are presumably included. These mechanisms incorporate interactions between perturbed systemic haemodynamics, activation of the vasoconstrictor systems and a decline in activity of the vasodilator systems **[4]**.

The dominant systems accountable for renal vasoconstriction are the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system (SNS). The effectiveness of both systems is expanded in decompensated cirrhosis and is magnified in HRS. Interestingly, a reverse relationship is present among the activity of the 2 systems with renal plasma flow and the GFR **[5]**.

Nitric oxide (NO) is a vasodilator that has a fundamental role in renal perfusion **[6]**. NO has a definitive role in the organization of the vascular tone and endothelial function in numerous organs including the kidney **[7]**.

NO half-life is incredibly short, approximately 1 s. consequently, endogenously generated NO is organized by NOS activity **[8]**. The end products of Nitric oxide metabolism in vivo are nitrite (NO2-) and nitrate (NO3-), which are indirect measures of the total NO concentration **[9]**. Various polymorphisms of the eNOS gene have been recognized, and their linkages with several diseases have been identified **[10]**. The eNOS gene G894T polymorphism at exon 7 which, also known as (Glu298Asp or rs1799983), influences the expression and the activity NOS3 **[11]**. Few studies have checked the role of the eNOS gene in HRS; therefore, we planned to elucidate the impact of polymorphism in eNOS G894T gene in liver cirrhosis and HRS and its link with nitrite levels and clinical parameters in these patients.

PATIENTS AND METHODS

This study was carried out by Tropical Medicine Department in collaboration with the Medical Biochemistry and Molecular Biology Department. It included 120 participants, classified as 80 patients with liver cirrhosis and 40 healthy participants without evidence of any liver or kidney disease as controls. Patients and controls were chosen from the outpatient and or inpatient departments of Tropical Medicine, Menoufia University Hospital, and Kafr El-Sheikh Liver Research Center Hospital in the period between June 2017 and August 2018. Patients with diabetes mellitus, hypertension, malignant diseases, acute gastrointestinal bleeding, or spontaneous bacterial peritonitis and patients with renal impairment due to any aetiology other than HRS were excluded.

Participants were classified into three groups; **Group I:** 40 decompensated cirrhotic patients without type II hepatorenal syndrome, **Group II:** 40 cirrhotic patients with type II hepatorenal syndrome, and **Group III:** 40 healthy subjects of matched age and sex as controls. Cirrhosis was proved by clinical examination, laboratory investigations and radiological evaluation. Diagnosis of type 2 hepatorenal syndrome was made according to the International Ascites Club criteria **[12]**. MELD scores were calculated for all cirrhotic patients. Our study was carried out in conformity with the Declaration of Helsinki. An informed consent was provided by all participants and the study protocol was notarized by the Ethics Committee of the Faculty of Medicine, Menoufia University.

Sample collection and assays

Ten-millilitre venous blood samples were obtained from all participants. Five millilitres of the blood was picked and left for thirty min in a plain tube to clot, and thereafter centrifugation done at 4000 rpm for ten min; the separated serum was utilized for measuring urea and creatinine by standard colorimetric tests by a Diamond Diagnostics Kit (Germany), with measurements of serum ALT by the kinetic UV-optimized method IFCC (LTEC Kit, England), serum albumin, and detection of serological markers of HCVAb and HBsAg by enzyme-linked fluorescent immunoassay (ELIFA) utilizing miniVIDAS systems (bioMerieux, Marcy l'Etoile, France); positive tests were confirmed by real-time PCR. Total and direct bilirubin were measured by a Diamond Diagnostics Kit (Germany), and serum Na and K were measured by an automated AU680 chemistry analyser (Beckman Coulter Inc; Brea, California, USA). Two millilitres fresh blood was obtained in ethylene diamine tetra-acetic acid (EDTA)- comprising tubes and was utilized for a complete blood count (CBC) by Sysmex XN-1000 (Japan, 19723, B.M Egypt company), and plasma was then separated by centrifugation at 4000 rpm for 10 min for measurement of nitrite levels (Biodiagnostic, Diagnostic and Research Reagents, Egypt). One millilitre of fresh blood was obtained in a citrate tube for measurement of prothrombin time (PT and INR) (BIOMED - LlQUIPLASTIN diagnostic kit, Germany). In EDTA-containing tubes, we collected two millilitres of fresh blood used for extraction of DNA and single-nucleotide polymorphism assays of eNOS G894T by real-time PCR.

SNP assay of eNOS G894T by RT-PCR

DNA was first isolated from whole blood utilizing a commercially available spin-column technique kit for DNA extraction (Invitrogen™, PureLink™ Genomic DNA Mini Kit, USA). DNA purity was subsequently tested by measuring the absorbance values at 260 nm, 280 nm, and 300 nm using a UV visible spectrophotometer. DNA purity was considered good if it had an A260 nm / A280 nm ratio of 1.8 and an A300 nm reading of 0.1 or less. At –20°C the purified DNA was stored until used.

TaqMan genotyping assay kits were used for analysis using a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). TaqMan probes to detect eNOS G894T were labelled with VIC and FAM fluorescent dyes, respectively, with the probe sequence:

CCCTGCTGCTGCAGGCCCCAGATGA[G/T]
CCCCCAGAACTCTTCCTTCTGCCCC GC.

Every Custom TaqMan® SNP Genotyping Assay was formed of a tube including 2 primers used to amplify the required polymorphic sequence in addition to two TaqMan® MGB probes used to distinguish between both alleles, that contain a reporter dye located at the 5' end of each probe, VIC® dye connected to the 5′ end of the Allele G probe, and FAM™ dye connected to the 5′ end of the Allele T probe. The alleles exist in the sample were indicated by the fluorescence signal generated by PCR amplification. Fluorescence of FAM-dye indicates homozygosity for only Allele T, fluorescence of VIC-dye denotes homozygosity for only Allele G, and both FAM- and VIC-dye fluorescence GT indicate heterozygosity (Fig. 1A).

A 20-µl reaction mixture was prepared with 10 µl from TaqMan Genotyping Master Mix, 1.25 µl from 20X SNP assay mixture containing both primers and probes, 3.75 µl of nuclease-free water, and 5 µl of template DNA. We multiplied the amount by the number of samples in each run forming the master mix of the run. Cycling conditions were carried out in 96-well plates: 50ºC for one min (Pre-PCR), thereafter 95 ºC (ten min) and 45 cycles of 95 ºC (fifteen s), 60 ºC for one min (cycling), and 60º C for one min (Post-PCR). Following the amplification of PCR, an endpoint plate was read utilizing the Applied Biosystems Real-Time PCR System. The fluorescence measurements produced during the plate read to plot fluorescence (Rn) values depending on the signals from every well by the Sequence Detection System Software. On the plot each well of the 96-well reaction plate was represented as an individual point.

Statistical analysis of the data

The IBM SPSS software package version 20.0 **(**Armonk, NY: IBM Corp**)** was used for analysis. Qualitative data are characterized using numbers and percentages. Quantitative data are characterized using range (minima and maxima), means, standard deviations and medians. The significance of the resulted values were judged at the 5%.

RESULTS

Patients included 53 males and 27 females (66.25% and 33.75%, respectively). Their ages ranged from 39 to 67 years. Forty healthy persons of matched age and sex were selected as a control group. In **Group I,** 40 cirrhotic patients without HRS were included, with 29 males (72.5%) and 11 females (27.5%), their mean age was 54.20 ± 7.66. **Group II included** 40 cirrhotic patients with HRS type II, with 24 (60%) males and 16 (40%) females, mean age was 53.95 ± 7.15.

Statistical data analysis revealed that age and sex were matched among the study groups (P = 0.094 and 0.404 respectively). Regarding the aetiology of cirrhosis in group I, 32 cases were related to chronic HCV, and 8 cases were related to chronic HBV. In group II, there were 30 and 10 cases related to HCV and HBV, respectively. CBC parameters showed significantly higher Hb ($p<0.001$), WBCs ($p<0.001$) and PLT ($p<0.001$) in the healthy participants compared with both patient groups, while they did not differ between patient groups. There were remarkable significant differences in serum electrolyte levels (Na and K) among and in-between groups, where serum K was higher and Na was significantly lower in HRS patients compared with other groups. Liver function tests, including INR (Fig. 1B), ALT, and total and direct bilirubin, were markedly higher while albumin was lower in both cirrhotic and HRS patients than in controls ($p<0.001$). Liver function tests analysis did not differ between cirrhotic patient with and without HRS (Table 1).

Cirrhotic patients with HRS had a higher MELD score than patients without HRS (p<0.001). Compared with both decompensated cirrhotic patients and healthy controls, HRS patients showed defective renal functions in the form of higher urea and creatinine levels (Fig. 1C) (p<0.001). Plasma nitrite was markedly higher in HRS patients than in cirrhotic ones and controls (p value =0.027 and p value <0.001, respectively); moreover, higher plasma nitrite levels were found in cirrhotic patients than in controls (p=0.024) (Table 1, Fig. 1D).

Regarding clinical manifestations of cirrhosis (jaundice, ascites, encephalopathy, upper GIT bleeding, Child-Pugh score, splenomegaly) that patients presented, no significant variations were detected between patient groups (p>0.05) (Table 1).

The distributions of eNOS G894T genotypes and allele frequencies among studied groups did not sidetrack from Hardy-Weinberg equilibrium and showed markedly higher homozygous mutant TT genotype and GT in both cirrhotic (22.5% and 50% respectively) and HRS (30% and 45% respectively) patients than in healthy controls (10% and 30 % respectively) (p=0.007) (Table 2, Fig. 2A) and excess frequencies of the mutant T allele versus the G allele in both cirrhotic (47.5%) and HRS (52.5%) patients than in healthy controls (25.0%) (p=0.001) (Table 2, Fig. 2B). However, eNOS G894T genotypes (P=0.748) and allele frequencies (p=0.527) were not different among cirrhotic and HRS patients (Table 2).

Table 3 shows the odds ratios (ORs) of eNOS G894T genotypes and alleles among studied groups. We observed that both GT and TT can increase the risk of cirrhosis by (OR 3.636 95% CI [1.32 – 9.99] and by OR 4.909 95% CI [1.24 – 19.46], respectively). Moreover, both GT and TT can increase the risk and predict the occurrence of HRS with cirrhosis (OR 3.60 95% CI [1.27 – 10.17] and OR 7.20 95% CI [1.86 – 27.77], respectively). In accordance with the genotype results, the T allele can also increase the risk of decompensated cirrhosis (OR 2.714 95% CI [1.39 – 5.30]) and HRS (OR 3.316 95% CI [1.70 – 6.48]).

The results of our study showed a distinct relationship between TT genotype and higher serum K (p<0.001) and lower plasma nitrite level (p<0.001) (Fig. 2C) in cirrhotic patients, while other studied parameters were not significant (Table 4). Furthermore, the TT genotype had an obviously lower plasma nitrite level (p<0.001) and hepatic encephalopathy (P= 0.012) compared with other genotypes in HRS patients (Table 5) (Fig. 2D).

Table (1): Demographic, Clinical and laboratory parameters among the studied groups.

	Group I (n=40)	Group II (n=40)	Group III (n=40)	Test	P	Sig.bet.Grps		
						I vs. II	I vs. III	II vs. III
Gender Male	29(72.5%)	24(60%)	24(60%)	$\chi2$=1.81				
Female	11(27.5%)	16(40%)	16(40%)		0.404			
Age (years) x ± SD	54.20 ± 7.66	53.95 ± 7.15	50.70 ± 8.91	F=2.41	0.094			
Hb% (gm/dl) x ± SD	10.12 ± 1.04	10.20 ± 1.12	13.67 ± 0.95	F=152	<0.001*	0.929	<0.001*	<0.001*
WBCs (10^3/mm^3) x ± SD	5.21 ± 1.06	4.82 ± 0.82	6.35 ± 1.06	F=25.96	<0.001*	0.189	<0.001*	<0.001*
PLT(10^3/mm^3) median	88 (51.0 – 230.0)	75.5 (58.0 – 200)	235(170.0 – 300)	K=82.25	<0.001*	0.223	<0.001*	<0.001*
Serum Na mEq/L x ± SD	127.2 ± 8.14	117.80 ± 7.15	139.7 ± 2.87	F=115.5	<0.001*	<0.001*	<0.001*	<0.001*
Serum K mEq/L x ± SD	4.65 ± 0.43	5.13 ± 0.45	4.32 ± 0.47	F=32.88	<0.001*	<0.001*	0.004*	<0.001*
INR x ± SD	1.62 ± 0.25	1.57 ± 0.16	1.01 ± 0.03	F=150.5	<0.001*	0.480	<0.001*	<0.001*
ALT (IU/L) median	47(32.0 – 105)	43(31.0 – 88.0)	22(21.0 – 24.0)	K=80.69	<0.001*	0.377	<0.001*	<0.001*
Albumin (gm/dl) x ± SD	2.48 ± 0.36	2.33 ± 0.36	4.12 ± 0.28	F=346.5	<0.001*	0.118	<0.001*	<0.001*
Total bilirubin (mg/dl) median	2.15 (1.20 – 9.0)	2.9(1.50 – 12.50)	1 (0.80 – 1.0)	K= 81.9	<0.001*	0.169	<0.001*	<0.001*
Direct bilirubin (mg/dl) median	0.9 (0.40 – 3.20)	1.15 (0.50 – 4.50)	0.30 (0.30 – 0.50)	K=82.06	<0.001*	0.085	<0.001*	<0.001*
MELD Score	12.61 ± 3.83	26.11 ± 3.21	-	U=3.0	<0.001			
Urea (mg/dl) x ± SD	27.60 ± 4.78	72.15 ± 14.59	24.0 ± 2.35	F=357.6	<0.001*	<0.001*	0.176	<0.001*
Creatinine (mg/dl) x ± SD	0.81 ± 0.18	3.03 ± 0.56	0.74 ± 0.14	F=559.19	<0.001*	<0.001*	0.642	<0.001*
Nitrite (umol/L)	8.51 ± 8.16	11.26 ± 7.03	4.83 ± 3.40	K=20.41	<0.001*	0.027*	0.024*	<0.001*
Jaundice Yes	22(55%)	28(70%)	-	$\chi2$=1.920	0.166			
No	18(45%)	12 (30%)	-					
Child Pugh scoring	**B** 12(30%	11(27.5%)	-	$\chi2$=0.061	0.805			
	C 28(70%)	29(72.5%)	-					
Encephalopathy			-	$\chi2$=2.581				
Yes	28 (70%)	21(52.5%)			0.108			
No	12(30%)	19 (47.5%)	-					
Upper GIT bleeding				$\chi2$=0.213				
Yes	26(65.0%)	24(60%	-		0.644			
No	14(35.0%)	16 (40%)	-					
Ascites moderate	22(55.0%)	20(50%)	-	$\chi2$=0.201	0.654			
marked	18(45.0%)	20 (50%)	-					
Splenomegaly Yes	33(82.5%)	35(87.5%)	-	$\chi2$=0.392	0.531			
No	7(17.5%)	5 (12.5%)	-					

*: Statistically significant at $p \leq 0.05$, Hb; hemoglobin concentration, WBCs; white blood cells, PLT; platelet count, INR; international normalized ratio, ALT; alanine aminotransferase; MELD; model of end stage liver disease . F for ANOVA test, k for Kruskal Wallis test, $\chi2$ for Chi square test, U; Mann Whitney test

Table (2): eNOS G894T genotypes and allele frequencies in studied groups.

	Group I		Group II		Group III		Test	P	Sig.bet.Grps		
	No.	%	No.	%	No.	%			I vs. II	I vs. III	II vs. III
eNOS G894T	**(n= 40)**		**(n= 40)**		**(n= 40)**						
G/G	11	27.5	10	25.0	24	60.0					
G/T	20	50.0	18	45.0	12	30.0	$\chi2$=14.1	0.007*	0.748	0.013*	0.004*
T/T	9	22.5	12	30.0	4	10.0					
Allele frequency	**(n= 80)**		**(n= 80)**		**(n= 80)**						
G	42	52.5	38	47.5	60	75.0					
T	38	47.5	42	52.5	20	25.0	$\chi2$=14.12	0.001*	0.527	0.003*	<0.001*

*: Statistically significant at $p \leq 0.05$, eNOS; endothelial nitric oxide synthase, $\chi2$ for Chi square test

Table (3): Comparison among the studied groups according to odds ratio.

	Group I		Group II		Group III		OR_1(95% C.I)	OR_2(95% C.I)
	No.	%	No.	%	No.	%		
eNOS G894T	**(n= 40)**		**(n= 40)**		**(n= 40)**			
G/G	11	27.5	10	25.0	24	60.0	1.000	1.000
G/T	20	50.0	18	45.0	12	30.0	3.636[1.32 – 9.99]	3.600[1.27 – 10.7]
T/T	9	22.5	12	30.0	4	10.0	4.909[1.24 – 19.46]	7.200[1.86 – 27.77]
Allele frequency	**(n= 80)**		**(n= 80)**		**(n= 80)**			
G	42	52.5	38	47.5	60	75.0	1.000	1.000
T	38	47.5	42	52.5	20	25.0	2.714[1.39 – 5.30]	3.316[1.70 – 6.48]

OR_1: (Group I vs Group III), OR_2 (Group II vs Group III), eNOS; endothelial nitric oxide synthase

Table (4): Relation between eNOS G894T genotypes and laboratory & clinical parameters in group I (n=40).

	GG (n = 11)	GT (n = 20)	TT (n = 9)	Test	p
ALT(IU/L) median	49 (32.0 – 59.0)	49 (32.0 – 60.0)	(34.0 – 105.0)40	K =0.84	0.655
Albumin (gm/dl) x ± SD	2.49 ± 0.36	2.55 ± 0.37	2.32 ± 0.32	F=1.20	0.312
INR x ± SD	1.67 ± 0.25	1.65 ± 0.22	1.47 ± 0.29	F=2.18	0.126
Total bilirubin (mg/dl) median	2.1 (1.20 – 9.0)	2.9 (1.20 – 8.0)	2 (1.50 – 5.50)	K=2.90	0.234
Direct bilirubin (mg/dl) median	0.6 (0.40 – 2.60)	0.9 (0.40 – 3.20)	1 (0.40 – 2.30)	K=1.80	0.405
Urea (mg/dl) x ± SD	29.82 ± 4.73	26.85 ± 4.80	26.56 ± 4.36	F=1.70	0.195
Creatinine (mg/dl) x ± SD	0.77 ± 0.20	0.79 ± 0.18	0.90 ± 0.17	F=1.45	0.246
Serum Na mEq/L x ± SD	125.64 ± 7.81	127.40 ± 6.78	128.67 ± 11.47	F=0.34	0.712
Serum K mEq/L x ± SD	4.62 ± 0.34	4.47 ± 0.42	5.10 ± 0.16	F=10.07	**<0.001***
MELD Score median	12.7(5.17 – 21.9)	12.1(6.63 – 19.3)	13.5(8.11 – 16.7)	K=0.76	0.681
Nitrite (umol/L) median	11(2.50 – 22.0)	6.25(1.50 – 34.0)	1.50(1.50 – 3.0)	K =18.38	**<0.001***
Child Pough Score	**No (%)**	**No (%)**	**No (%)**		
B	3(27.3%)	8(40%)	1(11.1%)	χ^2=2.37	^{MC}p=0.291
C	8(72.7%)	12(60%)	8(88.9%)		
Encephalopathy	**No (%)**	**No (%)**	**No (%)**		
No	8(72.7%)	14(70%)	6(66.7%)	χ^2=0.22	^{MC}p=1.000
Yes	3(27.3%)	6(30%)	3(33.3%)		

*: Statistically significant at $p \leq 0.05$, MC: Monte Carlo , F: F for ANOVA test , K for Kruskal Wallis test, χ^2 for Chi square test

Table (5): Relation between eNOS G894T genotypes and different parameters in group II (n =40).

	GG (n = 10)	GT (n = 18)	TT (n = 12)	Test	p
ALT(IU/L)	44.10 ± 7.26	44.11 ± 13.77	47.42 ± 11.41	K=1.214	0.545
Albumin (gm/dl)	2.38 ± 0.39	2.26 ± 0.34	2.39 ± 0.38	F=0.593	0.558
INR	1.64 ± 0.18	1.56 ± 0.18	1.53 ± 0.12	F=1.307	0.283
Total bilirubin (mg/dl)	4.13 ± 3.31	3.73 ± 2.61	3.06 ± 1.58	F=0.508	0.776
Direct bilirubin (mg/dl)	1.52 ± 1.18	1.52 ± 0.99	1.17 ± 0.49	F=0.515	0.774
Urea (mg/dl)	73.0 ± 7.24	71.28 ± 14.79	72.75 ± 19.28	F=0.056	0.945
Creatinine (mg/dl) x ± SD	2.99 ± 0.45	3.04 ± 0.56	3.04 ± 0.67	F=0.03	0.968
Serum Na mEq/L x ± SD	125.0 ± 8.54	124.89 ± 8.57	124.50 ± 7.59	F=0.01	0.988
Serum K mEq/L x ± SD	5.07 ± 0.60	5.80 ± 5.17	5.12 ± 0.54	F=0.16	0.847
MELD Score median	26.4 (21.6 – 30.8)	24.9 (20.7 – 31.1)	24.3(22.4 – 31.5)	K =1.21	0.544
Nitrite (umol/L) median	14.7 (9.50 – 28.0)	11.2 (3.50 – 24.0)	3.75(3.50 – 9.50)	K=19.9	**<0.001***
Child Pough Score	**No (%)**	**No (%)**	**No (%)**		
B	3(30%)	6(33.3%)	2(16.7%)	χ^2=1.07	^{MC}p=0.664
C	7(70%)	12(66.7%)	10(83.3%)		
Encephalopathy	**No (%)**	**No (%)**	**No (%)**		
No	7(70%)	12(66.7%)	2(16.7%)	χ^2=8.85	**0.012***
Yes	3(30%)	6(33.3%)	10(83.3%)		

*: Statistically significant at p ≤ 0.05 , MC: Monte Carlo , F: F for ANOVA test , K for Kruskal Wallis test, χ2 for Chi square test

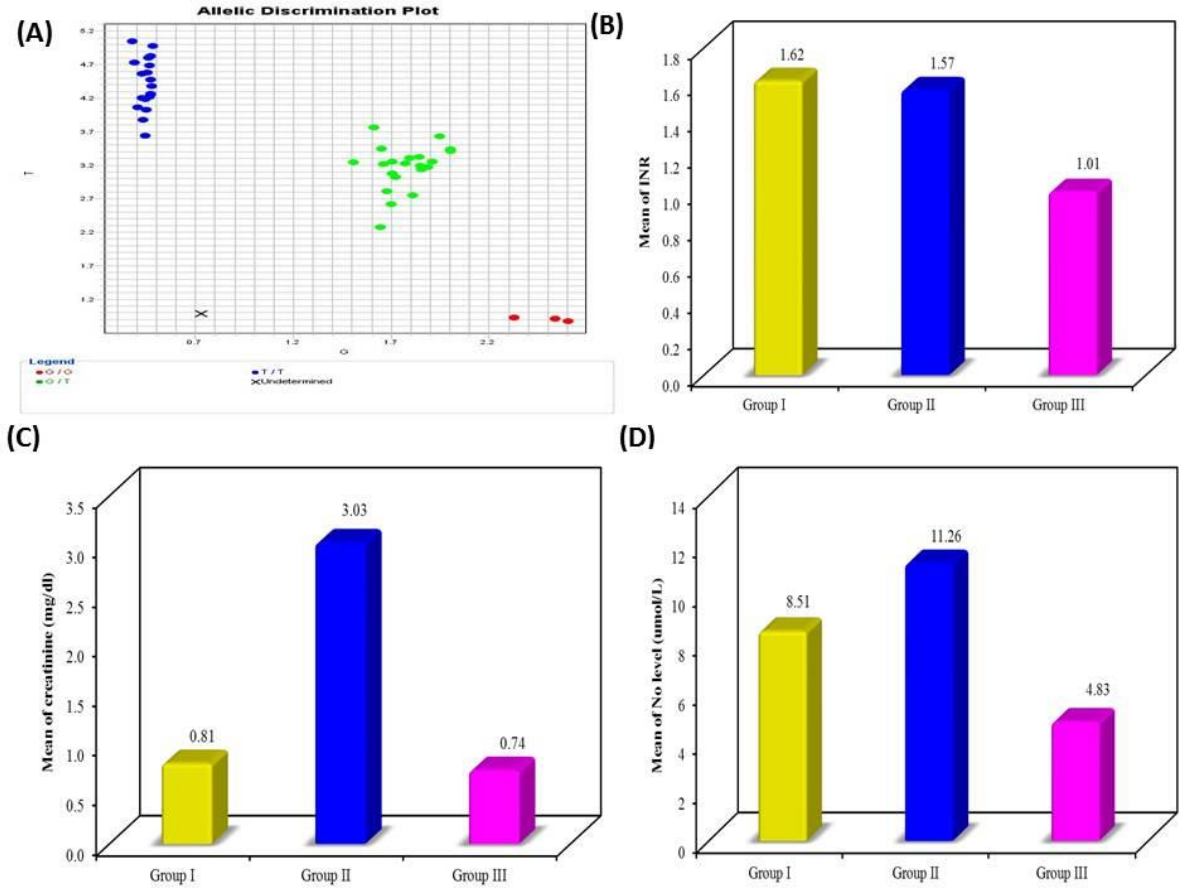

Figure (1): (A): Allele discrimination plot of eNOS G894T, (B): Comparison of INR among groups, (C): Comparison of creatinine among groups (D): Comparison of nitrites among groups.

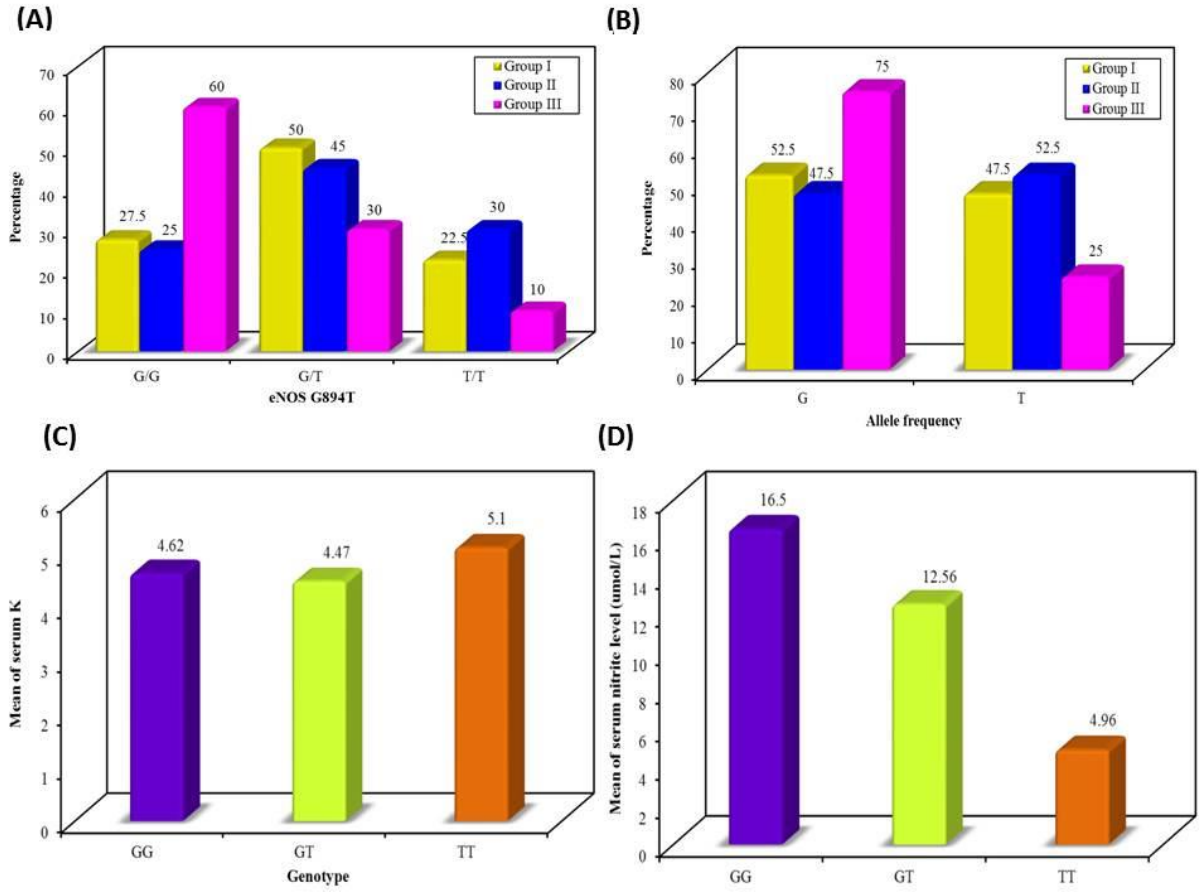

Figure (2): (A): Comparison of eNOS genotypes among groups, (B): Comparison of eNOS alleles among groups. (C): Comparison of serum K among genotypes. (D): Comparison of plasma nitrites among genotypes.

DISCUSSION

Renal impairment is widespread in decompensated cirrhotic patients and is linked with a poor prognosis, with life survival ranging from weeks to months. Hepatorenal syndrome is considered a functional form of acute kidney injury (AKI) described by renal vasoconstriction **[13]**. In the setting of cirrhosis, HRS is linked with the worst mortality amongst the different causes of AKI **[14]**.

The pathogenic pathway implied in HRS development of is not fully understood. HRS results from abnormal haemodynamics, which lead to systemic and splanchnic vasodilatation alongside renal vasoconstriction **[15]**. Distinctive molecules that are accountable for the splanchnic vasodilation in liver cirrhosis have been proposed as possible mediators, such as nitric oxide (NO) **[16, 17]**.

Four main isoforms of nitric oxide synthase (NOS) exist: the endothelial (eNOS), the neuronal (nNOS), the inducible (iNOS), and the mitochondrial isoform [8]. They are produced by different genes, with different localizations, regulations, catalytic properties and inhibitor sensitivities. NOS-3, also known as eNOS, is expressed in vascular endothelial cells **[18]**. It can inhibit aggregation of platelets, adhesion of leucocytes and vascular inflammation, it organize the proliferation vascular smooth muscle, and activate angiogenesis **[19]**.

It is known that the prevalence of HRS in liver cirrhosis varies among patients, and cirrhotic patients with similar liver function levels do not all develop HRS as a complication; thus, it could be assumed that genetic variations may comprise a hazard for development of hepatorenal syndrome.

The results of current study explored a significant link of the eNOS G894T polymorphism with decompensated cirrhotic patients with and without HRS compared with healthy subjects, with a predominance of the TT

genotype and the T allele in patients; thus, we assumed that the T allele increases the risk of cirrhosis (OR) 2.714-fold with 95% CI [1.39 – 5.30], while in cirrhotic patients with HRS the allele is present 3.316-fold more often with 95% CI [1.70 – 6.48]. These findings were supported by Seckin et al. **[20]**, who reported that the eNOS G894T homozygote (TT) and heterozygote (GT) mutants were significantly connected with a higher risk of development HRS. Furthermore, another case-control study has reported that the eNOS G894 T allele was deemed an independent hazard for portal hypertension in cirrhosis due to HBV infection **[21]** that, in turn, is believed the initial event in the pathogenesis of HRS.

However, Yildirim et al. **[22]** found no correlation between eNOS G894T polymorphism and the development of ascites in cirrhosis. They reported that the frequencies of TT, GT and GG genotypes did not differ ($p>0.05$) among cirrhotic patients with ascites, stable cirrhosis patients and healthy subjects.

Several previous studies have detailed significant associations of eNOS G894T with the danger of cardiovascular diseases & diabetes mellitus type 2 El-lebedy et al. **[23]**, diabetic nephropathy Dellamea et al. **[24]**, susceptibility with onset of end stage renal disease (ESRD) Yun et al. **[25]** and chronic kidney disease (CKD) Chand et al. **[26]**. The previous studies documented that polymorphism in gene G894T is linked with reduced activity of NOS enzyme and so the bioavailability of NO. The Glu298Asp variation in eNOS gene is quite widespread; with prevalence of the T allele (35–40%) in Caucasians and lower prevalence in Asians, African and Americans **[27]**.

Interestingly, we observed higher levels of plasma nitrites in cirrhotic patients with and without HRS than healthy subjects. Moreover, these levels were related to eNOS genotypes, where TT, GT genotypes had significantly lower levels of plasma nitrites than the GG genotype. Based on these finding, we can propose that G894T polymorphisms lead to lowering in NO production. This could imply that the presence of the mutant allele T is correlated with a lower level of enzyme activity and, consequently, a lower intrarenal NO level, which can lead to renal vasoconstriction. Previous studies support these findings **[20, 21]**. It was reported in randomized placebo-controlled that renal plasma flow diminished as a consequence of inhibition of NO, and that patients with cirrhosis NO is a vital renal vasodilator and natriuretic **[28]**.

Patients with liver cirrhosis and ascites are documented to have higher NO (nitrites) plasma levels than those with compensated cirrhosis and normal individuals. In addition, high serum NO levels correlate with high RAAS activity and antidiuretic hormone levels (ADH) **[29,30]**. Battista et al. found that NO levels in the portal venous plasma are higher than in peripheral venous plasma, suggesting increased production of NO in splanchnic areas **[31]**. Kone has reported that NO is a paracrine mediator that is produced and released in individual cells and penetrates the neighbouring cells and exerts its effects locally and transiently **[32]**.

In cirrhosis, clearance of amino acids by the liver, including L-arginine (the precursor of NO), is decreased, which may result in increased plasma NO due to higher levels of substrate **[33]**. In addition, plasma NO levels were increased in cirrhotic patients with renal imparment with highest levels observed in HRS patients together with increased levels of L-arginine in these patients **[34]**. These finding could explain the cause of elevated plasma NO levels in cirrhotic and HRS patients. This obviously marked increase in NO in HRS may be also likewise attributed to other forms of NOS, specifically iNOS. Unlike eNOS, iNOS is more widely expressed, as in vascular smooth muscle cells, macrophages, Kupffer cells and hepatic stellate cells (HSCs) following stimulation by lipopolysaccharide or inflammatory cytokines **[35]**. Lipopolysaccharide (LPS) detoxification is constrained in cirrhotic liver with portal hypertension, thereby increasing plasma levels of LPS. In the splanchnic circulation resident macrophages react to increased LPS with the generation of proinflammatory cytokines, like tumour necrosis factor-α (TNF-α) **[36]**, which induces iNOS in the extrahepatic vasculature **[37, 38]**.

In the present study we noted that TT genotype was obviously associated with hepatic encephalopathy. Multifactorial mechanisms are accountable for the occurrence and pathogenesis of hepatic encephalopathy in cirrhotic patients with portal hypertension. Although hyperammonaemia is pivotal, previous studies reported that abnormalities in cerebral blood flow also play a role in its pathophysiology **[39]**. These data could support our results regarding

the association of HE with TT genotype which at the same time associated with low nitrite level.

CONCLUSION

Striking results of this study are the marked association of the TT and GT genotypes and the T allele of eNOS G894T with the risk of decompensated cirrhosis and HRS. Moreover, higher nitrite levels were observed in cirrhotic patients and were specifically marked in HRS. In this way, we can support the genotyping of eNOS G894T in patients with liver cirrhosis and the frequent estimation of nitrites for the early prediction and rapid management of HRS.

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Ethical consideration:

Our study was carried out in conformity with the Declaration of Helsinki. An informed consent was provided by all participants, and, the study protocol was notarized by the Ethics Committee of the Faculty of Medicine, Menoufia University.

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