The interaction of sphingosine1 phosphate, adiponectin, and sex Hormones with sex disparity among patients with hepatocellular carcinoma

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Abstract:

Background: Previous studies showed contradictory data regarding sphingosine1-phosphate (S1P) levels in hepatocellular carcinoma (HCC) of HBV etiology and no data reported in HCV- related HCC. Role of S1P in liver fibrosis differs whether of HCV or HBV etiology. Experimental studies described interplay of S1P, adiponectin and sex hormones.
Aim: Studying sex disparity ad interplay of theses parameters in pathogenesis, diagnosis, clinic –morphological and staging of HCC.
Methods: Measurement of SIP, adiponectin and testosterone (T), estradiol (E) and sex hormone binding globulin (SHBG) among HCV–HCC group in comparing with HCV-related cirrhotic and healthy groups with their sex stratified subgroups.
Results: S1P was significantly higher in HCC patients with cut off value ≥ 113ng/l as screening test (sensitivity 95%, specificity 56%) for diagnosis of HCC diagnosis compared to sex -matched cirrhotic and healthy subjects. Compared to sex matched cirrhotic subgroups, male-HCC had significantly higher SHBG and lower adiponectin and estradiol while an opposite profile was observed in female-HCC. Female-HCC had significantly higher SIP and adiponectin than male-HCC. S1P level was positively correlated with adiponectin & testosterone and negatively correlated with E/T ratio in male and female HCC subgroups, adiponectin was negatively correlated with testosterone and SHBG and positively correlated with estradiol and E/T ratio among entire HCC group but reverse associations were observed in female-HCC. In females, large tumor size and higher T-class TNM staging were associated with higher adiponectin and testosterone and lower E/T ratio, and multiplicity was liked to higher estradiol. In males, an association between higher testosterone and higher T-class TNM staging was observed.
Conclusion: S1P is screening test for diagnosis of HCV-HCC which display sex disparity of association and interaction of S1P, adiponectin and, sex hormones in clinic-morphological features and staging

Keywords: hepatocellular carcinoma, gender disparity, sphingosine 1 phosphate, adiponectin, sex hormones and sex hormone binding globulin, bound and free testosterone.

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Background:

Hepatocellular carcinoma (HCC) is more frequently observed in males than in females with more aggressive behavior in males [1]. This sex disparity is multifactorial, hepatitis C viral infection (HCV), sex hormones and adiponectin play a contributing role [2,3]. The role of adiponectin in HCC is still unclear. It has diverse actions, and both protective and oncogenic actions have been previously reported [4,5]. Based on studies performed in cell lines, animal models, and epidemiological studies, testosterone may decrease adiponectin levels while estrogen may stimulate its release [3,6]. While many studies have supported the protective role of estradiol, others have referred to its oncogenic role in HCC. Enhanced aromatization with conversion of androgen to estrogen was described in HCC tissue and can be assessed by calculating the estradiol to testosterone (E/T) ratio [7,8]. Total testosterone (T) circulates in three forms, ~60% of which is bound to sex hormone-binding globulin (SHBG), which is synthetized by the liver. Both of the fractions bound to albumin (40-50%) and free testosterone (1%) represent active testosterone forms, and they are estimated by the bioavailable testosterone (BAT) and free androgen index (FAI), respectively [9]. Among HCV-cirrhotic males, FAI but not T was related to fibrosis and steatosis [10]. Despite extensive molecular studies, few clinical studies, with conflicting results, have described the role of testosterone (usually total and seldom free forms), estradiol, E/T and SHBG in males with HCC of various etiologies, and these studies were rarely stratified by sex or HCV etiology [11-16]

In addition, adiponectin receptors have intrinsic ceramidase activity that is increased 20-fold by adiponectin binding, particularly adiponectin receptor 2, which increases sphingosine 1-phosphate (S1P) production [17]. S1P is produced by the deacylation of ceramide to sphingosine by ceramidase, followed by phosphorylation by sphingosine kinase (SphK). S1P is a pleotropic molecule that enhances cell proliferation and mobility, immune cell recruitment, angiogenesis, and metastasis, and it is a diagnostic and prognostic biomarker for various cancers [18].

Animal and molecular studies have suggested the roles of SphK and S1P signaling in HCC, and these roles are involved in cell proliferation, epithelial mesenchymal transition, progression, angiogenesis, invasion and metastasis [19]. Sparse and inconsistent data exist about S1P tissue overexpression in HCC compared to normal adjacent tissues [20,21]. Alterations of its serum level were measured by high performance liquid chromatography-mass spectrometry in a heterogeneous population of Chinese HCC patients of mainly hepatitis B virus (HBV) etiology as compared to healthy subjects or non-comparable cirrhotic patients [22-24]. However, sphingosine is a biomarker of chronicity and fibrosis in HCV but not HBV [25,26]. The effects of sex and menopause on S1P level and its regulation by sex hormones are uncertain [27,28].

The purpose of the current study was to evaluate the serum levels of S1P, adiponectin, SHBG and sex hormones, including total testosterone, calculated BAT and FAI, estradiol and E/T ratio, in male and female subgroups of HCV-related HCC patients in comparison to sex-matched corresponding cirrhotic and healthy subjects. This study aimed to verify the possible interactions between the studied biomarkers and their associations with clinical and morphological data stratified by sex in HCC. This aspect not been studied previously.

Subjects and Methods:

In the current cross-sectional case-control study, 80 HCV-cirrhotic patients with HCC (males/females [m/f]: (40/40) formed the HCC group and aged 61.4±10 years old, 60 HCV-cirrhotic patients (m/f: 30/30) formed the cirrhotic group and aged 60±7.6 years old, and 50 healthy control subjects who aged 60.7±6.6 years old (m/f: 25/25) were included in our analysis. The three groups were age- and sex-matched, and each group was further subdivided into male and female subgroups. The study was performed in accordance with the Declaration of Helsinki and was permitted by the local ethics committee during the period from February 2019 to November 2020 at the Department of Internal Medicine, Minia University Hospital, Egypt. All participants signed written informed consent before inclusion in the study.

The diagnosis of chronic HCV infection was based on the presence of anti-HCV antibodies for ≥6 months and the detection of HCV RNA. The diagnosis of cirrhosis was based on abdominal ultrasound and laboratory data. The severity of liver dysfunction was assessed by the Child-Pugh and model end stage liver disease (MELD) scores [29]. The diagnosis of HCC was based on the European Association for the Study of the Liver (EASL) guidelines of 2012 and visualized by dynamic imaging CT or MRI [30]. Staging of HCC was based on The Barcelona Clinic Liver Cancer (BCLC) and tumor node metastasis (TNM) system according to The American Joint Committee on Cancer (AJCC) [31,32].

All participants underwent conventional assessments, including a thorough history, physical examination, and abdominal ultrasonography. They underwent routine laboratory tests. All females were menopausal.

Exclusion criteria were a history of malignancy elsewhere, diabetes mellitus, endocrine disorders, any organ failure, recent infection, fertile females, alcohol intake, any local or systemic treatment for HCC, other causes of cirrhosis, or hormonal treatment.

Biochemical assays:

After an 8-hour fast, venous blood samples were taken at 9 a.m. An EDTA -containing tube was used for the complete blood count. Prepared serum was used to assess liver function (aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin), viral infection status, renal function and fasting blood sugar. Citrated blood samples were used to separate plasma for prothrombin time and calculation of the international normalized ratio (INR). Routine biochemical analyses were done by auto-analyzer Kone-lab (2011). The Child-Pugh and MELD scores were calculated. Serum aliquots were stored at -80 °C for measurements of S1P, adiponectin, testosterone, estradiol and SHBG, which were quantitatively determined by ELISA kits supplied by Bioassay Technology Laboratory Biotech Co, Shanghai, China, according to the manufacturer's instructions. The principle for assessment of adiponectin, S1P, estradiol, and SHBG: the plate has been pre-coated with Human specific antibody. The specific measured parameter present in the sample is added and binds to antibodies coated on the wells, then biotinylated Human specific antibody and Streptavidin-HRP are added. After incubation, unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human specific measured parameter. The results were calculated by constructed a standard curve and drawing a best fit curve through the points on the graph. Testosterone assay is based on the principle of competitive binding between Testosterone in the test specimen and Testosterone-HRP conjugate for a constant amount of rabbit anti- Testosterone. BAT, FAI and E/T were calculated [8,9].

Statistical Analysis:

SPSS version 20 was used for data analysis. Normally distributed quantitative data are expressed as the mean ± SD and were compared by ANOVA test for comparison between more than two groups that was followed by post hoc Tukey test for comparison

between each two groups. Non-normally distributed quantitative data are expressed as the median and interquartile range (25% and 75% IQ) and were compared using the Kruskal Wallis test for comparison between more than two groups that was followed by the Mann–Whitney test for comparison between two groups. Comparing male to female subgroups in disease respective subgroups was done using the independent sample T test and the Mann–Whitney test for normally and non- normally distributed data respectively test. Qualitative variables are presented as percentages and were compared by the chi-square test. Correlation analysis was performed using the Spearman rank test. A receiver operating characteristic (ROC) curve was used to determine the diagnostic performance of S1P for HCC. Specificity, sensitivity, and cut-off values were detected via areas under the ROC curve (AUCs). $P < 0.05$ was considered to be statistically significant.

Results:

Clinical and laboratory characteristics of the studied groups and subgroups:

Although the HCC group was matched to the cirrhotic group with regard to the se

verity of liver disease (Child-Pough or MELD score), the former had significantly higher liver enzyme levels, leucocyte count, S1P levels and adiponectin levels. These two groups had significantly higher S1P levels and significantly lower adiponectin and estradiol levels than healthy subjects. The detailed laboratory characteristics and the comparisons between the three groups and each two groups were shown in Table 1.

In the next step, we apportioned the studied HCC, cirrhotic and healthy subject groups in the corresponding male and female subgroups, and the laboratory data are shown in Table (2). The male and female subgroups were similar in age, liver markers and severity in the HCC group and in the cirrhotic group. However, the ALT levels were significantly higher in the female HCC subgroup and significantly lower in the cirrhotic female subgroup compared to the male subgroups of their respective disease.

Comparing male subgroups, there were statistically significant differences between HCC patients, cirrhotic patients and healthy subjects groups with regard to Hb, AST, ALT, albumin, total bilirubin level, S1P, adiponectin, testosterone forms (total, BAT and FAI) SHBG and estradiol levels, and WBC and platelet count, and INR, and E/T ratio ($p<0.001$ for all except p values were 0.009 for HB, 0.03 for platelet count, estradiol level and E/T ratio, and 0.003 for ALT. Also, among the male subgroups, HCC patients had significantly higher S1P and SHBG levels and significantly lower adiponectin levels, estradiol levels and E/T ratios ($p =0.03$, <0.001, 0.03, 0.002, and 0.02, respectively) compared to cirrhotic patients. They had comparable testosterone forms (total, BAT and FAI). Moreover, comparing female subgroups, there were statistically significant differences between the HCC patients, cirrhotic patients and healthy subjects with regard to Hb, AST, ALT, albumin, bilirubin, S1P, adiponectin, SHBG, BAT, FAI, and estradiol levels, platelet count, INR, and E/T ratio ($p<0.001$ for all except p values were 0.008 for HB, 0.002 for SHBG and FAI, and 0.028 for BAT). Additionally, the female-HCC subgroup had significantly higher S1P levels, adiponectin levels, estradiol levels and E/T ratios and significantly lower total testosterone and SHBG levels ($p=0.002$, <0.001, <0.001, <0.001, 0.04, and 0.001, respectively) compared to the cirrhotic female subgroup. They had comparable levels of BAT and FAI.

In comparison to sex-matched healthy subjects, both the HCC and cirrhotic subgroups had significantly higher S1P levels and significantly lower adiponectin levels ($p <0.001$ for all). In addition, the HCC and cirrhotic male subgroups had significantly lower testosterone forms (total, BAT, FAI) and estradiol levels ($p<0.001$ for all) than healthy male subjects. Among the female subgroups, the FAI was significantly higher, and estradiol was lower in both HCC ($p=0.01$, 0.03) and cirrhotic patients ($p=0.001$, <0.001) compared to healthy subjects. Furthermore, in the female subgroups, the SHBG levels were lower in HCC patients, and the E/T ratio was lower in cirrhotic patients ($p=0.01$, <0.001) compared to healthy subjects.

When comparing males and females in their corresponding disease subgroups, the serum levels of S1P and adiponectin were lower among male HCC subgroup (0.001, <0.001). Additionally, testosterone, FAI, BAT, and SHBG levels were higher, and estradiol levels and the E/T ratio were lower in both the cirrhotic and HCC male subgroups ($p<0.001$ for all).

Morphological features and staging of HCC

We found tumors > 5 cm in diameter in 62.5% of HCC patients (50/80, m/f: 24/26). There were multiple tumor lesions in 53% (42/80, m/f: 20/22) of HCC patients, and portal vein thrombosis (PVT) in 27.5% (22/80, m/f: 12/10) of HCC patients. Neither lymph node metastasis nor distant metastasis was detected. The number of cases and m/f ratio according to the T-class (T1, T2 and T3) and TNM staging (I/II/IIIa) were similar (25 cases and 13/12; 32 cases and 16/16; and 23 cases and 11/12, respectively). According to the BCLC stages A, B, C, and D, these values were 16 cases and 8/8, 20 cases and 8/12, 16 cases and 10/6, and 28 cases and 14/14, respectively. Male and female subgroups had comparable morphological features and staging.

The correlation between sphingosine 1 phosphate, adiponectin and sex hormone levels in the HCC group

First, we studied the relationships among S1P, adiponectin and sex hormones in the entire HCC group and the sex-stratified subgroups. S1P was positively correlated with adiponectin and estradiol in the HCC group ($r= 0.56$, $p<0.001$; $r=0.25$, $p=0.02$) (Figures 1, 2). Among the male and female HCC subgroups (Table 3), S1P was positively correlated with adiponectin ($p=0.02$, 0.002), total testosterone ($p=0.04$, $p<0.001$), FAI ($p=0.01$, 0.01), and BAT ($p=0.03$, $p=0.001$), and it was negatively correlated with the E/T ratio ($p=0.02$, $p=0.001$). S1P was positively correlated with SHBG only in female HCC patients ($r=0.56$, $p<0.001$). Serum

adiponectin was negatively correlated with testosterone, SHBG, BAT and FAI and positively correlated with estradiol and the E/T ratio in the HCC group (r= -0.46, r= -0.59, r= -0.43, r= -0.44, r= 0.59, and r= 0.50, respectively, p<0.001 for all). In contrast, adiponectin was positively correlated with testosterone, BAT and FAI and negatively correlated with the E/T ratio in female HCC patients, with p<0.001 for all. S1P was positively correlated with age in male HCC patients (r=0.42, p=0.007) and positively correlated with HB levels in the male and female subgroups (r=0.46, p=0.003; r=0.35, p=0.02). However, it did not show any correlation with liver enzymes or severity of liver disease in the HCC subgroups.

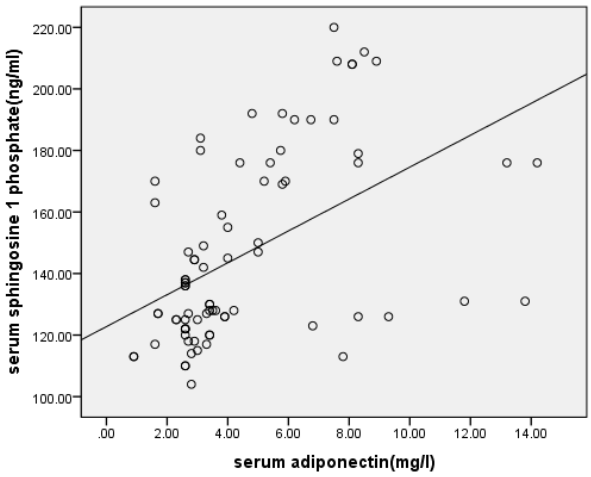

Figure (1): Correlation of serum sphingosine 1-phosphate with serum adiponectin in all HCC patients ((r= 0.56, p<0.001)

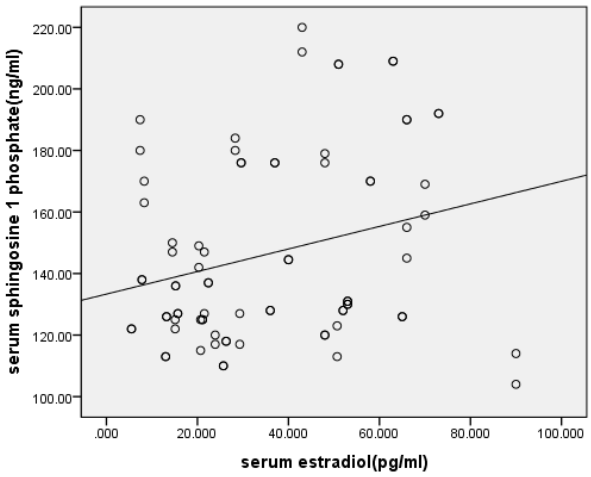

Figure (2): Correlation of serum sphingosine 1-phosphate with Serum Estradiol in all HCC patients (r=0.25, p=0.02)

Second, the associations between S1P, adiponectin and sex hormones and the tumor morphological features and TNM staging were analyzed (Table 4). In male HCC patients, the presence of PVT was associated with significantly lower levels of SHBG, and those with TNM stage II had higher total testosterone and BAT levels than those with stage I. Female HCC patients with large tumor size and advanced TNM staging had significantly higher adiponectin and testosterone levels (total, BAT, FAI) and a significantly lower E/T ratio compared stage IIIa to stage II. Additionally, the presence of multiple lesions and PVT were associated with higher estradiol and BAT levels, respectively. In comparison to TNM stage I, TNM stage II was associated with significantly higher estradiol levels while stage IIIa was associated with higher S1P, total testosterone and estradiol levels and a significantly lower E/T ratio in female HCC patients.

The diagnostic performance of S1P for the diagnosis of HCC was assessed by ROC analysis in the HCC patient group, male HCC patient subgroup, and female HCC patient subgroup. The AUCs were 0.79, 0.78, and 0.81, respectively, and the 95% confidence intervals (CIs) were 0.70–0.88, 0.65–0.92 and 0.69–0.93, respectively, (P < 0.001 for all) (Fig. 3). When the cutoff value was set at ≥ 113 ng/l as a screening test, the sensitivity was 95% for the HCC group and subgroups while the specificity was 56%, 52% and 60% in the HCC patient group, female HCC patient subgroup and male HCC patient subgroup, respectively. When the cutoff value was set at ≥ 125 ng/l as a diagnostic test, the sensitivity and specificity were 72% and 70% in the HCC patient group, 60% and 72% in the male HCC patient subgroup, and 85% and 64% in the female HCC patient subgroup, respectively.

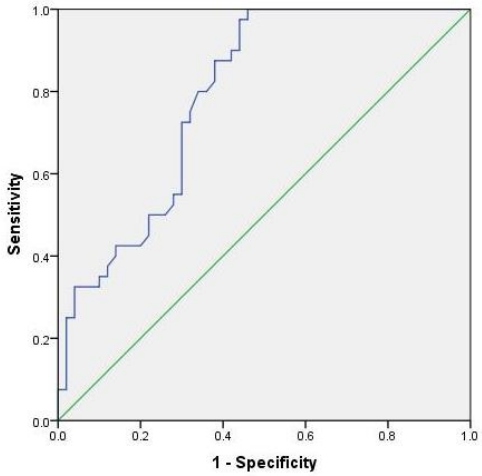

Figure (3): Roc curve analysis of sphingosine 1 phosphate for diagnosis of HCC among cirrhotic patients and healthy subjects. The AUCs were was 0.79, 95% CI (0.70–0.88) with P < 0.001. At cut-off value ≥ 113ng/l as a screening test, the sensitivity and specificity were 95% and 56% among all HCC patients.

Table (1): Clinical and laboratory Data of the Studied Groups.

Variables	**HCC Group (n=80)**	**Cirrhotic Group (n=60)**	**Healthy Group (n=50)**	**P value between the Three groups**
Age (years) [a]	61.4±10	60±7.6	60.7±6.6	0.9
Male gender (%)	40(50%)	30(50%)	25(50%)	1
Hemoglobin% (gm/dl) [a]	11.5± 2.3**	11.9±2.7*	13.2±1.2	**0.003**
White blood cells count(x103) [b]	6.1(4.5- 8.3) ‡‡	5.5(4.4-6.3) **	6.3(5.7 -7.7)	**0.003**
Platelet count (x103) [b]	153(131-225) **	163(143-212) **	219(179-271)	**<0.001**
Alanine aminotransferase (U/l) [b]	53(33-65) ‡‡‡***	34(21-49)	30(23-33)	**<0.001**
Aspartate aminotransferase (U/l) [b]	49 (33-78) ‡‡‡***	36(26-53.2) ***	25(23-33)	**<0.001**
Albumin (gm/dl) [a]	3.3±0.67***	3.3±0.8***	4.05±0.43	**<0.001**
Total bilirubin (mg/dl) [b]	1.3(0.9-1.9) ***	1.2(0.8-1.4) ***	0.56(0.4-0.8)	**<0.001**
International normalized ratio [a]	1.3±0.25***	1.4±0.32***	1.1±0.2	**<0.001**
Random blood sugar (mg/dl) [a]	130±17	128± 18	124±15	0.16
Creatinine (mg/dl) [a]	0.98±0.3	0.95±0.3	.93±0.3	0.58
Child class A/B/C n (%)	24/28/28 30/35/35	22/18 /20 36.7/ 30/ 33.3	------	------
Child score [a]	8.6±2.6	8 ±2.5	------	------
MELD score [a]	12.2±3.9	12±3.4	------	------
Sphingosine-1 phosphate (ng/l) a	146 ± 31‡‡‡***	129±22.4***	34.1.8±13.9	**<0.001**
Adiponectin (mg/l) [b]	3.4(2.6-6.5) ‡ ***	3(2.4-4.1) ***	12.1(8.5-15.8)	**<0.001**
Total testosterone (ng/ml) [b]	1.5(0.51-3.8)	1.2(0.66-3.4)	3.9(0.35-0.89)	0.16
Sex hormone binding globulin (mmol/l) [b]	15.2(10.2-29.6)	14.3(10.6-19.8) *	19.7(14.3-24.1)	0.23
Bioavailable testosterone (ng/ml) [b]	0.63(0.29-1.8)	0.73(0.36-1.7)	2.5(0.2-5.8)	0.19
Free androgen index (ng/ml) [b]	0.04(0.02-0.09)	0.04(0.02-0.09)	0.12(0.01-0.26)	0.48
Estradiol (pg/ml) [b]	29.4(16.8-22.7) ***	27.8(20.5-35) ***	57(49.5-66.7)	0.66
Estradiol to testosterone ratio $x10^{-3}$ [b]	17.3(5-10)	25.9(6.1-45.6)	31.9(5.6-47)	0.66

HCC= Hepatocellular carcinoma; MELD: Model of End stage Liver Disease; a= normally distributed quantitative data are expressed as mean ± standard deviation and compared using ANOVA test between the three groups followed by post hoc Tukey test between each two groups. Sex and child class are expressed as number (percentage) and compared by Chi square test

b= not- normally distributed quantitative data are expressed as median and interquartile (25%-75%) and compared using Kruskal Wallis between the three groups and was followed by Mann Whitney U test between each two groups. $P < 0.05$ is considered to be statistically significant. difference when HCC patients compared to cirrhotic patients $p <0.05$= ‡, $p<0.01$=‡‡, $p<0.001$=‡‡‡, Significant difference when compared to healthy subjects $p<0.05$= *, $p<0.01$=**, $p<0.001$=***

Table (2): Clinical and laboratory Data of the Studied Subgroups

Variables	Male subgroups			Female subgroups		
	HCC (n=40)	Cirrhotic (n=30)	Healthy (n=25)	HCC (n=40)	Cirrhotic (n=30)	Healthy (n=25)
Age(years) [a]	62. 8 ± 10.5	60.1±7.5	61.9±7.3	59.9 ±10.9	60±7.7	59.5±8.9
Hb (gm/dl) [a]	12.7±2.2**£$	13.3±2.4£$$	14.2±1.1£$$	10.7±2.2*	10.4±2.3**	12.1±0.4
WBCs(x103) [b]	6.7(5.4-8) ‡‡‡	5.5(4.5-6.1) **	6.9(5.6-7.9)	5.9(5.2-10.3)	5.6(4.5-7.7)	6.2(4.9-7.8)
Plat ($x10^3$)[b]	162(141-240)	160(140-179) **	207(173-243)	147(108-210) **	166(150-247) **	237(176-290)
ALT (U/l l)[b]	48(30-60) **£	39(24-75) *£$	31(26-35)	53(38-70) ‡‡‡***	29(16-35)	29(22-32)
AST(U/l)[b]	49(39-68) ‡ ***	73(29-58) **	26.5(20-38)	49(29-88) ‡‡ ***	33(20-53)	24(22.7-31)
Alb (gm/dl)[a]	3.4±0.7**	3.4±0.8**	4± 0.4	3.2±0.6***	3.2±0.9***	4.1±0.5
T bil (mg/dl)b	1.5(0.9-2.3)***	1.1(0.8-1.8) ***	0.58(0.4-0.7)	1.3(0.9-1.9)***	1.1(0.9-1.4) ***	0.5(0.3-0.7)
INR [a]	1.3 ± 0.2**	1.4 ± 0.3***	1.1 ± 0.2	1.3 ±0.3***	1.4 ±0.3***	1 ±0.1
RBS (mg/dl) [a]	132±16	127±18	127±15	128±18	128±18	121±15
Crea (mg/dl) [a]	1 ± 0.31	0.97 ±0.21	0.91 ±0.21	0.96 ±0.27	0.94 ±0.31	0.95 ±0.25
Child class n A/B/C %	12/14/14 30/35/35	11/9/10 36.7/33.3/30	-----	12/14/14 30/35/35	10/10/10 33.3/33.3/33.3	----
Child score [a]	9(6-11)	8(5-11)	---------	8(6-11)	8(6-11)	---------
MELD score [a]	12.5 (9-15)	12(10-18)	---------	13(8-14)	11(9-17)	---------
S1P(ng/l) [a]	134.3±22.1‡***£$	124.8±19.6***	36.7±15.7	157.5±33.7‡‡***	134.8±24.7***	31.5±12.1
Adipon (mg/l) [b]	2.6(2.4-3.2) ‡ ***£$$	3.3(2.5-4.1) ***	5.1(4.5-7) £$$	5.6(3.5-8.3)‡‡‡***	2.7(2.4-4.1) ***	12.2(9.8-14.5)
Test(ng/ml) [b]	3.8(2.9-5.1) *** £$$	3.4(1.4-5.9) **£$$	8.7(8.2-10.2) £$	0.51(0.34-0.82) ‡	0.66(0.49- 1.7)	0.48(0.17-0.82)
SHBG (nmol/l)[b]	29.4(21.8-32.8)‡‡‡**£$$	19.8(14.7-24.8) £$$	23.1(19.8-24.7) £$$	10.65(8.7-12.3) ‡‡*	12.7(9.9-14.2)	15.2(8.6-20.4)
BAT (ng/ml) [b]	1.7(1.12- 2.6) *** £$$	1.6(0.97-3.8) *** £$$	5.8(4.8-6.3) £$$	0.3(0.7-0.55)	0.36(0.31-0.7) *	0.26(0.9-0.5)
FAI (ng/ml) [b]	0.09(0.06-0.13) **£$$	0.09(0.05-0.19) *** £$$	0.25(0.22-0.3) £$$	0.02(0.01-0.03) *	0.02(0.01-0.03) **	0.01(0.004-0.02)
Estr (pg/ml) [b]	18(13-23) ‡ ‡***£$$	24(17.9-27.8) ***£$$	53(45.5-65)	52(45-64) ‡‡‡*	33(28.1-42.7) ***	63(52-67)
E/T $x10^{-3}$ [b]	5.3(2.3-7.8) ‡ £$$	6.6(4.35-11.2) * £$$	5.6(2.4-6.7) £$$	100(66-153) ‡‡‡	45(32.9-77.4) ***	185(47-393)

HCC= Hepatocellular carcinoma; HB= Hemoglobin; WBCs=White blood cells; Plat=platelet ,ALT=Alanine aminotransferase; AST= Aspartate aminotransferase; ALB= Albumin; T bil = total bilirubin; INR= International normalized ratio; RBS=Random blood sugar; crea = creatinine ; MELD: Model of End stage Liver Disease, S1P= Sphingosine 1- phosphate ;adipon= Adiponectin; Test= Testosterone; SHBG=Sex hormone binding globulin; BAT= Bioavailable testosterone ; FAI = Free androgen index; Estr = Estradiol ; E/T = Estradiol to testosterone ratio. .a= normally distributed quantitative data are expressed as mean ± standard deviation and compared using ANOVA test between the three subgroups of the same sex followed by post hoc Tukey test between each two groups and Independent sample T test when compared male to female of disease respective subgroups.b= not- normally distributed quantitative data are expressed as median and interquartile (25%-75%) and compared using Kruskal Wallis between the three subgroups of the same sex and was followed by Mann Whitney U test between each two groups. Mann Whitney U test was used to compare males to females of the disease respective subgroups.$P < 0.05$ is considered to be statistically significant.

. Child class is expressed as number (percentage) and compared by Chi square test $P < 0.05$ is considered to be statistically significant. Statistical significance vs. sex- respective matched cirrhotic patients ‡=P <0.05, ‡‡=P<0.01, ‡‡‡= p<0.001; Statistical significance vs. sex - respective matched healthy subjects *=P<0.05, **=p<0.01, ***= p<0.001; Statistical significance vs. corresponding group of females £=p<0.05, ££= p<0.01, £££= p<0.001

Table (3): Spearman's correlation coefficients of SIP and adiponectin in male and female HCC subgroups

Correlation of SIP and adiponectin with sex hormone							
variables	Adiponectin (mg/l)	Total T (ng/ml)	SHBG (nmol/l)	BAT (ng/ml)	FAI (ng/ml)	Estr (pg/ml)	E/T x10^{-3}
S1P	**0.36* (0.47**)**	**0.31*(0.66***)**	0.05**(0.56***)**	**0.34*(49**)**	**0.39*(0.4*)**	-0.24(-0.04)	**-0.36*(-0.49**)**
Adiponectin	----------	0.22 **(0.67***)**	-0.03(0.17)	0.22**(0.66***)**	0.15**(0.64***)**	0.11(0.14)	-0.06 **(-0.56***)**
Correlation of SIP with liver markers							
Liver markers	AST (U/l)	ALT(U/l)	Albumin (gm/dl)	Total bilirubin (mg/dl)	INR	Child score	MELD score
S1P (ng/l)	-0.24 (0.06)	-0.21 (0.16)	0.17 (0.11)	-0.07 (-0.08)	-0.05 (-0.24)	-0.06(-0.33)	0.11(-0.13)

Correlation coefficients are written first for male HCC patients followed by correlation coefficients in female HCC patients are shown within parentheses. HCC=Hepatocellular carcinoma; S1P=Sphingosine1phosphate; T= testosterone; SHBG=Sex hormone binding globulin; BAT= Bioavailable testosterone; FAI = free androgen index; Estr = Estradiol; E/T =Estradiol to testosterone ratio; AST=Aspartate aminotransferase; ALT=Alanine aminotransferase; INR= International normalized ratio; MELD: Model of End stage Liver Disease. Significant correlation coefficients are given in bold. ***=$p < 0.001$, **=$p < 0.01$,*=$p < 0.05$.

Discussion:

Our study is the first to demonstrate significantly elevated S1P levels in HCC and cirrhotic patients whose disease is related to HCV etiology compared to healthy subjects. The S1P levels were also elevated in HCC patients compared with the cirrhotic patients; this was found among the entire group and among the female and male subgroups. Moreover, we recommend serum S1P $\geq$ 113 ng/l as a screening test for HCC diagnosis with a high sensitivity (95%) but unfortunately a low specificity of 56%. On the other hand, with S1P $\geq$ 125 ng/l as a diagnostic test, the sensitivity and specificity were 72% and 70%, respectively, among all HCC patients. Notably, S1P as a diagnostic measure was more sensitive in females but more specific in males. S1P and adiponectin showed sex disparities with respect to HCC patients; they were significantly higher among females than males. Compared to sex-specific cirrhotic patients, female HCC patients had higher adiponectin, estradiol and E/T levels and lower SHBG levels, while the inverse profile was observed in male HCC patients. Our study is the first to describe interactions among S1P, adiponectin and sex hormones in HCC using data described by previous molecular studies. Although S1P demonstrated a significant positive correlation with adiponectin and testosterone forms and a negative correlation with the E/T ratio in both the male and female HCC subgroups. The adiponectin relationships showed a marked sex disparity. Adiponectin was negatively associated with testosterone forms and SHBG and was positively associated with estradiol and the E/T ratio among entire HCC group and an inverse association was found in female HCC patients. Additionally, only in female HCC patients a larger tumor size was associated with higher levels of adiponectin and testosterone forms and with significantly lower aromatization (lower E/T ratio). Furthermore, multiple lesions and PVT were associated with higher estradiol ad higher BAT, respectively. Advanced TNM staging was associated with higher testosterone in both sexes and with higher S1P levels, adiponectin levels, estradiol levels and the E/T ratio in females only.

The finding that S1P levels were higher in cirrhotic patients than in healthy subjects may be explained via several mechanism [33,34]. There is sparse previous research addressing the relationship between S1P and HCC, but three previous studies found a significantly higher level of S1P in HCC patients than in cirrhosis patients. However, these studies were restricted by either a very small sample number (n=10 for each group) with a lack of clinical, morphological and laboratory data [35] or a non-comparable cirrhotic group with regard to etiology, age, severity of liver disease and hemoglobin with the latter two variables were correlated with S1P levels in a retrospective study, which might convince us to accept their results [36]. A recent Chinese study found elevated S1P levels and indicated a diagnostic role for it in HCC patients who were mainly of HBV etiology [24]. Two Chinese studies reported conflicting data about the up-regulation of S1P in HCC patients, mainly of HBV etiology, compared to healthy subjects [22,23].

Table (4): Association of Sphingosine 1 Phosphate, adiponectin and Sex Hormones with Tumor Clinicopathological status and TNM Staging among female HCC subgroup

Clinicopathological and TNM staging		n	Sphigosine 1 phosphate	**Adiponectin**	Total testosterone	Sex hormone binding globulin	Bioavailable testosterone	Free androgen index $x10^{-3}$	estradiol	Estradiol to testosterone ratio ($x10^{-3}$)
Tumor	<5	14	130(120-192)	3.7(3.4 -5.1)	0.38(0.34-0.48)	11.4(9.5-2.8)	0.22(0.21-.21)	12(11-17)	63 (48-73)	152(102-165)
diameter (cm)	≥5	26	170(130-181)	7.5 (5 - 8.4)	0.75(0.37-0.83)	9.3(8.2-12)	0.48(0.23-0.57)	25(14-31)	51(42-59)	79(61-139)
P-value			0.21	**0.002**	**0.04**	0.10	**0.048**	**0.003**	0.07	**0.005**
Number of	S	18	131(128-176)	4.9(3.4-8.8)	0.36(0.32-0.79)	9.3(8.2-1.9)	0.20(0.22-0.54)	15(12-27)	51(37-53)	102(67-147)
Focal lesion	M	22	170(126-208)	5.8(3.9-8.3)	0.59(0.38-0.83)	12(8.9-12.3)	0.23(0.33-0.56)	25(12-29)	63(48-70)	98(62-165)
P-value			0.14	0.67	0.21	0.23	0.26	0.56	**0.003**	0.88
Portal vein	No	10	176(127-194)	6.8(4.5-8.6)	0.4(0.34-0.81)	11.4(8.9-12.3)	0.27(0.21-0.52)	14(11-29)	51(43-66)	137(62-165)
thrombosis.	Yes	30	150(128-176)	5.5(3.4-8.1)	0.75(0.49-0.84	9.3(7-12.8)	0.53(0.36-0.57	25(19-27)	63(48-65)	79(70-101)
P-value			0.47	0.41	0.08	0.86	**0.04**	0.19	0.46	0.30
TNM	I	12	130(128-144)	5.5(3.4-12.8)	0.34(0.29-0. 78	9.4(8.6-11.7)	0.21(0.19-0.57)	13(11-35)	45(36- 53)	120(65- 155)
	II	16	157(122-188)	4(3.4-5.7)	0.39(0.34-0.52	10.6(8.5-12.6	0.25(0.22-0.37)	14(11-19)	65(49- 72)	148 (88- 179)
	IIIa	12	184(170-208)	8.1(6.5-8.3)	0.82(0.75-.083	12.1(8.9-12.3)	0.52(0.48-0.65)	29(25-30)	54(48-65)	71(57- 86)
P-value		I vs II	0.3	0.32	0.42	0.47	0.2	1	**0.004**	0.2
		I vs III	**0.006**	0.38	**0.02**	0.17	0.17	0.17	**0.03**	**0.03**
		II vs III	0.07	**<0.001**	**<0.001**	0.8	**0.002**	**<0.001**	0.14	**0.001**

HCC =hepatocellular carcinoma; S= solitary, M=multiple Data are expressed as median (25-75%) quartile range and compared by Mann Whitney test.
Bold values indicate statistically significant results.

Our study overcame these obstacles and provides results from novel population designs, etiologies, regions, ethnicities and methodologies. We studied the effect of sex by constricting an equal number of postmenopausal female and age-matched male participants in sex-stratified HCV-related HCC subgroups that were comparable with regard to age, severity of liver disease, clinicopathological data and staging of HCC among both cirrhotic and HCC patients. The average age of HCC diagnosis was approximately 65 years [1]. The association between sphingosine and chronic hepatitis differs with regard to HCV or HBV infection status [25,26]. We used the standard antibody-based ELlSA method to measure S1P levels, as it is more sensitive than available conventional spectrometry in our area. However, it was more expensive and may show cross-reactivity. The previously mentioned studies used high-performance liquid chromatography tandem mass spectrometry.

Consistent with our result of higher S1P levels with higher T class or TNM staging only among female HCC patients, animal and molecular studies have suggested an enhancing effect of S1P on the initiation and progression of HCC [19]. The sex disparity of S1P that we observed is similar to its association with cardiac autonomic neuropathy only among diabetic females and not males [37]. In our study, S1P levels were positively correlated with age in male HCC patients and positively correlated with HB levels in the male and female subgroups. However, it was not correlated with liver disease markers, severity scores, tumor size or the multiplicity or presence of PVT. This was, to some extent, in agreement with previous studies [24,36]. S1P levels were elevated in female HCC patients compared to either male HCC patients or female cirrhosis patients. This finding may be attributed to higher adiponectin and estradiol levels in female HCC patients and its positive association with adiponectin and estradiol among all HCC patients. This association was in line with molecular studies; adiponectin receptors have intrinsic ceramidase activity that is markedly increased by adiponectin binding with increased S1P production [17]. Similarly, both overexpressing adiponectin receptors and adiponectin agonist administration in hepatocytes increased S1P levels [38]. This may be an oncogenic mechanism of action for adiponectin. In vitro and molecular studies have demonstrated that estradiol markedly improves S1P synthesis and export by activating SphK1 in normal and breast cancer cells. Compared to males, a higher level of S1P was reported in childbearing females in one study, and a higher level was reported in menopausal females in another study [27,28,39]. The positive association between S1P and various testosterone forms in male and female HCC patients is in line with previous experimental findings that T deprivation downregulated SphK1 expression but upregulated SphK2. Hence, SphK1 is a greater contributor to S1P synthesis than SphK2 [40]. S1P was correlated with estradiol in all HCC patients but was correlated with testosterone and E/T in only stratified male and female HCC patient subgroups. This may be attributed to different wide and narrow ranges of studied hormonal levels.

Adiponectin shows a complex dual tumor promoter or suppressor effect in hepatocarcinogenesis [5]. Tissue or serum adiponectin has been reported to be either elevated or decreased or even not associated with HCV-related HCC development compared to cirrhotic patients in global and Egyptian studies [4,41,42]. In our study, we observed a significant decrease in adiponectin levels in HCC and cirrhotic patients compared to healthy subjects in the entire groups and sex-stratified subgroups. Adiponectin showed sex disparity. It was elevated in female HCC patients compared to cirrhotic female patients, and Sadik et al. 2012 reported similar findings in both sexes. Additionally, we reported higher levels of S1P in female patients than in male HCC patients, which were similar to the findings of Shen et al. 2016 [5,41]. This can be explained by higher estradiol and E/T ratios and lower testosterone and SHBG levels compared to either HCC males or cirrhotic females. In this respect, we are the first to report significant positive correlations of adiponectin with estradiol and the E/T ratio and negative correlations of adiponectin with testosterone and SHBG in HCC patients. Our findings are supported by previous data. Manieri et al., 2019 reported physiologically higher adiponectin levels in females than males and observed faster growth of HCC allografts in male mice than in female mice but not in castrated or adiponectin knockout mice. Adiponectin activates AMPK and p38 in HCC cells through an adiponectin R2–dependent pathway, which is predominantly expressed in the liver as a protective mechanism of adiponectin against HCC. This study also suggested that testosterone enhances tumor growth through a reduction in adiponectin production via the activation of JNK in the adipocytes of humans and mice. This reduction prevents AMPK and p38 activation in HCC cells through an adiponectin R2–dependent pathway [6]. However, adiponectin demonstrated the opposite relationship with sex hormones in female HCC patients, and no relationship was detected in male HCC patients. These findings may be attributed to the narrow and different ranges or menopausal states. In line with our results, a higher androgen to estrogen ratio has been associated with lower adiponectin in both sexes, and estrogen administration in postmenopausal females decreases adiponectin levels [3]. In contrast to our findings, adiponectin was shown to increase hepatic SHBG production in males, and endogenous estrogen reduction causes a drop in adiponectin in postmenstrual females [3,43]. This conflict may be attributed to different behaviors in HCC pathology and normal physiology.

In our study, higher adiponectin levels were correlated with larger tumor size and advanced staging only in female HCC patients. This is consistent with previous reports in HCC tissues [44]. In our study, HCV-related cirrhosis in both sexes was accompanied

by significantly lower estradiol levels and insignificantly lower SHBG levels than corresponding healthy subjects. However, these parameters showed sex disparities with HCC development. Male HCC patients had significantly lower estradiol levels and E/T ratios and significantly higher SHBG levels, while female HCC patients showed the opposite changes compared to their respective sex subgroups in cirrhotic patients. This is an aspect that has not been studied before.

We reported a lack of association between the various testosterone forms and HCC development in the cirrhotic subgroups (except for total testosterone in HCC females). Most previous studies were longitudinal studies that involved males, and they showed conflicting results that may be attributed to ethnicity. Testosterone has been reported to not be a predictor, to be a positive predictor, and to be a negative predictor of HCC occurrence among healthy European individuals, HBV cirrhotic Asian individuals, and cirrhotic Egyptian individuals, respectively [12,13,15,16]. Among the males and females in our study, testosterone forms enhanced tumor growth and were associated with tumor size and staging. However, BAT was associated with PVT in females only. This result is similar to the results previously described in ovariectomized mice, which showed accelerated hepatocarcinogenesis upon testosterone administration due to upregulated cell cycle factors and downregulated apoptotic factors [45]. It is worth emphasizing that androgenic receptors are overexpressed in HCC. They demonstrate a "vicious circle" of androgenic signaling and tumor growth and a decline in HCC malignancy after androgenic receptor antagonism treatment [7].

In our study, a valid characterization of the association between testosterone and HCC requires concurrent measurement of SHBG synthesis in the liver and calculation of the FAI and BAT. SHBG synthesis may increase or decrease with liver damage depending on sex and etiology, for example, it was increased in HCV cirrhotic males but decreased in decompensated nonalcoholic cirrhotic males and HCV-related cirrhotic postmenopausal females [10,46,47]. We identified the sex disparity of SHBG with respect to HCC and its relationship with PVT in males, which developed mainly subsequent to tumor invasion. Recent studies have reported the role of SHBG in enhancing prostate and ovarian cancer. However, a protective role in breast and endometrial cancers has also been reported for SHBG, and it may even be expressed by tumor cells [48]. Little data, mainly in men, are available regarding the role of SHBG and HCC. Elevated SHBG may predict HCC development in males with cirrhosis of alcohol etiology but not of HCV etiology in longitudinal studies [12,13]. Similarly, it was a predictor among healthy subjects, even with sex-stratified groups. It was also associated with IGF-1 and liver damage markers in one longitudinal study but not in a cross-sectional study of Greece healthy males [14,15]. Elevated SHBG may have direct or indirect effects. The indirect effect occurs via a decrease in BAT, which either subsequently inhibits tumor growth and invasion to the portal vein, as was the case in our male HCC patients, or enhances fibrosis, steatosis and insulin resistance in HCV cirrhotic males. On the other hand, reduced SHBG in females leads to increased free testosterone, which enhances IR and tumor growth [3,10].

The protective effects of estrogen against HCC through IL-6 inhibition and STAT3 inactivation by binding to wild-type estrogen receptor (ER) are well recognized [7]. In line with our results, a previous study by Farinati et al., 1995 reported lower estradiol among virus-related HCC patients than patients with cirrhosis in a male predominant study, but others have reported the reverse for HBV-infected or alcoholic males [11,14]. Our results are in partial agreement with an Egyptian study that reported reduced estradiol levels in cirrhotic and HCC patients of both sexes [16]. Moreover, higher estradiol was associated with multiple focal lesions and advanced TNM staging in HCC females. No mechanistic evidence conclusively links local estrogen production to HCC growth, but the enhanced expression and activity of aromatase was observed in hepatocyte G2 cells and human HCC tissue. This aromatization has been associated with the degree of malignancy in liver tissue and liver cell lines. It also enhances the expression of an oncogenic ER called variant ER, which can induce tumor growth in contrast to wild-type ER [7]. This may contradict the suppressive role of estrogen and the oncogenic role of androgen, so further studies are required to verify this observation. The sex disparities in estradiol and the E/T ratio in HCC patients may be explained by sex differences in ERα expression in normal livers and altered subtype expression in HCV-related cirrhotic males progressing to HCC [49]. However, the role of increased estrogen in female HCC patients as a compensatory mechanism to protect and decrease morbidity and mortality in females cannot be excluded.

Our study had limitations. A cross-sectional study cannot describe causal relationships, and the study sample size was relatively small. We studied only elderly HCC patients and not younger age groups. Further studies may address this issue. We used the ELISA method for the quantitative measurement of S1P and did not use high-performance liquid chromatography-mass spectrometry, which allows for the quantification of many serum sphingolipid members. Despite these limitations, this study has many important strengths. First and foremost, this is the first study to examine sex disparities of S1P in respect to HCV-related HCC. It is also the first to support the ceramidase activity of adiponectin in both sexes and describe sex disparities in the interactions between adiponectin (but not S1P) and sex hormones. Second, male and female HCC patients were comparable with regard to age, severity of liver disease, clinicopathological and tumor staging. Additionally, cirrhotic patients and HCC patients (entire or sex-stratified groups) were matched for clinical and laboratory data as well as the severity of liver disease. Finally, elderly HCC patients were chosen as they are the most common age for its incidence.

Conclusion:

In conclusion, our study described sex disparities in S1P, adiponectin, and sex hormones (estradiol, total and free testosterone forms, SHBG, and E/T ratio) with respect to HCC development among HCV-cirrhotic patients and their clinicopathological features and staging. The cross talk of adiponectin with sex hormone parameters shows sexual dimorphism, but such cross talk with S1P was similar in both sexes. S1P could be used as a novel screening and diagnostic biomarker for the diagnosis of HCC among cirrhotic and healthy subjects with a cutoff diagnostic value that was more sensitive in females but more specific in males. The present study suggested that the enhanced ceramidase activity of adiponectin in HCC may explain the oncogenic role of adiponectin. Further mechanistic delineation of S1P, adiponectin, and sex hormones using a correct approach will be needed to shed light on HCC and its associated sex disparities. Further studies evaluate inhibition of the SpK/S1P/S1P receptor signaling axis as one arm of a combination therapy for the treatment of this HCC. Moreover, functional S1P receptor 1 antagonist FTY720 may also be a target for HCC treatment in the future. Further studies in young males and fertile females are recommended. This study described sex disparities that may impact the development, pathogenesis, progression and staging of HCC and may identify treatment modalities for response or relapse.

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The authors declare no conflict of interest.

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Availability of data and materials:

The datasets generated and analyzed during the present study are not publicly accessible due to concerns of participates confidentiality but are offered by the corresponding author on realistic request.

Ethics approval and consent to participate

Our study was approved by local ethical committee of our institute Faculty of medicine, Minia University, Egypt. All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki Declaration. All subjects gave informed written consent to be incorporated in the study.

Abbreviation:

HCC= Hepatocellular carcinoma; HCV =hepatitis C virus; E/T= estradiol to testosterone; T =Total testosterone; SHBG= sex hormone-binding globulin; BAT= bioavailable testosterone; FAI= free androgen index; S1P= sphingosine 1-phosphate; SphK= sphingosine kinase; HBV= hepatitis B virus; MELD= model end stage liver disease; EASL= European Association for the Study of the Liver; BCLC= Barcelona Clinic Liver Cancer; TNM =tumor node metastasis; AJCC= American Joint Committee on Cancer; AST =aspartate aminotransferase; ALT= alanine aminotransferase; INR= International normalized ratio; ROC= receiver operating characteristic; AUCs= areas under the ROC curve; PVT= portal vein thrombosis

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