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Antioxidants-based scores to predict Hepatocellular Carcinoma Patients among Chronic Hepatitis C individuals: correlation with Non-invasive Markers of Hepatic Fibrosis

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Abstract

Background High prevalence of Hepatitis C virus (HCV) in Egypt considers it as the major cause of hepatocellular carcinoma (HCC). So, several attempts have been used to predict HCC but still need more. In this regard, the predicting abilities of superoxide dismutase (SOD) and catalase (CAT) activities as well as total antioxidant capacity (TAC) were tested for estimating chronic hepatitis C (CHC)-mediated HCC. Patients and methods Blood samples from 79 patients (23 CHC and 56 HCC) were withdrawn to estimate these antioxidants and the routine analysis markers. Beside 15 healthy volunteers were included Results The medians of SOD activity and TAC values were significantly elevated in sera of HCC patients with positive correlation ($P<0.0001$, $r=0.334$) when compared with those of either CHC or healthy controls ($P<0.0001$). On contrary, the median of CAT activity was significantly lowered ($P<0.0001$) with negative correlation with SOD activity ($r=-0.146$, $P<0.04$). Stepwise logistic regression analysis of the last three markers produced a score; named "Antioxidants-diagnostic" which increased the AUCs values and their diagnostic activities ($P<0.0001$). By combining the three marker values with that of Age-platelet index (API) produced a score named "Antioxidants-API". Also, by combining them with that of the routine analysis values produced a score named "AAI-antioxidants". In each case the AUCs values were enhanced when compared with those of its native antioxidants ($P<0.0001$). Conclusion The assessment of these antioxidant activities and their combination in the last three scores can enhance their activities to more precisely predict HCC patients among CHC ones and control individuals.

Keywords: Superoxide Dismutase, Total Antioxidant Capacity, Catalase, chronic hepatitis C and Age-platelet index.

Introduction

Hepatocellular carcinoma (HCC) is a worldwide problem. HCC is considered the most common type of liver cancer and Lying in the fourth level of the neoplasms with a very poor prognosis and outcome **(Kanda *et al.*, 2017, Llovet *et al.*, 2021 and Ho *et al.*, 2020).**

On reviewing the literatures, more than one million patients will be expected to be affected by HCC annually before 2025. **(Akinyemiju *et al.*, 2017).** The risk of HCC related hepatitis C virus (HCV) was reported to be decreased after viral clearance using direct antiviral drugs**.** In spite, cirrhotic patients are still considered to be at higher risk for HCC even after HCV eradication **(Kanwal *et al*., 2017)**.

Non-alcoholic hepatic stellate cells, also exaggrvate faster growing of HCC **(Estes *et al.*, 2018)**. Moreover, aristolochic acid and tobacco are potentially pathogenic cofactors and mutational signatures in HCC **(Llovet *et al.*, 2021).**

The five-year growing HCC risk in HCV-related cirrhosis is 30% in Japan **(Yamashita *et al.*, 2011)**, 17% in Western countries **(Fattovich *et al.*, 2004; Bataller and Brenner, 2005) and** 14% in in Egypt **(Zekri *et al.*, 2015).** Moreover, HBV infection add more to the HCC incidence as was reported in persons with cirrhosis with 15% five-year cumulative incidence in endemic areas **(Fattovich *et al.*, 2004)**.

Extracellular matrix proteins deposition within hepatic parenchyma are markers of hepatic fibrosis **(Patel *et al.*, 2015).** The hepatic stellate cells and fibroblasts mediate these accumulations; including collagen **(Xu *et al.*, 2005).** Their distribution displays a variety of fibrosis patterns or even cirrhosis in an etiologically-based manner **(Bataller and Brenner, 2005).**

Serum alpha fetoprotein (AFP) use in HCC detection is limited. This may be related to its lower sensitivity . Therefore, new serological markers are of urgent demand. Higher accuracy and validity of these serological markers for early detection of such patients are targets **(Zhao *et al.*, 2013 and Zekri *et al.*, 2015).**

ROS was found to be constructed and liberated by non-parenchymal liver cells, such as Kupffer cells (resident macrophages), inflammatory cells, hepatic stellate cells (HSCs), and other immune effector cells **(Đorđević *et al.*, 2021)**. These ROS, might lead to destruct cell lipid components (phospholipid membranes), oxidatively modify proteins, and DNA and may also cause genomic instability, associated with the increased incidence of cell death and/or HCC **(Rebbani and Tsukiyama-Kohara, 2016)**. HCV infections mediate an increase in oxidative stress in the liver tissue. Latter, may be a causative factor damaging factor in liver injury and determine the severity of such injury. The complex hepatic fibrosis process includes death of hepatocytes and stimulation of lipocytes **(Rebbani and Tsukiyama-Kohara, 2016)** against those two processes and mediate hepatic carcinogenesis. A set of ROS is released throughout these disturbances including mitochondrial electron transfer chain and/or changes in oxidase activities following liver injury **(Rebbani and Tsukiyama-Kohara, 2016).**

Superoxide dismutases (SODs) are one of the metalloenzymes. These are found not only in eukaryotes but also in some prokaryotes. They are located in all organelles such as the cytosol as well as the mitochondrial intermembrane Cu/Zn-SOD (SOD1). The mitochondrial matrix and inner membrane contain Mn-SOD (SOD2), but the extracellular compartment contains Cu/Zn-SOD (SOD3). Their role ,as a major antioxidant defense، has been definitely recognized **(McCord and Fridovich, 1969)**. These isoenzymes catalyze the conversion of superoxide radical ($^{\bullet}O_2^-$) into hydrogen peroxide (H_2O_2) and O_2. Afterwards, catalase (CAT); a 240-kDa homotetrameric enzyme, and/or glutathione peroxidase (GPx) together with/or thioredoxin (Trx) reduce H_2O_2 to water. H_2O_2 might also undergo Fenton reaction to produce hydroxide ion ($^{\bullet}HO$). The latter is a more damaging species than the former superoxide anion **(Rosa *et al.*, 2021).**

Total antioxidant capacity (TAC) comprises the sum of endogenous and exogenous antioxidants. The TAC contents of extracellular fluids is an established way to evaluate the whole antioxidation defense process **(Soccio *et al.*, 2016)**. Balance must be found between pro-oxidant and antioxidant substances to provide homeostasis. The reactive oxygen and nitrogen species (ROS, RNS) are produced through physiological aerobic metabolism. Those species require a buffering capacity to evade molecules and tissue damages caused by their excess **(Prenesti *et al.*, 2021)**. TAC is an index of redox reactivity of a fluid in fixed experimental conditions. **Prenesti *et al*., (2021)** detect that TAC does not represent the concentration of a specific element. Also, its value is method dependent **(Prenesti *et al*., 2021).**

An increasing number of studies focus on ROS role in the pathogenesis of many diseases, including HCC. It has been indicated that higher antioxidants potential may protect the organism

against undesirable ROS activity. Accordingly, their dismutases, e.g via SOD can reduce disease incidence. In spite, evidences in this regard, are still deficient **(Petelin *et al.*, 2017).** In the current study, the aim is focused on predicting antioxidants-based scores to select HCC patients among those with CHC individuals and to correlate the developed scores with the non-invasive markers of hepatic fibrogenesis.

Patients and methods

Patients

23 CHC naive patients and (male, 14; female, 9 median of age, 51.0(43.0-59.0) years) and 56 HCC denovo patients(male, 44; female, 12 median of age, 61.00 (53.3-66.0) years) were selected from the outpatient hepatology clinics, in Egyptian Liver Research Institute and Hospital (ELRIAH), Sherpin, Aldakahlia, Egypt. All patients were positive HCV Ab and negative for any other related chronic liver diseases. They had normal kidney function, normal glucose levels and no liver transplantation. The patients were not treated with antiviral drugs. HCC patients were not subjected to treatments before liver Fibroscan or blood sampling. In line, 15 volunteers which had age and gender-matched with those of the patients were included (controls group). The controls had normal liver functions and were free from liver disorders. The individuals signed an informed written consent in each case.

Study Approval and patients, consent:

The study protocol was designed according to guidelines to the declaration of Helsinki, 1975. Also, prior approval by the institution's human research committee, Faculty of Medicine, Mansoura University was obtained. All participants were subjected to full medical history and complete clinical examination as well as laboratory and radiological examination.

Blood sampling

Six ml blood sample were withdrawn in each case. 4 ml were left to clot, centrifuged and the serum was separated and freshly used or stored at−80 °C. These samples were used to estimate HCV Abs and polymerase chain reaction for detecting HCV-RNA. Also, it was used to exclude infection of hepatitis B surface antigen (HBsAg) and assaying of TAC activity. The sample also used to routinely estimate the liver function tests. 1 mL was collected onto sodium citrate tube for detecting prothrombin time expressed as International Normalized Ratio (INR). The other 1 mL was collected onto ethylene diamine tetra acetic acid (EDTA) tube for the haematological assays then centrifuged and its plasma was used in assaying SOD and catalase activities.

Routine laboratory analysis

Hematological markers were done using D-cell 60 automated hematology analyzer (Sysmex X 1800 incorporation, Japan), international normalized ratio (INR) was performed using (Sysmex® CA-1500, Japan) auto analyser, Liver function tests; including serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin and total bilirubin using automated Biochemistry analyzer (Cobas Integra 400, Roch, Switzerland).

Serological markers and RT-PCR for detecting HCV infection

HCV Abs were evaluated using enzyme linked immunosorbent assay (ELISA, Merieux anti-HCV, version 4.0, Diasorin S.P.A. via Crescent no 13040 Saluggia (VC) - Italy). Manual extraction of RNA from 100 µl of plasma were reverse transcripted and followed by cDNA amplification using polymerase chain reaction (PCR). HCV RNA was quantized by quantitatively estimated (RT-PCR) using fully automated analyzer; (Cobas amplified, Taqman48 analyzer, Roche Switzerland).

Assessment of Antioxidant assays

Antioxidant assays were performed in-house according to those principles.

Assessment of superoxide dismutase (SOD) using colorimetric assay

Superoxide dismutase (SOD) was determined by the method of **(Nishikimi, 1972).** The optical densities of the developed colors were

measured at 560 nm using a recording spectrophotometer.

Assessment of catalase-like activity using colorimetric assay

Catalase-like activity (CAT) was determined by the method of **(Sinha, 1972)**. The optical densities of the developed colors were measured at 620 nm using a recording spectrophotometer.

Assessment of total antioxidant capacity (TAC) using colorimetric assay

Total antioxidant capacity (TAC) was measured according to the method of **(Koracevic, 2001)**. The optical densities of the developed colors were measured at 532 nm against deionized water using a recording spectrophotometer

Statistical analyses:

All statistical analyses were done by using SPSS software (version 24; IBM, USA). Continuous variables were expressed as mean ± standard deviation (SD) (if normally distributed) or median (IQR) if not normally distributed. Comparisons of markers as well as routine laboratory tests were analyzed using a two-sided P-value. Person's correlation coefficient was used in establishing correlation among parameters. Analyses were done for parametric quantitative variables using one-way ANOVA test and for non-Parametric quantitative variables using Kruskal Wallis test. Receiver-Operating Characteristic (ROC) curve was done to determine the cutoff point, area under curve (AUC), sensitivity (Sn), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV). A value of ($P<0.05$) was considered statistically significant.

Results

Patients' laboratory data

Our study was conducted on 79 include 23 CHC (male, 14; female, 9 median of age, 51.0 (43.0-59.0) years) and 56 HCC individuals (male, 44; female, 12 median of age, 61.00 (53.3-66.0) years). They were chosen from the outpatient's hepatology clinics of the Egyptian Liver Research Institute and Hospital, Dakahlia Governorate, Sherpin, Egypt. Beside 15 healthy volunteers (male, 8; female, 7 median of age, 25.0 (21.0-32.0) years) were included. The patients, controls, their sex and age were summarized in **table1**.

Table1. Patient characteristics

Total Number	Controls' group (n = 15)	CHC group (n=2)	HCC group (n=5)	p-value
Age (years)	25.0 (21.0-32.0)	51.0 (43.0-59.0)	61.00 (53.3-66.0)	<0.001
Male	8 (53.3)	14 (60.9)	44 (78.6)	0.087
Female	7 (46.7)	9 (39.1)	12 (21.4)	

N.B.: Data is presented as median (IQR) or n (%), at n: total number
By Chi-square test for qualitative data and Kruskal-Wallis test for quantitative data

Liver function tests and platelets count

The routine laboratory results of patients and controls were summarized in **table 2**. It was found that the median of activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly elevated in sera of HCC patients compared to CHC ones or healthy individuals (49.0 IU/L versus 36.0 IU/L and 16.0 IU/L) and (50.0 IU/L versus 23.0 IU/L and 21.0 IU/L, respectively). While, the median activity of Alkaline Phosphatase (ALP) of HCC patients was 100.0(IU/L) and that for CHC patients was 78.5 (IU/L) compared with those of the healthy individuals was 80.0(IU/L). In the same pattern, the median activity of International Normalized Ratio (INR) of HCC patients was significantly increase when compared with those of CHC or the healthy individuals (1.08, 1.01and 1.01, respectively) with ($P<0.001$). In addition, the difference in serum albumin was significant when its level in sera of HCC patients was compared with that of CHC patients or healthy individuals (3.8, 4.65 and 4.6 gm/dL) with ($P< 0.0001$). The same finding was reported for serum total bilirubin in both groups of patients ($P <0.0001$). Platelets count was significantly decreased in the blood of HCC patients with ($144.13\pm65.19(10^9/L)$) compared to that of patients CHC ($220.55\pm62.75\ (10^9/L)$) or healthy controls ($266.47\pm43.68(10^9/L)$).

Table 2. Routine laboratory investigations of patients and controls.

Groups / Parameters	Controls' group (n = 15)	CHC group (n=23)	HCC group (n=56)	p-value
ALT (IU/L) Median (Range)	16.0 (12.0-29.7)	36.0 (29.0-45.6)	49.0 (31.8-64.5)	< 0.003
AST (IU/L) Median (Range)	21.0 (18.3 - 26.7)	23.0 (21.0 - 26.0)	50.0 (30.0-70.0)	< 0.0001
ALP (IU/L) Median (Range)	80.0 (69.5-88.735)	78.5 (60.4 - 86.7)	100.0 (74.8-123.0)	< 0.004
INR Median (Range)	1.0 (0.926 - 1.01)	1.0 (1.01 - 1.01)	1.08 (1.0-1.65)	< 0.0001
Albumin (gm/dL) Median (Range)	4.6 (4.5-4.7)	4.65 (4.217-4.800)	3.8 (3.5-4.3)	< 0.0001
Total bilirubin (mg/dL) Median (Range)	0.8 (0.7-0.974)	0.65 (0.6-0.8)	1.1 (0.7-1.6)	< 0.0001
Platelets count (10^9/L) Mean ± SD	266.47±43.68	220.55±62.75	144.13±65.19	< 0.0001

n: total number; P: statistical significance between the control, CHC and HCC groups using the Mann–Whitney test. N.B.: Data is presented as mean ± SD or median (IQR) ,if not normally distributed, by ANOVA.

Antioxidants biomarker levels

The statistical analysis of Superoxide dismutase (SOD), catalase and total antioxidant capacity (TAC) activities in the three studied groups was carried out and the results were summarized in **table 3**.

Table3. Comparison of plasma SOD, Catalase as well as serum TAC among the different studied groups

Groups / Parameters	Controls' group (n = 15)	CHC group (n=23	HCC group (n=56	p-value
SOD (%inhibition) Median (Range) Mean ± SD	34.5 (21.3-40) 33.5 ± 4.98	38.7 (32.1-61.3) 44.3 ± 10.8	60.9 (13.83-99.26) 73.6 ± 20.9	<0.0001
Catalase (mU/mg) Median (Range) Mean ± SD	38.5(16.3-108.4) 51.8 ± 33.4	35.9 (3.9-64.9) 31.1 ± 18.9	23.7 (1.5-51.2) 24.6 ± 12.7	<0.0001
TAC (mmole/L) Median (Range) Mean± SD	1.36 (0.91-2.0) 1.4 ± 0.4	2.55 (2.1-3.3) 2.7 ± 0.4	2.95 (1.5-3.6) 2.8 ± 0.5	<0.0001

P: statistical significant when the results of CHC and HCC where compared with those of the control values using the Mann–Whitney test.

Plasma SOD was significantly increased (P<0.0001) in sera of CHC and cirrhotic patients with HCC compared to that of the control group (median 38.7, mean ± SD 44.3 ± 10.8), (median 60.9, mean ± SD 73.6 ± 20.88) and (median 34.5, mean ± SD 33.5 ± 4.98), respectively **(Figure 1)**.

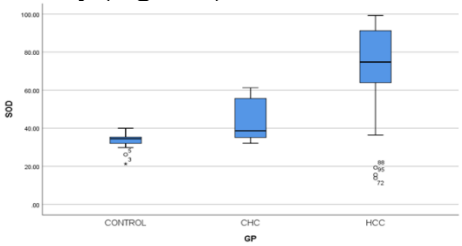


Figure 1. Medians of plasma SOD among CHC and HCC compared with that of the healthy control group.

In terms of catalase activity (**Figure 2**), it was significantly decreased in plasma of CHC patients (1.07 fold reduction) when compared to that of the healthy controls (median 35.85 vs. 38.49 mU/mg). Furthermore, it was found that catalase of CHC patients were significantly higher (1.5 fold) when compared with that of HCC (median 35.85 versus. 23.66 mU/mg) with p<0.0001.

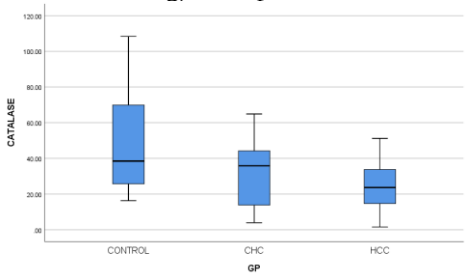


Figure 2. Medians of plasma catalase among CHC and HCC compared with that of healthy control group.

When comparing the results of SOD, Catalase and TAC of CHC patients with those of the

control group cut off values with the maximum Sn, Sp, PPV and NPV were presented in **table 4**. It was found that, at a cut-off value of 35.53% for SOD. The Sn, Sp, PPV and NPV were 68.42% and 80%, 81.3% and 66.7%, respectively with P<0.0001 and AUC 0.796. Whereas, catalase gave AUC of 0.663 at a cut-off value of 64.88mU/mg. The Sn, Sp, PPV and NPV were 100%, 33.33%, 66.7% and 100.0 %, respectively with P<0.08. For serum level of TAC, it gave AUC of 1.0 at a cut-off value of 1.97mmole/L. The Sn, Sp, PPV and NPV were 100%, 100 %, 100 % and 100 %, respectively with P<0.0001 (**Table 4).**

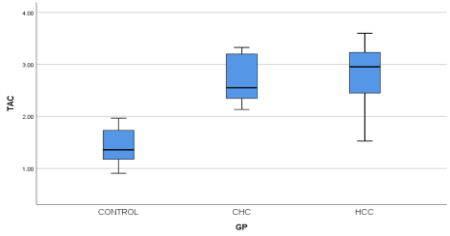


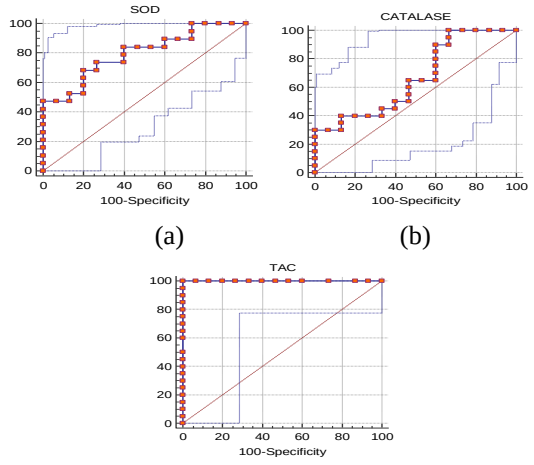
Figure 3. Medians of sera TAC among CHC and HCC compared with that of the healthy control group

Table 4. The diagnostic performance of candidate antioxidant biomarkers SOD, Catalase and TAC to differentiate between the CHC cases and healthy controls using ROC curve analysis.

Group	Discriminating CHC cases versus healthy controls						
Parameters	Cut off	AUC	Sn	Sp	PPV	NPV	P value
SOD (%)	35.53	0.796 (0.62 - 0.92)	68.42 (43.4 - 87.4)	80 (51.9 - 95.7)	81.3 (54.4 - 96.0)	66.7 (41.0 - 86.7)	<0.0001
Catalase (mU/mg)	64.88	0.663 (0.48 - 0.81)	100.0 (83.2 - 100.)	33.33 (11.8–61.6)	66.7 (47.2 - 82.7)	100.0 (47.8-100.0)	<0.08
TAC (mmole/L)	1.97	1 (0.90 - 1.00)	100 (83.2 - 100.0)	100 (78.2 -100.0)	100 (83.2 - 100.0)	100 (78.2 -100.0)	<0.0001

Data for Sn, Sp, PPV and NPV are presented as % (95% CI)

The ROC curve showed a higher AUC value for TAC than those of SOD or catalase antioxidant biomarkers when comparing CHC individuals from the healthy control ones (AUCs 1, 0.796 and 0.663, respectively) **(Figure 4).**



(a) (b)

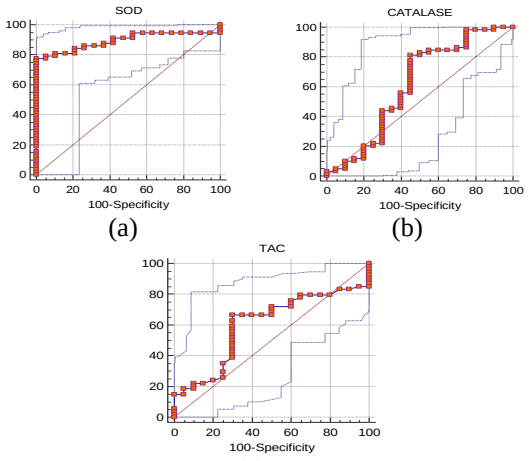
(c)

Figure 4. The ROC curve of the three antioxidant markers (A: SOD, B: catalase and C: TAC) between the two groups; the healthy controls and CHC cases.

Diagnostic performance of candidate antioxidant biomarkers comparing CHC group with HCC group

In the cirrhotic patients (CHC and HCC groups) different cut-off values of the SOD, Catalase and TAC with maximum Sn and Sp were evaluated for detecting early HCC. It was found that, at a cut-off value of 61.3% for SOD, the Sn, Sp, PPV and NPV were 77.59%, 100 %, 100% and 59.4% ,respectively with P<0.0001. Whereas for Catalase, it was found that at a cut-off value of 34.75mU/mg, the Sn, Sp, PPV and NPV were 81.36%, 55%,84.2 % and 50%, respectively with P<0.2. For sera TAC, it was found that, at a cut-off value of 2.72mmole/L, the Sn, Sp, PPV and NPV were 66.67%, 70%, 85.7% and 43.7 %, respectively with P<0.17 in **table 5.**

The ROC curve showed higher AUC value for SOD than those of catalase and TAC when differentiating CHC individuals from HCC ones (AUCs 0.895, 0.604 and 0.601), respectively **(Figure 5).**



(a) (b)

(c)

Figure 5. The ROC curve of (a: SOD, b: catalase and c: TAC) between the two groups; CHC and HCC cases.

Table 5. The diagnostic performance of the antioxidant biomarkers SOD, Catalase, and TAC to differentiate between the two patient groups CHC from those with HCC using ROC curve analysis.

Group	**Discriminating HCC cases versus CHC ones**						
Parameters	Cut off	AUC	P value	Sn	Sp	PPV	NPV
SOD (%)	61.3	0.895 (0.804 - 0.953)	<0.0001	77.59 (64.7 - 87.5)	100 (82.4 - 100.0)	100 (92.1 - 100.0)	59.4 (40.6 - 76.3)
Catalase (mU/mg)	34.75	0.604 (0.493 - 0.717)	<0.2	81.36 (69.1 - 90.3.)	55 (69.1 - 90.3)	84.2 (72.1 - 92.5)	50.0 (28.2-71.8)
TAC (mmole/L)	2.72	0.601 (0.480 - 0.713)	<0.17	66.67 (52.5 - 78.9)	70 (45.7 - 88.1)	85.7 (71.5 - 94.6)	43.7 (26.4-62.3)

Data for Sn, Sp, AUC, PPV and NPV are presented as % (95% CI).

Diagnostic performance of candidate antioxidant biomarkers comparing HCC patients with control group

When comparing the results of SOD, Catalase and TAC of HCC patients with those of the control group gave different cut-off values the Sn, Sp, PPV and NPV were presented in **table 6**. It was found that, at a cut-off value of 39.97% for SOD. The Sn, Sp, PPV and NPV were 91.38%, 100%, 100% and 66.7, respectively with P<0.0001. Whereas, for catalase it was found that at a cut-off value of 34.75mU/mg. The Sn, Sp, PPV and NPV were 81.38%, 60%,88.9% and 45.0 %, respectively with P<0.0004. For sera TAC level, it was found that at a cut-off value 1.91mmole/L, the Sn, Sp, PPV and NPV were 88.89%, 100 %, 100% and 71.4 %, respectively with P<0.0001 **in table 6.**

Table 6. The diagnostic performance of antioxidant biomarkers SOD, Catalase and TAC to differentiate between the healthy controls and HCC cases using ROC curve analysis.

Group	**Discriminating HCC cases versus healthy controls**						
Parameters	Cut off	AUC	Sn	Sp	PPV	NPV	P value
SOD (%)	39.97	0.941 (0.860 - 0.983)	91.38 (81.0 - 97.1)	100 (78.2 - 100)	100 (93.3 - 100)	66.7 (50.9 - 91.3)	<0.0001
Catalase (mU/mg)	34.75	0.759 (0.646 - 0.851)	81.38 (69.1 - 90.3)	60 (32.3 - 83.7)	88.9 (77.4 - 95.8)	45 (23.1-68.5)	<0.0004
TAC (mmole/L)	1.91	0.972 (0.900 - 0.997)	88.89 (77.4 - 95.8)	100 (78.2-100.0)	100 (92.6-100.0)	71.4 (47.8 -88.7)	<0.0001

Data for Sn, Sp, AUC, PPV and NPV are presented as % (95% CI)

The ROC curve showed higher AUC value for TAC was than those of SOD and catalase when differentiating HCC individuals from the healthy control ones (AUCs 0.972, 0.941 and 0.759), respectively **(Figure 6).**

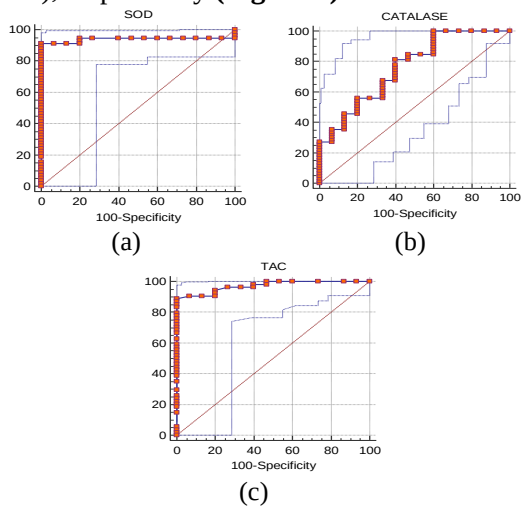


Figure 6 The ROC curve of (a: SOD, b: catalase and c: TAC) between the two groups; control and HCC cases.

Correlation coefficient among the candidate antioxidant biomarkers together

Significant positive correlation was found between individual activities of SOD and those of TAC (r = 0.497, P < 0.0001). On the other hand, catalase showed negative correlation with SOD and those of TAC (r = -0.290, P <0.005) and (r= -0.311, P<0.003) when the two last groups were compared with each other **in table 7 and figure 7.**

Table 7. Correlations of antioxidant biomarkers; SOD, Catalase and TAC.

	SOD	**Catalase**	**TAC**
Superoxide dismutase (SOD)		-0.290	0.497
r P	1	<0.005	<0.0001
Catalase (CAT)	-0.290		-0.311
r P	<0.005	1	<0.003
Total antioxidant capacity (TAC)	0.497	-0.311	
r P	<0.0001	<0.003	1

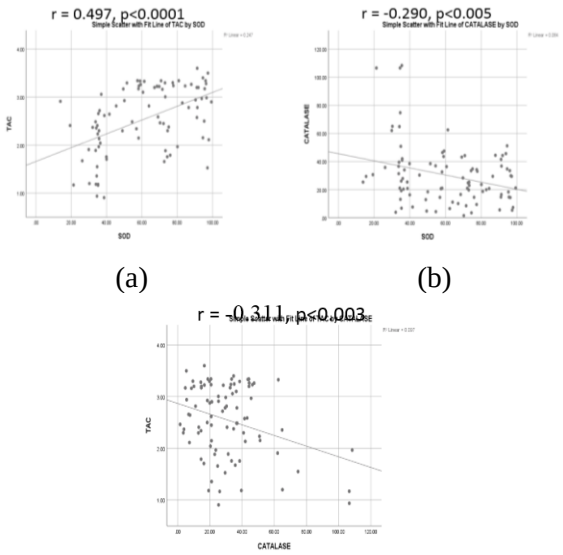


Figure 7 Correlations between the three antioxidant parameters; (a-c)

Correlation coefficient of the candidate antioxidant biomarkers with the parameters of liver function tests (LFTs), platelets count

There were correlations between age and liver function tests with SOD, Catalase and TAC. Age was found to be positively correlated with SOD and TAC (r= 0.532, p< 0.0001) and (r=0.669, p< 0.0001), respectively. Conversely, it was negatively correlated with catalase (r=-0.373, p<0.0001). AST showed positive correlation with SOD and TAC with high significance (r=0. 334, p<0.001), (r=0. 203, p<0.056), respectively. Conversely, it showed negative correlation with catalase (r=-0. 196, p<0.058). ALT showed positive correlation with SOD and TAC with high significance (r=0. 174, p<0. 098) and (r=0. 235, p<0.026), respectively. Conversely, it was negatively correlated with catalase enzyme (r=-0.151, p<0.146). ALP showed positive correlation with SOD (r=0.150, p<0.152) and TAC with high significance (r=0.233, p<0.028), while it showed negative correlation with catalase enzyme (r=-0.125, p<0.23). Albumin showed negative correlation with high significance with SOD (r=-0.480, p<0.0001) and TAC (r=-0.424, p<0.0001), while it showed positive correlation with catalase enzyme (r=0.261, p<0.011). Bilirubin showed positive correlation with high significance with SOD only (r=0.289, p<0.005), while it showed negative correlation with both TAC (r=-0.008, p<0.94) and catalase (r=-0.15, p<0.231). INR showed positive correlation with high significance with SOD (r=0.193, p<0.066) and TAC (r=0.173, p<0.105), while it showed negative correlation with catalase (r=-0.011, p<0.29). PLT showed negative correlation with high significance with SOD (r=-0.451, p<0.0001) and TAC (r=-0.287, p<0.006) while it showed positive correlation with catalase (r=0.156, p<0.134) **(Table 8).**

Table 8. Correlations between the individual levels of liver function tests as well as platelets count with oxidative stress parameters with the parameters.

	SOD	Catalase	TAC
Age (years)			
r	0.532	- 0.373	0.669
P	<0.0001	<0.0001	<0.0001
AST (IU/L)			
r	0.334	- 0.196	0.203
P	<0.001	<0.058	<0.056
ALT (IU/L)			
r	0.174	- 0.151	0.235
P	<0.098	<0.146	<0.026
ALP (IU/L)			
r	0.150	- 0.125	0.233
P	<0.152	<0.23	<0.028
Albumin (g/dL)			
r	- 0.480	0.261	- 424
P	<0.0001	<0.011	<0.0001
Bilirubin (mg/dL)			
r	0.289	- 0.15	-0.008
P	<0.005	<0.231	<0.94
INR			
r	0.193	- 0.11	0.173
P	<0.066	<0.29	<0.105
Platelets count (10^9/L)			
r	- 0.451	0.156	- 0.287
P	<0.0001	<0.134	<0.006

Levels of liver function tests as well as platelet counts were correlated with SOD, TAC and catalase using r-Pearson correlation coefficient, P: probability

Regression modeling

A novel non-invasive model was developed that consisted of incorporating the three antioxidant biomarkers for increasing the diagnostic accuracy for detection of HCC from those of CHC and healthy control individuals using multiple stepwise regression resulting in a complex score of the three markers called Antioxidants-diagnostic score.

$$Antioxidants\ diagnostic = \{SOD(\%) \times 0.015\} + \{TAC(mmole/L) \times 0.413\} - \{Catalase(mU/mg) \times 0.007\} + 0.671$$

The statistical analysis of the Antioxidants-diagnostic score in the three studied groups was carried out and the results were summarized in **table 9.**

Table 9. The statically analysis for the Antioxidants-diagnostic score among the studied groups.

Groups / Parameters	Controls' group (n = 15)	CHC group (n = 23)	HCC group (n = 56)	p-value
Antioxidants-diagnostic				
Mean ±S.D.	1.4±0.314	2.19±0.306	2.72±0.477	
Median (Range)	1.47(1.27 - 1.59)	2.22 (1.98-2.38)	2.81(2.62 -2.95)	P<0.0001

SD: standard deviation, n: total number, P: statistical significant when the results of CHC and HCC where compared with those of the control values using the Mann–Whitney test.

Antioxidants diagnostic score gave statistically significant increases in mean and median of its numerical values (P<0.0001) when the results of CHC and HCC patients were compared with those of the healthy controls. Their medians and mean ± SD were (2.22 and 2.19 ± 0.306), (2.81 and 2.72 ± 0.477) and (1.47 and 1.4 ± 0.314), respectively **(Figure 8).**

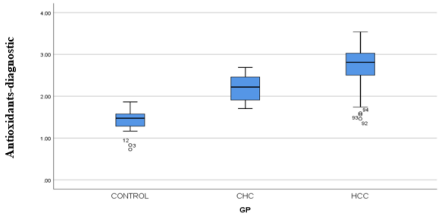


Figure 8. Medians of the Antioxidants-diagnostic score comparing CHC and HCC patients groups with those of the healthy controls group.

Diagnostic performance of the Antioxidants-diagnostic score comparing CHC patients with control group

By comparing CHC patients with healthy control group, the antioxidant diagnostic score gave AUC of 0.98 at cut-off value of >1.74 with more accurate Sn 95.0% and Sp 93.33%. The PPV was 95.0% and that NPV was 93.3 % with P<0.0001, (**Table 10 and Figure 9 a)**.

Table 10. The diagnostic performance of the Antioxidants-diagnostic score to differentiate between CHC cases and the healthy controls using ROC curve analysis.

Group	Discriminating CHC cases versus healthy controls						
Parameters	Cut off	AUC	Sn	Sp	PPV	NPV	P value
Antioxidants-diagnostic	>1.74	0.98 (0.865 - 1.000)	95.0 (75.1 - 99.9)	93.33 (68.1 - 99.8)	95.0 (75.1 - 99.9)	93.3 (68.1 - 99.8)	<0.0001

Data for Sn, Sp, AUC, PPV and NPV are presented as % (95% CI)

Diagnostic performance of the Antioxidants-diagnostic score comparing CHC patients with HCC ones.

In the cirrhotic patients (CHC group and HCC group), the Antioxidants-diagnostic score gave AUC of 0.837 at an optimal cut-off value of >2.19 with more accurate Sn 89.47% and Sp 50.0%. The PPV was 83.6% and that NPV was 62.5% with P<0.0001, **table 11 and figure 9b.**

Table 11. The diagnostic performance of the antioxidants-diagnostic score to differentiate between HCC and CHC cases using ROC curve analysis.

Group	Discriminating HCC cases versus CHC ones						
Parameters	Cut off	AUC	Sn	Sp	PPV	NPV	P value
Antioxidants-diagnostic	>2.19	0.837 (0.736 - 0.912)	89.47 (78.5 - 96.0)	50.0 (27.2 - 72.8)	83.6 (71.9 - 91.8)	62.5 (35.4 - 84.8)	<0.0001

Data for Sn, Sp, AUC, PPV and NPV are presented as % (95% CI)

Diagnostic performance of the Antioxidants-diagnostic score comparing HCC patients with control group

By comparing HCC patients with healthy control group, the Antioxidants-diagnostic score gave AUC of 0.98 at cut-off value of >1.86 with more accurate Sn 92.98% and Sp 100%. The PPV was 100.0% and that NPV was 78.9% with P<0.0001, **table 12 and figure 9c.**

Table 12. The diagnostic performance of the Antioxidants-diagnostic score to differentiate between HCC patients and healthy control individuals using ROC curve analysis.

Group	Discriminating HCC cases versus healthy control individuals						
Parameters	Cut off	AUC	Sn	Sp	PPV	NPV	P value
Antioxidants-diagnostic	>1.86	0.980 (0.914 - 0.999)	92.98 (83.0 - 98.1)	100.0 (78.2 - 100.0)	100.0 (93.3 - 100.0)	78.9 (54.4 - 93.9)	<0.0001

Data for Sn, Sp, AUC, PPV and NPV are presented as % (95% CI)

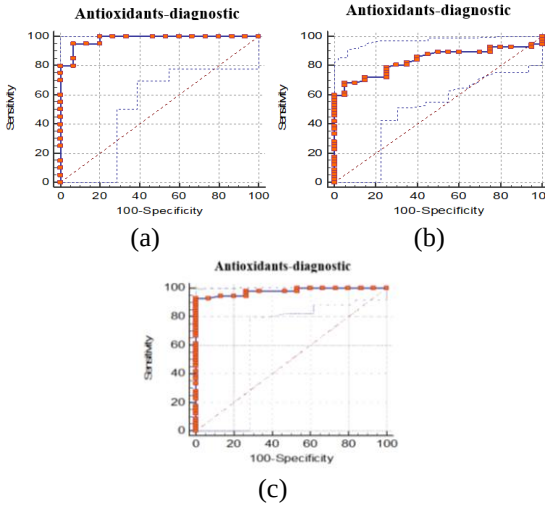


Figure 9 The ROC curve of the Antioxidants-diagnostic score (a: between the CHC cases and the healthy control ones, b: In the cirrhotic patients (CHC group and HCC group), and c: among the HCC cases and the healthy control ones)

Indirect serum markers of liver fibrosis levels

The statistical analysis of the indirect serum markers of liver fibrosis in the three studied groups was carried out and the results were summarized in **table 13.** It was found that the median of AAR (AST/ALT) score, and fibrosis 5 score (FIB5) were significantly decreased when both HCC and CHC patients were compared with that of healthy controls (1.28 and 0.737 versus 1.3) and (26.05 and 29.4 versus 35.42), respectively. As well as, Age-AST model (Age/AST) was decreased when HCC patients were listed against that of CHC and the healthy controls (0.94 versus 2.27 and 1.16), respectively. On contrary It was found that the median of Age-platelet index (API), AST to platelet ratio index (APRI), fibrosis 4 score (FIB4), FibroQ, Göteborg University Cirrhosis Index (GUCI), and KING'score were elevated in samples of HCC patients and CHC ones compared to healthy controls (0.46 and 0.24 versus 0.09), (1.84 and 0.33 versus 0.25), (4.93 and 0.97 versus 0.47), (4.035 and 2.79 versus 0.6), (67.8 and 10.6 versus 7.86), (38.7 and 5.94 versus 1.84), respectively with (P< 0.0001). As well as, Fibrosis-cirrhosis index (FCI) was significantly elevated in HCC patients compared to the CHC and the healthy controls values (0.33 versus 0.05 and 0.06), respectively with (P< 0.001).

Table 13. The indirect serum markers of liver fibrosis

Groups / **Parameters**	**Controls' group (n = 15)**	**CHC group (n = 23)**	**HCC group cases (n = 56)**	**p-value**
AAR score				
Median (Range)	1.3 (0.88 - 1.73)	0.737 (0.61 - 0.94)	1.28 (1.15 -1.45)	<0.0001
Age-AST model				
Median (Range)	1.16 (0.89- 1.656)	2.27 (1.54 - 2.67)	0.94 (0.86 - 1.16)	<0.0001
API				
Median (Range)	0.09 (0.07 - 0.125)	0.24 (0.16 - 0.27)	0.46 (0.4-0.51)	<0.0001
APRI				
Median (Range)	0. 25 (0. 2–0.36)	0. 33 (0.28 - 0.41)	1.84 (1.11-2.2)	<0.0001
FIB4				
Median (Range)	0.47 (0.40 - 0.61)	0.97 (0.7 - 1.23)	4.93 (3.64 - 5.68)	<0.0001
FIB5				
Median (Range)	35.42 (30.59 - 36.89)	29.4 (25.99 - 30.97)	26.05 (25.41- 27.87)	<0.0001
FibroQ				
Median (Range)	0.6 (0.55 -1.20)	2.79 (2.3 - 4.52)	4.035 (3.13-4.7)	<0.0001
GUCI				
Median (Range)	7.86 (5.91-11.48)	10.6 (9.17-13.3)	67.8 (48.46- 83.17)	<0.0001
King's score				
Median (Range)	1.84 (1.59 - 2.93)	5.94(4.17 - 7.01)	38.7(29.9- 47.89)	<0.0001
FCI				
Median (Range)	0.06 (0.04 - 0.07)	0.05 (0.04-0.07)	0.33 (0.238-0.43)	<0.001

Correlation coefficient of the candidate antioxidant biomarkers with the parameters of the indirect serum markers of liver fibrosis

A significant and positive correlation was found between individual activities of SOD and those of AAR score, API, APRI, FIB4, FibroQ, GUCI, King's score and FCI (r = 0.357, P<

0.001), (r = 0.484, P< 0.0001), (r=0.403, P< 0.0001), (r = 0.519, P< 0.0001), (r= 0.32, P< 0.002), (r=0.418, P< 0.0001), (r=0. 426, P<0.0001), (r=0.286, P<0.007), respectively. On the other hand, SOD shows negative correlation with Age-AST model and FIB5 (r = -0.115; P <0.288) and (r = -0.207; P<0.005), respectively **(Table 14)**.

However, Catalase showed statistically significant and negative correlation with those of API, APRI, FIB4, FibroQ, GUCI, King's score and FCI (r= -0.257, P<0.015), (r = - 0.254, p< 0.016), (r=- 0.255, p< 0.016), (r= -0.190, P< 0.072), (r= - 0.250, p< 0.018), (r= - 0.256, p<0.016) and (r=-0.217, p< 0.041), respectively. On the other hand, Catalase shows positive correlation with AAR score, Age-AST model (AGE/AST) and FIB5 (r= 0.024; P <0.826), (r= 0.118; P<0.27) and (r= 0.315, P<0.003), respectively **(Table14).**

TAC showed statistically significant and positive correlation with those of Age-AST model, API, APRI, FIB4, FibroQ, GUCI, King's score and FCI (r=0.066, P< 0.553), (r= 0. 381, P<0.0001), (r=0.301, P<0.005), (r=0.332, P<0.002), (r=0.41, P<0.0001), (r=0.328, P<0.002), (r=0.333, P<0.002) and (r=0.161, P<0.141), respectively. On the other hand, TAC shows negative correlation with AAR score and FIB5 (r = -0.099، P<0.369) and (r= -0.386, P<0.0001), respectively **(Table 14)**.

Table 14. Correlations between the individual levels of SOD, Catalase and TAC with the indirect serum markers of liver fibrosis.

	SOD	catalase	TAC
AAR score			
r	0.357	0.024	-0.099
P	<0.001	<0.826	<0.369
Age-AST model			
r	-0.115	0.118	0.066
P	<0.288	<0.270	<0.553
API			
r	0.498	- 0.262	0.397
P	<0.0001	<0.013	<0.0001
APRI			
r	0.389	- 0.247	0.320
P	<0.0001	<0.02	<0.003
FIB4			
r	0.306	-0.238	0.290
P	<0.004	<0.024	<0.007
FIB5			
r	-0.479	0.321	- 0.394
P	<0.0001	<0.002	<0.0001
FibroQ			
r	0.32	- 0.190	0.414
P	<0.003	<0.075	<0.0001
GUCI			
r	0.418	0.250	- 0.328
P	<0.0001	<0.018	<0.002
King's score			
r	0.426	-0.214	0.333
P	<0.0001	<0.003	<0.002
FCI			
r	0.289	-0.218	0.160
P	<0.006	<0.039	<0.14
LOK (Model 3)			
r	0.466	-0.131	0.168
P	<0.0001	<0.219	<0.127

Derivation of antioxidants-API score

A novel non-invasive model was developed that consisted of antioxidant biomarkers with one of the indirect serum markers of liver fibrosis for improving early HCC detection by adequately distinguishing HCC patients from healthy control individuals. The model is illustrated as follows:

$$\begin{aligned}\text{Antioxidants} - \text{API} &= \{SOD(\%) \times 0.011\} \\ &+ \{TAC(mmole/L) \times 0.377\} \\ &- \{\text{CAT}(mU/mg) \times 0.006\} \\ &+ \{API \times 0.811\} + 0.650\end{aligned}$$

The statistical analysis of the Antioxidants-API score in the three studied groups was carried out and summarized in **table 15.**

Table15. The statistical analysis for the Antioxidants-API score among the studied groups.

Groups / Parameters	Controls' group (n = 15)	CHC group (n =23)	HCC group (n =56)	p-value
Antioxidants-API				
Mean ±S.D.	1.33±0.258	2.121±0.283	2.793±0.395	P<0.0001
Median (Range)	1.38 (1.24 - 1.47)	2.15 (1.96 - 2.30)	2.8 (2.7 - 2.92)	

P: statistical significant when the results of CHC and HCC where compared with those of the control values using the Mann–Whitney test.

The median and mean numerical values of Antioxidants-API score for CHC and HCC patients were significantly increased when compared with the corresponding control values (P<0.0001). The median and mean± SD were (2.15 and 2.12 ± 0.283), (2.8 and 2.79±0.395) and (1.38 and 1.33±0.258), respectively **(Figure 10)**.

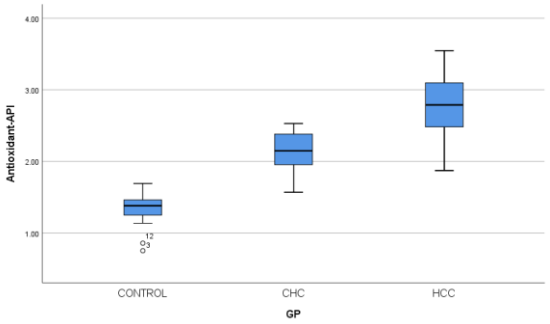


Figure 10 Medians of Antioxidants-API score in CHC and HCC in patients compared with that of healthy control.

Diagnostic performance of the Antioxidants-API score comparing CHC patients with control group

By comparing the CHC patients with that of the healthy control individuals, the Antioxidants-API score gave AUC of 0.987 (cut-off value of >1.69), Sn 90.0 % and Sp 100.0%. The PPV and NPV were 100.0% and 100.0 %, respectively with P<0.0001 (T**able 16 and Figure 11a)**.

Diagnostic performance of the antioxidant diagnostic score comparing HCC patients with CHC ones.

By comparing the cirrhotic patients with HCC with that of CHC ones, the Antioxidants-API score gave AUC of 0.910 (optimal cut-off value of >2.2), Sn 92.73% and Sp 70.0%. The PPV and NPV were 89.5% and 77.8 %, respectively with P<0.0001. (**Table 17 and Figure 11b).**

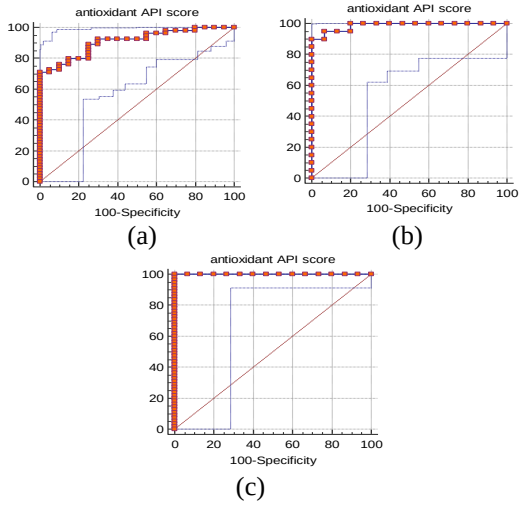


Figure 11. The ROC curve of HCC score (a: between the CHC cases and the healthy control ones, b: In the cirrhotic patients (CHC group and HCC group), and c: among the HCC cases and the healthy control ones).

By comparing HCC patients with healthy control group, the Antioxidants-API score gave AUC of 1.0, (optimal cut-off value of >1.69), with more accurate Sn 100.0 and Sp 100.0%. The PPV was 100.0% and that NPV was 100.0 % with P<0.0001 (**Table 18 and Figure 11c)**.

Table 16. The diagnostic performance of the Antioxidants-API score to differentiate between CHC cases and healthy controls using ROC curve analysis.

Group	**Discriminating CHC cases versus healthy control individuals**						
Parameters	Cut-off	AUC	Sn	Sp	PPV	NPV	P value
Antioxidants-API score	>1.69	0.987 (0.88 -1.00)	90.0 (68.3 - 98.8)	100.0 (78.2 - 100.0)	100.0 (81.5 - 100.0)	88.2 (63.6 - 98.5)	<0.0001

Data for Sn, Sp, PPV and NPV are presented as % (95% CI)

Table17. The diagnostic performance of the Antioxidants-API to differentiate between CHC and HCC cases using ROC curve analysis.

Group	**Discriminating HCC cases versus CHC ones**						
Parameters	Cut-off	AUC	Sn	Sp	PPV	NPV	P value
Antioxidants-API score	>2.2	0.910 (0.82 - 0.96)	92.73 (82.4 - 98.0)	70.00 (45.7 - 88.1)	89.5 (45.7 - 88.1)	77.8 (52.4 - 93.6)	<0.0001

Data for Sn, Sp, PPV and NPV are presented as % (95% CI)

Table18. The diagnostic performance of the Antioxidants-API score to differentiate between HCC patients and healthy control individuals using ROC curve analysis.

Group	**Discriminating HCC cases versus healthy control individuals**						
Parameters	Cut-off	AUC	Sn	Sp	PPV	NPV	P value
Antioxidants-API score	>1.69	1.0 (0.95- 1.00)	100.0 (93.5-100.0)	100.0 (78.2 -100.0)	100.0 (93.5-100.0)	100.0 78.2 - 100.0	<0.0001

Data for Sn, Sp, PPV and NPV are presented as % (95% CI)

Derivation of AAI-antioxidants-diagnostic score

The detection using a combination of the biomarkers aimed to increase the accuracy of the variables for predicting HCC. Based on, there is a week correlation among antioxidant

biomarkers with the single routine ones. They are not related and identified as independent predictive variables, which means that there is no redundancy and that they explore different biochemical abnormalities associated with the conditions of liver disease (HCC and CHC). It is expected to increase the diagnostic accuracy if multiple markers are used for detection of HCC.

For enhancing the diagnostic power of the AAI-antioxidants-diagnostic score, the significant variables in univariate analysis were inserted in a stepwise linear regression analysis with consequent development of a novel non-invasive model. It demonstrated that only AST, albumin and INR from the routine laboratory analysis retained significance when combined with those three antioxidant biomarkers. Under this analysis, a novel non-invasive was developed that consisted of incorporating the most discriminatory six-marker for improving early HCC detection by adequately distinguishing HCC patients from those of CHC ones or healthy control individuals. The model is illustrated as follows:

$$AAI - \text{antioxidants diagnostic} = 1.125 \times INR - \{0.005 \times \text{CAT}\ (mU/mg)\} - \{0.273 \times albumin(g/dl)\} + \{0.006 \times AST(IU/L)\} + \{0.353 \times TAC(mmole/L\} + \{0.009 \times SOD(\%)\} + 0.777$$

The statistical analysis of the AAI-antioxidants diagnostic score in the three studied groups was carried out and the results were summarized in **table 19.**

Table 19. The statically analysis for the AAI-antioxidants diagnostic score among the studied groups.

Groups / **Parameters**	**Control group (n = 15)**	**CHC group (n = 23)**	**HCC group (n = 56)**	**p-value**
AAI-antioxidants-diagnostic				
Mean ±S.D.	1.32±0.263	2.07±0.313	2.963±0.4305	
Median (Range)	1.33 (1.27 - 1.47)	2.08 (1.92-2.22)	2.9 (2.82 -3.01)	P<0.0001

n: total number, P: statistical significant when the results of CHC and HCC where compared with those of the control values using the Mann–Whitney test.

The median and mean numerical values of AAI-antioxidants diagnostic score for CHC and HCC patients were significantly increased when compared with the corresponding control values (P<0.0001). The median and mean± SD were (2.08 and 2.07 ± 0.313), (2.9 and 2.963±0.4305) and (1.33 and 1.32±0.263), respectively **(Figure 12).**

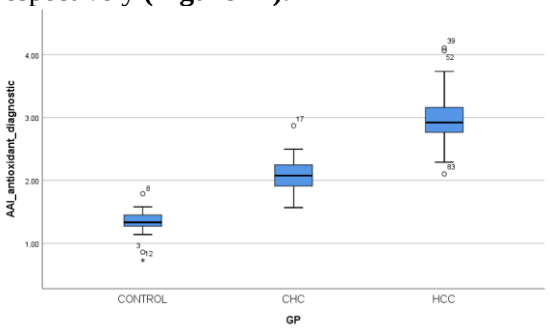


Figure 12. Medians of AAI-antioxidants-diagnostic score CHC and HCC patients compared with that of the healthy control group.

Diagnostic performance of the AAI-antioxidants diagnostic score comparing CHC patients with control group

By comparing CHC patients with healthy control group, the AAI-antioxidants diagnostic score gave AUC of 0.983 (optimal cut-off value of >1.52), with more accurate Sn 100.0% and Sp 86.67%. The PPV and NPV were 90.9% and 100.0%, respectively with P<0.0001 **(Table 20 and Figure 13 a)**.

Table 20. The diagnostic performance of the AAI-antioxidants diagnostic score to differentiate between CHC cases and the healthy controls using ROC curve analysis.

	Discriminating CHC cases versus healthy controls			**Groups**		
Parameters	Cut-off	AUC	Sn	Sp	PPV	NPV	P value
AAI-antioxidants diagnostic	>1.52	0.983 (0.87-1.00)	100.0 (83.2 - 100.0)	86.67 (83.2 - 100.0)	90.9 (70.8 - 98.9)	100.0 (75.3 - 100.0)	<0.0001

Data for Sn, Sp, PPV and NPV are presented as % (95% CI)

Diagnostic performance of the AAI-antioxidants diagnostic score comparing CHC patients with HCC ones.

In the patients' groups (CHC and HCC), the

AAI-antioxidants diagnostic score gave AUC of 0.957, (optimal cut-off value of >2.273), with more accurate Sn 98.18% and Sp 85.0%. The PPV and NPV were 94.7% and 94.4%, respectively with P<0.0001 (**Table 21 and Figure 13 b).**

Table 21. The diagnostic performance of the AAI-antioxidants diagnostic score to differentiate between HCC and CHC cases using ROC curve analysis.

	Discriminating HCC cases versus CHC ones Group						
Parameters	Cut-off	AUC	Sn	Sp	PPV	NPV	P value
AAI-antioxidantsdiagnostic	>2.273	0.957 (0.88 - 0.99)	98.18 (90.3 - 100.0)	85.0 (62.1 - 96.8)	94.7 (85.4 - 98.9)	94.4 (72.7 - 99.9)	<0.0001

Data for Sn, Sp, PPV and NPV are presented as % (95% CI)

Diagnostic performance of the AAI-antioxidants diagnostic score comparing HCC patients with control group

By comparing HCC patients with healthy control group, the AAI-antioxidants diagnostic score gave AUC of 0.98, (cut-off value of >1.86), with more accurate Sn 92.98% and Sp 100%. The PPV and NPV were 100.0% and 78.9 %, respectively with P<0.0001. (**Table 22 and Figure 13 c).**

Table 22. The diagnostic performance of the AAI-antioxidants diagnostic score to differentiate between HCC patients and healthy control individuals using ROC curve analysis.

Group	**Discriminating HCC cases versus healthy control individuals**					
Parameters	Cut off	AUC	Sn	Sp	PPV	NPV	P value
AAI-antioxidants-diagnostic	>1.7887	1.0 (0.949 to 1.000)	100.0 (93.5 - 100.0)	100.0 (78.2 - 100.0)	100.0 (93.4 - 100.0)	93.7 (69.8 - 99.8)	<0.0001

Data for Sn, Sp, AUC, PPV and NPV are presented as % (95% CI)

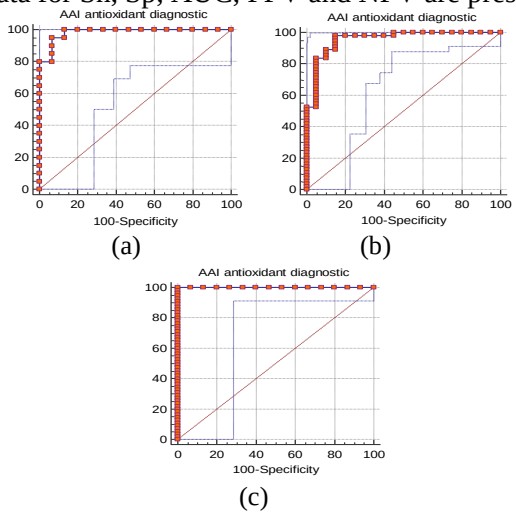


Figure 13 The ROC curve of AAI-antioxidants diagnostic score (a: between the CHC cases and the healthy control ones, b: In the cirrhotic patients (CHC group and HCC group), and c: among the HCC cases and the healthy control ones)

Discussion

Hepatitis C virus (HCV) is one of the etiological agent accounting for chronic liver disease in approximately 2–3% of the population worldwide. HCV infection often leads to liver fibrosis and cirrhosis. Also, various metabolic alterations including steatosis, insulin resistance and iron overload can help to develop HCC or other liver disorders **(Roehlen *et al*., 2020)**. There are multiple molecular mechanisms that trigger the emergence and development of each of these pathogenic. One of these involves marked induction of ROS in infected or damaged cells. The latter induce oxidative stress (**Ivanov *et al*., 2013)**.This is why markers of oxidative stress were included in this study.

HCV infection leads to ROS over production, in blood cells as well as liver parenchymal cells, thus induce oxidative stress in these cells. Almost all HCV proteins are included; core **(Ivanov *et al*., 2011 and Pal *et al*., 2010),** E1 **(Ivanov *et al*., 2011)**, E2 **(Ivanov *et al*., 2011 and Ming-Ju *et al*., 2011)**, NS3/4A **(Pal *et al*., 2010)**, NS4B (**Ivanov *et al*., 2011 and Li *et al*., 2009)**, NS5A **(Ivanov *et al*., 2011; Pal *et al*., 2010 and Garcia-Mediavilla *et al*., 2005).** In this regard, the core is the strongest regulator **(Ivanov *et al*., 2011 and Pal *et al*., 2010).** On the other hand, NS5A early induces ROS and reactive nitrogen species (RNS) **(Garcia-Mediavilla *et al*., 2005). Wang *et al.* (2010)** assigned that ROS production often result in cellular apoptosis. Thus, replication of HCV or its expression of its core protein can produce excessive ROS **(Piccoli *et al*., 2007).** In our opinion, the mechanism may involve inhibition of electron transport complex I activity as was previously reported by **Ando *et al*. (2008) and**

Wang *et al*. (2010).

Oxidative stress, is mainly reflects the imbalance between the rate of production of (ROS); or reactive nitrogen species and the rate of their elimination via antioxidants. This imbalance has been implicated in the pathogenesis of liver fibrosis; and thence in HCC **(Morry *et al*., 2017).** In fibrosis, ROS promote activation and proliferation of fibroblasts and myofibroblasts. This activation promote transforming growth factor beta (TGF-beta) pathway in an auotocrine manner **(Morry *et al*., 2017)**. TGF-β, plays a central role in the progression of fibrosis. Evidences indicate that the ROS exegravate TGF-β's signaling. As an auotocrine mechanism, TGF-β1 re-elevate the ROS production through mitochondrial impairment and NADPH oxidases induction. This may be the case in the present study. TGF-β also suppresses glutathione (GSH) and some antioxidant enzymes. These lead to oxidative stress and/or redox imbalance. The latter's, in turn induces TGF-β1 and mediates its fibrogenic effects **(Liu and Desai, 2015).** Unfortunately, the estimation of TGF- B was not carried out in this study but the reduction in the activity of blood and plasma catalase of HCV carrying individuals; CHC and HCC may lead one to accept the predominance of the latter mechanisms.

In the present study, the activity of SOD; as superoxide anion consumer, was increased in the blood plasma of HCC patients **(Table 3).** This dismutation is H_2O_2 travail **(Zelen *et al*., 2010).** Under the significant reduction in catalase activity in the plasma of HCC patients than that of the control, H_2O_2 can further degraded into the more reactive ROS; namely, hydroxyl radicals. In this way, both increased ROS production and decreased anti-oxidative defense are working in accord with inflammation to produce cell damage and hepatic genome instability **(Toson *et al*., 2016)**. Thus, the oxidative stress is exegravated and subsequently increase the genomic disturbance, apoptosis inhibition, proliferation as well as angiogenesis. All together are predisposers for HCC. These may be the case in the present study. These results confirm the results reported by others; including **Morry *et al.* (2017),** who reported that ROS mediate tumor cell invasion via invadopia formation and extravasation of the tumor cells into a distant sites. Unfortunately, metastatic studies are not included in the present study.

The elevation of SOD activity in plasma of HCC patients are in accordance with **Tamai *et* al. (2011)** who found that the serum MnSOD levels were significantly higher in patients with HCV-related HCC than that in patients without HCC. If this is the case, such elevation in SOD may reflect a state of excessive radicals' loads. This load can partially be counteracted via the elevated activity of SOD. The remaining free radicals are forced to be involved in a state of more oxidative stress followed by tumor overprogression of CHC into HCC. Somewhat similar results were reported by **Tamai *et al.* (2011)** in early HCC patients.

Catalase expression in HCC is decreased. A loss in catalase activity during HCC formation was found to be associated with the process of tumor formation, progression and finally metastasis. This is because the left H_2O_2 and/ or the formed hydroxyl radicals contribute to tumor characteristics via promoting either DNA damage and/ or alteration in cell signaling pathways. Further, ROS are involved in tumor metastasis. This mechanism involves a complex process including epithelial-to mesenchymal transition, angiogenesis, migration, and invasion either within the tumor microenvironment or outside**(Min *et al.*, 2010).** This reduction in catalase activity in plasma of HCC patients are in compatible with the result of **Ansari *et al*. (2015)** who demonstrated the decrease in the total antioxidative capacity. In view of catalase liver tissues are valuable to counteract oxidative stress or persistent of the chronic tissue damage **(Ansari *et al*., 2015).**

In the same pattern, TAC level of HCC patients was increased significantly when their levels were compared to the corresponding values of the healthy individuals ($P< 0.0001$). While, there was no significant difference in such level when compared with that of CHC. This lead one to suggest that the CHC and early HCC patients respond to the excessive increase in free radicals by increasing the serum level of the total antioxidant activity. These results confirm the results reported by others; including **Pomacu *et al.* (2021)**. These authors reported significant increases in serum markers of oxidative stress. All together showed that, oxidative stress is a continuous contributors of the pathogenic mechanisms in all stages of liver disease irrespective of their initial cause. **Pomacu *et al.* (2021)** reported that, the increase in the synthesis of thiobarbituric acid reactive substances (TBARS) and carbonylated protein

(PCARB) may be due to an imbalance between different agents viral; HCV and HBV or non-viral e.g ethanol. The imbalance between antioxidants and oxidants can trigger oxidative changes and reduce the body's ability to scavenge both ROS and RNS. Thus, the elevations in the free radicles load may be the causative factors of TAC elevation in sera of patients of the present study compared with that of the healthy controls **(P<0.0001 and Table 3)**. Involvement of oxidative stress in patients with liver diseases has been investigated. The impact of both ROS and RNS in the liver diseases pathogenesis has been reported **(Pomacu *et al.*, 2021).**

Savu *et al.*, (2012) demonstrated that, plasma TAC was elevated; despite the high levels of oxidative stress, in patients with uncomplicated type2 diabetes compared to that of the control subjects. Also, **Petelin *et al.* (2017)** found significantly higher levels of TAC in overweight subjects when compared with its level in subjects with normal weight. Therefore, **Petelin *et al.* (2017)** concluded that, the elevation of TAC in obese adults act as a compensatory response to the expected oxidative stress as a result generation of ROS and/or RNS.

Farhangi and Vajdi, (2020) demonstrated that in meta-analysis of prospective studies highest category of TAC was associated with reduced incidence of HCC. This study was a limited study as it used meta-analysis which included the results of observational studies which makes the causal inference impossible **(Farhangi and Vajdi, 2020).**

Biomarkers for surveillance, diagnosis and prediction of prognosis in patients with HCC are currently not ready for introduction into clinical practice. The cause is their limited sensitivities and specificities; especially for the early detection of small HCC. In this regard, novel biomarkers are needed to improve the current effectiveness **(Schütte *et al.*, 2015).** In the present study the individual capabilities of SOD, catalase and TAC to discriminate between the healthy controls and CHC patients as well as between the healthy controls and HCC patients were tested. The obtained AUCs were as follow: 0.796 (P<0.0001), 0.663 (P<0.08) and 1.0 (P<0.0001) and 0.941 (P<0.0001), 0.759 (P<0.0004) and 0.972 (P<0.0001), respectively. Further, their AUCs to discriminate between CHC and HCC were as follow: 0.895 (P<0.0001), 0.604 (P<0.2) and 0.601 (P<0.17).

Since a single biomarker "all-in-one" that fits all-surveillance, diagnosis, and/or prognosis has not been effectively found, a stepwise regression analysis was performed. In this regard combination between the three antioxidant biomarkers yielded a biomarker panel called Antioxidants diagnostic score.

$$Antioxidants\ diagnostic = \{SOD(\%) \times 0.015\} + \{TAC(mmole/L) \times 0.413\} - \{Catalase(mU/mg) \times 0.007\} + 0.671$$

Its ability to discriminate between the healthy individuals and patients with CHC as well as between the healthy controls and HCC patients was tested. The obtained AUCs were 0.98 (P<0.0001) and 0.837 (P<0.0001), respectively. This score was found to be significantly modify the discriminating power, the Sn and the Sp were (95.0%, 93.33%) and (89.47%, 50.0%), respectively. Further, its ability to discriminate between CHC and HCC was evaluated. In this regard, the AUC was 0.980 (P<0.0001). This value was with much higher sensitivity and specificity i.e 92.98% and 100.0% %, respectively when these values were compared with the individual values of these biomarkers **(Tables 9-11).**

Combination of non-invasive markers with each others is the future target. Such combination can include imaging and clinical parameters. Molecular profiling of tumorized and non-tumorized liver tissues may allow one to predict the prognosis for an individual patient to survive, thus, hopefully clear the way for individual treatment approaches. The calculated non-invasive markers evaluate liver fibrosis; namely API, APRI and FIB-4 were found to be significantly elevated in HCC compared to either CHC patients or healthy control group. The results of these markers are supported by **Sato *et al.* (2016).** These authors found higher values of both APRI and FIB-4 in patients with HCC when their values were compared to those with HCV but without HCC **(Sato *et al.*, 2016)**. Also, **Poynard and Bedossa (1997)** confessed that API index enabled accurate prediction of the presence of fibrotic activity in patients having HCV which come with the result of study as API index. In the present study, the mathematical values of API were significantly increased in patients with CHC if compared with that of the healthy individuals. Further, several studies showed that APRI could predict

the presence of hepatic carcinogenesis risk in chronic HCV patients; even after the viral eradication **(Ji *et al.*, 2017)**. However, **Nishikawa *et al.* (2017)** found that higher FIB-4 but not APRI can predict liver carcinogenesis after chronic hepatitis B patients treatment; if any.

In the present study, stepwise regression analysis was performed to combine the result of the three antioxidant biomarkers and those of the indirect fibrotic scores. Only API index; which include age and platelets in its formula, showed statistically significant difference and their mathematical values enhance their diagnostic performance; including its AUCs. The results of the present study showed that the combination of the three antioxidant markers with that of API index developed a score; named, Antioxidants-API score.

$$\begin{aligned}\text{Antioxidants} - \text{API} &= \{SOD(\%) \times 0.011\} \\ &+ \{TAC(mmole/L) \times 0.377\} \\ &- \{\text{CAT}(mU/mg) \times 0.006\} \\ &+ \{API \times 0.811\} + 0.650\end{aligned}$$

This score can discriminate between the healthy controls and that of CHC patients as well as between the healthy individuals and HCC patients. The AUCs of this score were: 0.987 (P<0.0001) and 1.0 (P<0.0001), respectively. Its sensitivities and specificities were 90.0%, 100.0% and 100.0%, 100.0%, respectively. Further, its ability to discriminate between CHC and HCC was tested and AUC was 0.91 (P <0.0001) with Sn of 92.73% and Sp of 70.0% **(Tables 16-18).** Thus, antioxidants-API score enhances the activities of the antioxidants to be more precisely predict patients with early HCC among control individuals or CHC among healthy persons **(Tables 10-12)**. The tests are convenient and inexpensive, and will be valuable addition to current options for the diagnosis of early HCC.

Platelets, as key factors in HCC, play a role in carcinogenesis recently, their count gained greater evidence. **Pavlovic *et al*. (2019)** added that, platelets promote proliferation of HCC and their invasion. In fact their involvement didn't directly effect on tumor cells but they play a role in pro-fibrinogenic signaling as well as in hepatic immune response. Therefore, anti-platelet therapy plays a role in the amelioration of liver injury and improve their outcome. However, platelets play a significant role in liver regeneration after organ damage **(Pavlovic *et al.*, 2019).** In the present study, platelets count was significantly decreased in the blood of HCC patients compared to that of CHC ones or healthy individuals **(P<0.0001, Table 2)** which comes with **Pavlovic *et al*. (2019).**

In the present study, the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were elevated in sera of HCC patients and CHC ones compared to those of the healthy control (P<0.001). These results are expected as these patients are viral hepatitis positive. These infections mediate hepatic disorders **(Table 2).** A somewhat similar results were reported by **Saad *et al*. (2017)** and **Toson *et al.* (2014).** Our findings also confirm those of **Sheta *et al.* (2021)** who demonstrated that there were significant increases in the activities of both ALT and AST among hepatocellular carcinoma patients versus those of cirrhotic patients. Therefore, their activities must be taken into our consideration as index of liver injury **(Saad, 2014)**. The presence of the ALT enzyme is restricted to the hepatocytes and to the renal tubular epithelium. In contrast, the highest activity of AST is in the heart tissue, liver HCC and kidney (**Rigato *et al.*, 2007**). AST inside the liver cells is localized mainly in the mitochondrial membrane (80%). Thus elevation of AST may reflect damage to several other organs while elevation of ALT is specific for hepatocellular injury **(Lala *et al.*, 2021).** Further, hepatic tumors secrete more AST than that of ALT **(Giannini *et al.*, 2005).**

Blood coagulation disorders are commonly found in the blood plasma of patients having cancer **(Zucker *et al.*, 2012).** Prothrombin time (PT) is the most frequently used coagulation test in routine laboratories as a measure of the extrinsic pathway International normalize ratio (INR), PT results variation among laboratories, has been used to standardize PT value in liver diseases. Also, its results has been included in several prognostic models of HCC as well as liver cirrhosis **(Zhang *et al.*, 2013).** In the present study, the levels of INR were elevated significantly in the plasma of HCC patients if compared with those of CHC ones or healthy control individuals **(P<0.0001, Table 2).** A somewhat similar results were reported by **Qamar *et al*. (2018)** who demonstrated that patients with advanced liver disease, particularly with a baseline elevation in INR, were considered to have an increased risk of bleeding and a low risk of thrombosis. However, recent data have contradicted the old dogma of cirrhotic "auto-anticoagulation" and

have recognized an increased prevalence of thrombotic complications in these patients **(Qamar *et al*., 2018).**

Albumin is the most important circulating protein found in plasma **(Moman *et al.*, 2021).** It is synthesized in the liver, which produce approximately 10 grams per day. **Lala *et al.* (2021)** relate albumin fall in serum to the decrease in the synthetic function of the liver, if any. Kidney damage is an exception. The results of the present study showed significant reduction of serum albumin levels in sera of HCC individuals with liver cirrhosis when compared to those of CHC or control individuals **(P<0.001, table 20).** These results consisted with those of **Gupta *et al.* (2009)** and **Lis *et al.* (2003)** who demonstrated that low levels of serum albumin are associated with poor outcome in cancer patients. Our findings also confirm those which were reported by **Bernardi *et al*. (2020)** who demonstrated that serum albumin in decompensated cirrhosis not only undergoes structural abnormalities but also functional ones. It is well known that albumin bind with bilirubin at its LYS 240 **(Jacobsen, 1978).** Such albumin-bound bilirubin was shown to act as an inhibitor of lipid peroxidation. Thus, the decrease in serum albumin of our patients can reduce the level of albumin -bound bilirubin of total antioxidants, and thus, activate lipid peroxidation. The latter can indirectly reduce the level **(Neuzil and Stocker, 1993).**Therefore, these authors added that both quantitative (hypoalbuminaemia) and the latter qualitative changes, the amount of the effectively circulating albumin can be dramatically reduced **(Bernardi *et al*., 2020).**

In the present study, the level of serum albumin was significantly reduced in blood sera of HCC patients compared to those of CHC or control individuals **(P<0.001, table 2)**. These results confirm those of **Bernardi *et al*. (2020)** who demonstrated that serum albumin in sera of decompensated cirrhosis undergoes structural, functional abnormalities or both. These endanger its non-oncotic properties such as antioxidant, scavenging, immune modulating and endothelium protection. As a result, due to hypoalbuminaemia and albumin qualitative changes, the amount of circulating 'effective' albumin can be dramatically reduced **(Bernardi *et al*., 2020)**.

Since liver enzymes, INR and serum albumin were correlated with the expected damage if the liver via HCV infection and hepatocarcinogenesis. Their values were tested to be combined with the mathematical values of the three antioxidant markers. The obtained score was named AAI- antioxidants diagnostic score.

$$AAI - \text{antioxidants diagnostic} = 1.125 \times INR - \{0.005 \times \text{CAT}\ (mU/mg)\} - \{0.273 \times albumin(g/dl)\} + \{0.006 \times AST(IU/L)\} + \{0.353 \times TAC(\frac{mmole}{L})\} + \{0.009 \times SOD(\%)\} + 0.777$$

AAI-antioxidants diagnostic score was found to discriminate between the healthy controls and CHC patients as well as between the healthy controls and HCC patients. The obtained AUCs were as follow: 0.983 (P <0.0001) and 1.0 (P<0.0001), respectively. This score can significantly improve the individual discriminating powe with much higher sensitivities and specificities as follow (100.0%, 86.67%) and (100.0%, 100.0%), respectively. Further, its capability to discriminate between CHC and HCC patients was as follow (P <0.0001) was with much higher sensitivity 98.18% and specificity 85.0%, and with an AUC of 0.980. These values were higher than any of the biomarkers alone **(Tables 20-22).** It enhanced their activities to more precisely predict patients with early HCC among those with control individuals or CHC with healthy controls than being combined alone or in the antioxidant diagnostic score **(Tables 16-18).** Additionally, this score was better than that of Antioxidants-API score in differentiating HCC patients from CHC ones. The included parameters are inexpensive convenient, and will be of valuable addition to the current diagnostic options used for HCC diagnosis.

ROS accounts correlated with genomic instability to apoptotic resistance, angiogenesis and proliferation in HCC **(Morry *et al*., 2017).** This actually may be the case in the present study. This is because angiogenesis is a pre-request for tumor growth. Since tumor is nutrients consuming site; a phenomenon which require excessive blood supply **(Nowak-Sliwinska *et al.*, 2018).** Further, proliferation is a criterion of the tumor cells formation irrespective of their origin or sites supply **(Welch and Hurst, 2019)**. Thus, ROS can act as proliferators under the presence of excessive viral load **(Paiva and Bozza, 2014).**

Since the origin of HCC in our patients may be HCV-based, one can expect the involvement of the process of oxidative stress in the hepatocarcinogenesis in our CHC patients. Since ROS is a good mediator of tumorgenesis, one can expect tumor overgrowth whenever these are available. Further, apoptotic resistance is an additive criterion of hepatocarcinogenesis **(Paiva and Bozza, 2014)**.

Conclusion

During the deduction of the results of the present study, an imbalance between the ROS production and anti-oxidative defense system in CHC patients and CHC-mediated HCC ones were reported. These results indicated that the oxidative stress may have a profound influence on liver disease progression with a final upregulation of HCC. Therefore, the combination between the three studied antioxidants (Antioxidants-diagnostic) score, or combined with API (Antioxidants-API score), or with the routine liver functions (AAI-antioxidants diagnostic) score may add more to the reduction of using the invasive liver biopsy. Also, they may be valuable in diagnosis of liver malfunction in the future; especially in CHC-mediated HCC patients, which is actually the aim of the present study.
Future studies should use larger sample sizes. As a result, more accurate sensitivity and specificity will be obtained.

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الملخص العربي

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يعد ارتفاع معدل فيروس الاتهاب الكبدى الوبائى سى (HCV) فى مصر السبب الرئيسي لانتشار مرض السرطان الكبدي الخلوى (Hepatocellular Carcinoma = HCC) . لذلك تم تجربة العديد من الطرق للتنبؤ به ولكن لايزال هناك حاجة إلى المزيد. فى هذا الصدد، تم تجربة القدرة التنبؤية لنشاط انزيم سوبر اكسيد الديسميوتيز (SOD) ومستوى نشاط انزيم الكاتاليز (catalase). وكذلك مستوى القدرة الكلية لمضادات الأكسدة (TAC) فى مصل الدم لدى المرضى المصابين بالسرطان الكبدي الخلوى الناشئ عن الاتهاب الكبد الوبائى سى. **المرضى والطرق المستخدمة** تضمنت هذه الدراسة ٧٩ مريضا منهم ٢٣ مريضا من مرضى الاتهاب الكبد الوبائى سى (CHC) فى حالته الأولية لم يتلقى اى من المرضى العلاج المضاد للفيروسات و ٥٦ مريضا بالسرطان الكبدي الخلوى الناشئ عن الاتهاب الكبد الوبائى سى (HCC based HCV) لتقدير المواد المضادة للأكسدة وكذلك الدلالات الروتينية. بالإضافة إلى ١٥ متطوعاً أصحاء تم إدراجهم بالدراسة للمقارنة. **النتائج** ارتفع مستوى نشاط انزيم سوبر اكسيد الديسميوتيز (SOD) وكذلك مستوى القدرة الكلية لمضادات الأكسدة (TAC) بشكل ملحوظ في مصل مرضى HCC وكان الارتباط ايجابيا (r=0.334،P <0.0001) عند مقارنتهما بقيم CHC أو مجموعة الأصحاء (P<0.0001). على العكس من ذلك، انخفض مستوى نشاط الكاتاليز (catalase) بشكل ملحوظ (P<0.0001) مع ارتباط سلبي مع نشاط انزيم سوبر اكسيد الديسميوتيز (SOD) (r= -0.146 ،P<0.04) . وأسفر تحليل الانحدار اللوجستي المتدرج للعلامات الثلاث الأخيرة عن معادلة سميت "مضادات الأكسدة - التشخيصية" والتي زادت من قيم المساحة تحت المنحنى (AUC) وأنشطتها التشخيصية (P<0.0001). من خلال الجمع بين قيم الدلالات الثلاث السابقة مع مؤشر الصفائح الدموية (API) اسمرت عن معادلة جديدة سميت "مضادات الأكسدة -API". أيضا، من خلال الجمع بينها وبين الدلالات الروتينية أنتجت معادلة سميت "مضادات الأكسدة AAI". في كل حالة تم تعزيز قيم AUC بالمقارنة مع مضادات الأكسدة الأصلية (P<0.0001). **الاستنتاج** إن تقييم هذه الأنشطة المضادة للأكسدة وتجميعها في المعادلات الثلاث الأخيرة يمكن أن يعزز من قدرتها للتنبؤ بشكل أكثر دقة بمرضى مرضى الاتهاب الكبد الوبائى سى(CHC) و السرطان الكبدي الخلوى الناشئ عن الاتهاب الكبد الوبائى سى (HCC) والتميز بينهم وبين مجموعة الأصحاء.