Serum oxidative-anti-oxidative stress balance is dysregulated in patients with hepatitis C virus-related hepatocellular carcinoma

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Running title: Oxidative stress balance in chronic hepatitis C

Abstract

Aim: Oxidative stress is associated with progression of chronic liver disease (CLD). This association is best established in chronic hepatitis C. However, the anti-oxidative state is not well characterized. The objective of the present study was to investigate the balance of oxidative and anti-oxidative stress in CLD patients.

Methods: We recruited a study population of **208** patients, including healthy volunteers (HV; n = 15), patients with hepatitis B virus (HBV)-related CLD without or with hepatocellular carcinoma (HBV-nonHCC, n = **25**, and HBV-HCC, n = **50**, respectively), and patients with hepatitis C virus (HCV)-related CLD without or with HCC (HCV-nonHCC, n = **49**, and HCV-HCC, n = **69**, respectively). Serum levels of reactive oxygen metabolites (ROM) and anti-oxidative markers (OXY-adsorbent test; OXY) were determined, and the balance of these values was used as the oxidative index. Correlations among ROM, OXY, oxidative index, and clinical characteristics were investigated.

Results: Patients with CLD exhibited elevated ROM and oxidative index compared to HV. Among patients with CLD, HCV-positive status correlated with increased ROM. **In CLD, HCV-HCC patients exhibited the highest ROM levels. Among HCV-related CLD patients, lower OXY correlated with HCC-positive status, but was recovered**

by eradication of HCC. In HCV-HCC, lower OXY correlated with high PT-INR. Conclusions: HCV-positive CLD patients displayed higher oxidative stress and HCV-HCC patients displayed lower anti-oxidative state. Anti-oxidative state depression was associated with liver reservoir-related data in HCV-HCC and could be reversed with HCC eradication.

Key words: antioxidant, chronic hepatitis C, hepatocellular carcinoma, oxidative stress

Introduction

Oxidative stress is increasingly recognized as a key factor in the progression of chronic liver disease (CLD). CLD usually results from hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, alcoholic liver disease, autoimmune hepatitis, primary biliary cirrhosis, and non-alcoholic steatohepatitis. Of these, chronic hepatitis C has been noted as a source of strong oxidative stress[1], resulting in a high rate of hepatocarcinogenesis among patients with cirrhosis[2]. The liver, a metabolically important organ, is a major reservoir of the mitochondria that serves as the source of reactive oxygen species (ROS). As a result, CLD could represent a major inducer of oxidative stress. In addition, HCV itself induces oxidative stress on hepatocytes[3, 4]. HCV-induced oxidative stress suppresses production of hepcidin, a negative regulator of iron absorption, thereby resulting in iron deposition. Chronic hepatitis C patients exhibit iron overload in the liver, and the accumulation of iron contributes to increased DNA damage and elevated lipid peroxidation[5, 6]. In addition, chronic hepatitis C patients show significant alteration of the mitochondria, and various HCV proteins elicit the generation of mitochondrial ROS *in vitro*[3, 7]. Levels of the oxidative stress marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) are reportedly elevated in the livers of patients with chronic hepatitis C compared to those from patients with other CLDs[2]. The

production of 8-OHdG is particularly elevated in patients with HCV-related hepatocellular carcinoma (HCC)[8]. Together, these *in vitro* and *in vivo* findings suggest the association of enhanced oxidative stress with chronic hepatitis C.

A major endogenous antioxidant produced by cells is glutathione (GSH). The liver is one of the primary sources of GSH. Hepatic and blood GSH levels are reduced in chronic hepatitis C patients, indicating that anti-oxidative status is attenuated in these patients[5]. Several *in vitro* experiments suggest that HCV core protein compromises the antioxidant system in the liver, including heme oxygenese-1 and NADH dehydrogenase quinone 1[9, 10]. However, several other groups have reported that antioxidant levels are increased upon expression of HCV core or polyproteins[11, 12]. These seemingly disparate effects could be due to the status of p53, aging, or the presence of other sources of ROS (e.g., inflammation), leading to a greater level of oxidative stress that overwhelms the antioxidant defenses *in vivo*[13]. Thus, although increased oxidative stress in chronic hepatitis C patients is widely accepted, anti-oxidative conditions in these patients remain unresolved.

The objective of the present study was to investigate the balance between oxidative stress and anti-oxidative stress in patients with chronic viral liver diseases. Serum levels of reactive oxygen metabolites (ROM) were determined as a marker of circulating

ROS[14, 15]. The OXY-adsorbent test was also performed to evaluate the corresponding anti-oxidative status[16]. We investigated possible correlations among ROM and OXY values and the clinical parameters and course of CLD in our patient population.

Methods

Subjects

The study group comprised 5 groups. The first group consisted of 15 healthy volunteers (HV). The second group (HBV-nonHCC) consisted of **25** patients with HBV-related CLD without HCC. The third group (HBV-HCC) consisted of **50** patients with both HBV-related CLD and HCC. The fourth group (HCV-nonHCC) consisted of **49** patients with HCV-related CLD without HCC. The fifth group (HCV-HCC) consisted of **69** patients with HCV-related CLD with HCC. **Twenty HCV-nonHCC patients and 25 HCV-HCC patients were followed for 16 months and 17 months, respectively, with data compared at a second time point after follow-up.** HV consisted of patients with no systemic diseases who were admitted to the Preventive Dentistry Clinic. Members of this group were age-matched with overall CLD patients, since aging is one of the main sources of oxidative stress. Serum levels of ROM and anti-oxidative OXY-adsorbent test (OXY) were determined (see below), and the balance between ROM and OXY was

used to define the oxidative index. Correlations between ROM, OXY, oxidative index, and clinical characteristics were assessed for all patients. All patients were recruited at the Clinic of Gastroenterology and Hepatology, and all HV were recruited at the Preventive Dentistry Clinic, Okayama University Hospital, between August 2008 and December 2010. The study protocols were approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. After obtaining written informed consent, a detailed medical questionnaire was completed by doctors or dentists.

Blood sample collection and preparation

Fasting blood samples were collected from patients and healthy volunteers. Serum was collected at the time of admission or at the outpatient clinic, meaning that no intervention had been performed before specimen collection. If not assayed immediately, serum aliquots were stored at -80°C until subsequent analysis. The obtained samples were used to determine biochemical data, including serum levels of ROM and OXY.

Measurement of ROM serum levels

Measurement of ROM serum levels was performed using a spectrophotometer (Diacron

International, Grosseto, Italy), as reported previously[14]. The measurement unit used was the Carratelli unit (CARR U), where 1 CARR U corresponds to 0.08 mg/dL of hydrogen peroxide.

Measurement of total serum anti-oxidant capacity

In order to determine total serum anti-oxidant capacity, OXY was performed using a spectrophotometer (Diacron International)[16]. This test evaluates the capacity of serum to oppose the massive oxidative action of a hypochlorous acid (HClO) solution. Total anti-oxidant capacity was expressed in terms of HClO (μmol) consumed by 1 mL of sample (μmol HClO/mL).

Calculation of oxidative-anti-oxidative balance

The balance between oxidative stress and anti-oxidative stress was calculated as an oxidative index. To incorporate parameters with differing measurement units, the standardized values of ROM and OXY were assessed using the formula of Vassale et al.[17]: *sv-var* = (*v-var* - *m-var*) / *ds-var*. In this formula, *sv-var* represents the standard value of a certain parameter, *v-var* corresponds to the original value, and *m-var* and *ds-var* represent the mean and standard deviation of the parameter, respectively. The

oxidative index was calculated by subtracting the OXY standardized variable from the ROM standardized variable. The HV group was used as the reference population for calculating the means and standard deviations of ROM and OXY.

Liver biopsy interpretation and immunohistochemistry for oxidative stress-related marker 4-hydroxy-2-nonenal (4-HNE)

Liver histology was available for 44 patients in the HCV-related CLD group. Liver tissues were fixed with 10% formalin and embedded in paraffin. Cross-sections (5-μm thick) were cut and stained using hematoxylin and eosin (HE) and Azan. All liver specimens were assessed by two hepatologists (T.Y. and A.T.) who were blinded to study group allocations. The METAVIR scoring system was used to analyze the stage of liver fibrosis in patients. Two patients had no fibrosis, 19 patients had stage 1 fibrosis, 5 patients had stage 2 fibrosis, 7 patients had stage 3 fibrosis, and 11 patients had stage 4 fibrosis.

For immunohistochemical staining of 4-HNE, sections were incubated with primary antibody against 4-HNE (1:100 dilution; Nikken Seil, Tokyo, Japan) at 4˚C overnight. Following rinsing with phosphate-buffered saline, sections were incubated with biotinylated secondary antibody (LSAB+System-HRP; DAKO,

Carpinteria, CA) for 30 min and the avidin/biotin system for 30 min and visualized using 3,3'-diaminobenzidine (DAB) solution (0.05% DAB and 0.003% hydrogen peroxide in 0.1M PB). After stopping the reaction with de-ionized water, sections were counterstained with hematoxylin. Positive staining was characterized by brownish staining in the cytoplasm of hepatocytes.

Statistical analysis

Statistical analysis was conducted using the JMP version 9.0.0 software (SAS Institute, Carry, NC). Continuous variables were expressed as median values (interquartile range), and the Mann-Whitney *U*-test or chi-squared test was used to compare parameters between HV and CLD groups. The multifactorial Steel-Dwass analysis was used to compare parameters among the HBV-nonHCC, HBV-HCC, HCV-nonHCC, and HCV-HCC groups. Distributions of oxidative stress-related markers in patient groups were compared using the chi-squared test. Follow-up data were compared using the Wilcoxon signed-rank test. Statistical significance was set at $P < 0.05$. Univariate analysis was performed in CLD patients to identify factors potentially correlated with oxidative stress-related markers. Age, which is widely accepted as an oxidative stress factor, and any variables yielding $P < 0.05$ in the univariate analysis were analyzed

further by multivariate analysis to identify independent factors correlated with oxidative stress-related markers.

Results

Baseline characteristics of the groups

The clinical characteristics of the study groups are shown in Table 1. No significant differences between HV and CLD groups were seen with regard to age or body mass index (BMI), although the sex distribution and data for multiple clinical parameters did differ significantly between groups (Table 1A). The clinical characteristics of CLD groups are shown in Table 1B. For further analysis, CLD patients were sorted into subgroups (HBV-nonHCC, HBV-HCC, HCV-nonHCC, and HCV-HCC) depending on viral and HCC status. **HBV-nonHCC group patients were younger than the others, but no age differences were apparent between other groups. Sex distribution and BMI were comparable between groups. In HCC-positive groups (*i.e.*, HBV-HCC and HCV-HCC), platelet counts were lower and PT-INR was longer than in nonHCC groups, suggesting splenomegaly and liver reservoir dysfunction in these patients. Among the 49 HCV-nonHCC patients, four patients with lower albumin levels developed HCC during follow-up after examination of oxidative stress-related markers (median, 39 months; Table 1C).**

The follow-up study of HCV-nonHCC (median interval, 16 months) and HCV-HCC (median interval, 17 months) revealed no change in clinical data over these

periods **(Table 1D)**. **The procedures performed most often for HCC treatments were surgery or radiofrequency ablation therapy (RFA) (15/25). Transarterial chemoembolization (TACE) was performed with epirubicin up to 20 mg/body mixed with lipiodol and arterial embolization with gelatin particles (7/25). Chemotherapies were oral sorafenib administration or 5-fluorouracyl (5FU) plus cisplatin infusion chemotherapy (3/25).** Treatment response was assessed with the Response Evaluation Criteria in Solid Tumors (RECIST). Fifty-two percent **(13/25)** of HCV-HCC patients showed complete response (CR) at the second time point. Only **4% (1/25)** of patients showed partial response (PR) and **24% (6/25)** showed stable disease (SD). **Twenty percent (5/25)** showed progressive disease (PD), including lung metastasis.

Correlation between intrahepatic and serum oxidative stress-related markers

To define the relationship between serum oxidative stress markers and intrahepatic oxidative stress-related conditions, we evaluated the correlation between expression of the intrahepatic oxidative stress marker 4-HNE and serum ROM levels (Fig. 1). Patients with positive intrahepatic 4-HNE expression showed higher serum ROM levels.

Oxidative stress-related markers in CLD patients

Both the oxidative stress marker ROM and calculated oxidative index were significantly higher in patients with CLD than in the HV group; these two groups did not differ significantly in the anti-oxidative stress marker OXY (Fig. 2A). Among CLD patients, ROM differed significantly for **HCV-nonHCC and HCV-HCC** vs. HBV-HCC, OXY differed significantly for HCV-HCC vs. HBV-HCC and HCV-nonHCC, oxidative index differed significantly for HCV-HCC vs. all others (Fig. 2B).

Oxidative stress-related markers and clinical characteristics

To define the impact of oxidative stress-related markers in CLD, correlations between clinical characteristics and oxidative stress-related markers were analyzed (Table 2A). Older (≥**62** years old), HCV-positive and lower serum albumin patients showed higher ROM values in univariate analysis; HCV-positive status was the only factor that exhibited a correlation in multivariate analysis. **Low OXY value, indicating diminished anti-oxidative function, correlated (in univariate analysis) with several factors, including HCC-positive status, reduced ferritin, reduced platelet counts, higher PT-INR and lower albumin. In multivariate analysis, lower albumin status**

was the only factor that correlated with low OXY value. The results of univariate analysis suggested that reduced OXY values were indicative of impaired liver function. The high oxidative index correlated with lower serum albumin in multivariate analysis.

To reveal the correlation between progression of CLD and reduction of OXY value, relationships between clinical parameters indicative of liver reservoir and OXY were determined (Fig. 3). Patients with advanced CLD as histologically proven F4 tended to show lower OXY, although no significant difference was apparent.

Oxidative stress-related markers in HCV-positive CLD patients

Since HCV positivity correlated with oxidative stress-related markers, we next analyzed stratified data in patients with HCV-related CLD (Table 2B). **Among patients with HCV, higher ROM correlated with higher HCV-RNA titer and lower PT-INR. Although multivariate analysis did not reveal any significant correlation, HCV-RNA titer showed the best correlation (*P* = 0.080).** HCC-positive status **correlated significantly with OXY in multivariate analysis**. Several liver reservoir-related parameters, including platelet counts, PT-INR and albumin levels,

demonstrated significant correlations with OXY in univariate analysis.

Oxidative stress-related markers in HCV-positive HCC patients

Since HCC-positive status correlated with OXY in patients with HCV-related CLD, we next analyzed data in HCV-positive HCC patients. In HCC patients, ROM did not correlate with tumor stage, tumor marker levels, or liver function parameters. **Higher HCV-RNA titer was the only factor showing a positive correlation with higher ROM, even in univariate analysis.** Conversely, OXY correlated with **PT-INR levels in multivariate analysis**. Anti-oxidative status thus correlated with liver function parameters even in HCV-positive HCC patients; markers of oxidative stress were elevated at all stages of HCV-positive HCC.

Follow-up of oxidative stress markers in HCV-positive CLD patients

Follow-up data after about one and a half years (median: 16 months for HCV-nonHCC; 17 months for HCV-HCC) were collected from HCV-positive CLD patients **(Fig. 4A).** In HCV-nonHCC, the three oxidative stress-related markers showed no change during follow up. **Three patients received pegylated interferon and ribavirin treatment that resulted in sustained viral eradication. Oxidative stress-related markers**

showed no change in these patients, while two patients showed OXY increase (Fig. 4B). In HCV-HCC, OXY was increased in the **CR group (Fig. 4C,D).**

Follow-up of HCV-nonHCC patients

All HCV-nonHCC group patients were followed for a median of 39 months at our hospital, with four developing HCC. To define whether oxidative stress affects hepatocarcinogenesis, oxidative stress markers were compared between patients who did and did not develop HCC later (Fig. 5). Although only 4 patients developed HCC later and no significant differences were identified, median OXY tended to be lower than that in patients who did not develop HCC later.

Discussion

In the present study, serum ROM reflected the level of intrahepatic oxidative stress and was higher in patients with CLD, particularly in HCV-positive patients with a high titer of HCV-RNA. In contrast, anti-oxidative status OXY was attenuated in HCV-positive HCC patients. **The follow-up data revealed that OXY attenuation in HCV-HCC could be reversed by eradication of HCC.**

ROM is considered to be a reliable indicator of circulating ROS[14, 15]. ROS

reportedly induce progression of HCC[18], inducing the synthesis and activation of a large series of cytokines and growth factors, which in turn lead to malignant transformation[19]. The present results suggest that oxidative stress is increased in **HCV-positive patients, particularly those with a high titer of HCV-RNA.**

This study also showed that serum levels of ROM were elevated in CLD patients compared to control subjects. These observations indicate that CLD, including that resulting from HBV-positive status, induces increases in systemic ROS. This result is consistent with a previous report demonstrating that HBV X protein induced ROS production *in vitro*[20], and with a previous report of increased serum levels of malondialdehyde (an indicator of lipid peroxidation) in HBV-positive patients[21].

In previous work, we demonstrated that HCC patients with periodontitis had increased levels of ROM, and that patients with advanced HCC had severe periodontitis[22]. In contrast, the present results revealed no correlation between ROM and tumor factors or any liver reservoir-related parameter in HCV-positive HCC patients. Presumably, additional factors or conditions (such as periodontitis) that induce ROS contribute to the distinction between early and advanced stages of HCC.

The main pathology of HCV-related disease is considered to reflect the immune reaction to virus-infected hepatocytes. The lack of strong Th1-type helper T cell

response and cytotoxic T cell response against HCV lead to chronic infection by this virus. Strong Th1-type helper T cell response and cytotoxic T cell response correlate with spontaneous recovery and interferon-induced sustained virological response[23]. Recent studies have focused on the direct effects of HCV on hepatocytes. Several studies have uncovered the distribution of viral proteins and revealed viral association with subcellular organelles, such as mitochondria, endoplasmic reticula (ER), and lipid droplets to facilitate replication and assembly of viral particles[24]. HCV core protein is closely associated with the mitochondria and causes increases in ROS, reactive nitrogen species production and lipid peroxidation[25]. HCV NS5A protein is involved in ER stress and disturbance of intracellular Ca^{2+} homeostasis, which leads to increased mitochondrial ROS production[26]. The non-structural proteins encoded by HCV induce strong mitochondrial injury, while production of the HCV full genome-length protein induces moderate mitochondrial injury, representing the cross-action of HCV core and non-structural proteins[27]. HCV-induced oxidative stress is thus implicated in the disease progression of chronic hepatitis. However, oxidative stress is physiologically important to protect cells from viral infection. ROS can rapidly inhibit HCV-RNA replication in HCV replicon-harboring cells[28], and antioxidants such as vitamin E, vitamin C, and coenzyme Q4 reverse oxidative effects and thereby permit HCV replication[29]. Clinical

studies of antioxidant therapies for chronic hepatitis C have yielded disappointing results, with no effects seen on ALT, viral load, or interferon sensitivity[30, 31]. Instead, providing a balance between oxidative stress and anti-oxidative stress might be important in assessing the potential use of antioxidants to treat CLD.

Total serum antioxidant levels, as assessed by OXY, were diminished specifically in patients with HCV-related HCC. This observation was consistent with the high ROM values in these patients. However, markers for liver reservoir function (e.g., albumin, PT-INR) or liver fibrosis (e.g., platelet counts) correlated with OXY in univariate analysis, **and albumin level was the only factor in multivariate analysis.** In HCV-positive patients, **HCC-positive status (*P* = 0.048) correlated with lower OXY in multivariate analysis.** In HCV-positive HCC patients, **higher PT-INR level** was the only factor that correlated with lower antioxidant capacity. Liver reservoir function might be involved in anti-oxidative state. In *in vitro* analysis, an HCV subgenomic replicon has been shown to induce the production of multiple antioxidant enzymes. Despite this effect, the overall redox environment was oxidative, since the oxidative stress induction was strong enough to overcome the antioxidant power[27]. Adequate oxidative-anti-oxidative balance is expected to be important for improved outcomes of chronic hepatitis C.

Stage progression of HCC did not correlate with ROM or OXY. Since our HCV-HCC subjects included mainly patients with early stage HCC (36% stage I, 28% stage II; Table 1B) and because HCV-HCC follow-up subjects included 48% of stage I and 28% of stage II patients (Table 1D), these results must be regarded as an early HCC phenomenon.

Indeed, our results revealed that oxidative index was independently elevated in HCV-positive and HCC-positive CLD patients. The balance of oxidative stress is more oxidative in HCV-positive HCC patients, and antioxidant therapy might be effective for such patients.

The follow-up study revealed that OXY reduction was recovered after complete remission (CR) of HCC. Since our follow-up HCV-HCC group comprised patients with early stage HCC (stage I, 48%; Stage II, 28%) following high CR frequency, these changes are characteristic of early stage HCC with good treatment response. **As the reduced anti-oxidative condition could be recovered by eradication of HCC, the anti-oxidative condition could be a result of HCV-positive HCC. However, relatively lower serum OXY was identified in HCV-nonHCC patients who developed HCC, suggesting a possible mechanism for the anti-oxidative state in hepatocarcinogenesis. To clarify whether oxidative stress is a cause or result of**

HCC, more patients with longer follow-up need to be accumulated.

In conclusion, oxidative stress was higher in HCV-positive patients compared to healthy controls and HBV-positive patients with HCC. Anti-oxidative stress (assessed as OXY) was lowest in HCV-positive HCC patients **and could be recovered by tumor eradication.** This distinction was most apparent in patients with **higher PT-INR,** suggesting that liver reservoir function might be important for maintaining anti-oxidative function. The oxidative-anti-oxidative balance was most biased towards the oxidative state in HCV-positive HCC patients.

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Figure Legends

Figure 1: Immunohistochemical staining of oxidative stress marker 4-HNE in liver biopsy specimens. (A) Negative staining. (B) Positive staining in the hepatocyte cytoplasm is shown by arrows. (C) The positive correlation between intrahepatic oxidative stress and serum oxidative stress state.

Figure 2: The distribution of ROM, OXY, and oxidative index in patient groups. (A) Comparison between HV and CLD: The ROM and oxidative index were higher in CLD patients. (B) Comparison among HBV-nonHCC, HBV-HCC, HCV-nonHCC, and HCV-HCC: Among the CLD groups, the ROM value was significantly higher ($P < 0.05$) **in HCV-nonHCC and HCV-HCC compared to HBV-HCC**. **OXY was significantly lower ($P < 0.05$) in HCV-HCC compared to that in HBV-HCC and HCV-nonHCC. Oxidative index was significantly higher ($P < 0.05$) in HCV-HCC than in other CLD groups.**

HV, healthy volunteers; CLD, chronic liver disease patients; HBV-nonHCC, hepatitis B virus (HBV)-related CLD without hepatocellular carcinoma (HCC); HBV-HCC, HBV-related CLD with HCC; HCV-nonHCC, HCV-related CLD without HCC; HCV-HCC, HCV-related CLD with HCC.

Figure 3: The relationship between OXY and clinical characteristics. (A) Positive correlation between OXY and both platelet count and albumin level. A negative correlation existed between OXY and PT-INR. (B) Serum OXY levels in histologically confirmed chronic hepatitis and liver cirrhosis. Although not significant, OXY was relatively decreased in histologically confirmed liver cirrhosis.

F, METAVIR fibrosis score.

Figure 4: Oxidative stress-related marker changes **after about 1.5 years.** (A) The follow-up data change in HCV-non HCC. No change was seen in this period **(median interval, 16 months)**. **(B) Follow-up data changes in the three HCV-nonHCC patients who eradicated HCV with pegylated interferon + ribavirin therapy in this period. Two of these patients tended to show increased OXY, although not statistically significant. (C) HCV-HCC with a treatment response of CR. OXY level increased after follow-up period (median, 17 months interval). (D) HCV-HCC with treatment response PD+SD+PR. No statistical differences were observed.**

Figure 5. Oxidative stress-related markers in HCV-nonHCC group patients who developed HCC (n = 4) and in those who did not develop HCC (n = 45) during 39 months of follow-up. Although not significant, median OXY of patients who developed HCC was relatively lower than that in patients who did not later develop HCC.

Tables

Table 1A Patient characteristics (healthy control vs chronic viral liver disease)

	HV (N=15)	CLD (N=193)	p
Age	65 (55–71)	62 (56–69)	0.335
Male SEX (%)	5 (33)	126 (63)	**0.021**
BMI	22 (19–23)	22 (20.5–24.5)	0.930
Platelet(x104/mL)	22 (21–26)	13 (8–18)	**<0.001**
PT-INR	0.89 (0.88–0.92)	0.98 (0.95–1.04)	**<0.001**
Total bilirubin (mg/dL)	0.5 (0.4–0.8)	0.8 (0.6–1.1)	**0.001**
Albumin (g/dL)	4.2 (4.0–4.5)	4.1 (3.4–4.3)	0.088
ALT (IU/L)	13 (11–19)	33 (22–49)	**<0.001**
Creatinine (mg/dL)	0.6 (0.6–0.9)	0.7 (0.6–0.8)	0.826
Ferritin (ng/mL)	109 (85–205)	115 (39–240)	0.649

HV; healthy volunteer, CLD; chronic liver disease patients, BMI; body mass index,

PT; prothrombin time, TBil; total bilirubin, ALT; alanine aminotransferase

Table 1B Patient characteristics and oxidative stress markers (Chronic viral liver diseases)

	HBV-nonHCC (N=25)	HBV-HCC (N=50)	HCV-nonHCC (N=49)	HCV-HCC (N=69)
Age	39 (35-60)*	61 (55-68)	62 (59-66)	67 (59-73)
Male SEX (%)	12 (48)	37 (74)	24 (44)	53 (76)
BMI	21 (19-23)	22 (20-24)	22 (20-24)	22 (20-24)
Platelet($x10^4/mm^3$)	19 (16-24)	13 (7-17)*	17 (12-22)	9 (6-12)*
PT-INR	0.94 (0.88-1.00)	1.00 (0.96-1.09)**	0.96 (0.94-1.00)	1.02 (0.96-1.08)**
Total bilirubin (mg/dL)	0.89 (0.65-1.05)	0.93 (0.68-1.19)	0.78 (0.55-1.01)	0.93 (0.62-1.23)
Albumin (g/dL)	4.6 (4.3-4.8)*	4.1 (3.6-4.4)	4.2 (3.8-4.3)	3.4 (3.0-3.9)*
ALT (IU/L)	24 (12-43)	25 (19-38)†	38 (22-65)	37 (27-49)
Ferritin (ng/mL)	202.6 (160.1-575.5)	88.4 (38.7-194.2)	177.5 (73.1-327.4)	101.5 (27.0-197.4)
HCV-RNA (LogIU/mL)	-	-	6.2 (5.2-7.0)	5.9 (4.4-6.5)
AFP (ng/mL)	2.4 (1.9-3.5)	22.2 (3.6-188)**	5.3 (2.4-8.6)	18.0 (7.8-85.3)**
DCP (mAU/mL)	18 (14-23)	32 (20-407)**	21 (17-28)	76 (24-251)**
HCC stage I/II/III/IV	-	20/14/7/9	-	25/19/16/9

* $p<0.05$ vs all

** $p<0.05$ vs HBV-nonHCC, HCV-nonHCC

† $p<0.05$ vs HCV-HCC

AFP; α-fetoprotein, DCP; des-γ-carboxy prothrombin

HBV-non HCC; hepatitis B related CLD without hepatocellular carcinoma (HCC),

HBV-HCC; hepatitis B related CLD with HCC,

HCV-non HCC; hepatitis C related CLD without HCC, HCV-HCC; hepatitis C related CLD with HCC

Table 1C The characteristics of the HCV-nonHCC patients who developed HCC during observation (median 39 months)

	Hepatocarcinogenesis (N=4)	No hepatocarcinogenesis (N=45)	p
Age	64 (57-69)	62 (58-66)	0.545
Male SEX (%)	2 (50)	18 (40)	0.698
Platelet(x104/mL)	12 (9-18)	18 (13-23)	0.093
PT-INR	1.07 (1.00-1.09)	0.96 (0.93-1.00)	0.052
Total bilirubin (mg/dL)	1.1 (0.6-1.9)	0.7 (0.5-0.9)	0.207
Albumin (g/dL)	3.4 (3.1-3.9)	4.2 (4.1-4.3)	**0.007**
ALT (IU/L)	56 (47-65)	33 (21-65)	0.158
Creatinine (mg/dL)	0.64 (0.56-0.72)	0.66 (0.57-0.76)	0.682
Ferritin (ng/mL)	95 (59-176)	132 (61-331)	0.436
HCV-RNA (LogIU/mL)	6.2 (5.7-6.3)	6.2 (5.0-7.0)	0.984
AFP (ng/mL)	6 (5-27)	3 (2-7)	0.147
DCP (mAU/mL)	14 (13-26)	22 (18-28)	0.281

Table 1D Patient characteristics for follow up study (HCV positive patients)

	HCV-nonHCC (N=20)		p	HCV-HCC (N=25)		p
	Time 1	Time 2		Time 1	Time 2	
Age	60.5 (55-73)	-	-	68 (62-72)	-	**(<0.001*)**
Interval (months)	-	16 (15-17)	-	-	17 (13-18)	(0.302*)
Male SEX (%)	8 (40)		-	20 (80)		**(<0.001*)**
Platelet($x10^4/mm^3$)	18.9 (16.4-24.0)	18.5 (16.4-22.4)	0.708	9.4 (5.7-12.6)	8.3 (7.1-11.5)	0.748
PT-INR	0.95 (0.90-0.97)	0.93 (0.89-0.98)	0.163	1.03 (0.96-1.09)	1.00 (0.96-1.13)	0.914
Total bilirubin (mg/dL)	0.77 (0.58-0.84)	0.72 (0.52-0.90)	0.876	0.93 (0.62-1.06)	1.02 (0.84-1.40)	0.052
Albumin (g/dL)	4.2 (4.2-4.5)	4.2 (4.1-4.4)	0.402	3.5 (3.2-3.9)	3.5 (3.2-3.7)	0.899
ALT (IU/L)	26 (20-39)	25 (17-32)	0.423	41 (35-57)	36 (25-61)	0.534
AFP (ng/mL)	3 (2-4)	3 (2-5)	0.236	11 (5-24)	11 (4-41)	0.872
DCP (mAU/mL)	21 (18-27)	19 (16-29)	0.916	32 (21-126)	30 (13-127)	0.146
Intereferon treatment		3/4 SVR				
HCC stage I/II/III/IV				12/7/5/1	16/4/1/4	**<0.001**
HCC treatment RFA+Ope/TACE/Chemo					15/7/3	
HCC outcome CR/PR/SD/PD					13/1/6/5	

*: Wlcoxon analysis between HCV-non HCC and HCV-HCC

RFA; radio frequency ablation therapy, TACE; transarterial chemoembolization

Table 2A Oxidative stress markers in patients with chronic liver diseases

	dROM, median (range)(CARR/U)	Univariate *P*-value	Multivariate *P*-value	OXY, median (range)(CARR/U)	Univariate *P*-value	Multivariate *P*-value	Oxidative Index, median (range)	Univariate *P*-value	Multivariate *P*-value
Age (years)									
≥62	443 (389–498)	**0.013**	0.195	291 (262–331)	0.064	0.316	0.533 (−0.542–1.443)	**0.002**	0.388
<62	408 (348–482)			304 (276–347)			−0.269 (−1.100–0.652		
sex									
Male	422 (366–492)	0.407		296 (266–339)	0.841		0.241 (−0.853–1.128)	0.909	
female	436 (381–492)			297 (267–347)			−0.182 (−0.812–1.272)		
Etiology									
HCV	454 (394–504)	**<0.001**	**0.001**	291 (261–348)	0.234		0.392 (−0.597–1.445)	**0.001**	0.079
HBV	394 (344–459)			304 (281–337)			−0.345 (−1.248–0.637)		
HCC									
HCC	436 (370–488)	0.801		291 (263–324)	**<0.001**	0.355	0.380 (−0.600–1.437)	**0.003**	0.643
Non-HCC	422 (367–495)			319 (278–358)			−0.329 (−1.098–0.633)		
Ferritin (ng/mL)									
≥115	428 (375–492)	0.933		306 (275–351)	**0.007**	0.199	0.005 (−1.112–0.935)	**0.035**	0.366
<115	433 (367–492)			291 (255–318)			0.547 (−0.563–1.439)		
Platelet(x10^4/mm^3)									
≥13	427 (371–494)	0.935		314 (281–350)	**<0.001**	0.228	−0.316 (−1.119–0.907)	**0.001**	0.461
<13	433 (368–489)			285 (253–317)			0.554 (−0.458–1.432)		
PT-INR									
≥0.98	417 (360–482)	0.111		288 (262–321)	**0.018**	0.284	0.224 (−0.688–1.215)	0.564	
<0.98	439 (379–505)			312 (276–348)			−0.097 (−0.856–1.334)		
TBil (mg/dL)									
<0.8	433 (379–500)	0.129		295 (261–340)	0.295		0.266 (−0.637–1.433)	0.126	
≥0.8	420 (362–482)			297 (270–344)			−0.034 (−1.075–0.871)		
Albumin (g/dL)									
≥4.1	414 (362–483)	**0.026**	0.141	318 (281–350)	**<0.001**	**0.010**	−0.460 (−1.245–0.617)	**<0.001**	**0.014**
<4.1	450 (385–506)			285 (259–313)			0.581 (−0.342–1.492)		
ALT (IU/L)									
<33	424 (361–488)	0.271		298 (276–338)	0.663		−0.182 (−0.851–0.927)	0.317	
≥33	436 (376–495)			296 (263–344)			0.335 (−0.726–1.280)		

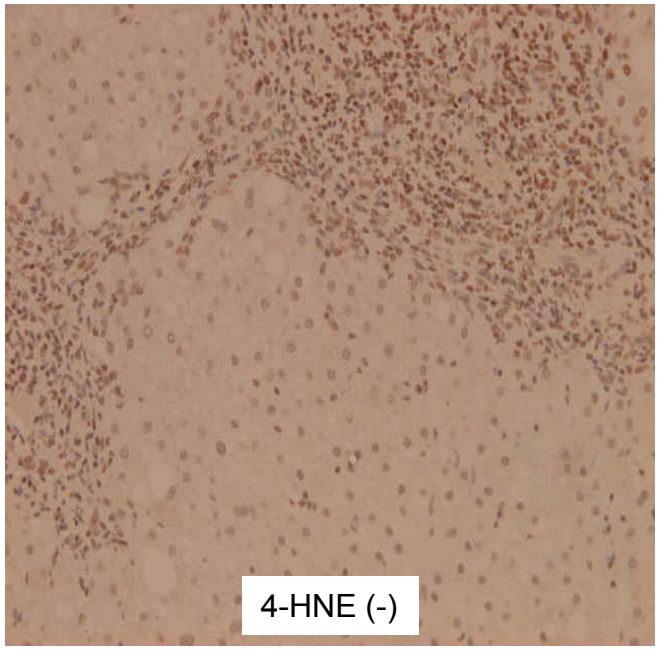
Table 2B Oxidative stress markers in patients with HCV related liver diseases

	dROM, medium (range)(CARR/U)	Univariate *P*-value	Multivariate *P*-value	OXY, medium (range)(CARR/U)	Univariate *P*-value	Multivariate *P*-value	Oxidative Index, medium (range)	Univariate *P*-value	Multivariate *P*-value
total	454 (394–504)			291 (261–348)			0.392 (−0.598–1.445)		
Age (years)									
≥64	463 (411–522)	0.217	0.353	281 (248–314)	**0.002**	0.149	0.982 (−1.63–1.555)	**<0.001**	0.128
<64	446 (384–497)			316 (269–361)			−0.311 (−1.030–0.714)		
SEX									
Male	455 (388–514)	0.761		289 (259–338)	0.243		0.642 (−0.535–1.441)	0.169	
female	446 (407–494)			301 (262–350)			−0.182 (−0.798–1.508)		
HCC									
HCC	455 (393–531)	0.271		277 (247–296)	**<0.001**	**0.048**	0.982 (−0.012–1.679)	**<0.001**	0.159
Non-HCC	446 (392–485)			342 (284–371)			−0.440 (−1.119–0.503)		
HCV-RNA (LogIU/ml)									
≥5.9	466 (415–521)	**0.029**	0.080	295 (264–352)	0.445		0.555 (−0.417–1.538)	0.393	
<5.9	432 (363–497)			291 (259–340)			0.335 (−0.688–1.341)		
Ferritin (ng/ml)									
≥115	451 (391–505)	0.435		297 (270–355)	**0.005**	0.066	0.059 (−0.808–1.013)	**0.002**	0.078
<115	466 (397–509)			279 (245–313)			0.733 (−0.104–1.646)		
Platelet(x104/ml)									
≥12	459 (411–508)	0.525		323 (286–361)	**<0.001**	0.202	−0.352 (−0.971–1.014)	**<0.001**	0.185
<12	450 (383–504)			276 (245–298)			0.702 (−0.080–1.570)		
PT-INR									
≥0.98	435 (373–495)	**0.039**	0.298	276 (249–314)	**0.005**	0.062	0.506 (−0.382–1.551)	0.501	
<0.98	465 (421–521)			309 (278–352)			0.420 (−0.635–1.452)		
TBil (mg/dl)									
<0.8	461 (412–528)	0.168		293 (256–344)	0.605		0.555 (−0.594–1.516)	0.250	
≥0.8	448 (385–490)			291 (262–353)			0.262 (−0.753–1.272)		
Albumin (g/dl)									
≥3.8	446 (393–494)	0.591		323 (289–363)	**<0.001**	0.088	−0.380 (−1.125–0.957)	**<0.001**	0.412
<3.8	455 (395–519)			278 (245–306)			0.702 (−0.175–1.617)		
ALT (IU/L)									
<37	452 (397–505)	0.692		295 (260–349)	0.967		0.275 (−0.408–1.500)	0.600	
≥37	454 (389–498)			291 (262–326)			0.392 (−0.707–1.422)		

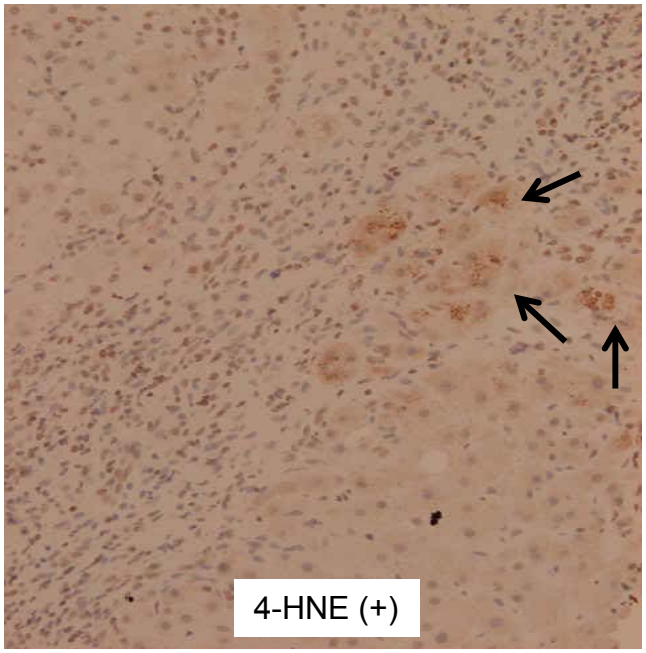
Table 2C Oxidative stress markers in patients with HCV related HCC patients

	dROM, medium (range)(CARR/U)	Univariate *P*-value	Multivariate *P*-value	OXY, medium (range)(CARR/U)	Univariate *P*-value	Multivariate *P*-value	Oxidative Index, medium (range)	Univariate *P*-value	Multivariate *P*-value
total	455 (393–534)			279 (247–300)			0.937 (−0.012–1.646)		
Age (years)									
≥67	463 (412–528)	0.571	0.626	270 (241–293)	0.155	0.390	1.241 (0.394–1.728)	0.268	
<67	445 (373–550)			280 (254–308)			0.702 (−0.273–1.633)		
HCV-RNA (LogIU/ml)									
≥5.9	479 (415–538)	**0.045**	0.234	280 (247–296)	0.772		0.982 (0.400–1.747)	0.158	
<5.9	432 (363–499)			276 (245–314)			0.698 (−0.374–1.537)		
Ferritin (ng/ml)									
≥101	446 (385–507)	0.217		279 (259–298)	0.373		0.707 (−0.367–1.542)	0.050	
<101	467 (397–541)			273 (244–297)			1.287 (0.540–1.806)		
Platelet($x10^4/mm^3$)									
≥9	459 (400–531)	0.563		282 (250–306)	0.293		1.133 (−0.031–1.625)	0.685	
<9	454 (382–529)			276 (245–292)			0.931 (0.148–1.736)		
PT-INR									
≥1.02	454 (381–504)	0.371		263 (245–282)	**0.008**	**0.010**	1.025 (0.110–1.744)	0.435	
<1.02	459 (396–536)			290 (265–316)			0.959 (−0.121–1.565)		
Albumin (g/dl)									
≥3.4	479 (397–553)	0.163		285 (259–312)	**0.048**	0.470	0.982 (−0.033–1.806)	0.903	
<3.4	433 (379–500)			265 (244–286)			1.100 (0.028–1.631)		
AFP (ng/mL)									
<18	437 (390–517)	0.425		276 (245–311)	0.801		0.982 (−0.039–1.471)	0.347	
≥18	479 (395–538)			279 (250–296)			0.937 (0.110–1.750)		
DCP (mAU/mL)									
<76	449 (396–488)	0.705		263 (239–295)	0.068		1.141 (0.269–1.609)	0.576	
≥76	489 (370–546)			281 (268–299)			0.926 (−0.340–1.731)		
Stage									
I+II	453 (396–527)	0.478		276 (245–297)	0.503		1.061 (0.033–1.559)	0.540	
III+IV	482 (378–614)			281 (254–305)			0.937 (−0.162–2.117)		

(A)



(B)



(C) ROM (CARR U)

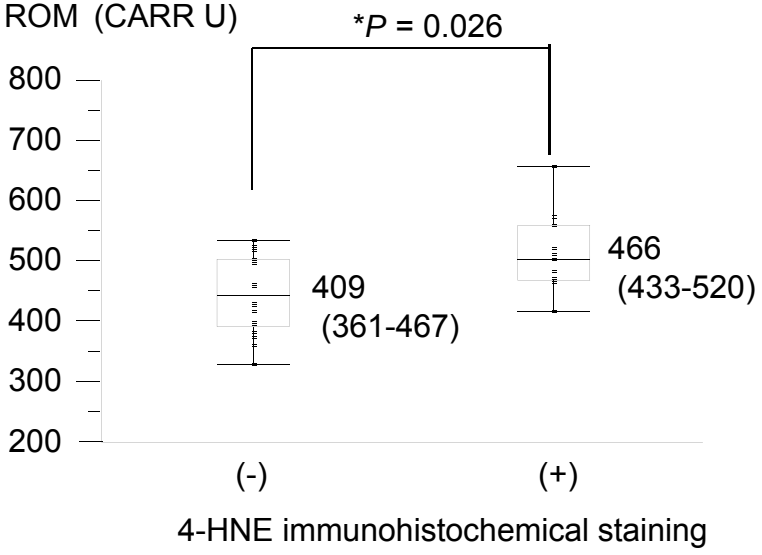
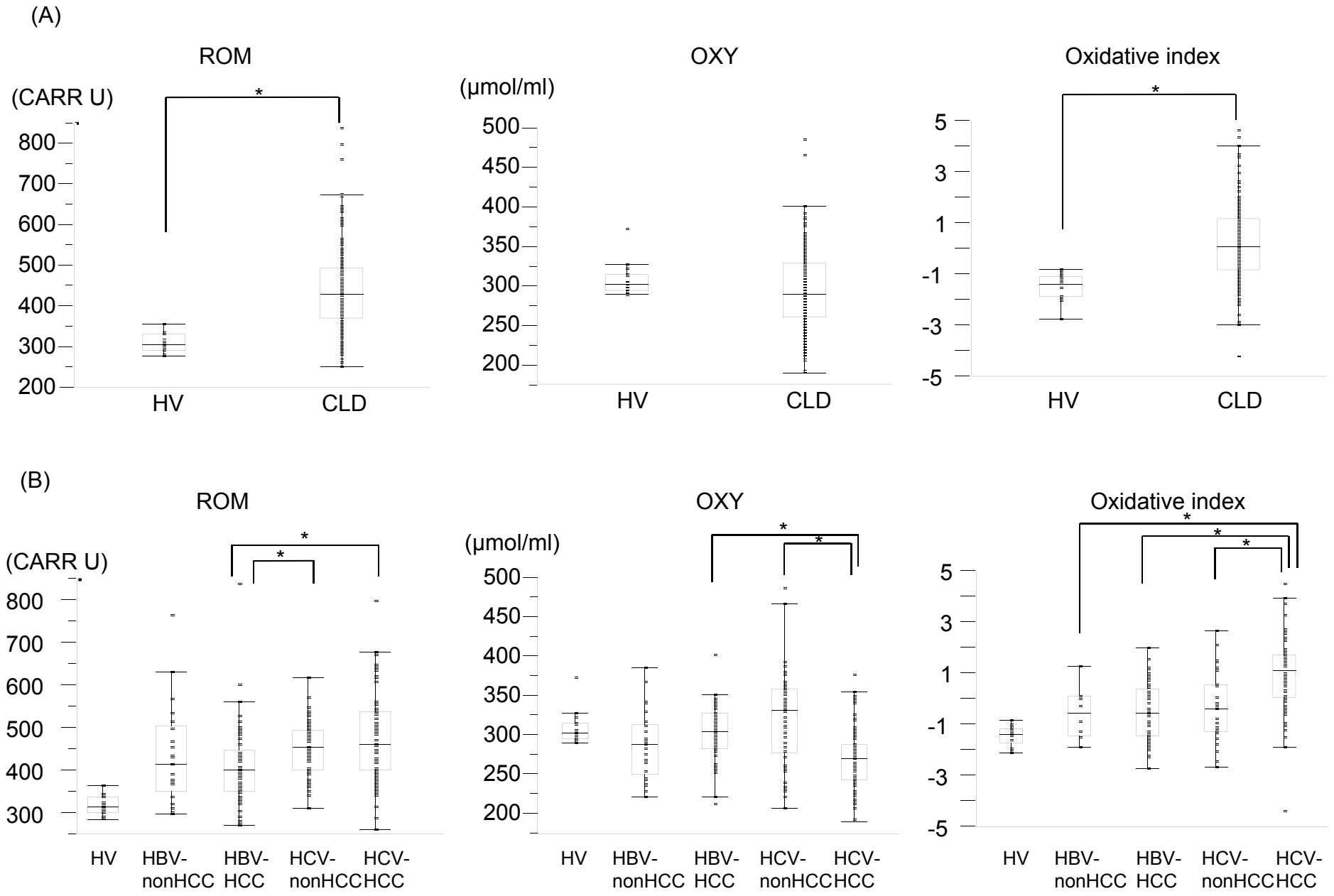


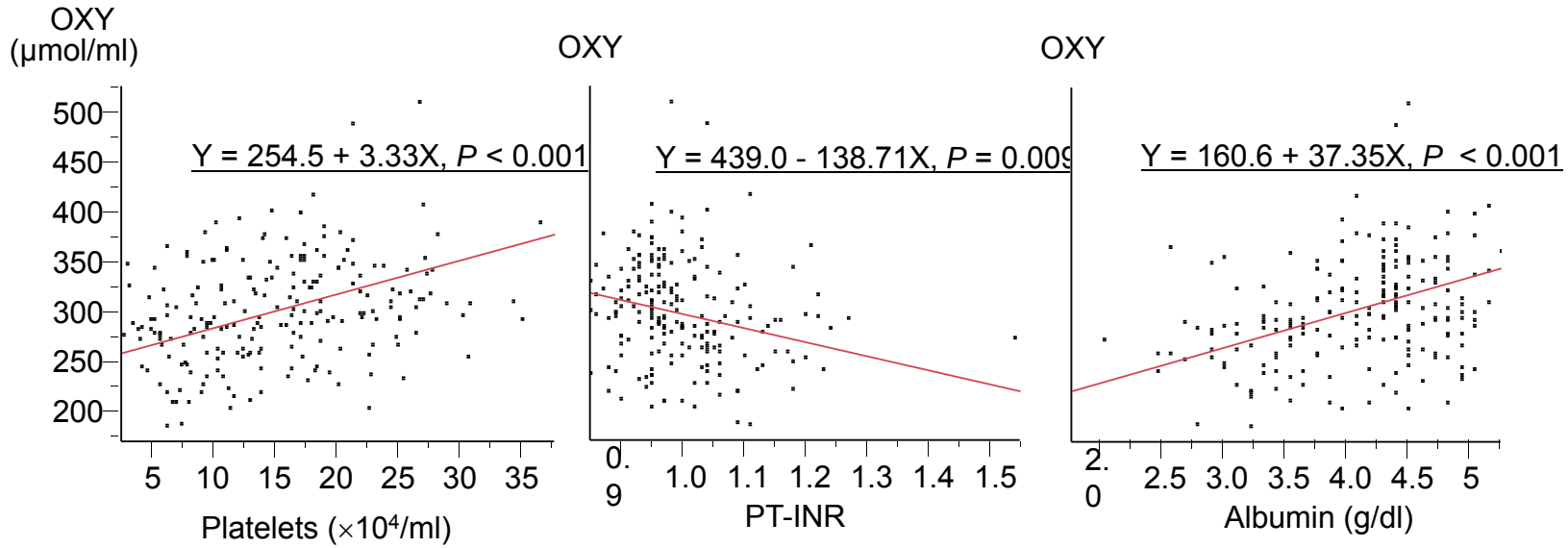
Figure 1



*P<0.05

Figure 2

(A)


OXY
(μmol/ml)
Y = 254.5 + 3.33X, P < 0.001
Platelets (×10⁴/ml)
OXY
Y = 439.0 - 138.71X, P = 0.009
PT-INR
OXY
Y = 160.6 + 37.35X, P < 0.001
Albumin (g/dl)

(B)

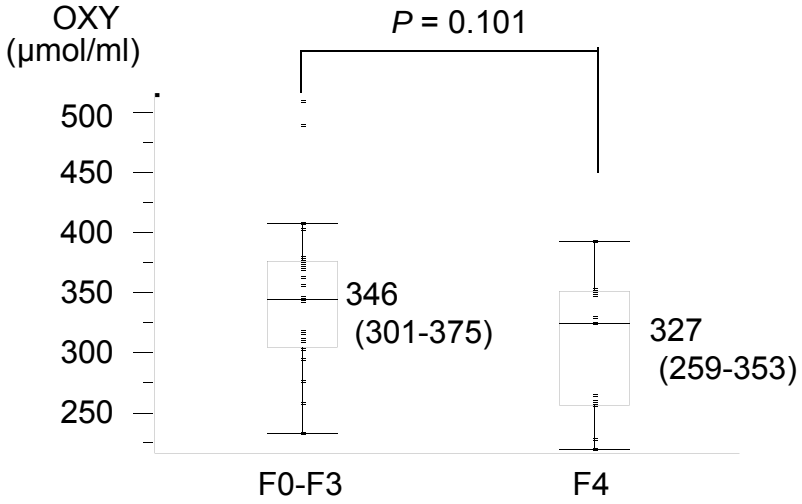
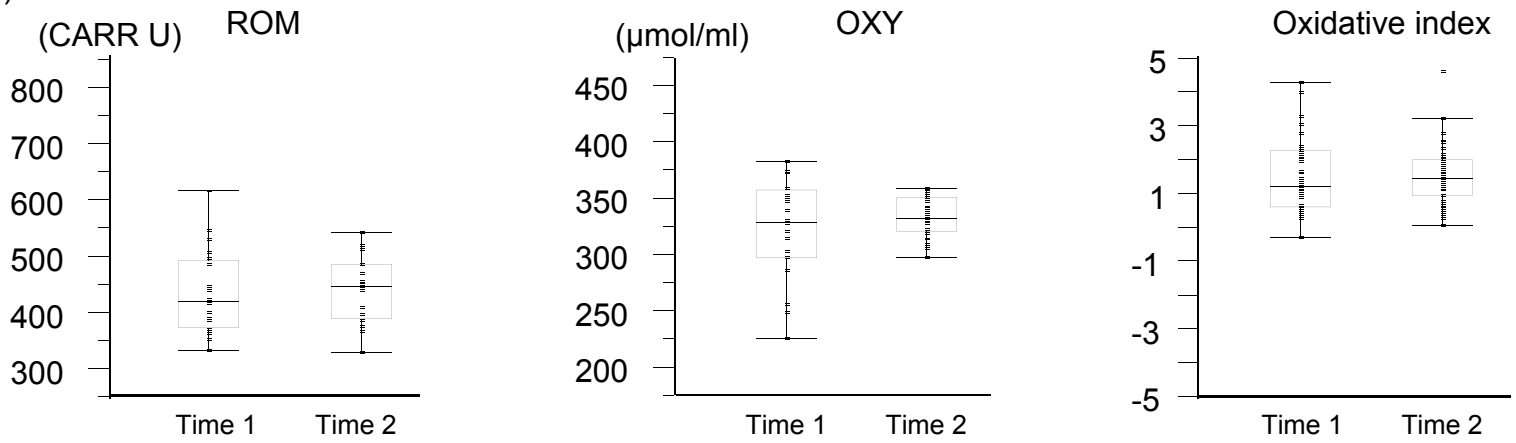

OXY
(μmol/ml)
P = 0.101
346
(301-375)
327
(259-353)
F0-F3
F4

Figure 3

(A) HCV-non HCC



(B) HCV-nonHCC who received pegylated interferon and ribavirin treatment resulting in Viral Responder status during Time 1 and Time 2

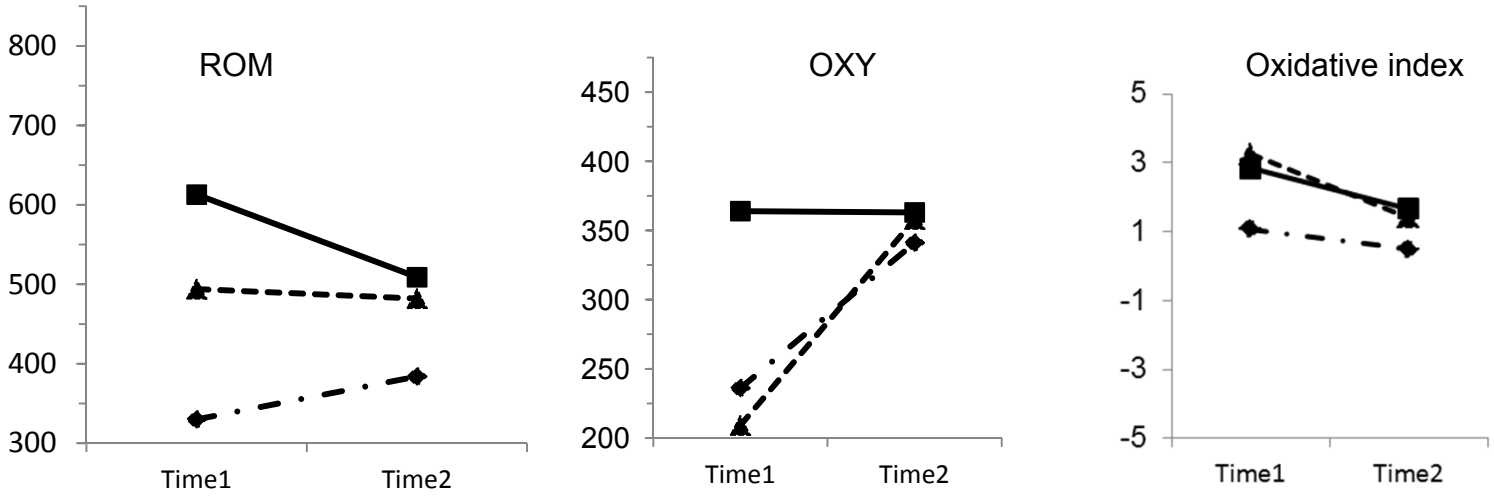
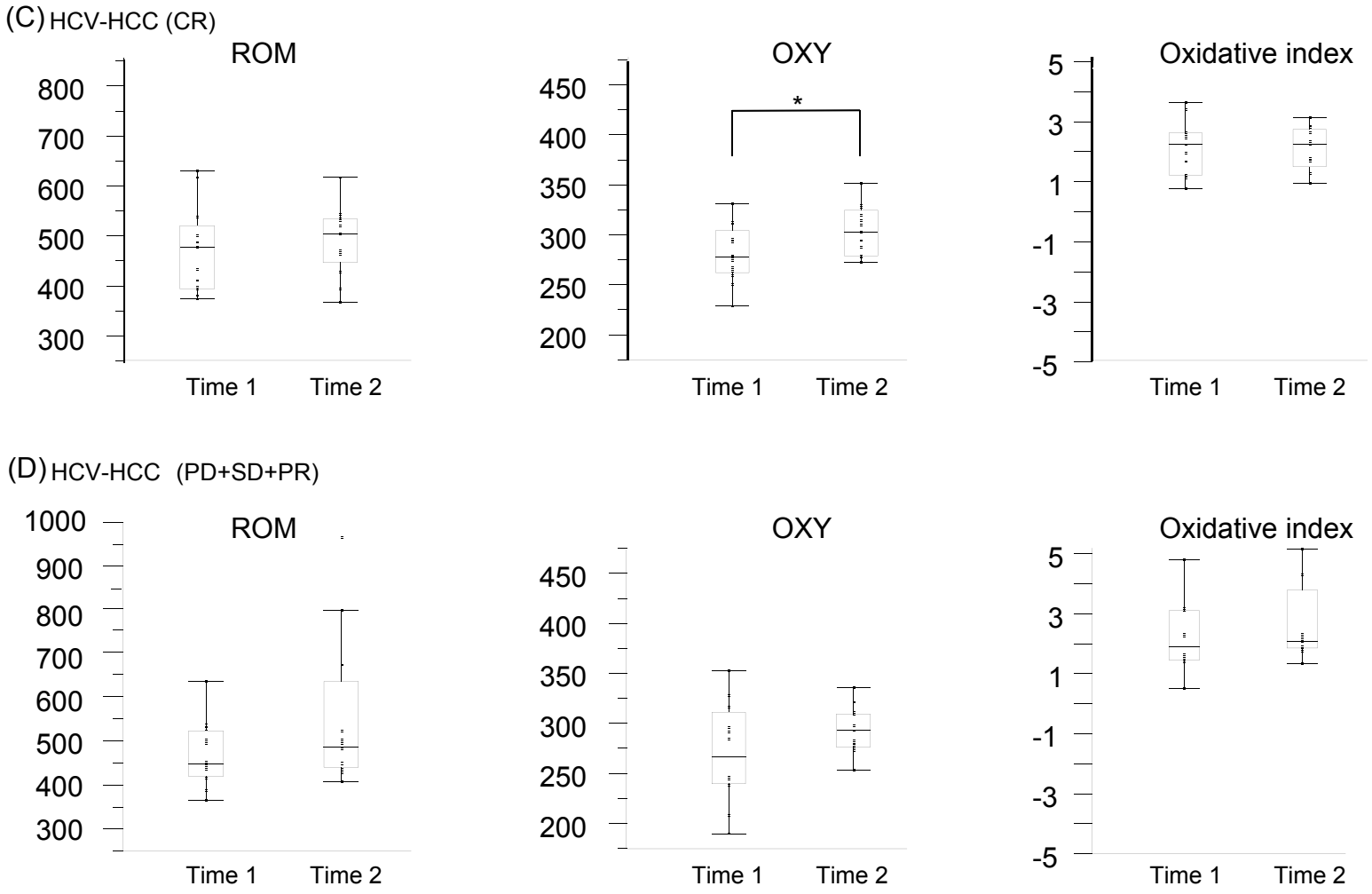


Figure 4



*P<0.05

Figure 4

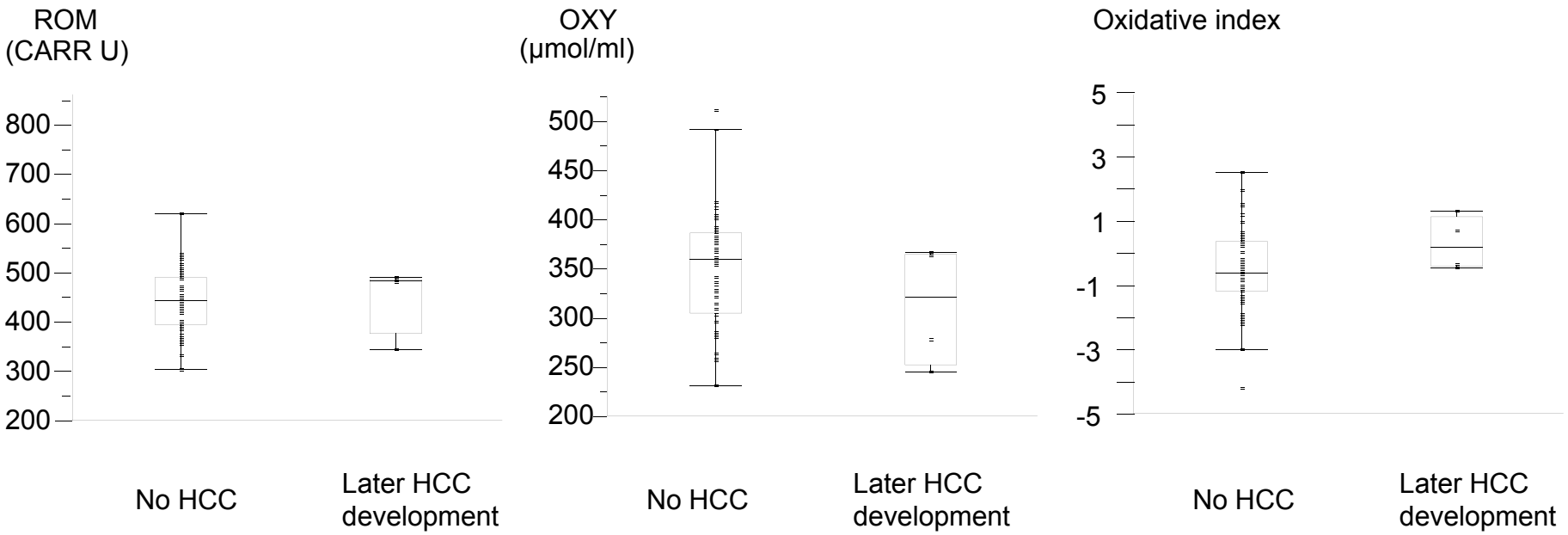

ROM
(CARR U)
800
700
600
500
400
300
200
No HCC
Later HCC development
OXY
(µmol/ml)
500
450
400
350
300
250
200
No HCC
Later HCC development
Oxidative index
5
3
1
-1
-3
-5
No HCC
Later HCC development

Figure 5