ANTICANCER RESEARCH *40*: 6853-6861 (2020)
doi:10.21873/anticanres.14707

Oxidative and Nitrosative Pattern in Circulating Leukocytes of Very Early/Early Hepatocellular Carcinoma Patients

PALOMA LLUCH[1,2,3], GLORIA SEGARRA[2,4], JOAN TOSCA[1,2], LAIA NAVARRO[1], JAVIER NAVARRETE-NAVARRO[3], GUADALUPE HERRERA[2], ANA SANAHUJA[1], SHERLY HERNANDEZ[1], SALVADOR LLUCH[2,4] and PASCUAL MEDINA[2,4]

[1]*Servicio de Medicina Digestiva, Hospital Clínico Universitario de Valencia, Valencia, Spain;*
[2]*INCLIVA, Research Institute Valencia, Hospital Clínico Universitario de Valencia, Valencia, Spain;*
[3]*Department of Medicine, University of Valencia, Valencia, Spain;*
[4]*Department of Physiology, University of Valencia, Valencia, Spain*

Abstract. *Background/Aim: In chronic liver disease, various immune cell subsets exert pro or anti-tumour effects by releasing reactive oxygen and nitrogen species (ROS, RNS). Here, we evaluated the oxidative and nitrosative pattern in peripheral blood leukocyte subpopulations of early hepatocellular carcinoma (HCC) patients compared with HCC-free cirrhotic patients. Materials and Methods: Venous blood samples from 18 HCC-free cirrhotic patients and 17 early stage HCC patients were collected to determine ROS, RNS and reduced glutathione levels in isolated leukocytes analyzed by flow cytometry. Results: Intracellular levels of ROS and glutathione were higher in lymphocytes, monocytes, and neutrophils from HCC patients as well as mitochondrial superoxide in neutrophils and monocytes whereas intracellular levels of nitric oxide were lower in lymphocytes, monocytes, and neutrophils. Conclusion: Early HCC alters intracellular levels of ROS and RNS of some circulating leukocytes subsets. This finding may represent a potential area of interest concerning the development of new treatments and prognostic markers.*

Despite significant improvements throughout the last two decades in the treatment and diagnosis of hepatocellular carcinoma (HCC) and the evolving understanding of its pathophysiology, fundamental aspects of HCC remain unknown. The molecular heterogeneity of HCC and the habitual presence of an underlying liver disease adds complexity to the classification, prognosis and treatment of this tumor. The Barcelona Clinic Liver Cancer (BCLC) is currently the most widely used staging system for HCC (1). According to the BCLC algorithm, patients with HCC in very early or early stage (BCLC 0-A) can benefit from potentially curative therapies (local ablation, resection or liver transplantation). One of the main concerns in this group of patients is the risk of recurrence after resection and local ablation that can exceed 70% at 5 years (2, 3). There is a lack of specific markers capable of predicting HCC prognosis in every stage. In stages 0-A no adjuvant therapies to local ablation or surgery have proven any benefit and hence, on behalf of recurrences, many patients in this group may show HCC progression and worse outcome.

A potential pathogenic role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in HCC has been previously suggested. The most important ROS include free oxygen radicals like superoxide (O_2-), hydroxyl radical (OH), nitric oxide (NO) radicals, as well as nonradical ROS like hydrogen peroxide (H_2O_2), organic hydroperoxides, and hypochloride. These compounds are released in response to different stress factors and cancer-related inflammation. ROS are known to damage DNA and membranes, induce mutations and apoptosis and finally induce carcinoma (4). Their relative excess or shortage is potentially deleterious. RNS are a family of molecules derived from nitric oxide (NO) and superoxide anion. NO reacts with ROS, releasing potent secondary intermediates, and is a key signaling molecule in inflammatory diseases including cancer, where NO can be involved in the promotion or prevention of tumor occurrence depending on the tumor microenvironment, NO concentration and time of exposure (5).

The central role of the immune system in the pathogenesis of HCC is also well known. Cancer immune response might show dual host-protective or tumor promoting actions on

Correspondence to: Paloma Lluch (ORCID number: 0000-0003-3192-3820), MD, Servicio de Medicina Digestiva, Hospital Clínico Universitario de Valencia, Av. Blasco Ibáñez 17, 46010 Valencia, Spain. Tel: +34 961973858, Fax: +34 963864642, e-mail: plluch15@hotmail.com

Key Words: Hepatocellular carcinoma, cirrhosis, oxidative stress, circulating leukocytes, nitric oxide, glutathione.

developing tumors (6). Studies in cancer immunotherapy have focused heavily on local immune responses in the tumor microenvironment, which contains various immune cell subsets exerting pro-tumor or anti-tumor effects (7). On the other hand, systemic immune response has been less studied and commonly related to systemic inflammation and paraneoplastic symptoms (8). A better understanding of systemic immune response in HCC, through the study of oxidative and nitrosative stress in different circulating immune cells, might help to improve prognosis and treatment of HCC.

The purpose of this study was to evaluate some aspects of the systemic immunity in HCC by the assessment of oxidative and nitrosative stress production in peripheral blood leukocyte subpopulations of HCC patients in early stages compared with cirrhotic patients without HCC. We determined ROS and RNS, specifically, intracellular and mitochondrial superoxide anion, nitric oxide and reduced glutathione in some circulating leukocyte subsets from very early/early-HCC cirrhotic patients and cirrhotic patients without HCC.

Materials and Methods

Patients. A total of 35 patients were recruited by the Hepatology Unit at the University Hospital of Valencia (Spain) between June 2017 and December 2018. The study protocols conform to the ethical guidelines of the Declaration of Helsinki and were approved by the local ethics committee. Informed consent was obtained from each patient. Two groups of patients were evaluated. The first group included 17 patients with cirrhosis and HCC in stages 0-A according to the BCLC staging system. The second group comprised 18 patients with cirrhosis without HCC (HCC-free cirrhotic patients). The diagnosis of liver cirrhosis was based on clinical, biochemical and ultrasonographic findings, and hepatic function was evaluated using the Child-Pugh classification. HCC was diagnosed according to radiological criteria as recommended by the American Association for the Study of Liver Diseases guidelines (9). The study was carried out when diagnosis of HCC was established, before any treatment was applied. Exclusion criteria were the following: 1) human immunodeficiency virus infection; 2) previous or actual history of other tumors; 3) patients with a history of inflammatory disease or active concomitant infection; 4) congestive heart failure and/or respiratory failure and 5) chronic kidney disease.

Blood collection and isolation of leukocytes. Samples of EDTA-anticoagulated peripheral venous blood were collected at diagnosis to determine a panel of hematologic and biochemical parameters and to isolate the leukocytes. Blood samples were kept in the dark at room temperature and processed within 1 h. Leukocytes were isolated from whole blood by sedimentation using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA), a density-gradient separation media. Histopaque-1077 and blood samples were added gently and sequentially into a 2-ml centrifuge tube in a volume ratio of 1:0.5, respectively. After 20 min, erythrocytes were precipitated, and a yellowish phase was formed in the upper layer, containing the leukocytes, which was carefully collected.

Table I. *Baseline characteristics of the patients with cirrhosis without HCC (HCC-free cirrhosis) and patients with very early-early HCC (HCC 0/A BCLC).*

	HCC-free cirrhosis	HCC 0/A BCLC	*p*-Value
Number of patients	18	17	
Gender (M/F)	8/10	10/7	NS
Age	71±11	69±8	NS
PS/ECOG			
0	-	14	
1	-	3	
Child-Pugh Score			NS
5	7	9	
	5	4	
7	4	3	
8	2	1	
Etiology			NS
HCV	9	13	
Alcoholic	4	2	
Others	5	2	
Comorbidities			
Diabetes	7	6	NS
Hypertension	8	8	NS
Cardiopathy	2	4	NS
Dyslipidemia	5	3	NS
Creatinine	0.85±0.06	0.76±0.02	NS
Urea	36.6±3.5	35.8±1.8	NS
Leukocytes ($\times10^6$/l)	3,992±455	4,296±378	NS
Lymphocytes ($\times10^6$/l)	893±119	1,191±132	NS
Platelets ($\times10^9$/l)	95±11	97±24	NS
AFP (UI/ml)	3.6±0.8	54.8±17.1	0.04
Total Bilirubin (mg/dl)	0.91±0.12	0.96±0.14	NS
Albumin (g/dl)	3.4±0.1	3.8±0.1	NS
Quick Index	81±4	82±5	NS
AST	53±8	99±12	0.003
ALT	34±6	104±19	0.001

Data are expressed as mean±SEM. PS/ECOG: Performance status/Eastern Cooperative Oncology Group; HCV: hepatitis C virus; AFP: α-fetoprotein; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

Fluorescence probes. The fluorescent probes 2',7'-dihydrodichlorofluorescein diacetate (DCF-DA), dihydroethidium (HE), MitoSOX™ Red (MitoSOX), 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate, and 5-chloromethyl-fluorescein (CMF) diacetate were purchased from Molecular Probes (Eugene, OR, USA). DCF-DA is one of the most commonly fluorogenic substrates used in studies related to ROS generation. Upon cleavage of acetate groups by intracellular esterases, the oxidation produces 2',7'-dichlorofluorescein (DCF), a fluorescent compound. DCF is an indicator of the intracellular levels of H_2O_2, but also of hydroxyl and peroxynitrite (10). HE is a cell-permeant redox probe selectively oxidized and hydroxylated by superoxide anion to a specific product, 2-hydroxyethidium, emitting orange fluorescence when bound to DNA (11). MitoSOX is a fluorogenic probe specifically targeted to mitochondria in live cells. MitoSOX is readily oxidized by superoxide but not by other ROS- or RNS-

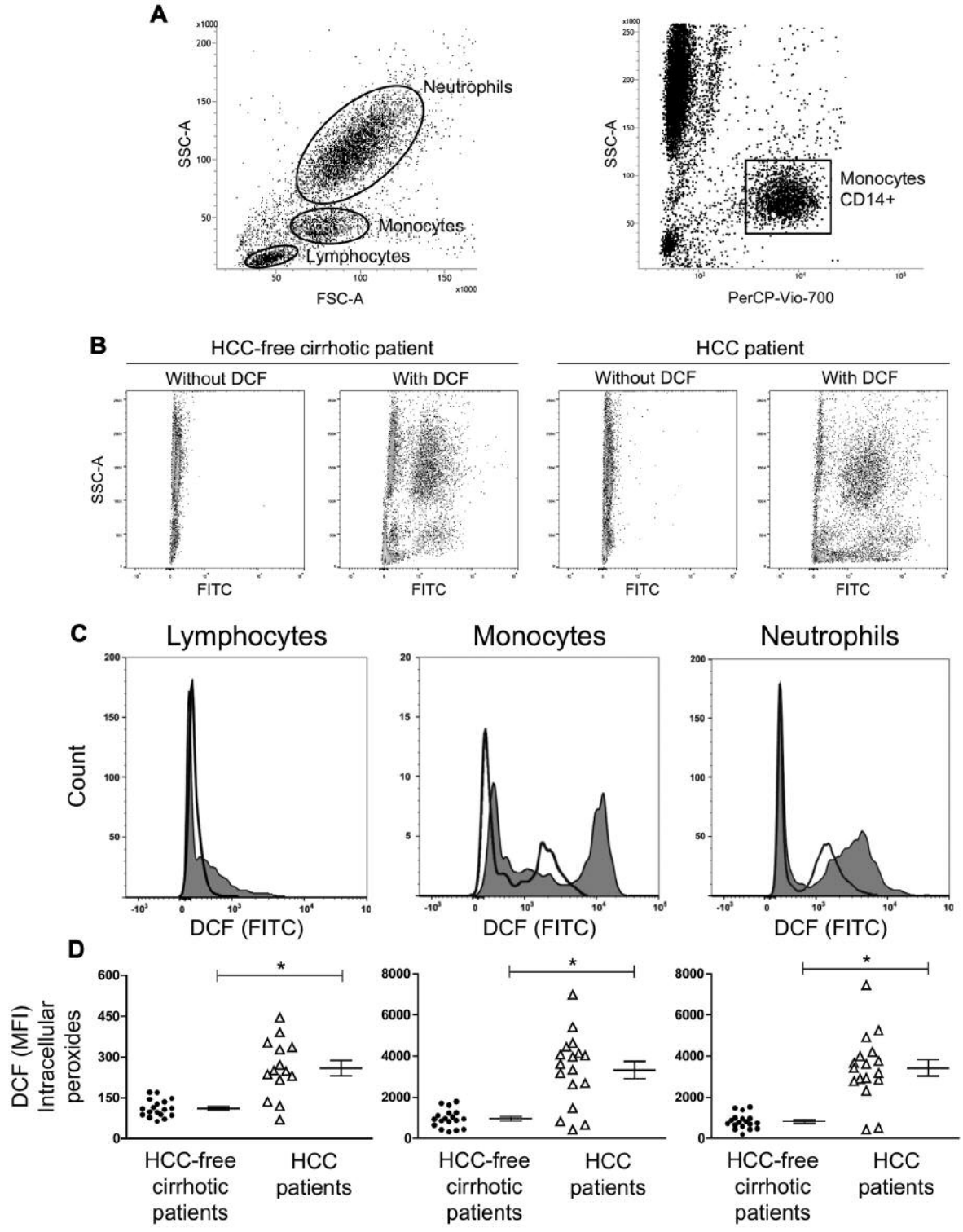

Figure 1. *Gating strategy used in flow cytometry analysis to detect different subsets of circulating immune cells. (A) Lymphocyte, monocyte and neutrophil populations from a sample of a hepatocellular carcinoma (HCC) patient were gated on a forward scatter (FSC)/side scatter (SSC) plot. Cells were then further gated to determine CD14+ monocytes. (B) Representative flow cytometry image of samples from one HCC-free cirrhotic patient and one HCC patients, in the absence or presence of the fluorescent probe 2',7'-dichlorofluorescein (DCF). (C) Representative histograms and (D) dot plots are shown in parallel with lines showing the mean±SEM of the mean fluorescence intensity (MFI). Empty histograms represent HCC-free cirrhotic patients and filled histograms represent HCC patients. *p<0.05 indicates significant differences between both groups.*

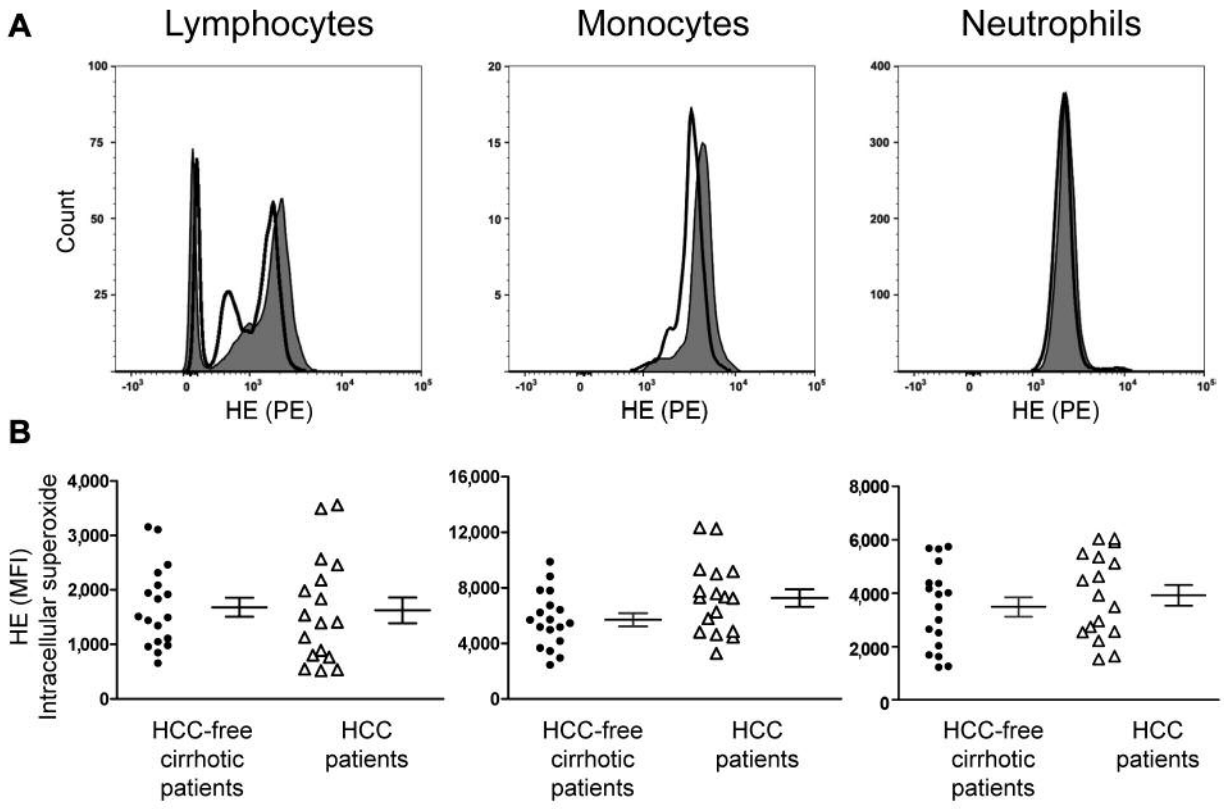

Figure 2. *Total intracellular superoxide anion in leukocytes from peripheral blood cells of hepatocellular carcinoma (HCC)-free cirrhotic patients and HCC patients measured through the fluorescent probe dihydroethidium (HE). (A) Representative histograms and (B) dot plots are shown in parallel with lines showing the mean±SEM of the mean fluorescence intensity (MFI). Empty histograms represent HCC-free cirrhotic patients and filled histograms represent HCC patients.*

generating systems to a fluorescent product with orange fluorescence emission (12). DAF-FM diacetate is a cell-permeable fluorogenic substrate that is used to detect and quantify intracellular NO (13). Lastly, CMF is a fluorescent indicator of intracellular thiol reduced status, including reduced glutathione, the main intracellular antioxidant (14).

Experimental protocol. An aliquot of 20 μl from the leukocyte layer was diluted with 250 μl of phosphate-buffered saline (PBS) solution and stained for 20 min in the dark at room temperature with one of the following fluorescence probes at the final concentrations of: DCF-DA 12 μM; HE: 19 μM; MitoSOX: 4 μM; DAF-FM: 2.5 μM; CMF: 0.06 μM. Then, the samples were fixed with 2% paraformaldehyde for 15 min and centrifuged and re-suspended with PBS. The samples were stained with CD14-PerCP-Vio700 to determine the monocyte population. The cells were washed, re-suspended in PBS, and then analyzed on a FACSVerse (BD Biosciences, San Jose, CA, USA). Background fluorescence was assessed using the appropriate fluorochrome-matched control to determine the positive cells.

The selection and cytometer setting were optimized for analysis using a FACSVerse cytometer equipped with three lasers emitting at 405, 488 and 640 nm. Fluorescence of CD14-PerCP-Vio700 was excited with the blue laser at 488 nm and collected through 700/40 nm. Fluorescence of DCF, DAF-FM, and CMF was excited with the blue laser at 488 nm and collected through 527/32 nm. Fluorescence of HE and MitoSOX was excited with the blue laser at 488 nm and collected through 586/42 nm. The lymphocytes, monocytes, and neutrophils from the leukocyte layer were initially gated by their characteristic forward and side scatter profiles, which represent size and granularity of the cells, respectively. The monocyte population was further evaluated with the CD14-PerCP-Vio700 antibody (MiltenyiBiotec, Madrid, Spain) and flow cytometry. Background fluorescence (in samples incubated without fluorochromes) was subtracted from total fluorescence to obtain the value from each individual sample. Off-line analysis of data was performed using FACSuite (BD Biosciences, San Jose, CA, USA) and FlowJo 10.0 (TreeStar Inc., Ashland, OR, USA) software.

Statistical analysis. Results are expressed as mean±SEM. The normality of the distributions of mean fluorescent intensities of the fluorochromes in the different subsets of leukocytes in HCC-free cirrhotic patients and HCC patients were determined using the Kolmogorov-Smirnov goodness-of-fit test. Mean fluorescence intensities of the cell staining in the two subject groups were compared using the Student's *t*-test for normally distributed populations. Relationships between parameters were examined using calculations of the Pearson's correlation coefficient. The level of

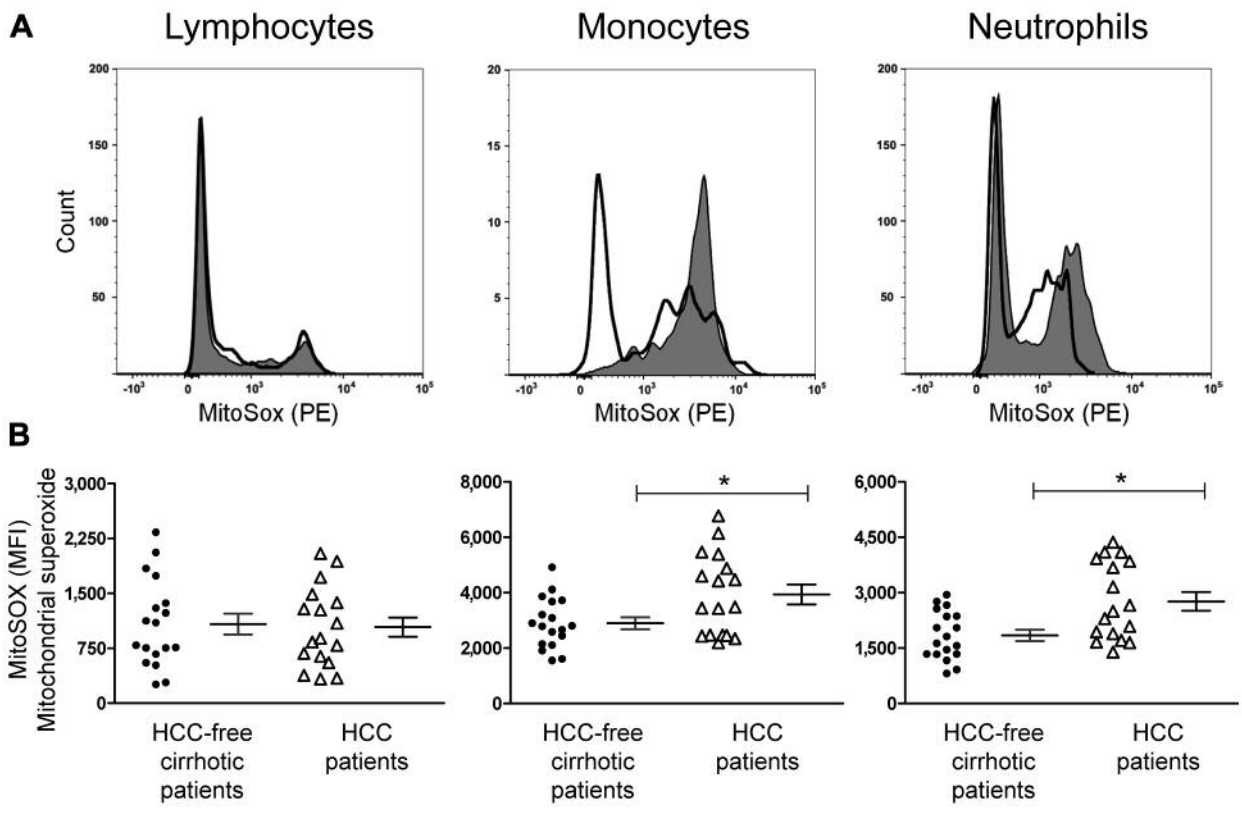

Figure 3. *Mitochondrial superoxide anion in leukocytes from peripheral blood cells from hepatocellular carcinoma (HCC)-free cirrhotic patients and HCC patients measured through the fluorescent probe MitoSOX™ Red (MitoSOX). (A) Representative histograms and (B) dot plots are shown in parallel with lines showing the mean±SEM of the mean fluorescence intensity (MFI). Empty histograms represent HCC-free cirrhotic patients and filled histograms represent HCC patients. *p<0.05 indicates significant differences between both groups.*

statistical significance was set at $p<0.05$. The statistical analyses were performed using SPSS software, version 19 (IBM Corp., Armonk, NY, USA).

Results

Baseline clinical and laboratory characteristics of the study subjects. Clinical and laboratory characteristics of patients are shown in Table I. Serum levels of alpha-fetoprotein, aspartate aminotransferase and alanine aminotransferase were significantly higher ($p<0.05$) in patients with HCC ($p<0.05$) than in HCC-free cirrhotic patients. Other hematologic and biochemical parameters considered were similar in both groups. There were no significant differences in baseline clinical characteristics including age, sex, Child-Pugh score and major coexisting diseases. The etiology of liver disease was mainly secondary to alcohol consumption or chronic hepatitis C. The group with HCC and the HCC-free cirrhosis group included 2 and 5 patients, respectively, with other etiologies (nonalcoholic liver disease, autoimmune hepatitis and hepatitis B).

Oxidative stress profile. DCF fluorescence was significantly increased in lymphocytes (132±25%), monocytes (243±44%), and neutrophils (316±48%) from HCC patients compared with HCC-free cirrhotic patients (Figure 1). Total superoxide anion levels were similar ($p>0.05$) among the different leukocyte subsets from patients with HCC and HCC-free cirrhotic patients (Figure 2). The study of the mitochondrial production of superoxide anion showed significantly higher levels in monocytes (36±12%) and neutrophils (50±14%) of patients with HCC than in the same subsets from the group of HCC-free cirrhotic patients; however, no differences between the two groups were found in lymphocytes (Figure 3).

Nitrosative stress profile. The pattern of nitrosative stress in leukocyte populations showed a significantly lower level of intracellular NO in lymphocytes, monocytes, and neutrophils of the peripheral blood of HCC patients than the leukocyte subsets from HCC-free cirrhotic patients (Figure 4). The greatest reduction in NO was observed in monocytes (47±14%), followed by lymphocytes (35±7%) and neutrophils (34±9%).

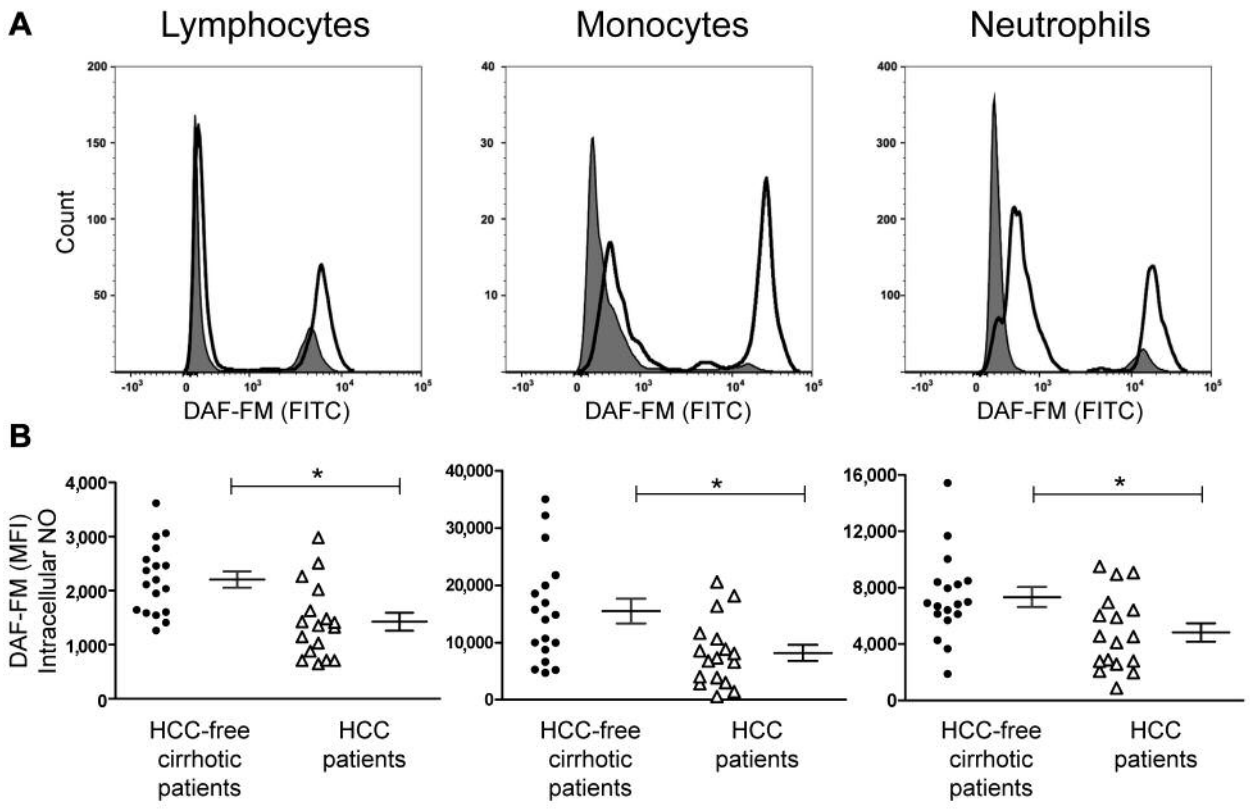

Figure 4. *Levels of intracellular NO in lymphocytes, monocytes, and neutrophils from hepatocellular carcinoma (HCC)-free cirrhotic patients and HCC patients measured through the fluorescent probe 4-amino-5-methylamino-2', 7'-difluorofluorescein (DAF-FM). (A) Representative histograms and (B) dot plots are shown in parallel with lines showing the mean±SEM of the mean fluorescence intensity (MFI). Empty histograms represent HCC-free cirrhotic patients and filled histograms represent HCC patients. *p<0.05 indicates significant differences between both groups.*

Glutathione levels. Levels of intracellular reduced glutathione in lymphocytes, monocytes, and neutrophils of the peripheral blood of patients with HCC, were significantly higher than in the same population from the group of HCC-free cirrhotic patients (Figure 5). The glutathione levels were markedly increased in lymphocytes (82±20%) and monocytes (70±15%), and to a lesser extent in neutrophils (45±10%). When assessing potential correlations in HCC patients by calculating the Pearson's correlation coefficient, it was found that the levels of ROS and RNS were not associated with Child-Pugh score and plasma hematological or biochemical parameters.

Discussion

The results of the present study demonstrate that patients with cirrhosis and early HCC stages present differences in oxidative and nitrosative stress of some circulating leukocyte subpopulations when compared with HCC-free cirrhotic patients. This finding could indicate that the presence of early HCC gives rise to an altered systemic immune response.

In physiological conditions, production of ROS and RNS maintains a balance with endogenous antioxidants (15). When considering the local HCC microenvironment, the overproduction of ROS and RNS is recognized as a key factor in hepatocarcinogenesis, regardless of the etiology of the underlying liver damage. An increase in both, ROS and RNS production, activates different signaling cascades that influence the regulation of cell growth (16), apoptosis, angiogenesis, and DNA damage (17). Martínez-Outschoorn *et al*. (18) have studied the secretion of hydrogen peroxide by cancer cells causing increased ROS production in the tumor microenvironment, what they call "the reverse Warburg effect". Propagation of ROS appears to be driving tumor-stroma co-evolution, leading to DNA damage and mutagenesis (19, 20). Fewer studies have focused their interest in peripheric redox immune response. The present report shows increased intracellular levels of hydrogen peroxide, hydroxyl and peroxynitrite in lymphocytes, monocytes and neutrophils, and increased mitochondrial superoxide anion levels in neutrophils and monocytes in the peripheral venous blood of HCC patients compared to HCC-

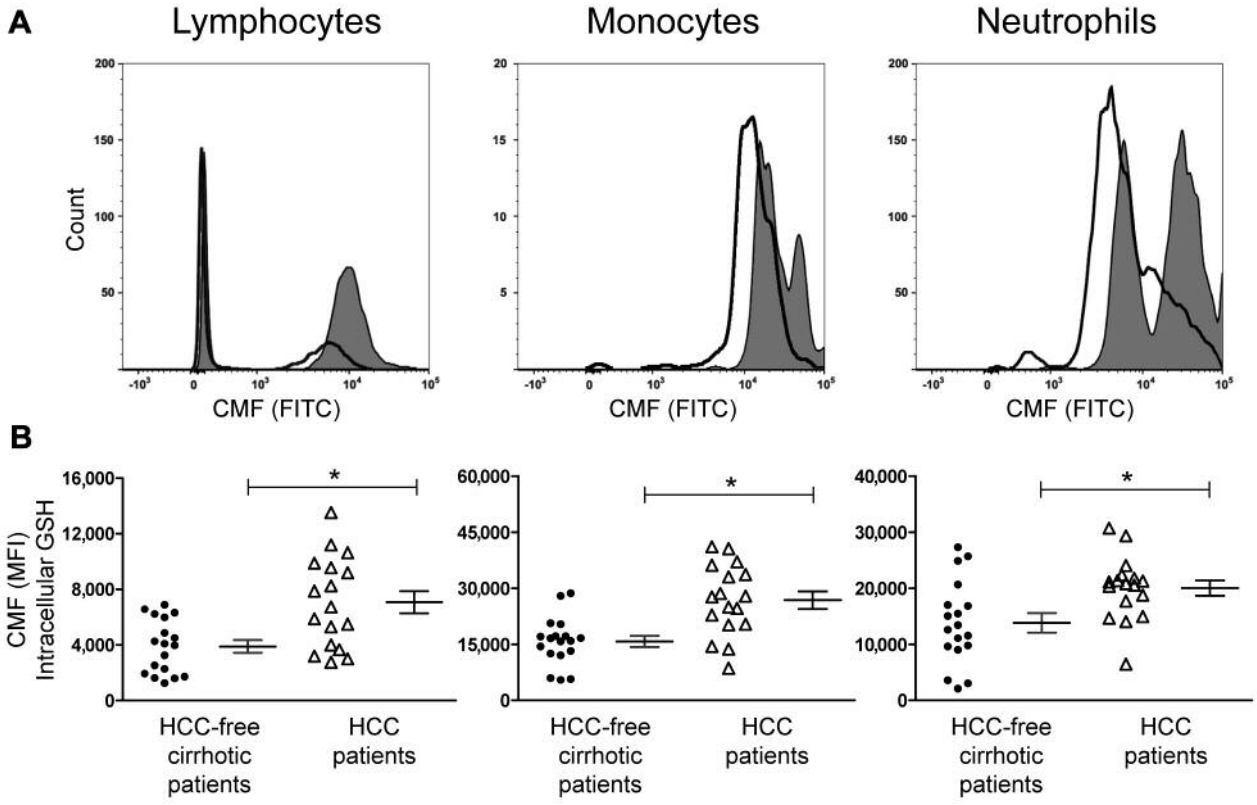

Figure 5. *Levels of intracellular reduced glutathione in lymphocytes, monocytes, and neutrophils from hepatocellular carcinoma (HCC)-free cirrhotic patients and HCC patients measured through the fluorescent probe 5-chloromethylfluorescein (CMF). (A) Representative histograms and (B) dot plots are shown in parallel with lines showing the mean±SEM of the mean fluorescence intensity (MFI). Empty histograms represent HCC-free cirrhotic patients and filled histograms represent HCC patients. *p<0.05 indicates significant differences between both groups.*

free cirrhotic patients. Wilson *et al*. (21) have suggested that there are phenotypic and biologically relevant changes in peripheral blood neutrophils from HCC patients when compared to healthy controls or cirrhotic patients. This is in accordance with the differences found in our study regarding the intracellular levels of circulating neutrophil ROS and RNS in HCC patients compared to those in HCC-free cirrhotic patients. Specifically, neutrophils have been classified into N1, exerting anti-tumor activity by cytotoxic effects, and N2, with pro-tumor activity (21). These phenotypic changes have also been described in macrophages (22), known as "macrophage polarization" (M1 macrophages and M2 macrophages). It seems that in the early stage of tumorigenesis, M1 macrophages exert their cytotoxic function by releasing ROS or toxic intermediates (23), while in advanced stages, M2 macrophages replace M1 macrophages promoting tumor development (24). In our study, we found increased intracellular ROS in circulating monocytes (which become macrophages in tissues) and neutrophils of HCC patients, that could reflect anti-tumor activity in early stages, mediated by increased ROS production (25).

On the other hand, Liaw *et al*. (26) described the dramatic down-regulation of oxidoreductase enzymes in HCC tissues, but the increased serum activity of these enzymes in cirrhotic patients who developed HCC. Clemente *et al*. (27) found increased levels of manganese superoxide dismutase, a key antioxidant enzyme, in the serum of patients with cirrhosis who developed HCC during a 7-year follow-up study, compared with HCC-free cirrhotic patients. They also found differences between healthy subjects and HCC-free cirrhotic patients. Our study also showed an increase in intracellular glutathione, another important antioxidant, in circulating monocytes, lymphocytes and neutrophils of patients with HCC. We suggest, that the concomitant rise in intracellular glutathione may counterbalance the increased oxidative stress of patients with cirrhosis and HCC.

In this study, the intracellular levels of NO in patients with HCC were decreased compared with those in HCC-free cirrhotic patients. NO is a small hydrophobic molecule that regulates several pathophysiological processes and a key signaling molecule in inflammation-driven diseases including cancer. The complexity of its biological effects explains the controversial

results obtained by different research groups regarding pro- or anti-tumorigenic properties. NO reacts with superoxide anion producing peroxynitrite and nitrogen dioxide, that may damage different cellular targets causing apoptosis and mutagenesis (28). NO is generated by three isoforms of NO synthases: neuronal, endothelial and inducible. Specifically, inducible NO synthase, can be induced in various liver cells and produce large amounts of NO for prolonged periods of time. The role of NO in cancer is controversial and might depend on its concentration. It has been described, that low concentrations of NO protect cancer cells from apoptosis and favor tumor growth (29). Conversely, high concentrations of NO induce apoptosis. Caraglia *et al*. (5) examined whether oxidative stress in serum and peripheral blood mononuclear cells could be predictive of a response to sorafenib in advanced HCC patients. They found that sorafenib induced a clear increase in NO levels and superoxide dismutase activity in responsive patients. Other studies (30) have also determined that sorafenib effectiveness is mediated by increasing intracellular superoxide anion, hydrogen peroxide and NO production in a dose-dependent manner. Our results show significantly lower levels of intracellular NO in leukocytes of the peripheral blood from HCC patients compared with HCC-free cirrhotic patients. We stress that our group of HCC patients was evaluated before any treatment was applied. Diminished levels of NO in circulating leukocytes could be secondary to NO consumption to generate ROS.

The present study demonstrates differences in the ROS and RNS pattern in some circulating leukocyte subpopulations of very early and early stage HCC when compared with cirrhotic patients. At present, early HCC stages are treated with percutaneous ablation, surgery or transplant, but the current guides do not encompass any adjuvant systemic approach. Improved understanding of the factors implicated at a systemic level can offer targetable aspects of HCC in order to ameliorate treatment strategies. Systemic immune response in early HCC involving ROS and RNS production could be evaluated as a prognostic indicator and as a new potential target for the development of adjuvant treatments. Nevertheless, it would be necessary to evaluate other stages of HCC to complement our findings.

Conflicts of Interest

The Authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' Contributions

Paloma Lluch: designed the study, selected the patients and wrote the manuscript. Gloria Segarra and Guadalupe Herrera: performed the experimental procedures. Joan Tosca, Laia Navarro and Javier Navarrete-Navarro: selected the patients and performed statistic procedures. Ana Sanahuja y Sherly Hernández: performed statistic procedures and data collection. Salvador Lluch: supervised the study and co-wrote the manuscript. Pascual Medina: co-designed the study, performed experimental procedures and co-wrote the manuscript.

References

1 Llovet JM, Bru C and Bruix J: Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis *19(3)*: 329-338, 1999. PMID: 10518312. DOI: 10.1055/s-2007-1007122

2 Llovet JM, Fuster J and Bruix J: Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. Hepatology *30(6)*: 1434-1440, 1999. PMID: 10573522. DOI: 10.1002/hep.510300629

3 Hsu HC, Wu TT, Wu MZ, Sheu JC, Lee CS and Chen DS: Tumor invasiveness and prognosis in resected hepatocellular carcinoma. Clinical and pathogenetic implications. Cancer *61(10)*: 2095-2099, 1988. PMID: 2834037.

4 Sasaki Y: Does oxidative stress participate in the development of hepatocellular carcinoma? J Gastroenterol *41(12)*: 1135-1148, 2006. PMID: 17287893. DOI: 10.1007/s00535-006-1982-z

5 Caraglia M, Giuberti G, Marra M, Addeo R, Montella L, Murolo M, Sperlongano P, Vincenzi B, Naviglio S, Del Prete S, Abbruzzese A and Stiuso P: Oxidative stress and ERK1/2 phosphorylation as predictors of outcome in hepatocellular carcinoma patients treated with sorafenib plus octreotide LAR. Cell Death Dis *2*: e150, 2011. PMID: 21525937. DOI: 10.1038/cddis.2011.34

6 Schreiber RD, Lloyd J and Smyth M: Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion. Science *331(6024)*: 1565-1570, 2011. PMID: 21436444. DOI: 10.1126/science.1203486

7 Mossanen JC and Tacke F: Role of lymphocytes in liver cancer. Oncoimmunology *2(11)*: e26468, 2013. PMID: 24498546. DOI: 10.4161/onci.26468

8 Diakos C, Charles K, McMillan D and Clarke SJ: Cancer-related inflammation and treatment effectiveness. Lancet Oncol *15*: e493-503, 2014. PMID: 25281468. DOI: 10.1016/S1470-2045(14)70263-3

9 Bruix J and Sherman M: Management of hepatocellular carcinoma. Hepatology *42(5)*: 1208-1236, 2005. PMID: 16250051. DOI: 10.1002/hep.20933

10 Kalyanaraman B, Darley-Usmar V, Davies KJ, Dennery PA, Forman HJ, Grisham MB, Mann GE, Moore K, Roberts 2nd LJ and Ischiropoulos H: Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. Free Radic Biol Med *52(1)*: 1-6, 2012. PMID: 22027063. DOI: 10.1016/j.freeradbiomed. 2011.09.030

11 Kalyanaraman B: Oxidative chemistry of fluorescent dyes: implications in the detection of reactive oxygen and nitrogen species. Biochem Soc Trans *39(5)*: 1221-1225, 2011. PMID: 21936793. DOI: 10.1042/BST0391221

12 Mukhopadhyay P, Rajesh M, Yoshihiro K, Hasko G and Pacher P: Simple quantitative detection of mitochondrial superoxide production in live cells. Biochem Biophys Res Commun *358(1)*: 203-208, 2007. PMID: 17475217. DOI: 10.1016/j.bbrc.2007.04.106

13 Kojima H, Sakurai K, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y and Nagano T: Development of a fluorescent indicator for nitric oxide based on the fluorescein chromophore. Chem Pharm Bull (Tokyo) *46(2)*: 373-375, 1998. PMID: 9501473.

14 Sarkar A, Mandal G, Singh N, Sundar S and Chatterjee M: Flow cytometric determination of intracellular non-protein thiols in Leishmania promastigotes using 5-chloromethyl fluorescein diacetate. Exp Parasitol *122(4)*: 299-305, 2009. PMID: 19393240. DOI: 10.1016/j.exppara.2009.04.012

15 Marra M, Sordelli IM, Lombardi A, Lamberti M, Tarantino L, Giudice A, Stiuso P, Abbruzzese A, Sperlongano R, Accardo M, Agresti M, Caraglia M and Sperlongano P: Molecular targets and oxidative stress biomarkers in hepatocellular carcinoma: an overview. J Transl Med *9*: 171, 2011. PMID: 21985599. DOI: 10.1186/1479-5876-9-171

16 Finkel T and Holbrook NJ: Oxidants, oxidative stress and the biology of ageing. Nature *408(6809)*: 239-247, 2000. PMID: 11089981. DOI: 10.1038/35041687

17 Calvisi DF, Conner EA, Ladu S, Lemmer ER, Factor VM and Thorgeirsson SS: Activation of the canonical Wnt/beta-catenin pathway confers growth advantages in c-Myc/E2F1 transgenic mouse model of liver cancer. J Hepatol *42(6)*: 842-849, 2005. PMID: 15885355. DOI: 10.1016/j.jhep.2005.01.029

18 Martinez-Outschoorn UE, Lin Z, Trimmer C, Flomenberg N, Wang C, Pavlides S, Pestell RG, Howell A, Sotgia F and Lisanti MP: Cancer cells metabolically "fertilize" the tumor microenvironment with hydrogen peroxide, driving the Warburg effect: implications for PET imaging of human tumors. Cell Cycle *10(15)*: 2504-2520, 2011. PMID: 21778829. DOI: 10.4161/cc.10.15.16585

19 Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, Chiavarina B, Pavlides S, Wang C,Whitaker-Menezes D, Daumer KM, Lin Z, Witkiewicz AK, Flomenberg N, Howell A, Pestell RG, Knudsen ES, Sotgia F and Lisanti MP: Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: A new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. Cell Cycle *9(16)*: 3256-3276, 2010. PMID: 20814239. DOI: 10.4161/cc.9.16.12553

20 Lisanti MP, Martinez-Outschoorn UE, Chiavarina B, Pavlides S, Whitaker-Menezes D, Tsirigos A,Witkiewicz A, Lin Z, Balliet R, Howell A and Sotgia F: Understanding the "lethal" drivers of tumor-stroma co-evolution: emerging role(s) for hypoxia, oxidative stress and autophagy/mitophagy in the tumor micro-environment. Cancer Biol Ther *10(6)*: 537-542, 2010. PMID: 20861671. DOI: 10.4161/cbt.10.6.13370

21 Wilson C, Mauricio J, McCain M, Littler P, Masson S, McPherson S, Reeves H and Mann DA: Mapping the heterogeneity of circulating neutrophils in hepatocellular carcinoma. HCC SUMMIT 2017, 2-5 February 2017, Geneva, Switzerland, pp. 126-127.

22 Mantovani A, Allavena P, Sica A and Balkwill F: Cancer-related inflammation. Nature *454(7203)*: 436-444, 2008. PMID: 18650914. DOI: 10.1038/nature07205

23 Brittenden J, Heys SD, Ross J and Eremin O: Natural killer cells and cancer. Cancer *77(7)*: 1226-1243, 1996. PMID: 8608497.

24 Noy R and Pollard JW: Tumor-associated macrophages: from mechanisms to therapy. Immunity *41(1)*: 49-61, 2014. PMID: 25035953. DOI: 10.1016/j.immuni.2014.06.010

25 Conde dlR, Schoemaker MH, Vrenken TE, Buist-Homan M, Havinga R, Jansen PL and Moshage H: Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases. J Hepatol *44(5)*: 918-929, 2006. PMID: 16310883. DOI: 10.1016/j.jhep.2005.07.034

26 Liaw KY, Lee PH, Wu FC, Tsai JS and Lin-Shiau SY: Zinc, copper, and superoxide dismutase in hepatocellular carcinoma. Am J Gastroenterol *92(12)*: 2260-2263, 1997. PMID: 9399766.

27 Clemente C, Elba S, Buongiorno G, Guerra V, D'Attoma B, Orlando A and Russo F: Manganese superoxide dismutase activity and incidence of hepatocellular carcinoma in patients with Child-Pugh class A liver cirrhosis: a 7-year follow-up study. Liver Int *27(6)*: 791-797, 2007. PMID: 17617122. DOI: 10.1111/j.1478-3231.2007.01485. x

28 Ying L and Hofseth LJ: An emerging role for endothelial nitric oxide synthase in chronic inflammation and cancer. Cancer Res *67(4)*: 1407-1410, 2007. PMID: 17308075. DOI: 10.1158/0008-5472.CAN-06-2149

29 Leon L, Jeannin JF and Bettaieb A: Post-translational modifications induced by nitric oxide (NO): implication in cancer cells apoptosis. Nitric Oxide *19(2)*: 77-83, 2008. PMID: 18474258. DOI: 10.1016/j.niox.2008.04.014

30 Coriat R, Nicco C, Chereau C, Mir O, Alexandre J, Ropert S, Weill B, Chaussade S, Goldwasser F and Batteux F: Sorafenib-induced hepatocellular carcinoma cell death depends on reactive oxygen species production *in vitro* and *in vivo*. Mol Cancer Ther *11(10)*: 2284-2293, 2012. PMID: 22902857. DOI: 10.1158/1535-7163.MCT-12-0093

Received September 20, 2020
Revised October 27, 2020
Accepted November 10, 2020