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Oxidative and antioxidant stress markers in cirrhosis

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Abstract
Chronic liver diseases are frequently associated with increased oxidative stress. The purpose of the study was to evaluate the correlation among oxidative and antioxidant stress markers with the cirrhosis etiology, food consumption and the liver disease severity. Transversal and prospective study, of patients with hepatitis C vírus (HCV) or alcoholic cirrhosis. There was a predominance of males in the alcoholic etiology group. The vitamin E consumption was associated with the MTT antioxidant increase. Levels of MTT, TBARS, NOX and AOPP were similar between the two etiologies. Increased AOPP levels were directly related to the liver disease severity in the HCV group. This study suggests an increase in oxidative stress according to the liver disease severity in HCV etiology. The increase of the MTT antioxidant in patients with higher intake of vitamin E suggests that food intake influences the amount of antioxidant in the plasma of patients with cirrhosis.

Keywords: oxidative stress; liver cirrhosis; hepatitis c; alcoholism; vitamin e

1. Introduction
Cirrhosis is considered a serious disease especially in its decompensated phase, being the main cause of hospitalization and mortality due to liver disease in Brazil [1]. Regardless of their etiology, chronic liver diseases are frequently followed by oxidative stress increase. Reactive oxygen species (ROS) participate in the liver fibrogenesis, contributing to ischemia / regeneration lesions, necrosis and apoptosis [2].

ROS are cellular metabolism byproducts with several functions (for example, cell signaling) that are essential for the cellular processes in low or moderated concentrations [3]. However, the imbalance between ROS generation and the antioxidant defense system provokes chronic oxidative stress, which contributes to the development of several chronic diseases and metabolic disorders [4]. The diet pattern and alcohol consumption have synergistic action to oxidative stress in the development of lipid and protein peroxidation, and ROS are one of the main resources to increase DNA damage and develop mutagenic patterns [5].

During the acute and chronic phases of HCV infection, the oxidative stress can play an important role in the disease pathogenesis [5]. Oxidative stress is also increased in the alcoholic liver disease. It is shown that chronic exposition to ethanol induces ROS production, reduces the antioxidant cellular levels, increasing oxidative stress in many tissues, especially in the liver [6].

The diet can be a source of antioxidants; in young women, it was demonstrated a positive correlation between the antioxidant capacity of a diet rich in vitamin C and polyphenols and the plasma antioxidant capacity [7].

This study aimed to evaluate the correlation among the oxidative and antioxidant stress markers with the cirrhosis etiology, the diet and the liver disease severity.

2. Patients and Methods
It was a prospective study with convenience sampling. All outpatients of a Gastroenterology clinic in a tertiary hospital in South of Brazil with cirrhosis infected by HCV or with alcoholic etiology, who attended the clinic between September of 2015 and October of 2017 were recruited.

Patients receiving enteral nutrition in the last six months, the ones with neurological diseases, hepatic encephalopathy, chronic renal failure, chronic pancreatitis, chronic diarrhea, inflammatory bowel diseases, human immunodeficiency virus (HIV) and patients with cancer in advanced stage were excluded.

Patients were interviewed to collect personal data and socio-demographic characteristics. Food registry was carried out using a 1-125g domestic scale (Plenna®) for food weighing, which was provided by the researchers. Patients were instructed to weigh and register all food consumed for three days (two days a week and one weekend day) in a detailed manner, including the trademark and preparation of each food and drink. Food registry was calculated using the Dietwin program. Caloric intake (energy), carbohydrates, proteins and lipids, and the median of A, B1, B9, B12, D, E vitamins, zinc, magnesium, potassium, sodium, calcium, iron and phosphate were also calculated. Patients did not receive nutritional supplementation.

Patients had lab tests to check the following: magnesium, potassium, sodium, ferritin, sodium, iron, albumin, total

bilirubin, prothrombin time, vitamin B12, phosphate, aspartate aminotransferase (AST), alanine aminotransferase (ALT), calciuria, proteinuria, creatinuria.

The oxidative stress evaluation was done by the following: a volume of 10 mL of blood was collected from the antecubital vein of the participants in heparinized tubes (BD, Brazil). Blood was centrifuged at 3500 rpm, aliquoted and frozen at -20° C until the analyses day. Thiobarbituric acid reactive substances (TBARS) are lipid peroxidation by products and represent an indirect oxidative stress marker. The TBARS concentrations (nmol/mL) in the plasma were spectrofluometrically determined following a previous described protocol [8]. Nitrite concentrations (NOX) were analyzed according to the methodology described by Miranda *et al*., with specific wavelength of 540 nm and the results were expressed in μM/L [9]. The advanced oxidation protein products (AOPP) in plasma were spectrophotometrically determined according to the method proposed by Witko-Sarsat *et al*. [10]. Briefly, 1 mL of plasma was diluted (1: 5) in phosphate-saline solution (10 mmol / L, pH 7,4) or standard chloramine-T solution (100 mmol / L) was mixed with 50 μL of potassium iodide (1,16 ml / L) followed by 100 μL of citric acid. The absorbance at 340 nm was immediately determined and the AOPP were expressed in mmol/L of chloramine-T equivalent. The plasma antioxidant status was determined through the direct reduction of 3 - (4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT - O.D.) in the plasma of subjects according to the technique described by Medina and collaborators [11]. For statistical analysis, the program Ms Excel 2000 was used to store data; the Statistical Package for the Social Science 21.0 (SPSS) was used to analyze the data. Quantitative variables were presented as mean and standard deviation or interquartile range and median. To compare the means, t-student test was applied for independent samples. In case of asymmetry, the Mann-Whitney test was used. The qualitative variables were shown as frequency and percentage. To verify the associations among these variables, the Pearson's chi-square test was applied. To evaluate the association among quantitative variables, Spearman or Pearson correlation tests were applied. The assumed significance level was 5%.

This research was approved by the Ethics Committee. All patients signed the informed consent.

3. Results

A total of 85 patients with cirrhosis were evaluated. HCV infection group was composed by 44 people, with mean age of 61, 9 ± 8, 3 years. In the alcoholic etiology group, 41 patients were included, mean age was 58, 4 ± 8, 8 years. There was a predominance of men in the alcoholic etiology group (p<0,001). There was no difference between the groups concerning the liver disease severity evaluated through the Child-Pugh or MELD classification (Table 1).

When food consumption and MTT, TBARS, NOX and AOPP levels were correlated, it was verified that there was a statistically significant association between vitamin E and MTT (p=0,008). On the other hand, it was shown the inverse association among the MTT levels and calorie intake (p=0,024), protein (p=0,010) and zinc (p=0,042) (Table 2).

It was observed and inverse association among TBARS and energy consumption (p=0,001), carbohydrates (p=0,001), protein (p<0,001), vitamin B1 (p=0,004), calcium (p=0,010), iron (p<0,001) and phosphate (p=0,001). It was also found an inverse association between NOX levels and sodium intake (p=0,029) (Table 2).

The mean MTT levels, TBARS, NOX and AOPP were similar between the two etiologies (Table 3).

Regarding the oxidants and antioxidants values analysis in relation to the biochemical variables, it was observed a correlation between MTT and AST. In the group with alcoholic cirrhosis, when the AST mean values were lower, highest were the MTT values (rs=-0,341; p=0,031) (Figure 1).

It was demonstrated the correlation between the increased AOPP levels and the most severe Child-Pugh classification in the HCV etiology (rs=0,448; p=0,003) (Figure 2).

Table 1: Sample Characterization (n=85).

Variables#	HCV*Group	Alchool Group	P
	(n=44; 51, 8%)	(n=41; 48, 2%)	
Age (years)	61,9 ± 8,3	58,4 ± 8,8	0,063
Male	14 (31,8)	36 (87,8)	<0,001
Caucasian	33 (75,0)	31 (75,6)	0,708
MELD*	11,1 ± 3,9	10,8 ± 2,9	0,672
Child-Pugh			0,066
A	28 (65,1)	32 (80,0)	
B	14 (32,6)	5 (12,5)	
C	1 (2,3)	3 (7,5)	
AST*	57,5 (30,5 - 102)	38 (27,7 – 48,5)	0,013
ALT*	43 (24,3 - 70,3)	28,5 (20,8 – 36,5)	0,008
GGT*	59 (33 – 101)	101 (51 – 169)	0,011

described by mean ± standard deviation, median (percentis 25-75) or n (%) * HCV: chronic hepatitis C virus, MELD: Model for end-stage liver disease, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GGT: Gamma-glutamyl transpeptidase

Table 2: Association of food consumption with oxidative and antioxidant stress markers (n=85).

Variables	MTT#	TBARS#	NOX#	AOPP#
Energy	-0,247*	-0,358**	-0,100	-0,174
CHO#	-0,206	-0,367**	-0,048	-0,147
PTN#	-0,280**	-0,437***	-0,076	-0,077
LIP#	-0,089	-0,093	-0,115	-0,138
VITB1#	-0,098	-0,312**	-0,041	-0,077
VITB9#	0,163	-0,013	-0,153	-0,078
VITB12#	-0,072	-0,033	0,006	-0,068
VITA#	-0,035	-0,062	-0,153	0,040
VITD#	0,024	-0,001	0,010	0,096

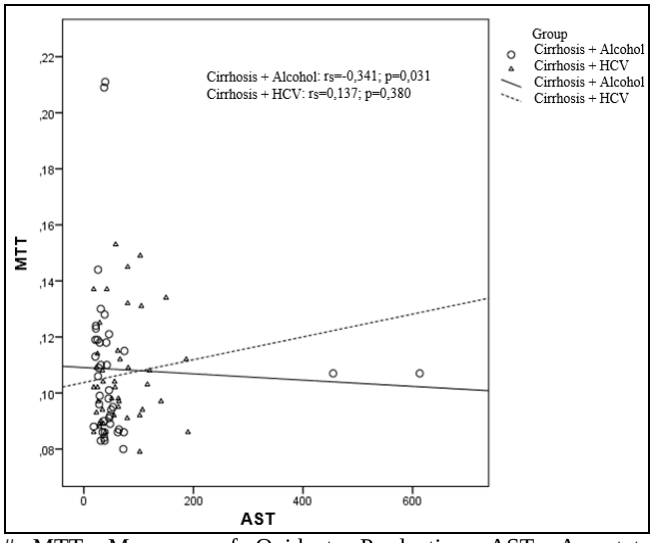
VITE#	0,287**	-0,013	-0,129	-0,077
Zinc	-0,224*	-0,106	0,001	-0,102
Magnesium	-0,179	0,143	-0,076	-0,199
Potassium	-0,172	0,115	-0,052	-0,209
Sodium	0,176	-0,004	-0,241*	-0,025
Calcium	0,049	-0,282**	-0,010	0,043
Iron	-0,170	-0,443***	-0,050	0,003
Phosphate	-0,200	-0,364**	-0,019	-0,019

*p<0, 05; ** p<0, 01; ***p<0,001 # MTT: Measures of Oxidants Production, TBARS: Lipid peroxidation, NOX: Nitrite, AOPP: advanced oxidation protein products, CHO: carbohydrates, PTN: proteins, LIP: lipids, VITB1: vitamin B1, VITB9: vitamin B9, VITB12: vitamin B12, VITA: vitamin A, VITD: vitamin D, VITE: vitamin E.

Table 3: Evaluation of oxidative and antioxidant stress markers according to the cirrhosis etiology (n=85).

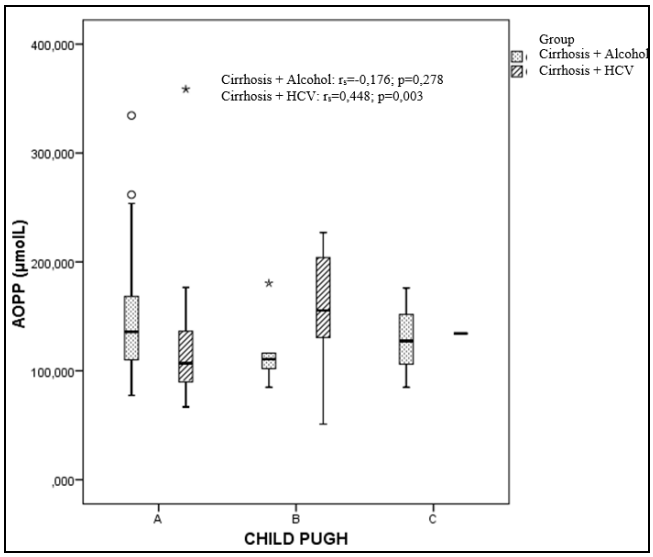
Variables	Alcoholic	HCV#	P
MTT# - mean ± DP	0,11 ± 0,03	0,11 ± 0,02	0,696
TBARS# - median (P25-P75)	12,1 (2,6-19,1)	15,6 (4,7-22,1)	0,141
NOX# - median (P25-P75)	8,8 (5,1-15,1)	11,7 (8,0-15,7)	0,222
AOPP# - median (P25-P75)	134,8 (103,6-168,4)	126,6 (96,6-151,2)	0,479

HCV: chronic hepatitis C virus, MTT: Measures of Oxidants Production, TBARS: Lipid peroxidation, NOX: Nitrite, AOPP: advanced oxidation protein products



MTT: Measures of Oxidants Production, AST: Aspartate aminotransferase

Fig 1: Correlation between MTT# and AST# in alcoholic cirrhosis (n=85)**.**



#AOPP: advanced oxidation protein products

Fig 2: Correlation between AOPP# and the HCV# severity (n=85)

4. Discussion

A total of 85 patients with cirrhosis were evaluated. HCV infection group was composed by 44 people Oxidative stress has been linked to the development of many diseases. Furthermore, the liver is one of the most susceptible organs to cell damage related to oxidation [12, 15]. In the current study, it was evaluated three substances that show the pro-oxidant state (TBARS, NOX and AOPP) and one antioxidant (MTT). In an interesting and unprecedent way, we demonstrated an association between the vitamin E intake, determined through diet evaluation, and the levels of MTT plasma antioxidant in patients with cirrhosis. Vitamin E is a fat-soluble vitamin with antioxidant properties that is frequently found in the following foods: olive oil, almonds, Pará nuts, peanuts, hazelnuts, vegetable oils, dark green leaves, among others [16]. In a study by Catanzaro *et al*., the daily oral supplementation of vitamin E was associated to the decrease of lipid oxidation products [12]. Moreover, Groenbaek *et al*. showed that supplementation with vitamin C, vitamin E and selenium increased the antioxidant condition of patients with HCV chronic hepatitis [17]. Therefore, the association showed in this study suggests the possibility of a simple diet intervention to improve health condition of patients with chronic liver disease.

Another finding of the present study, was the inverse association among MTT levels and calories, proteins and zinc. Zinc has potential cytoprotective effects against oxidative stress, apoptosis and inflammation [18]. Its deficiency can trigger oxidative stress in patients with chronic HCV-related hepatitis and liver cirrhosis [19]. It was observed a decrease in Zn serum levels in patients with chronic HCV-related liver disease in comparison to the Zn serum levels in patients presenting healthy conditions [20]. Medina *et al*. developed a simple technique to evaluate the plasma antioxidant status through measuring MTT reduction [11]. Besides, the same authors reported that theplatelets do not play an important role in the plasma antioxidant activity measured through MTT. This way, several effector antioxidants, such as ascorbate urate, sulfhydryl groups and micronutrients act in the non-enzymatic antioxidant protection [11, 20]. Metabolic and molecular changes due to progression of liver diseases change the food intake of cirrhosis patients, contributing to malnutrition in the

intermediate and final diseases' stages [21]. In addition, chronic viral infections are also associated with a condition of nutritional deficiency and the development of morbidities such as myocarditis, as well as impairments to the immune function [22]. Therefore, metabolic modifications and nutritional deficiencies may affect the systemic redox status of patients with cirrhosis. In fact, we identified an inverse correlation among lipid peroxidation and total energy, macronutrients, carbohydrate and proteins consumption. Likewise, the identification of negative correlations between MTT and total energy and protein intake may indicate an attempt to readjust the systemic antioxidant protection against changes in the redox state induced by malnutrition. When simple biochemical markers to evaluate the liver disease were analyzed, it was shown that while the AST levels were lower in the group of patients with alcoholic cirrhosis, the MTT levels were higher. AST is an enzyme that reflects the integrity of hepatocytes, increasing in diseases that result in hepatocyte necrosis and / or apoptosis. Alcoholic liver disease is associated with increased AST enzyme [23]. Some studies strongly suggest that liver damage produced by alcohol is mediated by oxidative stress. Ethanol abuse is directly involved in ROS generation, mitochondrial oxidative damage, apoptosis and hepatocyte necrosis [24, 27].

In the current study, TBARS plasma levels showed a negative correlation with both carbohydrate and daily protein intake. There are no data in the literature about macronutrient consumption and redox status in patients with cirrhosis. Previous studies have emphasized the need for adequate nutritional support to equalize changes in ROSgeneration. In rats submitted to hyper lipid diet, insufficiency in protein consumption increased lipid peroxidation together with a reduction in antioxidant defense systems [28]. In patients with renal disease, daily consumption of proteins and calories were inversely correlated with the altered redox state, emphasizing the important role of food consumption in the redox state protection [29]. In addition, since the liver is an extremely active metabolic organ, several liver diseases trigger cellular stress, with an increase in the systemic lipid peroxidation levels, which may be related to changes in dietary carbohydrate intake [2].

Micronutrient consumption has an impact on the systemic redox status of both healthy individuals and those with some chronic disease. In fact, micronutrients derived from diet may directly present antioxidant actions or work as cofactors of the enzymatic antioxidant system, leading to improvements in indicators of liver function and in the redox state [30]. Thus, we identified negative correlations of vitamin B1, calcium, iron and phosphate in relation to TBARS plasma levels.

Studies involving the micronutrients dietary pattern, especially minerals and vitamins, are complex because several of these micronutrients have various physiological functions that can inhibit or stimulate systemic reactions. While liver tissue is an important site for metabolism and biochemical reactions involving micronutrients, chronic diseases as cirrhosis alter their bioavailability (30). Deficiency in iron, calcium and / or phosphate may cause increased ROS generation and lipid peroxidation [31, 32]. Regarding vitamin B1, in vitro studies demonstrated that treatment of hepatocytes with vitamin B1 reduced protein damage, lipoperoxidation and DNA oxidation [33].

There is a proven increase in plasma and tissue levels of lipid peroxidation markers in patients with liver disease [34, 37]. It was demonstrated that lipid peroxidation may precede the initial fibrosis stages and be associated with increased TGF-β1 production (pro-fibrinogen) by Kupffer cells [25]. Peng *et al*. demonstrated that malondeido concentrations (MDA), reflecting increased lipoperoxidation, not only significantly increased in the alcoholic liver disease group, but were also with the duration of the alcohol addiction [38]. In the study by Khadem Ansari *et al*., it was observed that the MDA serum concentration was significantly high in HCV infected patients compared to controls; Cunningham-Rundles *et al*. also showed a correlation between MDA levels and severity of chronic hepatitis [39, 40].

Concerning NO, it is postulated to promote toxicity when in contact with the radical superoxide anion, leading to the formation of peroxynitrite anion and hydroxyl radical, potent oxidants that can cause lipid peroxidation [41]. There is evidence that NO is associated not only with the liver disease, but also with its severity. Nandeesha *et al*. demonstrated that NO serum and TNFα levels were significantly increased in patients with alcoholic cirrhosis compared to controls. A positive association was found between NO and Child-Pugh score (r = 0.391, P = 0.007) and MELD score (r = 0.311, P= 0.033) [42]. A similar study also found increased NO levels in patients with liver disease compared to the control group (P <0.001). In addition, NO levels proportionally increased with the liver cirrhosis severity as assessed by the Child-Pugh classification (P <0.05) [43]. In the study by Lluch *et al*., NO and plasma levels of asymmetric dimethyl-L-arginine (ADMA), an endogenous protein inhibiting nitric oxide synthesis, were also elevated in patients with decompensated cirrhosis compared to those with compensated disease and controls without hepatic damage [44]. In the present study, an inverse association between NOX and sodium intake was demonstrated. Patients with ascites, mostly classified as Child-Pugh B or C, were instructed to decrease sodium intake as an important measure for ascites control.

AOPP levels were correlated with the cirrhosis severity assessed by Child-Pugh classification only in the case of HCV etiology in the current study. Contrary, Zuwała-Jagiełło *et al*., found a correlation of this pro-oxidant only with alcoholic cirrhosis [45]. AOPPs reflect the pro-oxidant state and can be considered a proinflammatory molecule. Some studies have demonstrated its correlation with inflammation and fibrosis in chronic liver disease [45, 46]. Ozenirler *et al*. evaluated AOPP serum levels in 29 patients with chronic HCV infection without previous treatment, in whom hepatic transaminase levels were persistently elevated in the last six months. AOPP levels were found to be significantly higher in the HCV group compared to control (235, 0 ± 142, 8 vs. 116, 7 + 79, 5, respectively, p <0,001) [47].

Liu *et al*. evaluated AOPP levels in 50 patients with acute on chronic liver failure, 30 patients with compensated liver cirrhosis, 30 patients with chronic hepatitis B, and 50 healthy ones. AOPP levels were significantly higher in patients with acute on chronic liver failure compared to cirrhotic patients, chronic hepatitis B patients and healthy ones (69, 45 ± 29, 04 μmol / L vs. 19, 67 ± 7, 02 μmol / L, 26, 75 ± 5, 21 μmol / L and 21, 35 ± 6, 15 μmol / L, respectively, P <0,001). There was a correlation between increased AOPP levels and liver disease severity. In patients with acute on chronic liver failure, AOPP levels were higher

in those who died, suggesting that this marker may serve as an important biological marker of bad prognosis [48].

In conclusion, we demonstrated that AOPP level increases according to the liver disease severity in cirrhosis by HCV. Moreover, food consumption was able to influence the amount of antioxidant in the plasma of patients with cirrhosis. These finding emphasizes the importance of nutritional therapy to such patients.

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