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Assessment of lipid peroxidation and antioxidant capacity in non-alcoholic fatty liver disease

Alkole bağlı olmayan yağlı karaciğer hastalığında lipid peroksidasyonu ve antioksidan kapasite değerlendirilmesi

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Background/aims: *Lipid peroxidation/oxidative stress and/or endotoxin-induced cytokine release are implicated in the non-alcoholic fatty liver disease pathogenesis. Studies in the literature are experimental in nature and are based on histopathological evaluation and peripheral blood findings. In this study, we aimed to investigate the relationships between lipid peroxidation and antioxidant capacity in non-alcoholic fatty liver disease.* ***Methods:*** *Twenty-six patients with an ethanol consumption of less than 20 g/day who were diagnosed ultrasonographically and histopathologically as non-alcoholic fatty liver disease and 16 healthy control subjects with normal ultrasonographical findings were included in the study. All viral and autoimmune markers in patient and control groups included in the study were negative. Non-alcoholic steatohepatitis samples obtained by fine needle aspiration were evaluated according to Brunt et al. The levels of glutathione, catalase, superoxide dismutase, and malonyldialdehyde in peripheral blood and liver biopsy samples were measured.* ***Results:*** *Of patients with non-alcoholic fatty liver disease, 17 (65%) were detected to have mild, 7 (27%) moderate and 2 (8%) severe steatosis; portal inflammation was found in 17 patients (65%) and stage I fibrosis in 21 patients (80%). Minimal lobular inflammation was observed in all patients. In the patient group, the levels of erythrocytic glutathione, catalase, and superoxide dismutase were significantly lower but malonyldialdehyde levels were higher compared to the control group. It was revealed that hepatocytic reduced glutathione, catalase, and malonyldialdehyde levels were not correlated with peripheral blood levels, but there was a positive correlation between liver malonyldialdehyde level and liver reduced glutathione level. Plasma malonyldialdehyde level and liver glutathione had a negative correlation.* ***Conclusions:*** *It was discovered that lipid peroxidation and antioxidant capacity suppression due to its overuse were important in the non-alcoholic fatty liver disease pathogenesis, but antioxidant capacity was maintained well at tissue level in the early stages of the disease. Furthermore, it was identified that tissue lipid peroxidation and changes in antioxidant capacity were not reflected in the peripheral blood to the same extent.*

Key words: Non-alcoholic fatty liver disease, lipid peroxidation, antioxidant capacity

Amaç: *Lipid peroksidasyonu/ oksidatif stres ve /veya endotoksin bağımlı sitokin salınımı alkole bağlı olmayan yağlı karaciğer hastalığının patogenezinde sorumlu tutulmaktadır. Literatürdeki çalışmalar, deneysel nitelikte olup histopatolojik değerlendirme ve periferik kan bulgularına dayanmaktadır. Bu çalışmada alkolik olmayan yağlı karaciğer hastalığında lipid peroksidasyonu ve antioksidan kapasite arasındaki ilişkileri araştırmayı amaçladık.* ***Yöntem:*** *Çalışmaya etanol kullanımı 20g/günden az olan, ultrasonla ve histopatolojik olarak tanısı konulan 26 alkole bağlı olmayan yağlı karaciğer hastalığı olan hasta ve ultrason bulguları normal olan 16 sağlıklı kontrol alındı. Çalışmaya alınan hasta ve kontrol grubunun tüm viral ve otoimmun markerleri negatifti. İnce iğne biyopsisi ile alınan NASH örnekleri Brunt ve arkadaşlarına göre değerlendirildi. Periferik kan ve karaciğer biopsi örneklerinde glutatyon, katalaz ve süperoksit dismutaz ile malonildialdehid düzeyleri ölçüldü.* ***Bulgular:*** *Alkole bağlı olmayan yağlı karaciğer hastalığı olan hastaların 17'sinde hafif (%65), 7'sinde orta (%27), 2'sinde ağır (%8) steatozis saptanırken, portal inflamasyon 17 hastada (%65), stage I fibrozis 21 hastada (%80) saptandı. Minimal lobuler inflamasyon ise tüm hastalarda gözlendi. Hasta grubunda kontrol grubuna göre eritrosit glutatyon, katalaz ve süperoksid dismutaz düzeyleri anlamlı olarak düşükken malonildialdehid düzeyleri yüksekti. Karaciğer redükte glutatyon, katalaz ve malonildialdehid düzeylerinin periferik kan düzeyleri ile ilişkili olmadığı, karaciğer malonildialdehid düzeyi ile karaciğer redükte glutatyon düzeyi arasında pozitif; plazma malonildialdehid düzeyi ile karaciğer glutatyon düzeyi arasında negatif ilişki olduğu saptandı.* ***Sonuç:*** *Alkole bağlı olamayan yağlı karaciğer hastalığı patogenezinde lipid peroksidasyonunun ve antioksidan kapasitede fazla kullanıma bağlı baskılanmanın önemli olduğu; ancak hastalığın erken dönemlerinde doku düzeyinde antioksidan kapasitenin iyi korunduğu saptanmıştır. Ayrıca doku lipid peroksidasyonu ve antioksidan kapasite değişikliklerinin perifere aynı düzeyde yansımadığı da belirlenmiştir.*

Anahtar kelimeler: Alkole bağlı olmayan yağlı karaciğer hastalığı, lipid peroksidasyonu, antioksidan kapasite

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INTRODUCTION

Clinical and histopathological changes seen in patients who do not consume hepatotoxic levels of alcohol, characterized by development of steatohepatitis and cirrhosis from steatosis as in alcoholic liver disease, are referred to as non-alcoholic fatty liver disease (NAFLD) (1).

NAFLD, first described as non-alcoholic steatohepatitis (NASH) by Ludwig et al. in 1980, has been accepted within the past 20 years as one of the frequently seen important forms of chronic liver diseases (2). The pathogenesis of NAFLD has not been understood completely. Lipid retention in hepatocytes, predominantly triglycerides and fatty acids, is an important and early sign in the development of NAFLD. However, primary metabolic abnormalities that cause lipid accumulation are not yet exactly known. In the literature, it is suggested that, considering the risk factors for NAFLD, insulin resistance syndrome and the resultant metabolic changes may have a central role in the pathogenesis (3, 4). The clinical condition is attributed to lipid peroxidation/oxidative stress and/or endotoxin-induced cytokine release.

Most of the trials performed in NAFLD, because of its broad spectrum and the recent increases in its prevalence, have focused on clinical, biochemical or histopathological markers by which progressive disease may be determined (5, 6). Most of the clinical studies have involved comparison of peripheral blood samples and histopathological findings or follow-up of post-treatment changes based on risk factors and hypotheses. Most studies at the liver level are of experimental nature. In our study, it was aimed to investigate the role of lipid peroxidation and antioxidant capacity changes in peripheral blood and liver in the pathogenesis of NAFLD.

MATERIALS AND METHODS

This study was conducted prospectively in the Gastroenterology Department of the University of Osmangazi in Eskişehir in 2002-2003 after approval by our faculty's Ethics Committee.

A total of 42 cases (22 males, 20 females) who admitted to our Internal Medicine and Gastroenterology Clinics were included in the study.

Inclusion Criteria

1. No alcohol consumption or consumption of less than 20 g/day.

2. Presence of anti-HCV negativity, HBs Ag negativity, and negativity of specific autoantibodies for autoimmune liver disease.

3. No drug intake (glucocorticoids, estrogens, tamoxifen, amiodarone, perhexiline) leading to hepatosteatosis.

4. Absence of total parenteral nutrition, rapid weight loss, massive intestinal resection, gastropathy, and Wilson's disease, which cause hepatosteatosis.

5. Absence of diabetes mellitus (fasting blood glucose <126 mg/dl).

All patients were divided into two groups after ultrasonographical examination. The hepatosteatosis cases diagnosed ultrasonographically and confirmed by biopsy were considered Group I (26 cases) and healthy cases without detectable hepatosteatosis as Group II (16 cases). The blood samples from patients necessary for hematological and biochemical parameters were obtained following a 12-hour fasting. Biochemical parameters were studied using Böhringer Manheim-Hitachi 917 autoanalyzer and Böhringer Manheim-Hitachi original kits.

The parameters of oxidative stress and antioxidant capacity in the patients were studied in the Biochemistry Department of the University of Kocatepe in Afyon.

Blood samples transferred in EDTA tubes were centrifuged at 2000 rpm for 10 min and their plasma fractions were stored at -20°C to measure malonyldialdehyde (MDA) levels. Erythrocytes were washed with saline solution three times and erythrocyte packs were prepared and stored at -20°C to measure gluthatione (GSH) level and catalase and superoxide dismutase activities. The tissue samples were homogenized with Ultra Turrax (IKA T18 basic, Wilmington, NC, USA) homogenizator using 0.1 M phosphate buffer of pH 7.4. The homogenates were centrifuged at 5000 rpm for 10 min at +4°C and the supernatants were used as samples.

All chemical substances were obtained from Sigma Chemical Co (St. Louis, USA). UV-120 Shimadzu spectrophotometry was used for spectrophotometric measurements.

Tissue protein levels were measured by biuret method and the hemoglobin levels using Drabkin solution.

Modified method of Brunt et al. was used for NAFLD staging in biopsy samples (7, 8).

Statistical analyses were done using SPSS 10.0 statistics program, X^2 test, Student's *t* test, Spearman's correlation analysis, and Pearson's correlation analysis.

RESULTS

Table 1 depicts the general characteristics of patient and control groups. A statistically significant difference between the groups was observed only in gender distribution and transaminase levels.

Examination of biopsy samples in our patient group revealed balloon degeneration and minimal lobular inflammation in all cases. It was determined that 17 (65%) of the cases had mild, 7 (27%) had moderate, and 2 (8%) had severe steatosis. Portal inflammation was found in 17 (65%) of the cases. In steatohepatitis staging, 1 case was found to be consistent with stage II (moderate) and the other cases with stage I (mild). While stage II fibrosis was detected in 1, stage III fibrosis in 1, and stage I fibrosis in 21 of our cases, there was no fibrosis detectable in 3 cases. Lipogranuloma was found in 5 cases (19%). Minimal iron accumulation was determined in the liver in 10 (39%) of the cases.

Table 1. General characteristics of the cases

	Group I n=26	Group II n=16	p
AGE (years)	44.9±1.8	41.2±1.5	p>0.05
GENDER M	17	5	χ_2 =4.63
F	9	11	p<0.05
AST (U/L)	25.2±1.7	17.7±1.4	p<0.01
ALT (U/L)	40.9±4.8	20.36±2.3	p<0.001
GGT (U/L)	34.9±5.7	22.81±3.6	p>0.05
ALP (U/L)	221.0±20.2	174.0±8.1	p>0.05
T. BILIRUBIN (mg/dl)	0.88±0.08	0.65±0.07	p>0.05
D. BILIRUBIN (mg/dl)	0.27±0.03	0.19±0.02	p>0.05
T. PROTEIN (g/dl)	7.44±0.12	7.45±0.09	p>0.05
ALBUMIN (g/dl)	4.37±0.08	4.32±0.05	p>0.05

When the erythrocytic superoxide dismutase (SOD), erythrocytic GSH, erythrocytic catalase (CAT), and plasma MDA values in Groups I and II were compared, there were statistically significant differences between erythrocytic SOD (p<0.01), erythrocytic GSH (p<0.001), erythrocytic CAT (p<0.001), and plasma MDA (p<0.001) values (Table 2).

Table 2. Comparison of erythrocytic SOD, erythrocytic GSH, erythrocytic CAT, and plasma MDA values in Groups I and II

	Group I	Group II	p
E. SOD (U/gHb)	35.26±2.41	47.26±2.84	p<0.01
E. GSH (nmol/gHb)	34.09±2.19	56.90±5.03	p<0.001
E. CAT (U/gHb)	4676.19±209.07	6425.38±356.38	p<0.001
P. MDA (Umol/L)	7.86±0.47	5.69±0.32	p<0.001

There were no statistically significant differences between males and females in patients with NAFLD and controls in oxidative stress/antioxidant capacity markers (Table 3a-b). Erythrocyte SOD levels were lower and plasma MDA levels were higher than controls in female patients with NAFLD (Table 4). There was no correlation between oxidative stres/antioxidant capacity and age in female patients with NAFLD. However, catalase levels were correlated negatively with age in the control group (r= -0.726; p<0.05).

Table 3a-b. Comparison of the oxidative stress/antioxidant capacity markers in males and females in Groups I and II

A	Patients with NAFLD		
	Female	Male	p
E. SOD (U/gHb)	35.26±2.89	35.25±3.42	>0.05
E. GSH (nmol/gHb)	36.89±4.25	32.59±2.50	>0.05
E. CAT (U/gHb)	5071.11±248.3	4466.11±283.24	>0.05
P. MDA (Umol/L)	8.25±0.65	7.65±0.64	>0.05
B	**Controls**		
	Female	Male	p
E. SOD (U/gHb)	45.13±3.46	51.94±4.78	>0.05
E. GSH (nmol/gHb)	52.06±6.32	67.54±6.53	>0.05
E. CAT (U/gHb)	6259±502.73	6789.60±292.90	>0.05
P. MDA (Umol/L)	6.00±0.40	5.00±0.42	>0.05

There was no correlation between gender and erythrocyte SOD, GSH, CAT and plasma MDA in patients with NAFLD.

When the patients with normal alanine aminotransferase (ALT) levels were compared with those with high ALT levels in our patient group, there was a statistically significant difference only in erythrocytic GSH (p<0.05) and erythrocytic CAT (p<0.01) values (Table 5) (Figure 1). When comparing the group with normal alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT) levels to the group with high ALP and/or GGT levels in our patient group, a statistically significant

Table 4. General features of the females in the study

	Group I n=9	Group II n=11	p
	52.33±1.95	41.18±2.19	<0.01
E. SOD (U/gHb)	35.26±2.89	45.13±3.46	<0.05
E. GSH (nmol/gHb)	36.89±4.25	52.06±6.32	>0.05
E. CAT (U/gHb)	5071.11±248.37	6259±502.73	>0.05
P. MDA (Umol/L)	8.25±0.65	6.00±0.4	<0.01

difference was only seen in erythrocytic GSH levels (p<0.01) (Table 6).

In the liver-biopsied patients, a significant positive correlation was found between liver MDA level and liver GSH, liver CAT and liver SOD levels, and erythrocytic SOD and erythrocytic CAT levels

Table 5. Comparison of patients with normal ALT levels (Group I) to those with high ALT levels (Group II)

	Group I n=19	Group II n=7	p
E. SOD (U/gHb)	38.04±2.74	27.71±3.98	p<0.05
E. GSH (nmol/gHb)	37.06±2.58	26.01±2.23	p<0.05
E. CAT (U/gHb)	4998.37±200.94	3801.71±416.23	p<0.01
P. MDA (Umol/L)	7.99±0.58	7.51±0.84	p>0.05

in the correlation analysis of liver MDA, CAT, SOD and GSH values and erythrocytic SOD, erythrocytic GSH, erythrocytic CAT, and plasma MDA values. Moreover, a negative relationship was determined between tissue GSH and plasma MDA, and liver CAT and erythrocytic GSH (Table 7).

A positive correlation between serum ALP level and liver MDA (r:0.558; p<0.05) and catalase (r: 0.487; p<0.05) levels and between ALT level and liver GSH level (r:0.477; p<0.05) was found in our patient group. A similar correlation was not observed in the other parameters. There was positive

Table 6. Comparison of the patients with normal ALP and GGT levels (Group I) to those with high ALP and/or GGT levels (Group II)

	Group I n=18	Group II n=8	p
E. SOD (U/gHb)	35.02±2.62	35.79±5.48	p>0.05
E. GSH (nmol/gHb)	38.53±2.42	24.07±1.70	p<0.01
E. CAT (U/gHb)	4566.83±259.53	4922.25±356.07	p>0.05
P. MDA (Umol/L)	7.47±0.58	8.73±0.78	p>0.05

correlation between liver GSH and age in female patients with NAFLD (r=0.977; p<0.01).

DISCUSSION

Oxidative stress can be described as a condition resulting from an uncontrolled increase in free oxygen radicals or an insufficiency in the antioxidant system under certain pathological states. Free oxygen radicals have important toxic effects; chiefly the hydroxyl radical and to a lesser extent the superoxide anion lead to peroxidation of membrane lipids thereby causing production of MDA and 4HNE. These substances directly induce hepatocytic damage with generation of proinflammatory cytokines, activation of spindle cells, and fibrogenesis (9-11). The best known components of the endogenous antioxidant system are SOD, catalase, glutathione peroxidase, reduced glutathione, and glutathione transferase. GSH, an

Table 7. Correlation analysis of liver MDA, CAT, SOD, and GSH levels and erythrocytic SOD, erythrocytic GSH, erythrocytic CAT, and plasma MDA values in liver-biopsied patients (Group I)

	Tissue mda	Tissue cat	Tissue sod	Tissue gsh	Tissue tnf-α	E-sod	E-gsh	E-cat	P-mda
Tissue mda	r=1.00 n=18 p>0.05	r=0.16 n=18 p>0.05	r=0.07 n=18 p>0.05	r=0.48 n=18 p<0.05	r=0.44 n=18 p>0.05	r=0.09 n=18 p>0.05	r=0.16 n=18 p>0.05	r=0.18 n=18 p>0.05	r=0.2 n=18 p>0.05
Tissue cat	r=0.16 n=18 p>0.05	r=1.00 n=18	r=0.57 n=18 p<0.05	r=0.01 n=18 p>0.05	r=0.18 n=18 p>0.05	r=0.17 n=18 p>0.05	r=0.48 n=18 p<0.05	r=0.03 n=18 p>0.05	r=0.22 n=18 p>0.05
Tissue sod	r=0.74 n=18 p>0.05	r=0.57 n=18 p<0.05	r=1.00 n=18	r=0.02 n=18 p>0.05	r=0.16 n=18 p>0.05	r=0.25 n=18 p>0.05	r=0.47 n=18 p>0.05	r=0.02 n=18 p>0.05	r=0.10 n=18 p>0.05
Tissue gsh	r=0.48 n=18 p<0.05	r=0.01 n=18 p>0.05	r=0.02 n=18 p>0.05	r=1.00 n=18	r=0.44 n=18 p>0.05	r=0.31 n=18 p>0.05	r=0.30 n=18 p>0.05	r=0.14 n=18 p>0.05	r=0.6 n=18 p<0.05
E-sod	r=0.10 n=18 p>0.05	r=0.17 n=18 p>0.05	r=0.25 n=18 p>0.05	r=0.31 n=18 p>0.05	r=0.22 n=18 p>0.05	r=1.00 n=26	r=0.13 n=26 p>0.05	r=0.42 n=26 p<0.05	r=0.28 n=26 p>0.05
E-gsh	r=0.16 n=18 p>0.05	r=0.48 n=18 p<0.05	r=0.47 n=18 p>0.05	r=0.29 n=18 p>0.05	r=0.26 n=18 p>0.05	r=0.13 n=26 p>0.05	r=1.00 n=26	r=0.29 n=26 p>0.05	r=0.09 n=26 p>0.05
E-cat	r=0.18 n=18 p>0.05	r=0.03 n=18 p>0.05	r=0.02 n=18 p>0.05	r=0.14 n=18 p>0.05	r=0.15 n=18 p>0.05	r=0.42 n=26 p<0.05	r=0.29 n=26 p>0.05	r=1.00 n=26	r=0.24 n=26 p>0.05
P-mda	r=0.22 n=18 p>0.05	r=0.22 n=18 p>0.05	r=0.10 n=18 p>0.05	r=0.59 n=18 p<0.05	r=0.09 n=18 p>0.05	r=0.28 n=26 p>0.05	r=0.09 n=26 p>0.05	r=0.24 n=26 p>0.05	r=1.00 n=26

intracellular reducing tripeptide, exhibits its potent antioxidant effect by preventing the antioxidant damage from free radicals through non-catalytic direct reaction and allowing SH groups of proteins to remain continuously reduced.

In our study, the level of plasma MDA, which is the end product of lipid peroxidation, was determined to be 7.86 ± 0.47 Umol/L in NAFLD patients and 5.69 ± 0.32 Umol/L in the control group, with the difference between them detected to be statistically significant ($p<0.01$). Antioxidant capacity components of erythrocytic SOD ($p<0.01$), erythrocytic CAT ($p<0.001$) and erythrocytic GSH ($p<0.001$) were found to be at statistically significantly lower levels in the patient group than in the control group. In our patient group, erythrocytic GSH level was also found to be significantly lower in those cases with high enzyme levels.

Consistent with the literature, our findings show that an increase in lipid peroxidation and a suppression of antioxidant capacity because of its consumption play an important role in the development of NAFLD and NASH (9-11).

A number of studies have demonstrated that peripheral findings are not parallel with target tissue changes in clinical conditions arising from oxidative stress and an inadequacy in the antioxidant defense system. Therefore, measurement of lipid peroxidation and changes in antioxidant markers in peripheral and hepatic tissue would be an ideal approach in the NAFLD pathogenesis. However, most of the studies conducted on this issue are of experimental nature. In our study, the parameters of lipid peroxidation and antioxidant defense system were investigated in the liver samples from 18 of 26 patients diagnosed as having NAFLD and biopsied.

In our study, no statistically significant correlation was found between tissue and erythrocytic SOD, CAT, and GSH levels. Consistent with the literature, this finding shows that target tissue lipid peroxidation and antioxidant system changes are not reflected in the peripheral blood to the same extent (11, 12).

The most important antioxidant system in the liver is the GSH system. GSH plays a key role against lipid peroxidation through its influence both on enzyme catalyzing systems and other antioxidants such as vitamin E, vitamin C and selenium, which are crucial components of the non-enzymatic pathway (13).

In the reported studies, most of which are experimental, the results of those on antioxidant system changes in acute and/or chronic livers disease are controversial. The most extensive trials on this issue have been performed in alcoholic steatohepatitis (ASH). Chronic use of alcohol is suggested to cause depletion in hepatic GSH, particularly in mitochondrial GSH. GSH depletion is attributed to a defect in translocation of cytosolic reduced GSH to mitochondria and/or a decrease in GSH resynthesis capacity. This ethanol-induced depletion in the mitochondrial antioxidant defense mechanism is proposed to increase hepatic damage by rendering cells more sensitive to the oxidative stress from inflammatory cytokines such as tumor necrosis factor (TNF)-α and making them targets for free oxygen radicals (11). The studies in chronic hepatitis C patients also demonstrated depletion in antioxidant capacity, especially in cirrhotic cases, coupled with an increase in oxidative stress due to lipid peroxidation (14-16).

Clinical/experimental studies of lipid peroxidation and antioxidant capacity in NAFLD have involved assessment of response to treatment with antioxidant drugs with particular reference to the pathogenesis.

Most of the obese and experimental NASH models of NAFLD pathogenesis also showed decreases in hepatic GSH levels (17-21).

In our study, it was found that liver MDA, which is an indicator of lipid peroxidation, showed a significant positive correlation only with liver GSH levels. A negative correlation was determined between tissue GSH level and plasma MDA level.

In a study in which Yadav et al. assessed the non-enzymatic antioxidant capacity in 20 patients with chronic hepatitis C, they found depletion in the non-enzymatic capacity that was not to the same extent in the liver and serum, and an association of the decrease in capacity with fibrosis. In that study, it was shown that MDA levels in liver biopsy were higher in moderate-severe inflammation, but there was no substantial difference in antioxidant levels. These investigators explained their findings by the presence of an adequate antioxidant pool in the early stages of the disease before development of fibrosis (16).

This study supported that peripheral antioxidant capacity was influenced by age in females due to decrease of estrogen, despite GSH stores being unexpectedly well protected in the liver.

We determined in this study that all of our patients had steatosis with varying grades: 25 had grade I steatohepatitis, and 21 had a grade I fibrosis. Thus, our findings support that liver antioxidant capacity is well maintained in the early stages of NAFLD. Only those trials in new cases with histopathological findings at different stages will clarify this issue.

In conclusion, it was discovered that there is an increase in lipid peroxidation and a decrease in antioxidant capacity in NAFLD cases compared to the control group, and that tissue lipid peroxidation and antioxidant capacity changes are not reflected in the peripheral blood to the same degree. In addition, it was found that in the early stages of the disease, antioxidant capacity at the tissue level is well protected.

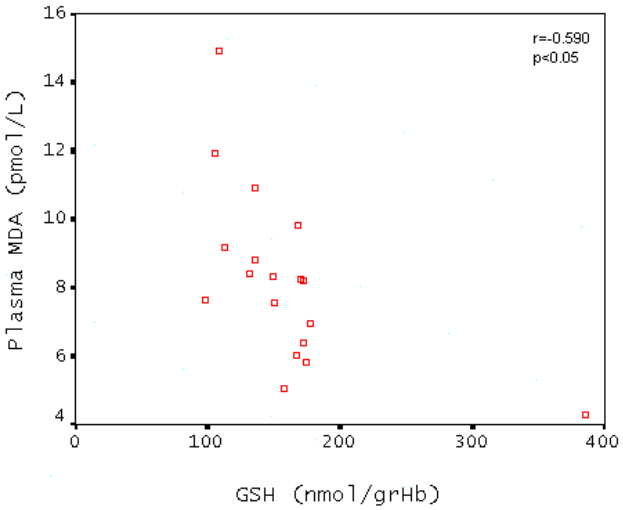

Figure 1. Correlation between tissue GSH and plasma MDA in NAFLD cases

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