

Malondialdehyde Levels and Ferric Reducing Ability of Plasma in Non-Alcoholic Fatty Liver Disease Patients: A Biomarker Study

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ABSTRACT

Background: Oxidative stress, defined as an imbalance between the production of free radicals and the body's ability to neutralize them with antioxidants, is a key contributor to the pathogenesis of Non-Alcoholic Fatty Liver Disease (NAFLD). Malondialdehyde (MDA), a marker of lipid peroxidation, and the Ferric Reducing Ability of Plasma (FRAP), a measure of total antioxidant potential, are critical biomarkers for assessing oxidative stress in NAFLD. **Objective:** This study aimed to evaluate serum MDA and FRAP levels in NAFLD patients to explore their potential as biomarkers for oxidative stress and antioxidant status. **Methodology:** A case-control study was conducted from December 2023 to March 2024, involving 120 NAFLD patients (mean age 51.62 ± 11.25 years) and 60 healthy controls (mean age 50.15 ± 9.56 years). Serum MDA levels were measured as an indicator of lipid peroxidation, while FRAP levels were assessed to evaluate total antioxidant capacity. **Results:** NAFLD patients demonstrated significantly lower FRAP levels compared to the control group ($p < 0.05$), indicating reduced antioxidant potential. Conversely, MDA levels were significantly higher in the NAFLD group, reflecting increased oxidative stress and lipid peroxidation. **Conclusion:** Elevated MDA and reduced FRAP levels are strongly associated with oxidative stress in NAFLD patients. These findings highlight the potential utility of MDA and FRAP as biomarkers for monitoring disease progression and evaluating therapeutic interventions targeting oxidative stress in NAFLD.

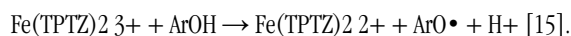
Keywords: NAFLD, Malondialdehyde (MDA), Ferric-reducing ability of plasma (FRAP) and Reactive oxygen species (ROS).

INTRODUCTION

NAFLD is a heterogeneous disease which is associated with varying severity and clinical course. These variations may be due to the several main mechanisms that underlie the development of the disease [1]. NAFLD is the liver manifestation of a group of diseases associated with metabolic dysfunction [2]. NAFLD is a disease that has many features including the different rates of NAFLD progression and different clinical results. These factors may be influenced by the various main mechanisms which have been known to cause the development of the disease. This study has also revealed that the prevalence of NAFLD is rather high in Asian countries [3]. Nonalcoholic steatohepatitis (NASH) related cirrhosis or HCC is a rare complication, affecting only 2.5% of the patients. This evolution is usually less rapid than in the other chronic liver diseases. Also, more than 70% of patients with cryptogenic cirrhosis have at least one major criterion for NAFLD [4]. NAFLD patients have increased the levels of serum oxidative stress markers including MDA and decreased antioxidants activities [5]. Insulin resistance, inflammation and oxidative stress are the major factors in the development of the disease and should be considered as the key pathological mechanisms [6]. Oxidative stress (OS) is a state of disequilibrium which results from the overproduction of reactive oxygen species (ROS) and the inability of antioxidant mechanisms to detoxify them [7]. The process of the generation of ROS starts with the formation of the superoxide anion (O_2^-) which is a byproduct of standard metabolic processes and then leads to formation of other ROS. The toxic impact of ROS becomes a concern because they are capable of reacting with bio-molecules including protein, to produce carbonyls. In addition, ROS may interact with lipids to form 4-hydroxynonenal and malondialdehyde, respectively. Also, ROS and

DNA may interact to form 8-hydroxylation of guanine (8-OHdG) that alters DNA structure and its function [8]. MDA emerged in the last two decades as one of the potent markers of lipid peroxidation. Thus, the determination of MDA levels in biological materials of patients with different diseases has been widely used [5]. Malondialdehyde (MDA) is the primary and extensively researched compound that results from the peroxidation of polyunsaturated fatty acids [4]. Lipid peroxidation produce a number of pro-inflammatory chemicals that are involved in the progression of the disease. MDA is one of the most used markers of the oxidative damage and appear as a by-product of lipid peroxidation. Also, MDA exerts its effect on hepatic stellate cells whereby they are stimulated to produce collagen and cause fibrosis [9]. Patients with NAFLD have been reported to have increased levels of serum oxidative stress markers including MDA while the antioxidant activity has been reported to be reduced. Several researches have shown high plasma MDA levels in diabetic patients or those with obesity associated non-alcoholic fatty liver disease (NAFLD) than normal subjects [10]. The possibility of lipid peroxidation as a destructive process for the cellular membranes and its potential importance in biology was first discussed in the sphere of radiation injury [11]. Due to the generation of free radicals through the process of water ionization and in the sphere of nutrition due to deficiency of vitamin E. [12]. When biological membranes are exposed to oxidative stress, they undergo a gradual deterioration of their structure and a decrease in their activity. Lipid peroxidation is the broad term used to describe the destruction of molecules such as cholesterol and phospholipids that contain unsaturated fatty acyl moieties, which are susceptible to oxidative attack [13]. The FRAP assay is a new approach for evaluating

the antioxidant strength of plasma by measuring its ferric-reducing ability [14]. The reduction of ferric ions to ferrous ions at low pH results in the formation of a complex with tripyridyltriazine, which exhibits a distinct colour [14]. FRAP values could be calculated by comparing the difference in absorbance at 593nm between test reaction mixtures and mixtures with known amounts of ferrous ions [14].



MATERIAL AND METHOD

Blood Sample Collecting

The samples of blood from all patients were collected while they were in a fasting nation, between 9 and 11 A.M., and they had not consumed any alcohol. The clinical investigation was conducted from February 2024 to October 2024.

Study Design

The present study comprised a total of 120 participants, with 60 of them being healthy controls and the remaining 60 being patients with non-alcoholic fatty liver. The study excluded participants who consumed alcohol. The participants were classified into:

G1: control group., **G2:** non-alcoholic fatty liver patients.

Inclusion and Exclusion Criteria

Inclusion

The study involved collecting the medical history of each participant, which encompassed their family history, duration of the disease, and the therapies they received. Additionally, a mention was made of a participant who had been diagnosed with another disease that could potentially impact the study. Weight and height measurements were taken in order to compute the body mass index of the study participant. The present investigation focused on individuals diagnosed with non-alcoholic fatty liver disease. Healthy adult volunteers were chosen as control subjects. Each subject provided informed written consent. At first, all participants were interviewed to gather information about their medical history and underwent a physical examination.

Exclusion

Alcoholics were excluded as both cases and controls.

Chemicals

All The chemicals were used as provided without any sort of purification.

Assessment of Ferric-Reducing Ability of Plasma (FRAP)

In this study, we measured Total Antioxidant Capacity (TAC) using a Ferric-Ferro zine reagent. The proposed assay depends on reducing a Ferric-Bipyridine reagent with antioxidants to the stable Ferrous-Bipyridine chelate in the buffered medium. The methods vary greatly concerning the free radical generated, the generation process's reproducibility, and the endpoint used. The reductive techniques do not generate free radicals but measure the serum's ability to act against an oxidizing agent, Fe^{3+} , for the FRAP assay. The FRAP (Ferric Reducing Antioxidant Power) approach measures the ability of a sample to reduce iron, using the method established by Benzie and Strain. The quantification of results was performed by measuring the

absorbance at 595 nm using a standard curve of known concentrations of Trolox 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Aldrich Chemical Co., Gillingham, Dorset, UK), in accordance with our laboratory protocol.

Assessment of Malondialdehyde (MDA)

Malondialdehyde was obtained from serum samples with the application of 20 percent TCA. The serum samples underwent centrifugation at a speed of 8000 revolutions per minute and were subsequently passed over a membrane with a pore size of 0.2 micrometres. The liquid portion was subjected to treatment with Thiobarbituric acid at a concentration of 20 millimolar and heated in a water bath for a duration of 60 minutes. A 10µL sample was introduced into high-performance liquid chromatography (HPLC) using a Merck Hitachi instrument. The sample was separated using a C-18 Phenomenex column, specifically the RP-18 model. Detection was performed using an HPLC fluorescence detector from Shimadzu, Japan, with excitation set at 532nm and emission at 553 nm. The mobile phase was composed of a mixture of methanol and 0.050M potassium phosphate buffer, with a volume ratio of 35:65 (v/v). The pH of the buffer was 7. The flow rate of the mobile phase was set at 1mL/min. Standard solutions from the MDA were utilised to create a standard curve.

Statistical Analysis

The data analysis was performed using SPSS 24 for Windows, a software developed by SPSS Inc. in Chicago, IL, USA. The data were displayed as the average, standard deviation (SD), and range, and were evaluated using a T-test. The Kolmogorov-Smirnov test was used to determine if the data followed a normal distribution. The statistical significance level was established with a value of $P < 0.05$.

Results and Discussion

The biochemical and clinical features of the participating groups as shown in (Table 1). The results of current study show an elevation in the levels of liver enzymes. Also, there was a significant elevation in serum lipids and serum glucose levels (measured by HbA1c).

Table 1: The Biochemical and Clinical Characteristics of Participating Groups

Parameters	Healthy subjects	NAFLD patients
Number	60	60
Age (yrs)	50.146 ± 9.56	51.62 ± 11.25
BMI (kg/m2)	30.38 ± 4.435	29.69 ± 4.576
HbA1C (mmol/mol)	4.70 ± 0.740	6.80 ± 2.106
Liver function tests		
TSB (µmol/L)	0.68 ± 0.462	1.41 ± 2.655
AST (U/L)	23.78 ± 16.233	50.51 ± 47.574
ALT (U/L)	24.71 ± 9.868	70.38 ± 112.134
ALP (U/L)	156.44 ± 64.580	251.85 ± 213.457
Lipid profile		
T. Cholesterol (mg/dL)	170.14 ± 40.908	179.74 ± 53.137
LDL (mg/dL)	88.51 ± 31.774	111.13 ± 41.553
HDL (mg/dL)	52.73 ± 28.274	35.91 ± 23.378
TG (mg/dL)	137.61 ± 58.446	170.14 ± 88.617

When comparing the patient group's level of FRAP to that of the control group, Table 2 demonstrates a considerable drop. In contrast to people in the control group, the NAFLD patient group had a significant rise in malondialdehyde. Also, there was a significant elevation in the concentration of GSH and GPx concentrations relates to oxidative stress impact in tissues of NAFLD patients. Elevation of ROS concentration relates to the occurrence of oxidative stress in the tissues of individuals with non-alcoholic fatty liver disease (NAFLD).

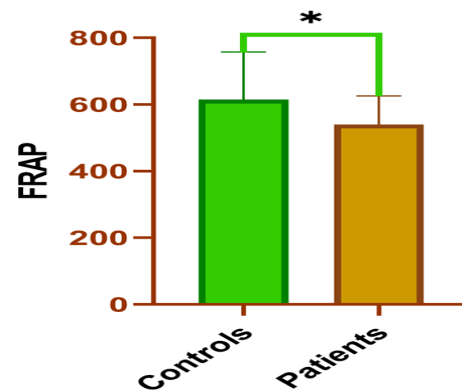
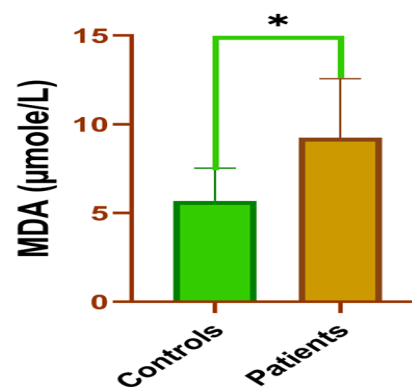
Table 2: The Clinical Parameters Measurements in the Current Study

Parameters	Healthy subjects	NAFLD patients
FRAP	614.87 ± 142.89	540.36 ± 85.39
MDA	5.69 ± 1.839	9.26 ± 3.311
GSH	12,301 ± 4,312	9,58 ± 2,218
GPx	325.9 ± 109.387	263.17 ± 126.519

Discussion

Diabetes type 2 is linked to numerous issues that result in harm to various organs in the human body [16]. Globally, the occurrence of (NAFLD) among peoples have type 2 diabetes has been documented to range from 60% to 86% [17]. Prior research has elucidated the connection between oxidative stress and the development of diabetes. In cases of diabetes, the excessive generation of free radicals occurs as a consequence of the oxidation of glucose. The atypical increase in the number of free radicals is followed by a noticeable decrease in the human body's anti-oxidant defense mechanisms. The presence of oxidative stress causes cellular organelles to accumulate damage, increases lipid peroxidation, and leads to the development of insulin resistance [18]. The association between circulating total bilirubin levels and the development of non-alcoholic fatty liver disease (NAFLD) is still unclear. Two separate investigations were conducted in China [19] and the Netherlands [20], to evaluate the correlation between total bilirubin levels and the likelihood of developing new-onset NAFLD [21]. According to the findings of our study, the serum total bilirubin (TSB) level was elevated in the Non-alcoholic fatty liver group compared to the healthy group. The high prevalence rate of NAFLD is inferred from elevated aminotransferase levels [22]. In our study, serum alanine amino transferase and aspartate amino transferase were higher in the patient group than in the control (healthy donors). In another case of NAFLD, there is a slight increase in ALP levels along with an increase in triglyceride and cholesterol levels [23]. Further work is needed to determine the prognostic importance of serum ALP in patients with NAFLD, especially in obese individuals following metabolic surgery, as well as its potential as a predictor of liver fibrosis [24]. NAFLD is associated with dyslipidaemia, which is marked by increased levels of triglyceride and LDL cholesterol, as well as reduced levels of HDL cholesterol [25]. In our study, levels of total cholesterol, LDL and triglycerides were high. Our findings also show that FRAP value in NAFLD patients was significantly lower than that of the controls. It is seen that the FRAP levels are significantly raised in the NAFLD group, thus suggesting that about 60% of the FRAP activity could be due to uric acid. It is possible that hyperuricemia, which is a component of the metabolic syndrome, is responsible for the increased FRAP levels observed in the NAFLD group [26]. The plasma ferric-reducing ability in patients with NAFLD was substantially greater than in both healthy controls and control groups of patients. The findings suggested that patients with NAFLD experienced a notable rise in lipid peroxidation and oxidative stress [27]. The FRAP assay quantifies the substance's capacity to convert Fe^{3+} to Fe^{2+} and was first suggested for assessing the overall antioxidant capacity of plasma. [28]. Our study findings indicate that NAFLD patients have increased levels of Malondialdehyde. Elevated serum MDA levels provide evidence for the theory that heightened oxidative stress, specifically lipid peroxidation, plays a role in the development of sepsis. Oxidative stress arises when the generation of free radicals surpasses the ability of naturally occurring chemicals to counteract them through antioxidants. In non-alcoholic fatty liver disease (NAFLD), the accumulation of lipids in liver cells stimulates the generation of elevated quantities of reactive

oxygen species (ROS), resulting in lipid peroxidation [29]. Lipid peroxidation refers to a biochemical process in which free radicals initiate a chain reaction. Both liver cells and circulating lipids experience abnormal peroxidation, resulting in lipotoxicity and metabolic dysfunctions [30, 31]. Oxidative stress has been proposed as a key mechanism that contributes to liver damage, triggering the progression from simple steatosis to non-alcoholic steatohepatitis (NASH) [32]. Research has indicated that NAFLD can cause mitochondrial dysfunction in liver tissue, which disrupts the balance of lipids in the liver, increases the formation of reactive oxygen species (ROS), and leads to lipid peroxidation and cell death [33]. Elevated serum MDA levels provide evidence for the theory that heightened oxidative stress, specifically lipid peroxidation, plays a role in the development of sepsis. Oxidative stress arises when the formation of free radicals surpasses the antioxidant capability of naturally occurring molecules including glutathione, superoxide dismutase, thioredoxin, and vitamin E. Lipid peroxidation occurs when fatty acids combine with reactive oxygen species (ROS), leading to the formation of various reactive aldehydes, such as MDA. Mitochondria are a significant contributor of intracellular reactive oxygen species (ROS) due to the incomplete reduction of oxygen to water during the electron transport chain, resulting in the production of superoxide and hydrogen peroxide [34].

**Figure 1:** Comparison of FRAP between Patients and Healthy Controls**Figure 2:** Comparison of MDA between Patients and Healthy Controls.

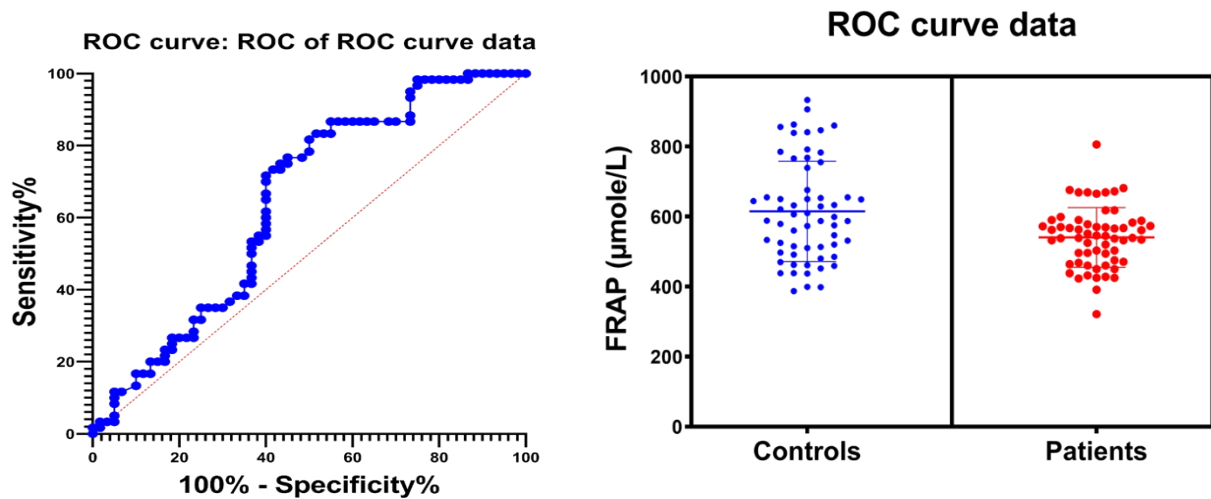


FIGURE 3: FRAP Levels in Participants Classified as Patients/Healthy Controls Based on the ROC Analysis

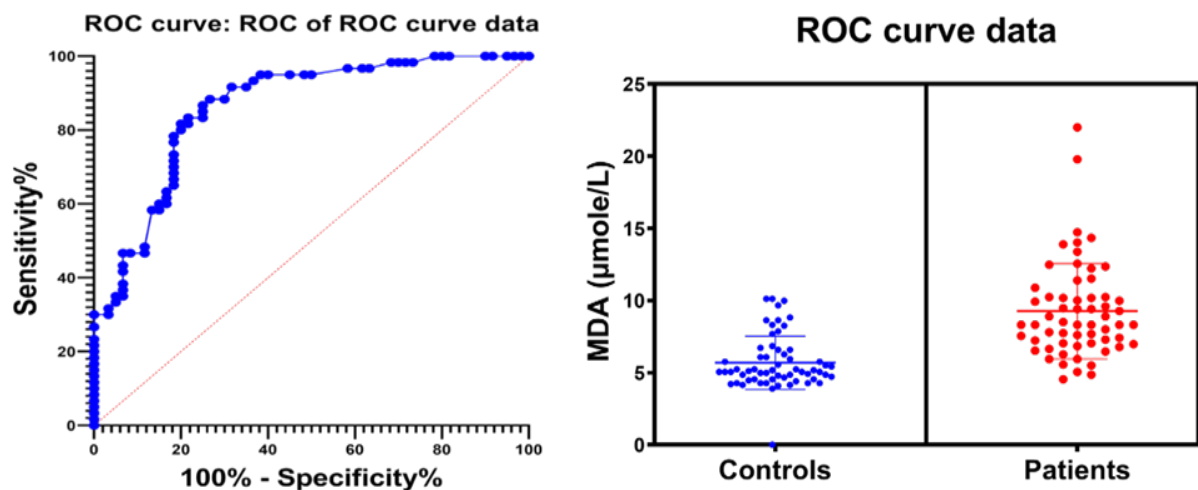


FIGURE 4: MDA Levels in Participants Classified as Patients/Healthy Controls based on the RC Analysis.

Conclusion

In conclusion, the current investigation reveals that individuals with nonalcoholic fatty liver disease exhibit a substantial reduction in plasma ferric-reducing capacity levels when compared to the healthy control group. Conversely, the levels of malondialdehyde (MDA) were elevated in patients compared to the healthy control groups. These

findings indicate that individuals with NAFLD experience an increase in oxidative stress, leading to elevated amounts of free radicals, which in turn result in higher levels of MDA and reduced FRAP levels.

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