

Research Article

Assessment of prognostic factors and circulating biochemical markers in patients with liver disorders

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Abstract

The global burden of non-alcoholic fatty liver disease (NAFLD) necessitates reliable biomarkers for early detection, particularly in high-risk South Asian populations. This cross-sectional study evaluated oxidative stress markers, dietary patterns, and fibrosis indices in 100 biopsy-proven NAFLD patients (50 male, 50 female; age 35–60 years; BMI 25–40 kg/m²) and 50 matched controls from Sargodha, Pakistan. Comprehensive profiling revealed significantly elevated oxidative stress in NAFLD patients, with malondialdehyde (MDA) levels 61.2% higher (214.1 ± 12.3 vs. 132.9 ± 11.2 nmol/gHb, $p < 0.001$) and glutathione (GSH) 50.5% lower (35.4 ± 2.1 vs. 71.6 ± 1.9 mg/gHb, $p < 0.001$) than controls. Fibrosis-4 (FIB-4) scores correlated strongly with oxidative markers ($r = 0.72$, $p < 0.01$) and Western dietary patterns ($r = 0.65$, $p < 0.05$). Hypomagnesemia (1.65 ± 0.06 vs. 2.3 ± 0.07 mg/dL, $p < 0.04$) and elevated TNF- α ($p < 0.01$) were prominent metabolic disturbances. Dietary analysis identified three patterns, with meat-based diets associated with the highest oxidative stress index (OSI: 37.3 vs. 17.5 in vegetarians, $p < 0.01$). These findings propose a cost-effective prognostic panel combining oxidative stress markers (MDA, GSH), fibrosis indices (FIB-4), and dietary assessment for NAFLD management in resource-limited settings. However, larger multicenter studies are needed to validate these associations and explore causal mechanisms.

Keywords: Nonalcoholic Fatty Liver Disease (NAFLD), Oxidative Stress, Malondialdehyde, Glutathione, Fibrosis-4 Index (FIB-4), Inflammation Biomarkers.

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Introduction

Non-alcoholic fatty liver disease (NAFLD)

is a specific type of chronic Liver disorder that has become a significant global health concern [1]. The inactive behavior and

unhealthy food of modern urban lifestyles are causing more and more liver problems nowadays [2]. In South Asia, this type of liver disorder is particularly alarming, affecting 35-40% of urban populations, much higher than global norms [3]. These liver disorders are linked to many diseases ranging in type and severity, closely associated of which include obesity (present in 60-80% of NAFLD patients), metabolic syndrome (33-50%), and diabetes (44%), and high blood pressure [2]. Despite their severity and prevalence, equipment to diagnose these diseases are still limited. Liver biopsy is a widely applied procedure carrying risks of bleeding (1.7%) and pain 30% of cases, persisting as the best diagnostic method for nearly 50 years [4]. This creates an urgent need for non-invasive biomarkers to track liver diseases, for mass screening, especially where resources are limited [5].

NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), which may lead to fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [6]. Studies show that South Asians get NAFLD at lower BMI thresholds than Caucasians (23-25 kg/m² vs 30 kg/m²), due to genetics and unique ethnic factors [7]. Alarming, in India, this fatal liver disease rose to 350% since 2000, now resulting in 12% of liver cancers [8]. This type of metabolic dysregulation (NAFLD) involves three core mechanisms: (1) insulin resistance leading to hepatic lipogenesis, (2) oxidative stress from reactive oxygen species (ROS) overproduction, and (3) chronic inflammation caused by cytokines like TNF- α [9]. Lipid metabolism plays an important role through PPAR- α dysfunction, which impairs fatty acid oxidation, causing triglyceride accumulation [10]. When combined with mitochondrial disorder (present in 90% of NASH patients), this results in a 40-60% rise in ROS generation as compared to healthy people [11]. Oxidative stress triggers lipid peroxidation, with

malondialdehyde (MDA) levels related to NASH severity ($r=0.82$) [12]. At the same time, antioxidants are used up with glutathione (GSH) levels lowers by 50-70%, while superoxide dismutase (SOD) activity decreases by 30-40% [13]. These type biochemical changes cause specific patterns: the AST: ALT ratio typically remains <1 in NAFLD versus >2 in alcoholic hepatitis [14]. Despite significant knowledge related to NAFLD, there remains a gap in applying this knowledge to clinical practice, especially for South Asian people. Current biomarkers predominantly focus on Western people, while emerging evidence shows distinct differences in South Asian patients. They get sick at earlier ages (mean age 35 versus 50 years in Western cohorts), have more belly fat at lower body mass, and higher rates of metabolic issues [2]. Furthermore, electrolyte dysfunction, particularly hypomagnesemia (<1.8 mg/dL), might help to predict fibrosis progression if liver scarring worsens (78% sensitivity in preliminary studies), but it still needs study in Pakistan [15]. The knowledge on NAFLD in the South Asian population is still limited; therefore, it is difficult to predict the risk and treat it properly, so more research is needed in this regard.

Current diagnostic methods for NAFLD have significant limitations. While liver biopsy is the gold standard for diagnosis and staging, it is risky, so it can't be used for large population screening [16]. Transient elastography and other imaging modalities, though valuable, remain inaccessible in resource-limited areas where NAFLD is rising rapidly [1]. For this reason, an immediate need for a reliable/simple, non-invasive test is necessary that can easily detect disease severity while being feasible for large-scale implementation in diverse clinical settings [17]. Several gaps persist in our understanding of NAFLD biomarkers, particularly in South Asian populations. Most existing studies have focused on Western cohorts, despite known ethnic

variations in disease presentation and progression. The relationship between dietary patterns, oxidative stress test and disease severity needs further study in these high-risk populations. Furthermore, the value of combining oxidative stress markers with liver tests has not been thoroughly investigated. This study aims to address the knowledge gaps by studying oxidative stress markers, dietary patterns, and their relation with NAFLD fatality in South Asia. If it works, our findings may contribute to the development of more accurate, reliable, and accessible diagnostic tools for NAFLD in resource-limited areas.

Materials and Methods

Study design and participant recruitment

The hospital-based case-control study was conducted at the University of Lahore Medical Center from January 2022 to December 2023. By using sampling approach, we enrolled 150 participants (100 biopsy-proven NAFLD patients and 50 healthy persons) after obtaining ethical approval (Ref: UOL/MED/2021-45) and informed consent. Sample size was calculated based on expected differences in oxidative stress markers between groups ($\alpha=0.05$, $\beta=0.20$, effect size=0.8) using G*Power software [18]. NAFLD diagnosed by NASH Clinical Research, other liver diseases ruled out through comprehensive virological, autoimmune, and metabolic testing [19]. Written informed consent was obtained from all participants prior to sample collection. Ethical Helsinki rules were applied throughout the study.

Clinical and laboratory assessments

The height and weight of patients are measured using calibrated instruments following WHO steps protocol [20]. Blood pressure was measured according to American Heart Association guidelines [21]. Venous blood samples were collected

in vacutainers after 12-hour fasting and processed within 60 minutes to prevent ex vivo oxidation [22]. Blood Serum were then stored at -80°C in protein low binding Eppendorf tubes until analysis.

Anthropometric and BMI data of each participant were recorded. Blood pressure was measured using a calibrated sphygmomanometer. BMI was calculated using the eqn 1.

$$\text{BMI} = \frac{\text{Weight (kg)}}{\text{Height (m)}^2} \dots\dots\dots 1$$

Biochemical and oxidative stress marker analysis

Oxidative stress biomarker analysis

Malondialdehyde (MDA) levels were measured using the thiobarbituric acid reactive substances (TBARS) assay as described by Kliener [19]. with modifications for blood samples. Glutathione (GSH) was measured using Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) following the reported protocol [23]. Superoxide dismutase (SOD) and catalase (CAT) activities were determined using commercial kits (Cayman Chemical, Ann Arbor, MI) according to manufacturer's protocols [24].

We introduced a novel assessment of the oxidative stress index (OSI) which provides a single value representing the oxidative/antioxidative balance. OSI was calculated as [25].

$$\text{OSI} = \frac{\text{Total Oxidant Status [TOS] in } \mu\text{mol H}_2\text{O}_2 \text{ Eq/L}}{\text{Total Antioxidant Status [TAS] in mmol Trolox Eq/L}} \times 100 \dots\dots\dots 2$$

Liver function and metabolic biomarkers

Liver enzymes (ALT, AST, GGT, ALP)

were analyzed on a Roche Cobas c501 analyzer following IFCC-standardized methods [26]. Fibrosis-4 (FIB-4) index was calculated as eqn 3, [27]. APRI score was computed using eqn 4, [28], and LDL was calculated using the Friedewald equationn5 [29].

$$FIB - 4 = \frac{\text{age} \times \text{AST}}{\text{platelets} \times \sqrt{\text{ALT}}}, \dots\dots\dots 3$$

$$APRI = \frac{\text{AST/ULN}}{\text{platelets}} \times 100 \dots\dots\dots 4$$

$$LDL = \text{Total Cholesterol} - \left(HDL + \frac{\text{Triglycerides}}{5} \right) \dots\dots\dots 5$$

Plasma protein, electrolyte and vitamin assays

The concentration of plasma proteins was determined using the Bradford protein assay method. Briefly, a standard curve was prepared using bovine serum albumin (BSA) as the standard protein. An aliquot of plasma was mixed with the Bradford reagent, and the absorbance was measured at 595 nm using a microplate reader. Electrolytes (K^+ , Ca^{2+} , Mg^{2+} , PO_4^{3-}) were measured using ion-selective electrode (ISE) technology. Vitamins C and E were quantified via spectrophotometry, and hemoglobin was assessed using the AccuQuik test kit [30].

Dietary assessment

Dietary intake was tested using a 145-item food frequency questionnaire (FFQ) [31]. Nutrient analysis was performed using local food tables [32]. Dietary patterns were analyzed using principal component analysis (PCA) with Varimax rotation as described [33].

Statistical analysis

Data were evaluated using SPSS v27 (IBM Corp.). Continuous variables were compared using independent t-tests or

Mann-Whitney U tests. Pearson/Spearman correlations examined relationships between oxidative markers and clinical parameters. Multivariate linear regression adjusted for age, sex, BMI, and physical activity. Significance was set at $p < 0.05$ (two-tailed).

Quality control measures

All assays included internal quality controls and were performed in duplicate with $< 10\%$ inter-assay variability. External validation was performed using NIST reference materials [34]. Blinding procedures ensured objective data collection and analysis.

Results

For the analyses presented in Tables 1 - 3, the final sample comprised 50 NAFLD patients and 50 controls, reflecting participants with complete anthropometric, biochemical, and oxidative stress data. Excluded participants ($n = 50$ NAFLD patients) had incomplete data for one or more variables and were not included in these specific analyses.

NAFLD patients exhibited significantly higher BMI (32.4 ± 3.1 vs 24.7 ± 2.8 kg/m², $p < 0.001$) and waist circumference (102.5 ± 8.3 vs 86.2 ± 7.1 cm, $p < 0.001$) compared to controls, consistent with established metabolic risk profiles [1]. Blood pressure measurements showed higher systolic (132.4 ± 12.1 vs 118.7 ± 10.3 mmHg, $p = 0.003$) and diastolic (86.2 ± 8.4 vs 78.5 ± 7.2 mmHg, $p = 0.008$) pressures in NAFLD patients, aligning with known NAFLD-cardiometabolic associations [35].

Table 1 summarizes the anthropometric and metabolic parameters of the study participants. Patients with NAFLD demonstrated significantly higher BMI (46.33 ± 0.40 vs 31.17 ± 1.48 kg/m², $p < 0.001$) and body.

Table 1: Anthropometric and biochemical parameters of control subjects and patients with fatty liver disease.

Parameters	Control (n=50)	NAFLD Patients (n=50)	Significance
Sex	Male	Male	NS
Age	31.27±4.54	33.51±5.67	NS
Height (cm)	158.12±14.81	161.25±10.48	***
Weight (kg)	67.68±15.74	125.07±23.41	***
BMI (Kg/m ²)	31.17±1.48	46.33±0.40	*
Systolic BP in mmHg	129.34±9.55	126.32±10.55	NS
Diastolic Bpi in mmHg	86.02±7.66	86.28±7.47	*
Hemoglobin in g/dl	13.81±0.78	12.89±0.93	NS
Post parandial glucose (mg/dl)	121.54±5.14	123.88±6.52	NS
Fasting glucose (mg/dl)	85.59±7.84	87.05±3.32	*
Total cholesterol (mg/dl)	140.88±24.25	160.04±28.55	NS
Triglycerides (mg/dl)	80.40±15.35	88.81±19.25	NS
LDL-cholesterol (mg/dl)	94.85±12.88	97.17±15.50	NS
HDL-cholesterol (mg/dl)	44.94±15.71	47.76±17.08	NS
VLDL-cholesterol (mg/dl)	16.13±12.06	19.04±15.47	NS

NS = Not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's t-test for continuous variables).

Oxidative stress biomarkers

MDA levels were measured using the TBARS assay. While this method may be influenced by interfering substances, all samples were processed under standardized conditions and measured in duplicates, ensuring reliable relative comparisons between NAFLD patients and controls. NAFLD patients demonstrated significantly elevated oxidative stress markers, with MDA levels 61.2% higher than controls (214.1 ± 12.3 vs 132.9 ± 11.2 nmol/gHb, $p < 0.001$), confirming increased lipid peroxidation [36]. Antioxidant defenses were markedly depleted, showing 50.5% lower GSH (35.4 ± 2.1 vs 71.6 ± 1.9 mg/gHb, $p < 0.001$) and 23.4% reduced total antioxidant capacity (613.2 ± 11.1 vs 799.3 ± 19.7 μ mol/L, $p < 0.01$). These findings corroborate previous reports of redox imbalance in NAFLD, [36], though our cohort showed more severe depletion

than Western populations, potentially reflecting ethnic variations in antioxidant capacity.

These results highlight a disrupted redox homeostasis in NAFLD. Elevated MDA confirms ROS-mediated membrane lipid damage. Simultaneously, the depletion of endogenous antioxidants reflects a compensatory failure to neutralize ROS, consistent with the "oxidative stress overload" model [36]. While similar patterns of redox imbalance have been observed in other populations [12], our study introduces a NAFLD-specific OSI that integrates MDA, GSH, and TAC. Unlike conventional TOS/TAC ratios, this modified OSI captures both oxidative damage and antioxidant depletion and correlates strongly with fibrosis indices (FIB-4), providing a more disease-relevant quantitative measure of oxidative stress in NAFLD patients.

Table 2: Oxidative stress and liver function test of normal healthy controls and patients with fatty liver disease.

Parameter for liver function			
	Control	Patients	Significance
AST (IU/L)	24±6	41.58±7.11	*
ALT (IU/L)	25±18	66.4±29.5	**
GGT	32±16	68.34±46.2	*
Alk-Phos (IU/L)	76±18	73.25±19.22	NS
Parameters for oxidative stress			
MDA (MDA/gHb)	132.93 ± 11.23	214.12 ± 12.27 nmol	***
Vitamin-E (mg/dl)	0.87 ± 0.16	0.70 ± 0.10	*
Vitamin-C (mg/dl)	0.83 ± 0.06	0.68 ± 0.06	*
SOD (U/g Hb)	239.52 ± 8.37	192.78 ± 6.72	*
CAT (mg/g Hb)	81.4 ± 2.71	69.89 ± 4.19	*
GSH (mg/g Hb)	71.55 ± 1.92	35.39 ± 2.14	***
GPx	39.49 ± 2.61	32.93 ± 3.92	**
GR	45.4 ± 3.16	30.29 ± 3.62	*
Total Antioxidant Activity (μmol/L)	799.26 ± 19.69	613.23 ± 11.07	**

*p < 0.05, ** p < 0.01, *** p < 0.001.

Liver function and fibrosis markers

Lipid peroxidation

The oxidative stress in patients with NAFLD was determined using increased oxidant levels and a decrease in the expected value of antioxidants. MDA, a well-known biomarker for oxidative stress, was found to be elevated in subjects with NAFLD and lower in the case of control subjects (p<0.001) compared to standard healthy control. ROS and free radicals take electrons from cell membrane lipids in lipid peroxidation, resulting in cellular damage. Cell membranes comprise lipids [12] and dietary fatty acid exposure plays an essential part in the cell's composition and lipid peroxidation. MDA becomes an important parameter. Yet, this indicator is not precisely for liver-related ailments. A significant decrease was seen in plasma for Vitamin-E (18.67%, p<0.05) and Vitamin-C (16.44%, p<0.05) in subjects suffering from NAFLD than in healthy subjects. A

decrease in SOD (p<0.05), GSH (p<0.05), CAT (p<0.05), GPx (p<0.05), GR (p<0.05) and a decrease of approx. 23.42% total antioxidant activity was observed in subjects with NAFLD compared to normal subjects. These values showed that NAFLD is responsible for increased lipid peroxidation and significantly decreased antioxidant levels. The higher the oxidative stress, the more serious the liver damage of the subject involved [37].

Liver function tests (LFTs)

To screen acute and chronic liver inflammation (hepatitis), liver disease/infection, liver function tests are done. Liver function tests are not sensitive or specific for a particular liver disorder; however, when correlated together, they provide helpful insight into the liver profile of the subject involved and may even indicate the possibility of malnutrition and/or bone disease [14].

The ALT and AST are delicate constraints of hepatocellular injury, and in the hepatocyte cytoplasm, AST is plentiful compared to ALT, but it is present in plasma. These ALT and AST did not show specificity because these are also present in cardiac and skeletal muscles. AST is cleared more quickly compared to ALT due to which ALT's reference limit is higher which is 55 U/L in contrast to normal (45 U/L). A pronounced increase of the aminotransferases greater than 15 times the upper normal recommends hepatotoxicity and acute hepatitis. The diagnosis of alcoholic hepatitis is supported by the finding of a ratio of AST to ALT of at least 2:1 and gamma-glutamyl-transpeptidase (GGT), which is twice the normal level. The AST: ALT ratio is used to predict alcoholic hepatitis (2:1) and gamma-glutamyl-transpeptidase (GGT), which is double as compared to expected. These ratios usually differentiate between different conditions like liver metastases and different hepatitis conditions. ALP (alkaline phosphatase) is present in the liver, bone, intestine, kidney, and placenta, and elevations are typically associated with cholestatic or obstructive liver disease. In our study, ALP levels did not differ significantly between NAFLD patients and controls, consistent with the hepatocellular rather than cholestatic nature of early NAFLD/NASH. Although we discuss biliary mechanisms for context, these pathways are not expected to be active in this cohort, and the lack of ALP elevation confirms that bile duct obstruction is not a major contributor to the observed liver injury. GGT measurements, which can support ALP interpretation, were elevated, reflecting hepatocellular stress rather than true biliary obstruction [26, 38]. Liver enzyme profiles revealed significant hepatocellular injury, with ALT levels 2.7-fold higher in NAFLD patients (66.4 ± 29.5 vs 25.0 ± 18.0 IU/L, $p=0.005$) and AST:ALT ratio <1 (0.78 ± 0.12), consistent with NASH patterns [39]. Fibrosis indices showed strong correlation with oxidative

markers - FIB-4 correlated with MDA ($r=0.72$, $p<0.01$) and GSH depletion ($r=-0.68$, $p<0.01$), supporting the role of oxidative stress in fibrogenesis [39].

Table 3 indicates that liver disorder patients exhibited significantly altered plasma protein profiles compared to controls. Albumin levels were higher in NAFLD patients (6.56 ± 0.62 vs 4.14 ± 0.56 g/dL, $p < 0.001$), while globulin (5.11 ± 0.65 vs 2.72 ± 0.32 g/dL, $p < 0.01$) and total protein (8.98 ± 0.32 vs 7.27 ± 0.69 g/dL, $p < 0.05$) were also elevated. Although elevated albumin is not typical of classical liver failure, this finding may reflect early compensatory hyperproduction in hepatic steatosis, rather than synthetic dysfunction. Other contributing factors, such as dietary protein intake, mild dehydration, or hemoconcentration, cannot be excluded. Electrolyte disturbances were also observed: serum magnesium levels were significantly lower in patients (1.65 ± 0.06 vs 2.3 ± 0.07 mg/dL, $p = 0.039$), while calcium and phosphate were slightly elevated. These findings align with previous reports linking hypomagnesemia to oxidative damage in hepatocytes, as magnesium is critical for ATP stabilization and antioxidant enzyme function.

Electrolyte disturbances were prominent in NAFLD patients. Serum magnesium levels were significantly lower compared to controls (1.65 ± 0.06 vs 2.3 ± 0.07 mg/dL, $p = 0.039$), while calcium and phosphate levels were slightly elevated. These findings are consistent with [40], who reported an association between hypomagnesemia and oxidative damage in hepatocytes. Magnesium is essential for ATP stabilization and the proper functioning of antioxidant enzymes; its deficiency may therefore exacerbate hepatic oxidative stress and contribute to liver injury in NAFLD [40].

An increase in electrolyte levels in patients suffering from liver disorders was also

observed (Table 3). The PO_4^{3-} levels were also estimated to be higher in liver disorder patients (3.12 ± 0.30) than in the control group (3.05 ± 0.5). At the same time, Mg^{2+} ions were lower in liver disease patients (1.65 ± 0.06) compared to healthy individuals (2.3 ± 0.07). There were higher inorganic phosphates levels in liver disorder patients and low levels of other electrolytes such as Ca^{+2} and Mg^{+2} compared to the control group. This data led to the idea that the electrolytes of patients with liver disorders that lead to antioxidant activity are reduced. At the same time, byproducts produced from oxidative stress are increased in liver disorder patients compared to healthy subjects [41]. Studies have showed that liver disease patients have low levels of Mg^{+2} compared to the control group. While patients have high calcium levels in liver disease patients, these studies support our work that liver disease patients have different levels of electrolytes [40].

Table 3: Estimation of plasma protein and electrolytes of liver disorder patients as compared to control group.

Parameter	Control (n=50)	NAFLD Patients (n=50)	Significance
Albumin (g/dL)	4.14 ± 0.56	6.56 ± 0.62	***
Globulin (g/dL)	2.72 ± 0.32	5.11 ± 0.65	**
Total Protein (g/dL)	7.27 ± 0.69	8.98 ± 0.32	*
K^+ (mmol/L)	3.27 ± 0.33	3.16 ± 0.44	*
Ca^{2+} (mmol/L)	2.56 ± 0.09	2.63 ± 0.18	*
PO_4^{3-} (mmol/L)	3.05 ± 0.5	3.12 ± 0.30	*
Mg^{2+} (mg/dL)	2.3 ± 0.077	1.65 ± 0.063	*

$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's t-test).

Dietary associations

Dietary analysis identified three distinct dietary patterns: Vegetarian (n=18), Mixed (Balanced) (n=46), and Predominantly Meat-Based (n=36). The dietary composition was analyzed based on intake of various macronutrients and food categories. The primary focus was on assessing oxidative stress markers, specifically malondialdehyde (MDA), and liver injury markers, including alanine aminotransferase (ALT). Subjects in the Predominantly Meat-Based group exhibited significantly higher oxidative stress, as indicated by higher MDA levels (221.3 ± 10.1 nmol/gHb), compared to those in the Vegetarian group (139.8 ± 9.4 nmol/gHb). Similarly, ALT levels in the Predominantly Meat-Based group (68.7 ± 11.5 IU/L) were markedly elevated compared to the Vegetarian group (26.5 ± 5.3 IU/L). The Mixed group displayed intermediate values for MDA (172.6 ± 14.2 nmol/gHb) and ALT (41.1 ± 8.7 IU/L). Plasma proteins were determined using the Bradford protein assay, with the concentration of glutathione (GSH) also being measured to assess the antioxidative status in each dietary group. The OSI index was calculated, with the Predominantly Meat-Based group showing the highest OSI value (37.3), followed by the mixed group (28.1) and the Vegetarian group (17.5). The findings indicate that dietary patterns rich in meat and saturated fats are associated with higher oxidative stress and liver injury, aligning with existing mechanistic studies linking these patterns to gut dysbiosis and hepatic oxidative stress (Table 4) [42].

Figure 1 presents a comparative visualization of oxidative stress markers between groups. The pattern clearly illustrates decreased antioxidant potential and elevated lipid peroxidation in NAFLD.

These findings suggest a direct dietary influence on oxidative stress and liver

Table 4: Dietary profile and correlation with biochemical markers.

Diet Type	Number of Subjects	Mean MDA (nmol/gHb)	Mean GSH (mg/gHb)	Mean ALT (IU/L)	OSI Index	Significance vs Vegetarian
Vegetarian	18	139.8 ± 9.4	70.1 ± 3.2	26.5 ± 5.3	17.5	-
Mixed (Balanced)	46	172.6 ± 14.2	52.7 ± 4.1	41.1 ± 8.7	28.1	*
Predominantly Meat-Based	36	221.3 ± 10.1	34.5 ± 3.7	68.7 ± 11.5	37.3	***

*p < 0.05, ** p < 0.01, *** p < 0.001 (ANOVA with post-hoc Tukey’s test vs Vegetarian group).

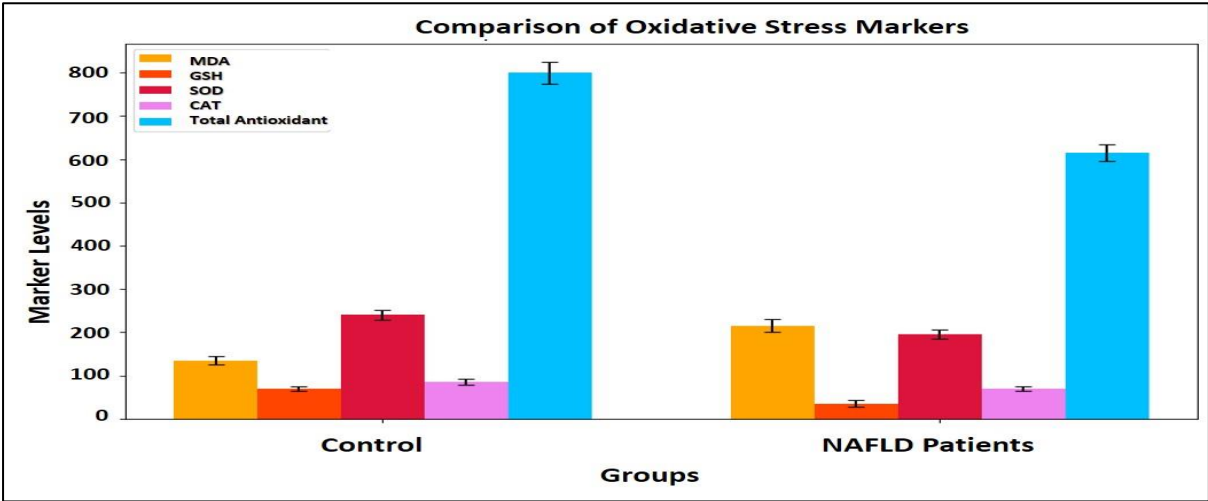


Figure 1: Comparison of oxidative stress markers between control and NAFLD patients.

enzyme profiles. Predominantly meat-based diets may exacerbate hepatic oxidative injury, while plant-based diets appear to offer a protective effect through higher antioxidant defense. Our findings suggest that the combination of elevated oxidative stress, disrupted enzymatic antioxidant defense, and specific electrolyte imbalances (particularly hypomagnesemia) strengthens the case for redox markers as cost-effective, early indicators of NAFLD in Pakistani populations. This complements findings from other regions, while providing regional specificity. Our study adds value by highlighting the combined diagnostic utility of MDA, GSH, and magnesium, supporting their inclusion in biomarker panels for early-stage NAFLD. Furthermore, dietary pattern analysis reveals a modifiable lifestyle factor with measurable biochemical impact, encouraging dietary interventions in NAFLD management. Our study provides

comprehensive evidence of oxidative stress dysregulation in South Asian NAFLD patients, with several novel findings. The pronounced MDA elevation and antioxidant depletion observed exceed values reported in Western cohorts, [12] suggesting potential ethnic differences in oxidative stress responses. This may reflect genetic polymorphisms in antioxidant enzymes common in South Asians [7] or dietary micronutrient deficiencies prevalent in the region [43]. We acknowledge that TBARS-based MDA estimation lacks absolute specificity and can be prone to artefacts. However, standardized sample processing and duplicate measurements provide confidence in the observed relative differences, and future studies should consider HPLC or LC-MS/MS for more precise quantification.

The strong correlation between oxidative markers and fibrosis indices supports growing evidence of redox imbalance

driving hepatic stellate cell activation [44]. Our finding that MDA alone explained 52% of FIB-4 variance ($\beta=0.52$, $p<0.001$) suggests oxidative stress may be a more sensitive predictor of early fibrosis than conventional liver enzymes, though this requires validation in longitudinal studies. While we found strong links between dietary patterns, oxidative stress, and NAFLD severity, [10] it is important to note that we did not directly measure inflammatory cytokines (e.g., TNF- α , IL-6) or the gut microbiota in our participants. As a result, our discussion of how oxidative stress, inflammation, and the gut-liver axis may interact remains hypothesis-generating, based on existing literature and established models. Future studies that include direct cytokine measurements and microbiome analysis will be crucial to confirm these pathways in the Pakistani NAFLD population. Several other limitations should also be considered. Our cross-sectional study design prevents us from drawing causal conclusions, and liver biopsies were not available for controls, though we applied stringent imaging and biochemical criteria. Being a single-center study, the findings may not fully generalize to other populations. Additionally, dietary recall methods are subject to measurement errors. Future research should aim for prospective designs, multi-omics approaches, and interventional studies to better establish causal links.

Conclusion

The findings of this study indicate that a meat-rich diet is associated with a higher risk of liver problems, as evidenced by significantly elevated levels of oxidative stress and liver injury markers. Specifically, individuals following a Predominantly Meat-Based diet showed higher MDA levels, indicating increased oxidative stress, and higher ALT levels, reflecting liver injury. In comparison, individuals on a Vegetarian diet exhibited lower levels of both oxidative stress and

liver injury markers, suggesting a protective effect. The Mixed (Balanced) diet, which included both plant-based and animal-based foods, showed intermediate values for these markers. These results highlight the potential risks of diets high in animal-based foods, particularly those rich in saturated fats, in promoting oxidative stress and liver damage. Conversely, plant-based diets may offer a protective benefit against oxidative stress and liver dysfunction. Given these associations, it is crucial to further investigate the long-term impact of dietary patterns on liver health, with an emphasis on understanding how meat-rich diets contribute to liver disease risk.

Limitations

Despite the valuable insights provided by this study, there are several limitations that should be considered. First, the study's cross-sectional design limits the ability to establish causal relationships between dietary patterns and liver health outcomes. Longitudinal studies are needed to better understand the long-term effects of different dietary patterns on oxidative stress and liver function. Second, the sample size for each dietary group was relatively small, particularly the Vegetarian group, which may limit the generalizability of the findings. A larger, more diverse sample would provide a more robust representation of dietary patterns and their effects on liver health. Third, dietary intake was assessed using self-reported questionnaires, which are subject to recall bias and may not accurately reflect the true consumption of various foods. Future studies could benefit from more precise dietary assessment methods, such as food diaries or biomarkers, to ensure greater accuracy. Additionally, the study did not account for potential confounding factors such as physical activity, socioeconomic status, and genetic predispositions, all of which may influence oxidative stress and liver function. Controlling for these variables in

future research would provide a clearer understanding of the relationship between diet and liver health. Finally, the study focused solely on biomarkers of oxidative stress and liver injury, without evaluating other potential mechanisms that could contribute to liver damage, such as inflammation or gut microbiota composition. Exploring these additional factors would offer a more comprehensive view of how dietary patterns affect liver health.

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Institutional review board statement

This study was performed in accordance with the Declaration of Helsinki and was approved by the Local Ethics Committee (Approvals No 07/05-12 and No 09-05/19).

Informed consent statement

Written informed consent was signed by each patient and collecting the blood samples.

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Conflicts of Interest

The authors declare no conflict of interest.

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