Research Article

Evaluation of Adropin, Irisin and Cytokeratin 18 as Biomarkers in Metabolic Dysfunction-Associated Steatotic Liver Disease: A Comparative Clinical Study

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Keywords

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Abstract

Background: Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) has become the leading cause of chronic liver disease globally, affects more than one-third of the adult population and includes a spectrum of conditions ranging from simple steatosis of liver to metabolic dysfunction–associated steatohepatitis (MASH), progressive fibrosis, cirrhosis and, in some cases, hepatocellular carcinoma. Early detection and accurate staging are important to prevent disease progression and studies have recently identified metabolic and apoptotic markers such as Adropin, a peptide hormone secreted by the liver that is involved in energy homeostasis; Irisin, a myokine that is linked to exercise and metabolic regulation; and CK-18, a biomarker of hepatocyte apoptosis.
Methods: Using FibroScan for the diagnosis and staging of MASLD, CAP scores were used for steatosis and liver stiffness measurements for fibrosis. Quantification of serum adropin, irisin, and CK-18 was done, and independent t-tests, correlation analysis, and ROC curve analysis were used for statistical analysis to assess the diagnostic potential.

Results: Adropin levels were lower in MASLD cases than in controls and decreased further with the severity of the disease. The association was highly significant ($p < 0.001$), indicating a very high negative correlation between Adropin levels and hepatic dysfunction. Levels of CK-18 were greatly increased in MASLD patients and were highly positively correlated with the degrees of fibrosis and steatosis ($p < 0.001$), which supports the hypothesis that it is a marker of hepatocyte apoptosis.

Conclusion: The significant changes in their levels observed in MASLD patients suggest their possible application in multimarker diagnostic strategies. Nonetheless, the inconsistent behavior of Irisin in this study requires more conclusive evidence from future studies involving larger samples. Such biomarkers may help in identifying the disease at an early stage and improve the management of the disease.

Introduction

MASLD has emerged as a significant global health concern [1], characterized by excessive fat accumulation in the liver (more than 5%) in the absence of substantial alcohol intake or other liver diseases. The condition encompasses a spectrum of hepatic disorders ranging from simple steatosis to more severe forms such as MASH, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [2]. The rise of MASLD can primarily be attributed to the increasing sedentary lifestyle, urbanization, and the ongoing obesity epidemic [3, 4]. Globally, the prevalence of MASLD is alarmingly high, currently estimated to affect approximately 32.4% of the population [5]. Reports suggest that men are more significantly affected than women, with prevalence rates of 39.7% and 25.6%, respectively. In India, the situation is particularly concerning, with adult prevalence reported at 38.6%. Studies reveal that children are not immune to this condition, showing a prevalence of 35.4%, and particularly alarming is the rate among obese children [6], which stands at 63.4%. Urban populations in India exhibit substantially higher MASLD rates than their rural counterparts, further emphasizing the influence of lifestyle on liver health. For example, studies have depicted a stark contrast, noting a prevalence of 34.8% in Goa's urban settings versus 16.6% in rural South India.

The implications of MASLD extend beyond liver health, as the condition is closely associated with numerous metabolic complications, including Type 2 Diabetes Mellitus (T2DM), metabolic syndrome, and cardiovascular diseases [7]. Research indicates that individuals diagnosed with MASLD are at a 57% higher risk of overall mortality, particularly due to cardiac deaths [8]. Alarmingly, the rates of T2DM and chronic kidney disease (CKD) are also reported to be doubled among those affected by MASLD. Primary risk factors contributing to MASLD emergence include insulin resistance, obesity, and diabetes [9]. Insulin resistance, a hallmark of metabolic dysfunction, is associated with fat accumulation in the liver, resulting in steatosis. Obesity, especially central obesity, stands out as a critical risk factor; approximately 80% of obese individuals exhibit hepatic fat accumulation, indicating a strong link between adiposity and MASLD [10]. For instance, the prevalence of MASLD is remarkably high (78.09%) in the morbidly obese, while lower rates of 52.655% and 12.01% are reported in overweight and normal-weight populations, respectively. Moreover, central obesity not only heightens the risk of MASLD but also exacerbates insulin resistance and contributes to intrahepatic triglyceride (IHTG) accumulation, a key marker of metabolic disturbances [11]. While obesity is a predominant risk factor, MASLD is not exclusive to overweight individuals; about 5-10% of the MASLD population is lean. Genetic predisposition and the presence of abdominal obesity among normal-weight individuals are major risk factors for "lean" MASLD [12]. Insulin resistance is the primary mechanism linking T2DM and MASLD, with MASLD affecting around 55.5% of T2DM patients [13, 14]. Notably, the occurrence of cardiovascular complications in MASLD patients is significantly higher (1.87 times) when T2DM coexists. The risk of progression from MASLD to MASH is also notably elevated in diabetic patients [15]. Metabolic syndrome - a complex involving central obesity, insulin resistance, dyslipidemia, and hypertension - compounds the challenges faced by MASLD patients, as it is present in 70-90% of affected individuals [16]. Endocrine disorders further exacerbate the risk of MASLD, particularly in women with polycystic ovary syndrome (PCOS), where hormonal alterations and metabolic disturbances contribute to hepatic fat accumulation. Decreased estrogen levels in postmenopausal women are linked to increased hepatic fat, highlighting the role hormones play in MASLD pathogenesis [17]. Similarly, low testosterone levels in men correlate with heightened MASLD risk due to their engagement in lipid metabolism and anti-inflammatory actions [18]. Additionally, thyroid hormone deficiencies are known to significantly influence lipid metabolism, promoting increased fat deposition in the liver. Genetic disposition emerges as a crucial factor in MASLD susceptibility, with the PNPLA3 gene variant being notably implicated in the disorder [19]. This genetic variant leads to an enzyme dysfunction that controls triglyceride accumulation in the liver, resulting in increased susceptibility to MASLD and worsened disease progression. The complexity of MASLD pathogenesis is further appreciated through the "Multiple Hit Hypothesis," which integrates metabolic, genetic, and environmental factors affecting disease progression [20].

As MASLD often goes undiagnosed until advanced stages, there is a critical need for early identification and management strategies. Early-stage symptoms may remain subtle, leading to delayed diagnosis. Utilizing medical history, physical examinations, and non-invasive biomarker tests is essential. For instance, the initial laboratory tests may indicate elevated liver enzymes (ALT and AST), yet these findings are not specific to MASLD [21, 22]. Imaging methods, particularly ultrasonography, are commonly employed to assess liver steatosis, albeit with limitations in distinguishing between steatosis and MASH [23, 24]. Several novel biomarkers are being explored to enhance the diagnosis and monitoring of MASLD. Adropin and Irisin, both emerging peptides implicated in metabolic regulation, are garnering attention [25]. Adropin, a hormone produced in the liver and brain, plays a critical role in lipid metabolism and insulin sensitivity [26]. Lower Adropin levels are associated with metabolic conditions such as T2DM and obesity, indicating its potential as a biomarker for MASLD severity [27]. Similarly, Irisin, secreted by skeletal muscle, impacts energy metabolism and has shown promise in influencing liver health; however, research results on Irisin levels in MASLD have been variable [28]. Cytokeratin-18 (CK-18) fragments are also recognized for their potential as non-invasive markers to differentiate MASH from simple steatosis, while reflecting hepatocyte apoptosis and necrosis [29-31]. Elevated CK-18 levels have been correlated

with liver inflammation and fibrosis severity, reinforcing its relevance in clinical practice [32]. The emerging biomarkers such as Adropin, Irisin [33], and CK-18 underscore promising avenues for improving diagnosis, assessment of disease severity, and therapeutic strategies. Continued research into these biomarkers and their roles in advancing MASLD management remains essential to mitigate the burden of this complex disease on individuals and healthcare systems worldwide, ultimately reducing the risks of severe liver complications like HCC.

Materials and Methods

Ethical approval

Ethical clearance was obtained from the Institute Ethics Committee of AIIMS Bhopal (IHEC/SR/2024/Apr/10 dated: 23/04/2024), and all participants gave their informed consent before participation. From the Department of Medicine, AIIMS Bhopal, MASLD patients were recruited based on FibroScan to participate in the study, after fulfilling the inclusion and exclusion criteria and after obtaining consent. All studies involving human subjects must indicate that they are in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

Study design and participants

It is a case control study, Adropin, Irisin and CK18 ELISA was performed in the blood of cases diagnosed through FibroScan, and the same was done for age and gender matched apparently healthy controls obtained from AIIMS Bhopal. All participants were told about the study and were asked to participate in the study. Only participants who gave their consent were enrolled and had their blood samples and anthropometric measurements taken.

Inclusion and exclusion criteria

Patients aged 18 and older with MASLD diagnoses confirmed by FibroScan who lived in Madhya Pradesh, India comprised the case group. Patients needed to provide written consent for blood sample collection and ELISA testing and routine laboratory examination for research purposes. Patients with diabetes mellitus and Hepatitis B or C infection and polycystic ovarian syndrome (PCOS) and ischemic heart disease and congestive hepatopathy and active malignancy and cancer treatment and secondary fatty liver causes were excluded from the study. Patients with overt cirrhosis who showed gross ascites and hepatic encephalopathy were excluded from the study as well as patients who took hepatotoxic drugs in the previous six months exceeding 4 g/day of acetaminophen or methotrexate or nitrofurantoin or rifampicin and those with more than 10 years of heavy alcohol use exceeding 140 g/week for men and 70 g/week for women according to NIAAA standards. The study excluded all participants younger than 18 years old. For the control group, apparently healthy adult individuals matched for age and gender with the cases, residing in Madhya Pradesh, and willing to give consent for ELISA and routine laboratory tests were included. Exclusion criteria for controls included any current or past history of liver disease, alcohol intake, diabetes mellitus, hyperlipidemia, or age below 18 years.

Sample size, sampling method and data collection tools

As regards the worldwide incidence of Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) and the suitability of the sample for its analysis within the time available for the study, 186 samples were collected. This included 92 cases (patients with MASLD) and 97 controls (apparently healthy individuals), all recruited from the Outpatient Department (OPD) of AIIMS Bhopal, Madhya Pradesh, India. Sample size was calculated using OpenEpi software and the required number of participants to achieve sufficient statistical power was calculated to be 186. Participants were purposefully selected to match the criteria of the study, thus guaranteeing the appropriateness of the study population.

Data collection tools

It is a printed case record form, which the investigator filled in after obtaining informed consent from the participants. The case record form consisted of the following information that was gathered: demographic details- age, gender, residential address, and contact number. Biochemical investigation reports were done with other parameters in relation to liver function and metabolic status. Anthropometric measurements were performed with a standard weighing machine and a measuring tape for height, waist circumference, and so on. FibroScan results are used for MASLD staging in cases. Sample collection from each subject were done with five milliliters of whole blood, obtained in a yellow-top BD vacutainer (serum separator tube) for serum analysis. Furthermore, 5 ml of blood was drawn into a purple-top EDTA vacutainer containing 4mg of EDTA using a Buck-type tourniquet and BD vacutainer blood collection needle. All the tubes were properly marked with the name of the participant and the group to which the sample belonged (case or control) to avoid any mix-up during processing.

Measurement of serum levels of Adropin and Irisin

CK18 (FineTest, EH2820, India), Adropin (Lablisa® Human Adropin ELISA Kit, India. LAB 6444) and Irisin (Lablisa® Human Irisin ELISA Kit, India. LAB 6625) antibodies and other reagents for ELISA were purchased from a commercial supplier. ELISA was performed at room temperature and the protocol was established in the Department of Biochemistry. All plastic ware and deionized water were autoclaved. Same protocol and steps were used for Adropin and Irisin. The ELISA plate was setup with Standards, Blank and Serum Samples in designated wells. 100 µL of each Standard dilution or 100 µL

of serum sample was added to the appropriate wells. The plate was covered with a Plate Sealer and the plate was incubated at 37°C for 80 minutes. When the incubation was over, the liquid from the wells was decanted and each well was washed with wash Buffer and then aspirated. This step was then repeated three times to make sure that all the unwanted materials were removed from the wells. The last wash was completely removed by turning the plate upside down and blotting it on absorbent paper. After washing, biotinylated antibody working solution was added to each well and the plate was covered and incubated at 37°C. Streptavidin-HRP working solution was added to each well and the plate was covered and incubated at 37°C. After the final wash, TMB Substrate Solution was added to each well and the plate was covered and incubated at 37°C in the dark. The presence of the target analyte caused a blue color development by enzymatic reaction and color changing from blue to yellow. The absorbance was then immediately measured at 450 nm using a microplate reader (BioTek, India).

Quantitation of CK-18 (M65)

CK-18/M65 was quantified using a sandwich ELISA kit (FineTest, EH2820) as per the manufacturer's guidelines. All reagents were warmed up to room temperature before use in the assay. The concentrated wash buffer was diluted with distilled water and used within 48 hours. Lyophilized CK-18 standards were dissolved in sample dilution buffer and diluted in a series of concentrations (5000, 2500, 1250, 625, 312.5, 156.25, 78.125, and 0 pg/mL). The biotin-labelled antibody was made up to a concentration of 1:99 with the antibody dilution buffer immediately before use. Similarly, SABC HRP-streptavidin conjugate was diluted 1:99 in SABC dilution buffer and used within 30 minutes.

Each standard, sample and blank control was added to a pre-coated at ELISA plate and incubated at 37°C for 90 minutes in the assay. Then the biotin labeled antibody solution was added and incubated at 37°C and then washed three times. Then, HRP-streptavidin conjugate was added and left to incubate for 30 minutes at 37°C before five washes. TMB substrate was added to the wells, the plate was left to incubate in the dark at 37°C and then the reaction was stopped with stop solution. The microplate reader was used to measure absorbance at 450 nm (BioTek, India).

The data were further processed by taking the mean of the duplicate samples for OD450. A four-parameter logistic standard curve was constructed from the OD values of the standards and the concentration of the samples was determined from this curve and the dilution factor applied. The assay was performed in such a way that disposable pipette tips were used to prevent cross-contamination and washing steps were appropriately done to minimize background signals, and the standard and the working solutions were made right before the assay.

Statistical analysis

Continuous data such as age, CK18, Adropin and Irisin were presented as either mean and standard deviation (SD) or median and range according to the data distribution. Age, gender, BMI, Adropin and Irisin levels were considered to have differences between the groups and were tested. Correlation analysis was performed to examine the relationship between Adropin and Irisin levels based on the normality of the data. ROC curve analysis was used to establish the cutoff point, sensitivity and specificity of Adropin and Irisin levels in the different stages of MASLD. All statistical analyses were carried out at 5% significance level and a p value of less than 0.05 was considered to be statistically significant. P-values calculated using Mann–Whitney U test.

Results

A total of 182 participants were analyzed, comprising 92 MASLD cases and 90 healthy controls (7 controls were excluded due to incomplete data). The age of participants in both groups was similar (MASLD: 39.5 years vs. Controls: 37.5 years; $P = 0.54$). The gender distribution was comparable between groups, with 43.3% females in the control group and 39.1% females among MASLD cases. The difference was not statistically significant ($P = 0.67$), thus indicating a balanced gender representation across the study population. MASLD patients showed higher BMI measurements (median: 27.25 kg/m²) than the control participants (24.41 kg/m²; $P < 0.001$). FibroScan was used for classifying cases and controls based on liver fibrosis and hepatic steatosis evaluations for every participant in the study. The MASLD group consisted of 92 participants in which the patients had Grade 1 steatosis in 16 patients (17.4%), Grade 2 steatosis in 22 patients (23.9%) and Grade 3 steatosis in 46 patients (50.0%). The 8 participants (8.7%) who had Grade 0 steatosis received inclusion based on their fibrosis results indicating fibrotic progression. The liver stiffness measurements revealed that 39 patients (42.4%) had Stage F0 fibrosis while 31 patients (33.7%) had Stage F1 and 5 patients (5.4%) had Stage F2 and 2 patients (2.2%) had Stage F3 and 7 patients (7.6%) had Stage F4. The fibrosis staging data were missing for 8 study participants. The Fibro Scan liver stiffness measurements were higher in MASLD participants (6.85 vs. 5.0 kPa; $P < 0.001$) and the CAP score steatosis measurements were higher (290.5 vs. 217.0 dB/m; $P < 0.001$). The MASLD group showed elevated liver enzymes ALT and AST compared to controls (ALT: 53.15 vs. 23.85 U/L; AST: 53.45 vs. 25.55 U/L; both $p < 0.001$) which indicated more hepatocellular injury. The MASLD group presented significantly lower Adropin levels when compared to controls (245.0 vs. 414.4 pg/mL; $p < 0.001$) and CK-18 levels were significantly higher in MASLD patients (1030.5 vs. 533.1 pg/mL; $p < 0.001$) which supports the role of CK-18 as a marker of hepatocyte apoptosis. The Irisin concentration levels did not show any significant variation between study groups ($p = 0.17$). The anthropometric measurements of MASLD patients versus

controls showed inconsistent results among participants older than 60 years. The BMI and weight measurements of MASLD patients exceeded those of controls but these results failed to achieve statistical significance possibly because of aging body composition alterations or insufficient participant numbers. Anthropometric parameters in MASLD grouped by age and sex are shown in Table 1.

Table 1: Anthropometric parameters in MASLD - stratified by age and sex.

Age group	Gender/ Sex	Parameter	MASLD pg/mL	Control pg/mL	P-value
18–30 years	M	BMI (kg/mÂ²)	28.69±1.02	23.14±6.35	0.0008
	F	BMI (kg/mÂ²)	28.32±0.96	24.76±5.96	0.0617
	M	Hip-to-Waist ratio	0.93±0.053	0.88±0.12	0.0327
	F	Hip-to-Waist ratio	0.9±0.03	0.85±0.22	0.0211
	M	Weight (kg)	79.0±6.2	68.0±6.47	0.0017
	F	Weight (kg)	77.0±8.3	68.5±5.98	0.2639
	M	Height (cm)	170.0±12.5	171.0±14.6	0.3933
	F	Height (cm)	162.0±11.6	167.0±12.9	0.1441
31–60 years	M	BMI (kg/mÂ²)	26.21±2.03	24.58±3.5	0.0027
	F	BMI (kg/mÂ²)	28.31±2.5	24.28±2.5	0.0031
	M	Hip-to-Waist ratio	0.9±0.85	0.88±0.22	0.3221
	F	Hip-to-Waist ratio	0.89±0.74	0.86±0.23	0.0012
	M	Weight (kg)	78.0±10.2	73.5±6.5	0.0113
	F	Weight (kg)	76.0±11.63	68.0±6.85	0.0003
	M	Height (cm)	170.0±15.2	169.5±9.98	0.9194
	F	Height (cm)	165.0±11.63	164.5±10.55	0.0902
More than 60 years	M	BMI (kg/mÂ²)	26.86±6.35	24.38±5.89	0.4762
	F	BMI (kg/mÂ²)	20.94±8.65	23.51±4.78	0.2013
	M	Hip-to-Waist ratio	0.9±0.56	0.89±0.21	0.7619
	F	Hip-to-Waist ratio	0.81±0.63	0.84±0.19	0.8102
	M	Weight (kg)	79.0±9.52	75.5±5.74	0.7476
	F	Weight (kg)	60.5±8.34	72.0±8.33	0.2102
	M	Height (cm)	171.0±15.21	172.0±15.3	0.7619
	F	Height (cm)	170.0±14.96	175.0±14.69	0.8002

*Values are expressed as medians. P-values calculated using Mann–Whitney U test.

The analysis showed no statistically relevant variations in total protein, albumin or globulin concentrations between the groups. The main clinical and laboratory characteristics of the study population are summarized in Table 2.

Table 2: Clinical and biochemical characteristics of study participants.

Factor	All samples (n = 182)	Control (n = 90)	MASLD (n = 92)	P-value*
Age (years)	39.0 (29.0, 48.0)	37.5 (29.0, 47.0)	39.5 (29.8, 49.0)	0.54
Female gender	75 (41.2%)	39 (43.3%)	36 (39.1%)	0.67
BMI (kg/m²)	25.3 (23.6, 28.0)	24.4 (22.8, 25.5)	27.3 (25.0, 29.4)	<0.001
Liver stiffness (kPa)	5.7 (5.0, 7.9)	5.0 (4.8, 5.3)	6.85 (5.5, 9.5)	<0.001
CAP Score (dB/m)	262.0 (214.0, 315.0)	217.0 (203.0, 240.0)	290.5 (252.0, 330.0)	<0.001
AST (U/L)	30.6 (22.8, 53.5)	23.5 (18.8, 32.6)	44.5 (28.0, 110.6)	<0.001
ALT (U/L)	30.4 (22.4, 59.8)	23.9 (19.1, 32.2)	53.1 (29.1, 84.0)	<0.001
GGT (U/L)	34.1 (22.0, 65.5)	23.6 (18.8, 36.4)	59.6 (24.9, 86.5)	<0.001
A/G Ratio	1.40 (1.20, 1.50)	1.43 (1.25, 1.55)	1.37 (1.19, 1.48)	0.11

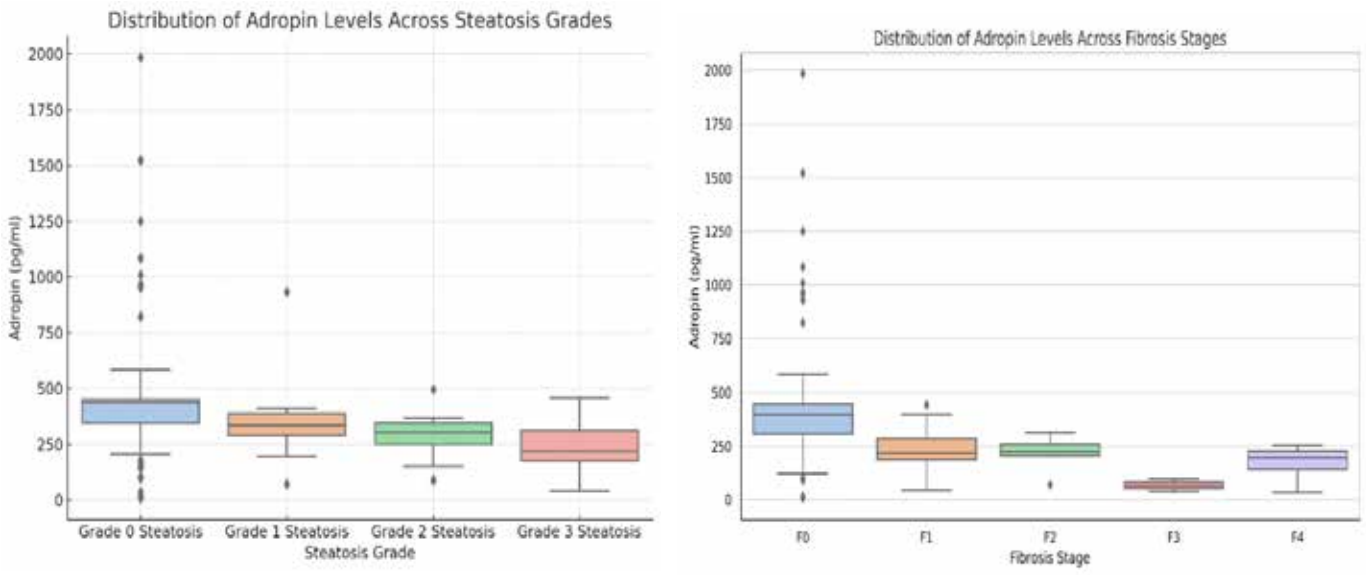
Factor	All samples (n = 182)	Control (n = 90)	MASLD (n = 92)	P-value*
CK-18 (pg/mL)	801.5 (548.2, 1101.8)	533.1 (408.7, 704.4)	1030.5 (836.4, 1352.9)	<0.001
Adropin (pg/mL)	329.6 (194.8, 455.4)	414.4 (276.9, 545.4)	245.0 (161.6, 309.4)	0.001
Irisin (pg/mL)	1375.6 (724.1, 1901.4)	1482.1 (826.4, 2004.6)	1290.2 (668.3, 1798.1)	0.17
Total protein (g/dL)	7.4 (6.9, 7.8)	7.4 (6.9, 7.8)	7.3 (6.9, 7.8)	0.78
Albumin (g/dL)	4.3 (4.0, 4.6)	4.3 (4.1, 4.6)	4.3 (4.0, 4.5)	0.62
Globulin (g/dL)	3.0 (2.6, 3.3)	3.0 (2.6, 3.2)	3.0 (2.6, 3.3)	0.79

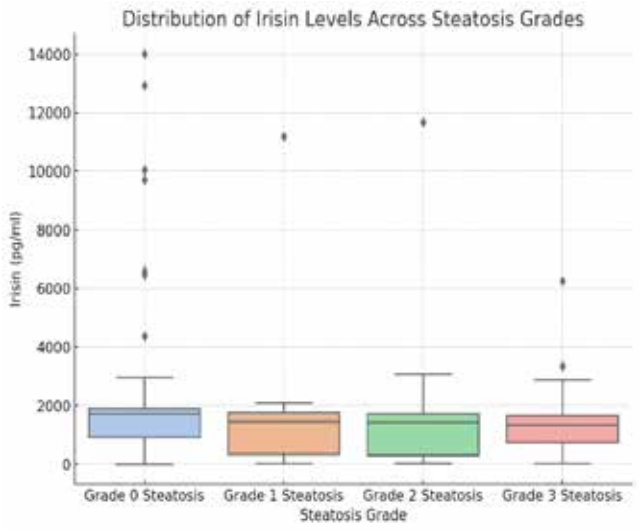
*Values expressed as median (IQR). P values were calculated using Mann–Whitney U test. $P < 0.05$ considered statistically significant. ALT – Alanine aminotransferase; AST – Aspartate aminotransferase; GGT – Gamma-glutamyl transferase; ALP – Alkaline phosphatase; TB–Total bilirubin; A/G– Albumin to globulin ratio; CK-18– Cytokeratin 18.

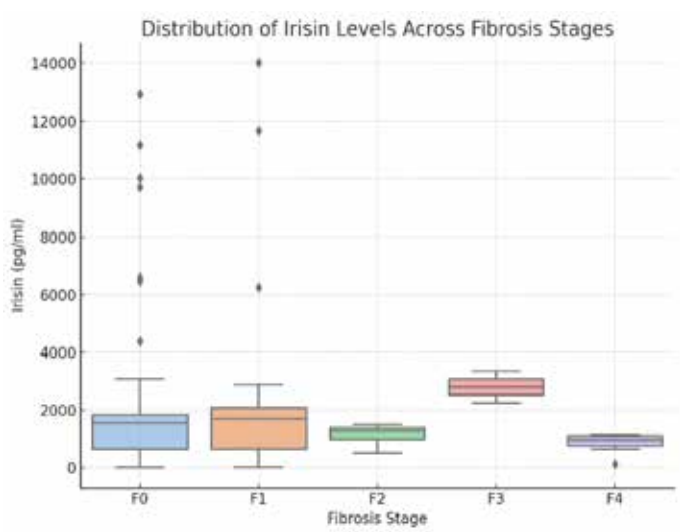
The MASLD group showed significantly higher serum ALT (60.10 ± 36.58 U/L vs 29.75 ± 23.37 U/L, $p < 0.0001$) and AST (68.71 ± 50.63 U/L vs 28.03 ± 16.90 U/L, $p < 0.0001$) levels which indicated greater hepatocellular injury. The ALP levels were significantly higher in MASLD cases (73.96 ± 29.57 U/L vs 117.76 ± 67.42 U/L, $p < 0.0001$). The MASLD group showed significantly lower Adropin serum levels compared to controls (271.98 ± 121.61 pg/mL vs 451.63 ± 278.64 pg/mL, $p < 0.0001$) which indicates this peptide hormone may play a protective or regulatory function in liver pathology. The A/G ratio showed no significant statistical difference between the groups (1.37 ± 0.27 vs 1.43 ± 0.40, $p = 0.21$). Most parameters showed non-normal distribution so the Mann–Whitney U test determined statistical significance.

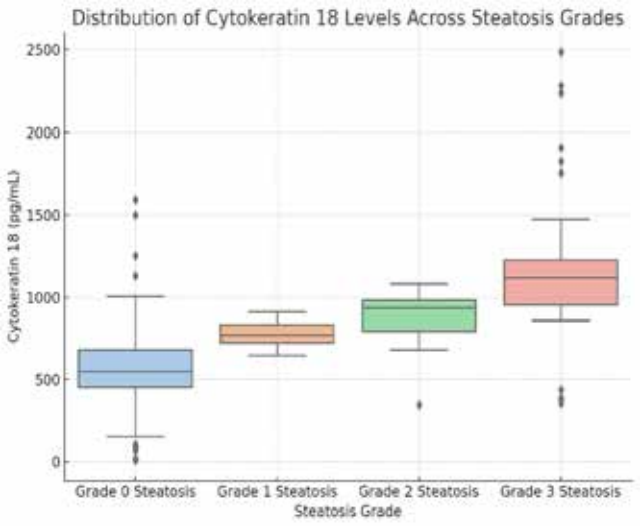
The analysis with Spearman's correlation examined the relationship between candidate biomarkers and MASLD severity clinical indicators such as liver enzymes and CAP steatosis grade and FibroScan kPa. The serum CK18 (CK18) showed a moderate positive correlation with CAP ($r = 0.666$) which indicates a strong relationship with hepatic steatosis. In the Figure 1 we have shown the distribution of Adropin, Irisin and Cytokeratin 18 levels in fibrosis and steatosis grades. The positive correlations between CK18 and ALT, AST, and GGT support its function as a marker of hepatocyte apoptosis and liver injury. The inverse relationship between Adropin and MASLD progression confirms previous research showing Adropin acts protectively against metabolic dysfunction.

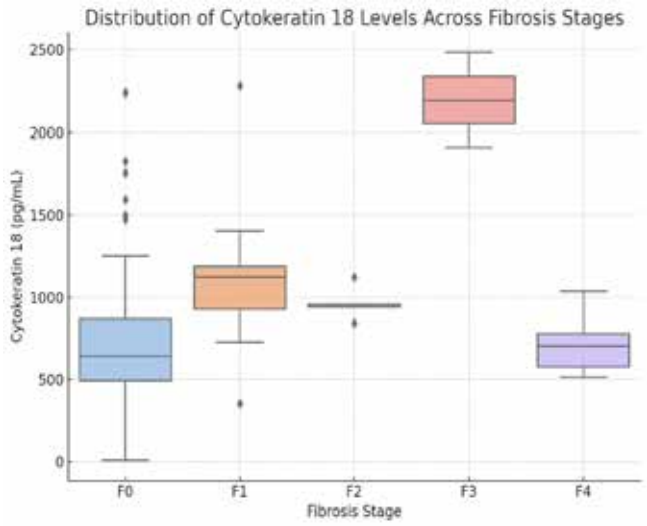
Figure 1: Distribution of Adropin, Irisin and Cytokeratin 18 levels in fibrosis and steatosis grades.







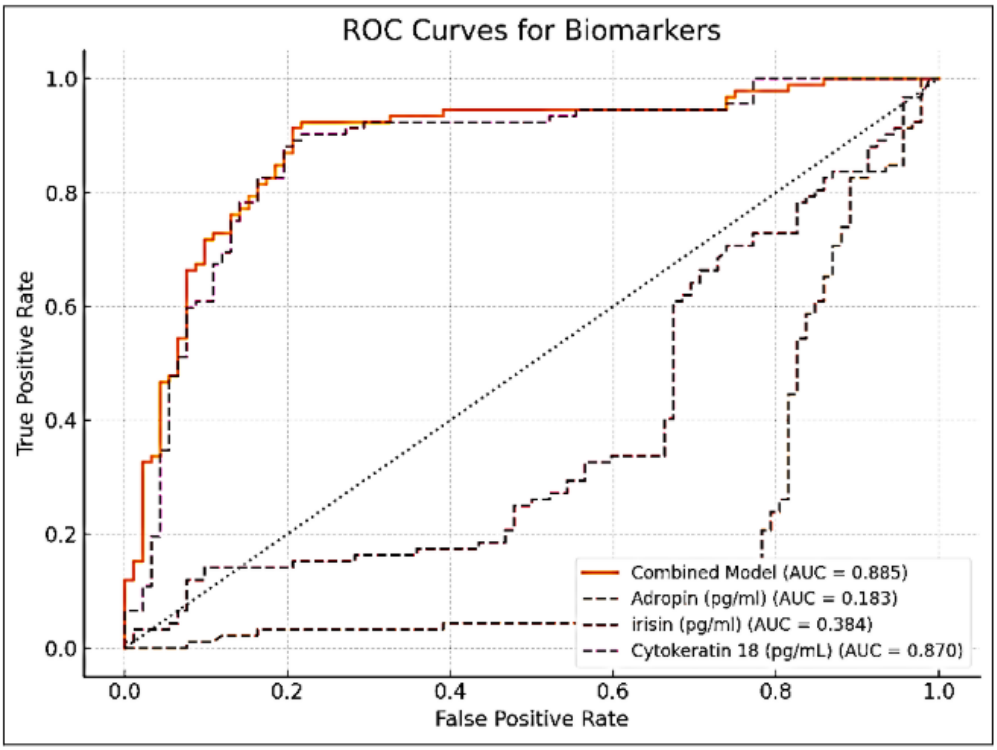




The MASLD patient group had lower Irisin levels but the results did not show a reliable relationship with CAP or FibroScan kPa values which suggests its function might be complicated or dependent on disease stages. ALT and AST liver transaminases showed a strong correlation with each other (r = 0.814) and also had moderate correlations with CK18 and GGT which indicated hepatocellular damage and enzyme leakage patterns.

The research evidence supports the use of CK18 and Adropin as diagnostic markers for steatosis and fibrosis but Irisin needs additional assessment in bigger or time-based studies. The diagnostic capabilities of serum biomarkers Adropin, Irisin, and Cytokeratin 18 (CK18) to distinguish MASLD patients from healthy controls were evaluated through receiver operating characteristic (ROC) curve analysis and multivariate logistic regression (Figure 2). Among the individual biomarkers, CK18 (CK18) demonstrated the highest diagnostic accuracy, with an area under the curve (AUC) of 0.87, indicating excellent ability to distinguish MASLD cases from controls. In contrast, Irisin exhibited limited discriminatory power (AUC = 0.384), and Adropin showed poor performance (AUC = 0.183) when assessed independently.

Figure 2: ROC curves comparing the diagnostic accuracy of Adropin, Irisin, Cytokeratin 18, and their combined model for detecting MASLD.



The orange line represents the combined model (AUC = 0.885), the black dashed line shows Adropin (AUC = 0.183), the brown dash-dot line indicates Irisin (AUC = 0.384), and the purple dotted line represents Cytokeratin 18 (AUC = 0.870). The diagonal grey line denotes the reference (AUC = 0.5).

A logistic regression model that included all three markers (Adropin, Irisin, and CK18) was used to evaluate the diagnostic performance when biomarkers were combined. The combined model yielded a superior AUC of 0.885, suggesting that multivariate assessment may provide added value in identifying MASLD, particularly in its earlier stages where traditional liver enzymes may remain within normal limits. The results indicate that CK18 functions as a strong single marker and demonstrate how biomarker panels could enhance non-invasive MASLD diagnosis.

The participants were further stratified by age and sex to explore differential patterns in MASLD. In the 31–60-year male subgroup, MASLD patients exhibited significantly higher levels of ALT, AST, GGT, and ALP compared to controls ($P < 0.05$ for all). A similar pattern was observed in females aged 31–60, particularly for ALT and AST. Interestingly, while ALT and AST levels were also elevated in younger males and females (18–30 years), the AST/ALT ratio remained close to 1 across all subgroups, without significant variation (Table 3). Total bilirubin was significantly elevated in younger males (18–30 years) with MASLD compared to controls ($P = 0.0136$), suggesting early cholestatic involvement in some individuals. Albumin levels remained stable across groups, with no significant difference observed, indicating preserved synthetic function in the majority of cases.

Table 3: Key liver function test (LFT) markers in MASLD - stratified by age and sex.

Age group	Gender/ Sex	Parameter	MASLD pg/mL	Control pg/mL	P-value
18–30 years	Male	ALT	80.0±9.3	26.2±6.37	0.0007
		AST	69.3±5.84	24.8±5.68	0.0004
		AST/ALT ratio	1.41±0.51	0.89±0.2	0.1711
		GGT	75.9±7.3	22.1±5.32	0.0006
		ALP	125.4±10.2	65.7±6.34	0.0075
		TB	1.0±0.2	0.61±0.2	0.0136
		Alb	3.79±0.5	4.4±0.96	0.1293
	Female	ALT	78.3±6.41	29.05±6.31	0.0021
		AST	55.9±8.3	21.9±5.21	0.0017
		AST/ALT ratio	0.88±0.24	0.95±0.27	0.4817
		GGT	65.5±5.3	22.85±9.32	0.0124
		ALP	121.0±9.56	73.2±8.68	0.007
		TB	1.21±0.3	0.75±0.2	0.0687
		Alb	4.0±0.42	4.37±2.12	0.3953
31–60 years	Male	ALT	49.1±5.8	23.35±6.85	0.0003
		AST	43.2±6.9	23.2±3.21	0.0003
		AST/ALT ratio	1.0±0.01	0.99±0.2	0.8748
		GGT	54.6±9.5	23.85±6.3	0.0019
		ALP	86.1±9.47	74.5±8.62	0.0363
		TB	0.93±0.2	0.75±0.99	0.0252
		Alb	4.39±0.8	4.44±0.95	0.8697
	Female	ALT	41.5±6.5	20.1±5.74	0.0635
		AST	40.9±8.52	21.5±6.5	0.0002
		AST/ALT ratio	1.16±0.36	1.06±0.8	0.8364
		GGT	54.66±13.2	29.45±5.2	0.0231
		ALP	112.5±17.2	78.35±9.5	0.0335
		TB	0.63±0.9	0.6±0.84	0.2149
		Alb	4.2±0.81	4.39±.091	0.0334
More than 60 years	Male	ALT	44.55±6.34	20.92±3.49	0.6095
		AST	63.55±9.35	24.1±3.53	0.3524
		AST/ALT ratio	2.06±0.8	1.47±0.62	0.3524
		GGT	19.0±1.02	17.43±1.34	0.9143
		ALP	112.25±9.06	53.15±8.35	0.1143
		TB	0.72±0.49	0.58±0.12	0.3923
		Alb	3.26±0.51	4.15±1.37	0.1344
	Female	ALT	41.4±6.34	45.5±6.92	0.8135
		AST	47.35±8.35	23.2±6.38	0.8032
		AST/ALT ratio	1.15±0.42	0.68±0.47	0.2001
		GGT	63.0±5.97	36.5±6.35	1.0235
		ALP	188.85±9.35	75.5±3.34	0.2021
		TB	0.58±0.28	0.5±0.02	0.8329
		Alb	3.71±0.34	4.1±0.63	0.835

*Values are expressed as mean ± SD. P values were calculated using Mann-Whitney U test. ALT–Alanine aminotransferase; AST– Aspartate aminotransferase; GGT– Gamma-glutamyl transferase; ALP– Alkaline phosphatase; TB– Total bilirubin; Alb– Albumin.

The research showed different metabolic dysregulation patterns in MASLD patients when lipid profile markers were analysed by age and sex subgroup. Males aged 31–60 with MASLD showed higher total cholesterol (201.6 vs 176.1 mg/dL; P = 0.0457), triglycerides (131.3 vs 95.5 mg/dL; P = 0.0240), and VLDL levels (33.5 vs 20.17 mg/dL; P = 0.0021) than controls without significant differences in HDL and LDL levels. The lipid profile results for females in this age group showed that triglycerides (146.5 vs 96.5 mg/dL; P = 0.0141) and VLDL (30.4 vs 21.1 mg/dL; P = 0.0193) were higher than controls but HDL and LDL levels were similar.

Young participants aged 18–30 years with MASLD showed increased triglycerides and VLDL levels but the differences became statistically significant only in males (TG: 140.0 vs 87.0 mg/dL; P = 0.0189, VLDL: 33.6 vs 18.5 mg/dL; P = 0.0157). The VLDL levels of females in this group were higher than other lipid parameters but did not show significant variation. More than 60 years age group showed no statistically significant differences between MASLD and controls for any lipid markers which may be due to metabolic blunting with age or limited subgroup size (Table 4).

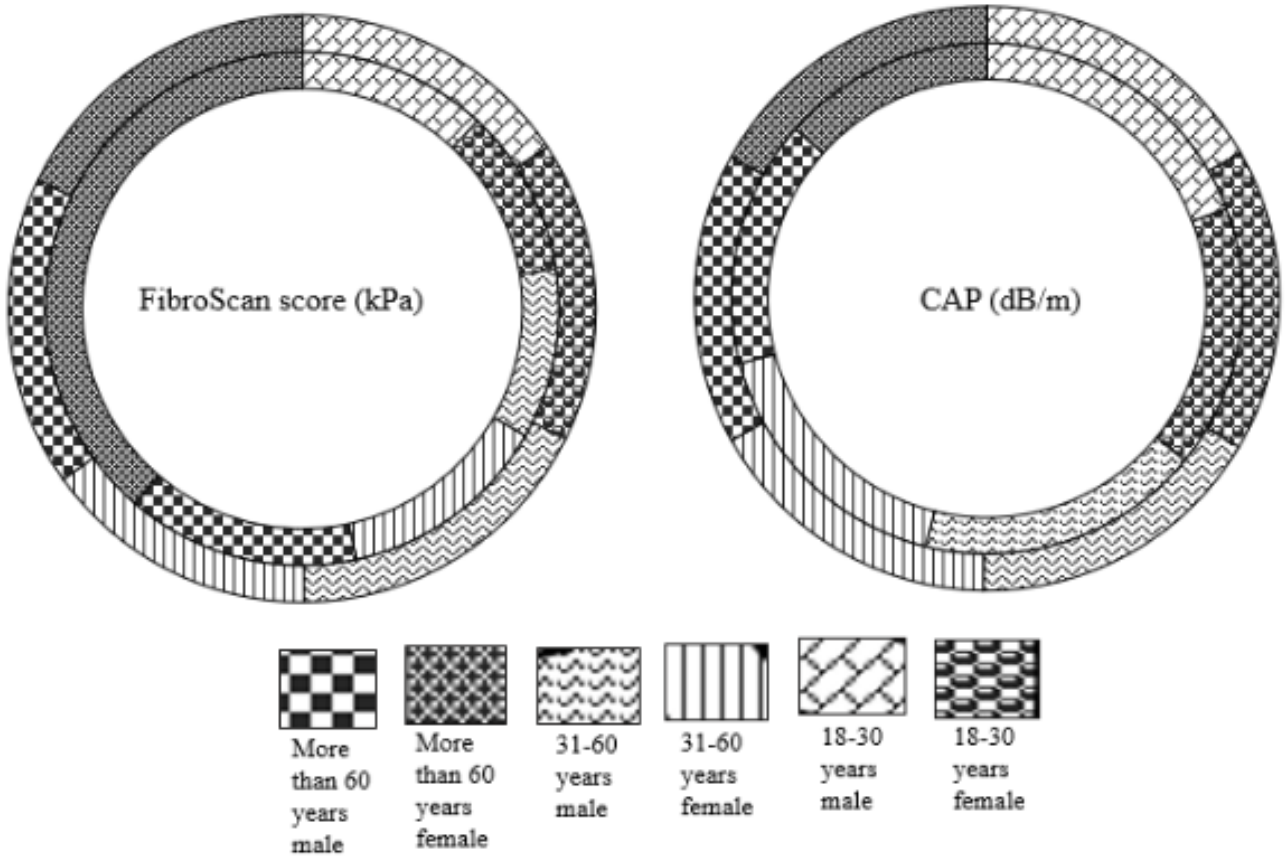
Table 4: Lipid profile comparison in MASLD - stratified by age and sex.

Age group	Gender/ Sex	Parameter (mg/dL)	Patient with MASLD	Control	P-value
18–30 years	Male	TC	200.7±15.2	168.11±11.2	0.0028
		TG	165.0±12.4	95.0±9.2	0.0225
		HDL	36.0±6.52	45.0±5.2	0.0649
		LDL	114.94±12.4	98.9±6.3	0.0062
		VLDL	36.24±5.3	23.2±7.2	0.1585
	Female	TC	210.0±15.32	176.96±8.2	0.044
		TG	150.0±14.23	110.65±9.7	0.0553
		HDL	35.4±8.37	41.05±8.2	0.1387
		LDL	110.5±5.38	99.35±7.15	0.1147
		VLDL	36.0±6.32	28.28±6.25	0.1264
31–60 years	Male	TC	201.6±17.25	176.1±10.9	0.0457
		TG	131.3±13.2	95.5±5.32	0.0241
		HDL	42.95±8.3	43.85±9.2	0.659
		LDL	117.14±9.35	107.65±11.5	0.1942
		VLDL	33.5±9.2	20.17±6.3	0.0021
	Female	TC	219.52±12.3	167.52±11.9	0.003
		TG	143.0±15.3	89.38±5.3	0.0279
		HDL	42.0±6.35	44.3±6.23	0.6618
		LDL	126.98±6.5	99.35±11.2	0.0003
		VLDL	25.94±5.3	18.37±5.2	0.1143
More than 60	Male	TC	216.94±22.3	167.06±12.2	0.3524
		TG	105.8±16.3	97.45±9.2	0.7619
		HDL	41.2±10.85	36.53±4.5	0.4762
		LDL	105.02±11.25	109.52±6.5	0.9143
		VLDL	22.4±9.3	29.15±6.7	0.7619
	Female	TC	158.55±10.2	154.52±9.5	0.9412
		TG	184.33±13.45	98.6±8.7	0.2136
		HDL	35.96±8.3	42.56±5.8	0.4012
		LDL	85.72±7.9	88.5±6.2	0.8231
		VLDL	36.88±6.35	32.3±5.2	0.8027

*Values are expressed as medians. P-values calculated using Mann–Whitney U test. Bold values indicate statistically significant differences (P < 0.05). TC– Total cholesterol; TG– Triglycerides; HDL– High-density lipoprotein; LD–Low-density lipoprotein; VLDL– Very-low-density lipoprotein.

The research examined BMI together with body weight and height and hip-to-waist ratio in specific age and sex groups to determine their association with MASLD. The BMI and weight measurements of 31–60-year-old male MASLD patients were significantly higher than those of controls (26.21 vs 24.58 kg/m² and 78.0 vs 73.5 kg respectively; P = 0.0027 and P = 0.0113). The BMI measurements of female patients with MASLD were substantially higher than controls in this age group (28.31 vs 24.28 kg/m²; P < 0.0001) which indicated central adiposity as a primary risk factor in this specific age group. The BMI and body weight measurements of MASLD patients within the 18–30-year age group demonstrated significant increases when compared to controls (P < 0.05) yet these differences were not as significant as those found in the older age group. The research demonstrates how small amounts of body fat accumulation during young adulthood can lead to liver fat deposition which results in the development of MASLD. FibroScan score and CAP score of the different age group are shown in the Figure 3.

Figure 3: FibroScan-based fibrosis and steatosis scores in MASLD - stratified by age and gender.



The assessment of liver stiffness (fibrosis) and steatosis (fat accumulation) was done using FibroScan and was stratified by age and sex to determine the patterns of disease severity in MASLD. The most significant differences were observed in the 31–60-year group, where both males and females with MASLD had significantly higher fibrosis scores (6.10 vs 5.15 kPa in males, P = 0.0021; 7.60 vs 4.45 kPa in females, P = 0.0002) and higher CAP scores indicating steatosis (298.0 vs 217.0 dB/m in males, 284.0 vs 217.5 dB/m in females; both P < 0.0001).

Among participants aged 18–30 years, MASLD patients also had significantly higher liver stiffness (6.80 vs 4.60 kPa in males, P < 0.0001; 6.28 vs 4.80 kPa in females, P = 0.0128), and higher CAP scores, although the magnitude of difference was less than in the older group. These findings are noteworthy, as they indicate that fibrotic and steatotic changes are already present in younger individuals with MASLD, suggesting early onset of hepatic involvement. On the other hand, in the >60-year age group, there were no statistically significant differences in fibrosis or CAP values between MASLD cases and controls. This may be due to age-related changes that diminish disease discrimination or smaller sample size in this subgroup.

Therefore, these results confirm the progressive nature of fibrosis and steatosis with age in MASLD and the diagnostic usefulness of FibroScan as a non-invasive tool to assess the stage of liver involvement, especially in early-to mid-adult populations. The study analyzed biomarkers related to

metabolic regulation and hepatocyte apoptosis through age and sex stratification to determine their diagnostic value across the MASLD spectrum. The 31–60-year age bracket showed MASLD patients of both sexes to have significantly lower Adropin levels and extremely elevated CK-18 levels when compared to controls ($P < 0.0001$ in all cases) which indicated severe metabolic and apoptotic disturbances in this population. The 18–30-year subgroup showed similar patterns to older age groups with MASLD patients displaying reduced Adropin levels compared to controls ($P = 0.0001$ in males and $P = 0.0002$ in females) and elevated CK-18 levels ($P = 0.0004$ in males and $P = 0.0035$ in females) at lower levels than observed in older participants. Biomarker disturbances appear to emerge in young MASLD patients as they indicate the beginning of their disease. The >60-year-old participants showed minimal and non-significant variations between MASLD patients and controls regarding CK-18 and Adropin levels. The smaller study population and metabolic changes that accompany aging might explain these results. The 31–60-year age bracket showed statistically significant lower Irisin levels in MASLD patients compared to controls. The study confirms that Adropin and CK-18 serve as effective early biomarkers for MASLD especially among younger and middle-aged adults while suggesting Irisin has a limited influence on disease mechanisms (Table 5).

Table 5: Biomarker levels in MASLD - stratified by age and sex.

Group	Gender	Parameter (pg/ml)	Patient with MASLD	Control	P-value
18–30 years	Male	Adropin	297.46±16.2	427.81±14.5	0.0037
		Irisin	1422.44±32.2	1768.62±21.8	0.6186
		Cytokeratin 18	993.34±21.48	496.45±15.4	0.0125
	Female	Adropin	257.76±18.2	433.34±13.9	0.0108
		Irisin	1311.03±32.8	1573.59±19.8	0.3565
		Cytokeratin 18	959.63±21.5	549.42±12.2	0.0005
31–60 years	Male	Adropin	308.28±19.7	439.96±11.7	0.0021
		Irisin	1405.57±32.8	1759.76±28.5	0.0278
		Cytokeratin 18	929.79±24.2	541.3±12.9	0.0102
	Female	Adropin	267.67±20.1	442.09±12.5	0.0201
		Irisin	1403.79±26.6	1789.16±19.8	0.0119
		Cytokeratin 18	942.3±21.5	526.65±11.5	0.0098
More than 60 years	Male	Adropin	266.58±20.7	428.21±9.54	0.3524
		Irisin	1505.22±27.6	1743.64±14.5	0.9143
		Cytokeratin 18	695.35±22.4	548.47±11.5	0.4762
	Female	Adropin	275.76±15.2	258.2±12.7	0.8102
		Irisin	1642.41±28.6	125.7±9.6	0.4032
		Cytokeratin 18	744.56±23.4	950.1±10.61	0.8087

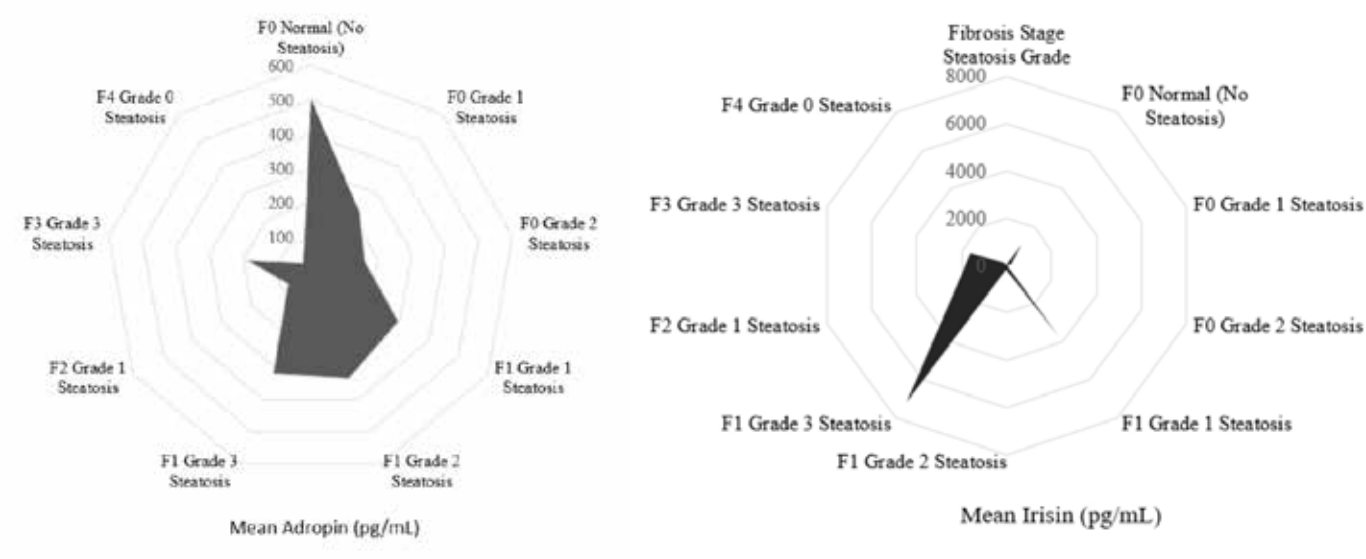
*Values are expressed as medians. P-values calculated using Mann–Whitney U test.

The table 6 and figure 4 allows for insights into how Adropin and Irisin concentrations correlate with steatosis and fibrosis. Higher concentrations of Irisin in certain groups (especially in F1 Grade 3 Steatosis) may indicate potential metabolic changes or responses to increased fat accumulation in the liver.

Table 6: Adropin and Irisin concentrations correlate with steatosis and fibrosis.

Fibrosis stage	Steatosis grade	Count of patients	Mean Adropin concentration (pg/ml)	
F0	Normal (no steatosis)	45	508.98±21.2	1085.13±34.67
F0	Grade 1 Steatosis	10	224.25±15.4	205.21±15.3
F0	Grade 2 Steatosis	4	162.17±12.3	52.81±10.55
F1	Grade 1 Steatosis	4	294.29±18.95	3010.64±42.85
F1	Grade 2 Steatosis	5	332.66±21.97	185.32±10.2
F1	Grade 3 Steatosis	5	319.82±24.35	7156.07±56.95
F2	Grade 1 Steatosis	2	77.41±9.35	2096.30±24.3
F3	Grade 3 Steatosis	3	186.41±10.52	1629.09±19.35
F4	Grade 0 Steatosis	1	33.95±6.35	114.61±12.54

Figure 4: Adropin and Irisin concentrations with steatosis grade and fibrosis stages.



Discussion

This research study investigated the diagnostic and staging abilities of three circulating biomarkers - Adropin, Irisin, and CK18 - in patients suffering from MASLD. MASLD represents a growing global public health concern, characterized by the accumulation of fat in the liver in the absence of excessive alcohol consumption [34-37]. Its progression can lead to more severe liver conditions, including MASH, fibrosis, and eventually liver cirrhosis or liver cancer [38, 39]. Therefore, accurate diagnosis and staging of MASLD are essential for timely management and treatment. To assess these biomarkers, the researchers employed recognized reference standards, namely Controlled Attenuation Parameter (CAP) and FibroScan, which are widely used for measuring liver fat content and fibrosis stage non-invasively [39, 40]. This method allows for the evaluation of liver conditions without the need for invasive liver biopsies, which can be risky and uncomfortable for patients.

The study's primary focus was on understanding how well each biomarker could identify the presence and severity of MASLD, as well as their potential role in tracking the disease's progression. The results of the study provided insights into the performance of each biomarker. Among the three biomarkers, CK18 stood out for its diagnostic capabilities [41, 42]. The findings indicated that CK18 demonstrated a strong discriminatory power for identifying MASLD and also for determining its progression. The biomarker revealed significant correlations with various parameters, including the grade of steatosis (the amount of fat in the liver) and the stage of fibrosis (the level of scarring in the liver). Additionally, CK18 levels were found to correlate well with liver enzyme levels, which are often indicative of liver inflammation and damage [43-45]. This suggests that CK18 could potentially be a reliable biomarker for clinicians, allowing for improved diagnosis and monitoring of MASLD in patients [46]. The second biomarker, Adropin, displayed a more complex role in the context of MASLD [47-50]. While its diagnostic accuracy as a standalone marker was limited - meaning it may not be sufficiently reliable on its own - it exhibited a negative correlation with the severity of MASLD. This suggests that lower levels of Adropin might be associated with more severe forms of the disease. Although Adropin was not robust enough to be used independently, its potential as a complementary biomarker became apparent. When combined with other markers, Adropin may enhance the overall diagnostic accuracy for MASLD, making it a valuable addition in the context of multimodal approaches to biomarker assessment [51].

The third biomarker, Irisin, showed inconsistent patterns in its relationship with MASLD disease parameters [52]. This inconsistency raises doubts about its utility as a reliable standalone biomarker for MASLD. Unlike CK18, which provided clear correlations with disease severity, Irisin's variability limits its effectiveness in both diagnosing and staging MASLD. As such, while it may have potential, further investigation into its role in MASLD is necessary before it can be considered a reliable biomarker. One of the study's significant findings was the advantage of employing a multimodal approach to biomarker assessment. The logistic regression model that included all three biomarkers - CK18, Adropin, and Irisin - resulted in improved diagnostic accuracy for MASLD. This highlights the importance of using multiple biomarkers together when assessing patients. By employing a comprehensive approach that leverages the strengths of various biomarkers, clinicians may be better equipped to diagnose and stage MASLD effectively [53-55]. The clinical implications of these findings are noteworthy. While CK18 appeared to be the most promising biomarker for both detection and staging of MASLD, there are opportunities to enhance diagnostic

accuracy by incorporating metabolic markers like Adropin into clinical evaluations. The complementary nature of these biomarkers may lead to a more thorough understanding of an individual's liver health status, enabling more informed clinical decisions regarding the management of MASLD.

While the results are promising, the researchers highlighted the need for additional studies to validate these findings further. Future research should involve larger and more diverse patient populations, as well as longer follow-up periods. This would ensure that the biomarkers' performance is consistent across different demographic and clinical contexts [56, 57]. Through such efforts, it will be possible to establish clinical reference values for CK18, Adropin, Irisin, and their combinations, thereby improving their utility in everyday clinical practice.

Ultimately, the research underscores the critical need for reliable biomarkers in the diagnosis and management of MASLD. Identifying patients at risk for progression to more severe liver disease is essential for implementing preventative strategies and therapeutic interventions [58, 59]. The study contributes to the evolving landscape of liver disease diagnostics, highlighting biomarker research's importance in enhancing patient outcomes and advancing therapeutic approaches in liver health [60-63]. In inference, the research into Adropin, Irisin, and CK18 provides valuable insights into biomarkers for MASLD. With CK18 showing the most promise for diagnosing and staging MASLD, supplemented by Adropin's potential, the combination of these biomarkers through a multimodal approach represents a significant advance in the assessment of this increasingly prevalent condition. Continued investigation in this area will be vital for refining diagnostic tools and improving strategies for managing MASLD in diverse patient populations [64-70].

The study contains a number of drawbacks. First off, the results may not be as broadly applicable to a variety of populations due to the limited sample size. Second, evaluation of longitudinal changes and causal links between biomarkers and disease development is not possible due to the cross-sectional methodology. Furthermore, even while non-invasive techniques like FibroScan and CAP are safer, they might not fully capture the histological features that can be obtained through biopsy. Inconsistent relationships and variations in Irisin levels also point to possible measurement errors or biological variability. Lastly, a thorough grasp of biomarker roles is restricted by the absence of broad demographic representation and the scant investigation of underlying molecular mechanisms, underscoring the necessity of larger, multicenter, and mechanistic studies in subsequent research.

Future direction

Large-scale prospective studies could be the main focus of future research on Irisin and Adropin to determine their predictive potential for cardiovascular and metabolic disorders. The therapeutic potential of these peptides may be clarified by interventional trials that investigate the effects of altering them with pharmacological treatments or lifestyle modifications. Furthermore, molecular mechanistic research is necessary to comprehend their functions in cellular signaling cascades, inflammation, and energy management. Investigating how genetic differences affect their activity or levels may provide information about a person's sensitivity. Targeted therapies and customized medicine tactics may be made possible by combining omics methodologies and cutting-edge imaging tools to better understand their roles in tissue-specific situations. Furthermore, examining how they interact with hormones and other metabolic regulators may help us better understand their integrative functions. Translational applications will be improved by researching their impact in a variety of populations and disease situations. All things considered, thorough investigation will be essential to maximizing the therapeutic and diagnostic potential of Adropin and Irisin.

Conclusion

This research aimed to evaluate the diagnostic and staging capabilities of three circulating biomarkers - Adropin, Irisin, and CK18 - in patients with Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD), using Controlled Attenuation Parameter (CAP) and FibroScan as reference standards for comparison. The study yielded significant insights into the effectiveness of these biomarkers in identifying MASLD and assessing its progression. The results indicated that CK18 demonstrated a robust discriminatory power, effectively distinguishing MASLD patients from those without liver disease. This was further supported by its significant correlations with steatosis grade, fibrosis stage, and liver enzyme levels, highlighting CK18's reliability as a diagnostic tool. Its strong performance in identifying disease severity makes it a promising candidate for clinical application in the management of MASLD.

In contrast, the diagnostic utility of Adropin as a standalone marker was somewhat limited. However, its negative correlation with MASLD severity suggests it may have potential as a complementary biomarker, especially when used in conjunction with other diagnostic tools. The combined approach could enhance overall diagnostic accuracy, making the identification of MASLD more effective. The role of Irisin as a standalone biomarker remains uncertain, as inconsistent patterns were observed with disease parameters, which raises questions about its utility in MASLD diagnostics. Importantly, the study employed a logistic regression model that integrated all three biomarkers, demonstrating improved diagnostic accuracy for MASLD assessment through a multimodal biomarker approach. While CK18 exhibits considerable potential for clinical application in liver disease diagnostics, supplementing it with metabolic markers like Adropin may further enhance detection and staging accuracy. Future research is essential to confirm these findings, ideally involving larger and more diverse patient groups, alongside extended follow-up

periods to establish comprehensive clinical reference values for these biomarkers in the context of MASLD. This research serves as a foundational step towards optimizing the diagnostic framework for liver disease management.

Ethics and consent to participate

Ethical clearance was obtained from the Institute Ethics Committee (IHEC/SR/2024/Apr/10 dated: 23/04/2024) of AIIMS Bhopal, India. and all subjects gave their informed consent before participation. From the Department of Medicine, AIIMS Bhopal, MASLD patients were recruited based on FibroScan to participate in the study, after fulfilling the inclusion and exclusion criteria and after obtaining consent. All studies involving human subjects must indicate that they are in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

Consent for publication

All participants gave their informed consent before participation from the Department of Medicine, AIIMS Bhopal after fulfilling the inclusion and exclusion criteria and after obtaining consent.

Declaration of conflict of interests

No.

CRediT authorship contribution statement

DR and SM conceptualized the manuscript. DR, ZS, SR and SKR wrote the first draft. DR and SKR drew the figures. SKR, AK, JRK and SM edited it and given valuable inputs. All the authors agreed and approved the final manuscript.

Author's Disclosures

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