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Assessment of oxidative stress index in patients with non-alcoholic fatty liver disease using spectroscopy and Immunoassay

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Abstract:-
In the process of oxidative stress, fats are oxidized by reactive oxygen species, replacing hydrogen atoms with molecular oxygen, and eventually the formation of low molecular weight oxidized products in the blood, such as Malondialdehydehyde (MDA), which is a non-invasive and vital indicator of the oxidative stress process.
Objective: Comparison of accuracy and sensitivity in the detection of very low concentrations of malon dialdehyde MDA using two analytical methods, the enzyme linked immunoassay (ELIZA) method, forming a yellow malondialdehyde derivative measured by a microplate reader, and the method of reaction with thiobarbityric acid (TBA), forming a pink malondialdehyde derivative measured spectrally, and then finding the relationship with non-alcoholic fatty liver disease.
Methods: - The study were applied to 90 patients suffering from fatty liver(a 44 males and 46 females). Based on the degree of steatosis, they were divided into three groups: (n=29) healthy, (n=29) mild steatosis and (n=32) moderate to severe D2 steatosis. Serum was collected after it's processing for detection malondialdehyde (MDA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, total bilirubin and vitamin "D". Body mass index (BMI) was calculated by the ratio of weight to height (in kilograms/square of length in meters).
Study results: The results of the comparison between the two analytical methods showed significant statistical differences ($p < 0.0001$), and according to the quantitative analyses of the blank solution, the detection limit was respectively (1.5 x 10-3 nmol/ml) (2.9 x 10-6 nmol/ml) and quantitative estimation (4.5 x 10-3 nmol/ml) (8.8 x 10-5 nmol/ml). Quantitative analyses showed different trends when patients compared to healthy controls. There was a high trend in the concentration of MDA ($p < 0.05$), ALT and AST, especially in males, but a low trend in the concentration of total bilirubin with increasing body mass index, especially in females , but the concentration of both vitamin D, ALP and albumin was not statistically significant ($p>0.05$).
The results of this study confirm the active role of AST, ALT, total bilirubin and BMI in the development of non-alcoholic fatty liver disease, while the enzyme ALP and albumin and vitamin D was not associated with non-alcoholic fatty liver disease. The results were in the spectroscopy method with a linear rate of 99.93%, although it is the least accurate and sensitive, but it is the most specific to detect MDA compared to the immunoassay method (ELIZA), thus highlights the Influential MDA relationship in non-alcoholic fatty liver disease.
Keywords: Enzymatic antioxidants, malonedidehyde, oxidative stress, fatty liver diseases.

1. INTRODUCTION:-

The incidence of non-alcoholic fatty liver disease (NAFLD) among adults increases in most countries of the world with the large number of cases of obesity, insulin resistance and a disorder in the metabolic process, and it is accompanied by damage to liver cells and fatty tissue outside the liver, which often leads to chronic diseases such as fibrosis and liver cancer, and the burdens of this disease in the future lead to the world being in front of major economic and health challenges [1]. Non-alcoholic fatty liver disease is prevalent in the elderly, especially males, and is usually accompanied by serious diseases such as heart, atherosclerosis, diabetes and polycystic ovaries in females, due to factors including genetics, genes and oxidative stress, and the latter is related to the accumulation of fat in the liver, and over time leads to the secretion of inflammatory causes and damage to liver cells. The diagnosis of the disease is usually late due to the suppressed clinical

characteristics of the disease [2] .Therefore, the best tool to predict the accumulation of liver fat in people with obesity is to use an ultrasound machine [3] , and examination of the abdominal area because obesity is associated with non-alcoholic fatty liver disease, and has a significant relationship with an increase in mortality in people with heart disease and atherosclerosis [4].There is another method of diagnosis, which is called the liver biopsy method, where it was found that more than 5% of the percentage of accumulated fat in patients with non-alcoholic fatty liver was diagnosed by adipose tissue, through which it was reached to accurately detect cases of non-alcoholic fatty hepatosis that develops over time to the stage of cirrhosis [5].There are several cases of the emergence of non-alcoholic fatty liver disease, as it begins gradually from simple liver fat to more severe liver fat ,and comes as a result of stimulating and releasing free fatty acids and it is associated with the secretion of inflammatory cytokines, disruption of the function of adipose tissue, mitochondrial weakness and weakness in the oxidative phosphorylation process, and for these reasons more serious symptoms of non-alcoholic fatty liver disease have appeared [6]. The free fatty acids are formed as a result of the breakdown of fats in foods and also present in adipose tissue, and it has been found in individuals with insulin resistance that it recreates free fatty acids, so it is said about new fats that accumulate and accumulate in the form of triglycerides in the liver. The mechanism in which hepatic fat accumulates is the production of large amounts of free radicals of reactive oxygen types leading to an imbalance in the balance of the antioxidant system called the stress process. oxidative reaction, and it seems that through its oxidative products, many living systems are damaged, including impaired normal liver function [7] , such as the enzyme alanine aminotransferase (ALT), which is an enzyme found in liver cells, works to transport the amino group of alanine acid to form glucose [8] , it is an indicator of liver health, as high levels usually indicate that liver cells are mainly damaged [9]. Liver enzymes ALT are concentrated in the cytoplasm and aspartate aminotransferase AST in the mitochondria [10] , high AST enzyme is an indication of mitochondrial damage in the liver and other organs outside the liver, including the muscles and heart, and may reach the kidneys and pancreas due to pathological disorders [11]. The process of oxidative stress is associated with type II diabetes, cardiovascular disease, metabolic syndrome [12] , polycystic ovaries in women [13] , hepatic cancer and kidney disease [14]. Under normal conditions, cells can protect themselves from oxidative stress through their enzymatic antioxidant system, but the production of reactive oxygen species (ROS) is a natural process that occurs in the mitochondria during respiration and although it occurs at low levels, increasing this process may precipitate the onset of many chronic conditions [15]. Unsaturated fats such as arachidonic acid are highly affected by oxidants, so they turn them into lipid peroxides (unsaturated fatty radicals) that decompose by complex processes into water and dialdehyde products such as: - Malondialdehyde MDA, which has very high toxicity and effectiveness properties with biological molecules ,thus the spread of oxidative damage. So MDA was used as a marker of oxidative stress due to it is produced in large quantities from the oxidation of unsaturated fats [16].The Malonedialdehyde MDA has physical properties such as solubility in water, methanol and ethanol, so MDA is found in biological samples naturally in both forms: - free but nonexistent in human plasma. As for its chemical properties, it is noted that it is subject to nucleophilic reactions with biological molecules, so it exists in the associated form, which is circulating and most commonly used as a sign of oxidative damage . MDA is produced experimentally and naturally , many studies have found that it can be produced in laboratory under strong acidic conditions, so it is usually detected by reaction with Thiobarbituric acid with a pH (pH< 7) [17]. As for its naturally production, it is inside the body of the organism, as a result of the oxidation of unsaturated fatty acids by the of action of various types of free oxygen radicals (•OH/•O2-) , where the free radical works by withdrawing hydrogen from the methylene molecule in the fatty acid RCOOH and then the molecular oxygen binding and the formation of fatty radicals called lipid peroxyl ROO• , which in turn draws another hydrogen from another lipid molecule, and so the reaction continues until the formation of stable compounds represented by lipid hydroperoxide (ROOH) is reached, and the fatty radicals are decomposed by complex processes and mda is derived from them [18]. The capacity of MDA to degrade biological molecules is attributable to a balanced system involving its production and degradation. There have been very limited studies on how MDA is degraded in the organism's body; a study was conducted on mice and found that MDA quickly decomposes

into CO2 within about 12 hours. Another study confirmed that the MDA derivative of acetaldehyde Malonyl Co-A is quickly degraded into CO2 in adipose tissue by the enzyme aldehyde dehydrogenase LDH, which together play in the fat production process [19]. MDA levels are usually detected by spectroscopic methods and Immunoassay [20] , In addition to many other methods, some of which may provide inaccurate sensitivity and selectivity for the determination of MDA, additional methods that are more sensitive and selective may be required in the future for its determination [21]. Due to the lack of studies that use the method of comparison between techniques in the detection of very low concentrations and high selectivity when detecting MDA this on the one hand, and on the other hand for the increasing importance in estimating Malon dialdehyde MDA in monitoring the severity of patients with non-alcoholic fatty liver associated with many chronic diseases. Therefore, this current study aimed to detect very low concentrations of Malondialdehyde MDA by comparing the analytical properties between the method of interaction with thiobarbituric acid (spectroscopy) and enzyme-linked immunoassay (ELIZA), and finding the relationship with non-alcoholic fatty liver disease.

2. EXPERIMENTAL METHODS: -

2 .1 Place of study -:

This pilot study was conducted in Kirkuk Governorate in Iraq, and approval was taken from the Department of Health in Kirkuk to facilitate the work in collecting blood samples from Kirkuk General Hospital, and the study began during November of 2024 and continued to February of 2025 .

2.2 Data Collection:

2.2.1 Persons included in the study: -

Patients with non-alcoholic fatty liver disease were included in addition to concomitant chronic diseases such as type II diabetes and blood pressure diseases because they are diseases associated with non-alcoholic fatty liver disease.

2.2.2 Persons excluded from the study:

This study excluded people with the following medical conditions: - viral liver disease, cirrhosis, alcoholic fatty liver patients, pregnant women, lactating women, cancer, autoimmune diseases, obesity, neurological diseases, chronic heart disease, anemia , because these patients show abnormal levels of oxidative stress indicators and do not represent the correct sample for non-alcoholic fatty liver disease.

3.2.2 Selection of participants:

Participants who were attending the hospital were selected for diagnosis and treatment. They were examined with an ultrasound of the abdominal area by the diagnostic doctors at the hospital, and accordingly the participants were interviewed directly and filled out the questionnaire form prepared forward, which included questions about their age, family history of the disease, clinical symptoms, diet pattern used daily and physical activity, and after completing the collection of demographic and clinical data and obtaining the consent of the participants, venous blood was drawn from the patients. The study included (n=90) males and females aged between thirty to seventy years and were divided based on their ultrasound examination into the healthy group, the group with light fat accumulation, and the group with fatty accumulation between moderate to severe.

3.2 Sample preparation and measurement procedure:

In the morning, after fasting at night for 9 hours, blood was drawn from the participants by pricking venous blood about 5 mL and transferred to centrifuge tubes containing a gel to accelerate blood clotting and to separate the serum, and after letting it clot for 20 minutes, it was expelled with a centrifuge for 10 minutes at a speed of 6000 cycles / minute. Keeping the serum in small tubes called Microtube with a size of 1.5 mL, It was frozen at a temperature of -20 ° C until the analysis procedures were completed. The specific parameters in this study were measured after purchasing the special materials from (Sunlong biotech, China) to analyze

MDA using the enzyme-linked immunosorbent assay (ELIZA) method from (Paramedical-PKL, Italy) and the reaction method with thiobarbituric acid from (Bijing Solarbio, China) and measured by using a spectrophotometer from (Shanghai Yoka Instrument, China). Also, the biomarkers of liver function and vitamin D were measured (from Roche Diagnostics, Germany) and analyzed using the Cobas 6000 device from (Hitachi High technologies Corporation, Japan).

4.2 Calculation of accuracy and sensitivity in analytical methods : -

Analytical methods aim to test accuracy and sensitivity to ensure reliable detection of target substance concentration at very low levels. In this study, the accuracy and sensitivity of two analytical methods were calculated based on measuring the signal of the blank solution without adding the target sample. Six samples were taken for the empty solution and the signal was read for each sample six times and on three different days, and the signal readings were taken at 450 nm in the ELIZA-linked immunoassay method , and in the spectroscopic analysis method at 532 nm. The detection limit was calculated by taking the mean and standard deviation of the blank sample readings and then multiplying the standard deviation (SD) by K = 3.3 to obtain the lowest signal measured by the device [23] , [22]

Lowest analytical signal = mean of the blank signal + K * SD of the blank samples.

Detection limits = Lowest analytical signal - mean of the blank signal

In the same way, the quantitative estimate was calculated, which is equal to ten times the standard deviation [24].The relative coefficient of variance (CV% < 2%) was found for the values of the signal of the blank solution . In Bearer-Lambert's law When drawing the relationship between absorption and concentration, we get the equation of the straight line, and the molar absorption coefficient in the length of the path passing through the sample represents the slope in the equation of the straight line, so the slope was found from the molar absorption coefficient given in the protocol [25]. In the method of immunoassay (ELIZA) the molar absorption coefficient was calculated from the equation: - (m = ε L) in L/mol and a path length of 0.6 cm, but in the spectroscopy method it was given in the protocol, from which the slope was calculated in L/mol.

5.2 Detection of MDA level in serum: -

The MDA level was detected using two common analytical methods which are :

1.5.2 Method of reaction with thiobarbituric acid (spectroscopy): -

In acidic conditions and high temperatures MDA condenses with thiobarbituric acid and the formation of a reddish-pink complex called 3,5,5-threemethylsulfametoxazole-2,4-twoketo [26]. In a sealed (Blank solution) test tube, added 300 µL of MDA working reagent with 100 µL of distilled water and 100 µL of Reagent III, while in the tubes for MDA samples in serum, 100 µL of the sample are added in each tube with 100 µL of Reagent III and 100 µL from the MDA working reagent . The mixture is incubated for both Blank and MDA samples in a water bath with a temperature of (90-100) degrees Celsius for a full hour, taking into account the tight closure of the pipes to prevent moisture loss. After incubation, a colloidal solution of almost reddish-pink color is obtained, cooled on ice and then centrily expelled at a speed of 10,000 rpm (from Thermo fisher scientific German) at room temperature to remove insoluble substances, then we take 200 µL of the upper liquid and It is added to the cuvette (measured in microliters), and the absorbance is measured at two wavelengths, 532 nm and 600 nm, then the absorbance reading of the blank solution is subtracted from the samples readings at both wavelengths. The concentration is then calculated using the equation: - **nmol/ml = 32.258 x ΔA**

2.5.2 Enzyme linked immunoassay (ELIZA) method:
Under almost mild acidic conditions, MDA in serum reacts with antibodies in wells made of polystyrene that includes 96 wells and then interacts with enzyme-linked reagents and as a result of the reaction gives a blue-colored complex called 3,3,5,5-tetramethylbenzidinediimin After adding the stop solution, it turns yellow [27]. To draw the standard curve, first five tubes are numbered to prepare a series of standard dilute solutions for MDA by adding a standard concentrated solution of MDA at a concentration of 540 ng/ml with the standard diluent solution. added 150 µL of the standard dilute solution to each of the five tubes and then add 300 µL of the concentrated standard solution to tube (1) only. After blending 300 µL is taken from this mixture and added to tube No. (2). After mixing, 150 µL of this mixture is taken and added to tube No. (3). After mixing, 150 µL of this mixture is taken and added to tube No. (4). After mixing, 150 µL of this mixture is taken and added to tube No. (5). By applying the law of dilution of concentrations, we obtain the standard dilute concentrations of MDA (360, 240, 120, 60, 30) ng/ml. These concentrations are placed in wells that includes 96 wells, then add 40 µL of the sample diluent to each well and 10 µL of the sample and close with a transparent adhesive, and then incubate the mixture at a temperature of 37 ° C for half an hour, then wash the wells with washing solution for 5 minutes (to remove non-bound materials). Added 50 µL of HRP-Conjugate detector to every well except Blank well and brood for half an hour at 37°C. Added 50 µL of chromogen A and 50 µL of chromogen B to each well, the solution is colored bright blue and then the mixture is incubated again for 15 minutes at 37 ° C. 50 µL of stop solution was added and the blue color turned to yellow and then absorption is measured by the plates reader at a wavelength of 450 nm.

6.2 Measurement of diagnostic indicators: -
The diagnostic indicators were calculated using an analytical method based on the principle of Electrochemiluminescence, which includes the interaction of the target material with photoluminescence and electrically stimulated particles, which does not require a light source, which prevents interference and improves the detection process, in addition to that it is considered one of the fast, simple and selective methods accurate, at low cost and with high quality. This phenomenon is known as the emission of light visually as a result of the excitement of electrons and their return to their stable electronic state [28]. The analytical performance power of the COBAC 6000 is based on the principle of Electrochemiluminescence and combines three analytical systems including clinical chemistry, immunohistochemistry, electrode and ion selectivity. Therefore, it was used in this study to evaluate the level of routine chemistry tests such as liver enzymes and immunological tests [29].

7.2 Statistical Analysis:
Through the tools and programs used, the data was simplified in a way that allows seeing the general direction of the data, using the Excel 2024 program for calculations such as percentages and column graphs to quickly detect the natural and unnaturaltrends of the studied variables. Using One-Way ANOVA Analysis by Minitab (17) program to compare the data presented as the arithmetic mean and standard deviation of three groups participating in the study, with the use of the Dunkin' test that determines how different the groups are with each other. The t-test was used to compare the mean and standard deviation in each analytical method and consider statistical significance important at the level of ($p < 0.05$). Use the Spearman relationship to examine the associations between oxidative stress index, liver function and vitamin D and their relationship to nonalcoholic fatty liver disease.

3. Review data: -
The results of this research are derived from the master's thesis entitled "Evaluation of the level of Malon dialdehyde and some enzymatic antioxidants in the serum of patients with non-alcoholic fatty liver disease". Table (1) shows the percentage in each group of both males and females participating in this study, which shows in detail the percentage of healthy people and people with mild and moderate to severe liver fatty accumulation.

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Table (1) :- Shows the percentage of male and female partici- pation in the patient and healthy groups.

Groups	N.o. Males	%	N.o. Femals	%
Con	13	44	16	55
D1	17	58	12	41
D2	14	43	18	56
Total	44	% 49	46	% 51

*Con represents the healthy group, D1 represents the group of patients with mild fatty liver, and D2 represents patients with moderate to severe fatty liver.

Table (2) shows the ratio of males to females in each group, and the percentages show that most patients have high blood pressure compared to type II diabetes. The percentages also show that healthy people do not suffer from any chronic diseases, but the D1 group who suffer from mild fatty accumulation and the D2 group who suffer from moderate to severe fatty accumulation, suffer from cases of high blood pressure in almost the same proportion but more compared to cases of type 2 diabetes.

Table (2): Number of participants with chronic diseases associated with non-alcoholic fatty liver disease.

Chronic diseases associated with NAFLD disease	Con		D1		D2	
	Males 13	Females16	Males 17	Females 12	Males 14	Females 18
Type II diabetes	0:0		0:1		1:2	
Hypertension	0:0		3:6		3:7	
Type II diabetes with hypertension	0:0		1:1		4:5	

***Con healthy group, D1 group of patients with mild fatty deposition, D2 group of patients with moderate to severe fatty deposition.**

Table (3) shows the comparison of accuracy and sensitivity in analytical methods based on the blank solution . It was found that the detection limit, quantitative estimation and coefficient of variation between CV% values by the enzyme-linked immunoassay method (ELIZA) were more accurate and sensitive compared to the method of interaction with TBA acid and the difference between the two methods was statistically significant ($p < 0.0001$). The coefficient of determination R2 Very high, indicating a high compatibility between the measurements in both methods is what enhances the reliability of the results

Table (3): Summary of the results of the analysis (accuracy and sensitivity characteristics) using the blank solution in the two analytical methods.

	Analytical criteria	A- Spectroscopy	B- ELIZA
1	Iterativity (n=18) , CV < 2%	1.3	0.9
2	Mean	0.033	0.068
3	Standard deviation (SD)	4.47×10^{-4}	6.32×10^{-4}
4	ΔMean (B-A) ± SEM	0.03483 ± 0.0002687	
5	Probability value (p-value)	< 0.0001	
6	Coefficient of determination (R^2)	0.99	
7	inclination L/mol	1.55×10^5	4.32×10^5
8	Molar absorption coefficient (ε) L/mol.cm	1.55×10^5	7.2×10^5
9	Limit of Detection (L.O.D)	(0.11 ng/ml) (1.5×10^{-3} nmol/ml)	(2.1×10^{-4} ng/ml) (2.9×10^{-6} nmol/ml)

Table (4): Measurement of clinical indicators with the Cobas 6000 device.		
Clinical indications	Range of values	Natural limits
ALT (U/L)	3.7 - 65.9	Males ≤ 41 & Females ≤ 33
AST (U/L)	10.9 - 55.6	Males ≤ 40 & Females ≤ 32
ALP (U/L)	43 - 267	Males (40 – 129) Females (35 – 104)
Albumin (ng / dL)	3.2 - 4.9	3.5 - 5.2
Bilirubin (mg / dL)	0.09 - 1.26	Males ≤ 1.4 & Females ≤ 0.9
Vitamin D (ng / mL)	4.52 - 90.9	30 - 75
BMI (kg / m^2)	20.4 – 50.7	18.5 - 24.9
*Alanine aminotransferase (ALT) , aspartate aminotransferase (AST) , alkaline phosphate (ALP) and body mass index (BMI).		

10	Limit of Quantification (L.O.Q)	(0.32 ng/ml) (4.5 x 10^{-3} nmol/ml)	(6.32 x 10^{-3} ng/ml) (8.8 x 10^{-5} nmol/ml)
* ΔMean:- Difference between averages , SEM -: Mean standard error , CV < 2% -: The coefficient of variation is less than 2% ELIZA :- Enzyme-linked immunoassay .			

Table (4) shows the natural limits of routine measurements (dependent variables) as found in the protocol, which are usually considered as a reference for comparing the natural and pathological state to see the extent to which they are affected by the independent variable MDA .

Three sets of duplicate data were matched based on age and table (5) shows the analysis of liver enzymes, vitamin D, oxidative stress index and body mass as averages and standard deviations within the level of statistical significance (p< 0.05).

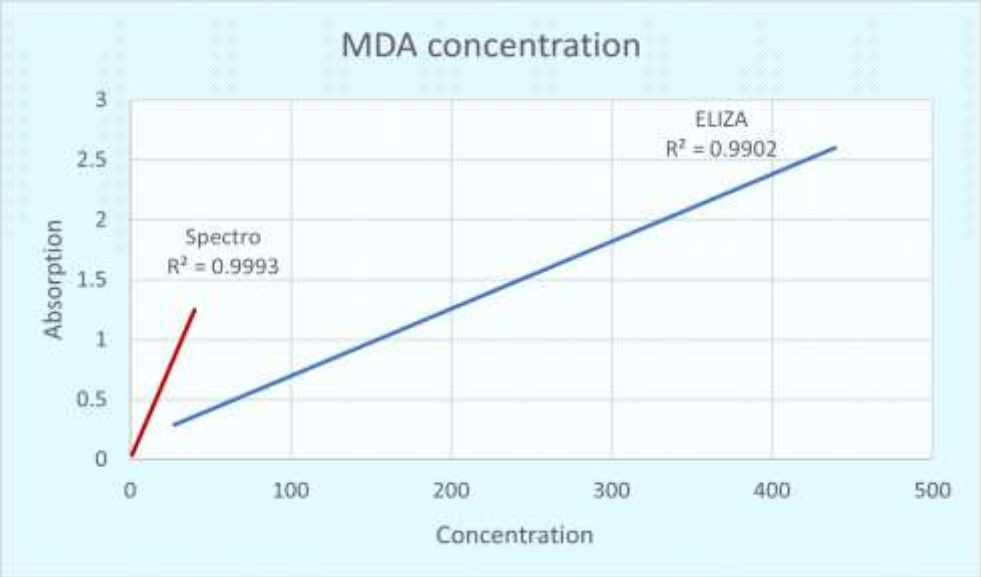
Figure No. (5) :- Age-specific analysis of variables in each group .							
Variables	Con=29		D1		D2		p-value
	30-50=18	51-70=11	30-50=16	51-70=13	30-50=20	51-70=12	P < 0.05
ALT	11.406 ± 3.107 c	11.400 ± 2.032 c	17.630 ± 3.600 b	11.820 ± 3.870 c	24.650 ± 4.120 a	12.830 ± 2.140 c	0.0003***
AST	16.894 ± 2.597 c	16.680 ± 3.450 c	21.310 ± 4.120 b	15.985 ± 1.377 c	25.010 ± 5.110 a	16.440 ± 4.270 c	0.0002***
ALP	82.09 ± 18.77 a	94.85 ± 17.20 a	93.34 ± 17.61 a	95.60 ± 17.20 a	94.50 ± 17.00 a	84.50 ± 14.28 a	0.609
Albumin	4.2220 ± 0.4290 a	4.1818 ± 0.2639 a	4.3750 ± 0.2236 a	4.1385 ± 0.2873 a	4.2950 ± 0.2502 a	4.1083 ± 0.2314 a	0.149
Bilirubin	0.3567 ± 0.1324 bc	0.6650 ± 0.3350 a	0.4331 ± 0.2092 b	0.5038 ± 0.2126 ab	0.3625 ± 0.1426 bc	0.2692 ± 0.1030 c	0.0003***

Vitamen D	18.32 ± 1.69 a	19.67 ± 3.64 a	17.22 ± 1.61 a	17.52 ± 1.38 a	19.29 ± 3.56 a	17.11 ± 2.06 a	0.779
MDA ELIZA	59.47 ± 9.85 bc	56.50 ± 10.94 c	64.92 ± 8.95 a	58.98 ± 16.75 bc	66.18 ± 9.89 a	61.68 ± 8.94 ab	0.042*
MDA Spectrophotometer	15.69 ± 3.40 c	16.88 ± 4.00 bc	16.15 ± 3.83 bc	19.08 ± 3.96 a	18.50 ± 5.55 ab	14.43 ± 2.96 c	0.029*
BMI	26.35 ± 4.16 d	30.52 ± 4.97 c	30.30 ± 4.40 c	31.07 ± 4.45 bc	31.77 ± 5.20 bc	33.89 ± 8.25 a	0.006**

*The letters c, b, and a indicate the presence/absence of differences between groups, with the letter a taking the highest value. The compound letters ab and bc indicate no differences between groups. The data above represent the mean ± standard deviation at the statistical significance level of $p < 0.05$.

1.3 Measurement of Malondialdehyde (MDA) in (nmol/ml): -

Graph (1) shows results (n=90) of the detection of MDA by analytical methods, , and absorption versus concentration of unknown samples it was linear 99.93% in the method of interaction with theobarbituric acid (spectroscopy) and by 99.02% by Enzyme linked immunoassay (ELIZA) method .



Graph 1: Shows the linear ratio when detecting malondialdehyde (MDA) using the Spectral analysis method and the ELIZA immunoassay.

The results of the t-test for ninety samples as a comparison of the change between the averages in the two methods showed that it had a significant difference and a high statistical significance ($p < 0.05$) and a very small relative error, which indicates that the difference is clear between the two methods, and the compatibility of the data to a large degree (R^2 = 99%), as shown in Table (5):

Table (6) :- Summary of the results of MDA analysis in the two analytical methods.

	Analytical criteria	A – Spectroscopy	B – Immunoassay ELIZA
1	Mean , n=90	16.19	61.49
2	ΔMean ± SEM	45.30 ± 1.285	

3	p-value	<0.0001	
4	Practical Linear Ratio % (R^2) - (n=90)	99.93	99.02
5	Practical linear range	29.9 - 1.29 nmol/ml	97.7 - 50 ng/ml
6	Model size (µL)	100	10
7	Model/Hour	3 / 90	2 / 90
8	Reaction Temperature (°C)	95	37
9	pH of the reagents	3.6	7.4
10	Maximum Wavelength (λ_{max})	532 nm	450 nm
11	Light source	التنكستن	التنكستن
ΔMean :- Difference in absorption , SEM :- Standard error of the mean .			

In Table (5), the analysis according to age for three groups (healthy and two patient groups) showed a probability value (p< 0.05) in the interaction with Thiobarbituric acid less than in the (ELIZA) method, so the results were relied on according to the analysis by interaction with Thiobarbituric acid. MDA concentration is independently associated with non-alcoholic fatty liver disease. MDA concentration was increased differently among males and females in patients compared to healthy subjects, however when comparing patients it was higher in males compared to females by 4%. To find out that an increase in MDA concentration affects any of the dependent variables,the correlations showed statistical significance (p < 0.05) a strong and positive between the concentration of MDA and alt (p = 0.55) and with the concentration of AST (p = 0.56).

2.3 Measurement of the enzyme alanine aminotransferase ALT (U/L): -

In Table (5) when analyzing the data according to age, the results were acceptable and statistically significant at the level of (p< 0.05), which indicates a very strong association between ALT concentration and non-alcoholic liver fatty accumulation. The concentration of ALT enzyme increased in patients in both sexes compared to healthy people, however when comparing patients it reached higher concentrations in males compared to females by 18%. To determine the extent to which ALT concentration is affected by the independent variant MDA Other dependent variables showed associations in patients of very strong statistical and significant significance (p< 0.05) between the concentration of ALT and AST (r=0.72) and with MDA (r = 0.55) and with ALP (r = 0.37) and in an inverse relationship with albumin (r = 0.44).

3.3 Measurement of the enzyme aspartate aminotransferase (AST) (U/L):

In Table (5) analysis by age, the results were acceptable and statistically significant at the level of (p< 0.05), which indicates a very strong association between AST concentration and non-alcoholic liver fatty accumulation. AST concentration was higher in patients of both sexes compared to healthy people, however when comparing patients it was 12% higher in males compared to females. To determine the extent to which AST concentration is affected by the independent variable MDA The associations in patients showed statistical significance (p< 0.05) and very strong positivity between the concentration of AST and ALT enzymes (r = 0.72) in addition to the association with MDA (r = 0.56) and ALP (r = 0.55).

4.3 Measurement of alkaline phosphate enzyme ALP (U/L): -

In Table (5) analysis by age the results were unacceptable and were not statistically significant (p > 0.05). This indicates that ALP concentration is not associated with non-alcoholic liver fatty accumulation. ALP concentration was higher in patients of both sexes compared to healthy people, however, when comparing patients in males and females, the difference between them was equal. To see how the ALP concentration is

affected by the independent variable MDA Dependent variables showed associations in healthy people that there was strong statistical and positive significance ($p< 0.05$) with total bilirubin ($r = 0.37$), but in patients they were strong negative with albumin ($r = -0.50$).

5.3 Measurement of albumin (ng/d L): -

In Table (7) analysis by age, the results were unacceptable and were not statistically significant ($p> 0.05$). This indicates that albumin concentration is not independently associated with non-alcoholic hepatic fatty accumulation. The concentration of albumin was higher in patients compared to healthy people, however when comparing patients it was 2% higher in males than in females. To find out the extent to which albumin is affected by the independent variable MDA and the dependent variables, the correlations showed statistical significance ($p < 0.05$) Strong positive with ALT ($r=0.44$) and negative with ALP ($r=-0.50$).

6.3 Measurement of total bilirubin (mg/dL):

In Table (5) analysis by age the results were acceptable and statistically significant ($p < 0.05$). This indicates that bilirubin concentration is very significantly independently associated with the accumulation of non-alcoholic hepatic lipids. Total bilirubin concentration was lower in patients of both sexes compared to healthy people, however, when comparing patients, females had 12% lower concentration compared to males. To determine the extent to which total bilirubin is affected by the independent variant MDA The correlations showed statistical significance at the level of significance ($p< 0.05$) and strong negative between the concentration of total bilirubin with the enzyme ALP ($r =- 0.37$).

7.3 Measurement of vitamin-D (ng/mL): -

In Table (5) analysis by age, the results were unacceptable and were not statistically significant ($p> 0.05$). This indicates that vitamin D concentrations are not independently associated with nonalcoholic fatty liver disease. The majority of healthy and sick participants had a lower than normal vitamin D concentration. However, when comparing patients in males and females, the difference between them was in equal proportions. To find out how vitamin D is affected by the independent variant MDA Other dependent variables showed no statistical significance ($p>0.05$) between vitamin D concentration and measured variables.

8.3 Calculation of body mass-BMI (Kg/m2):

In Table (5) the results according to the statistical significance ($p < 0.05$) showed that BMI is independently associated with non-alcoholic fatty liver disease. Body mass was higher in patients of both sexes compared to healthy people, especially in women with the highest mean was in group D2. Correlations showed no statistical significance ($p>0.05$) between body mass and measured variables. The percentage of body mass within the normal limits of the participants was unanimously about 12% and within the abnormal limits about 87 % . However, the highest percentage of increase in body mass and obesity was found in the D2 group, especially in 17 females aged 30-70 years and 14 males aged 30-50 years[30] .

Table (7) :- Comparison of BMI ratios in each group by category greater than or less than 30.

Groups	Con		D1		D2	
	Males 13	Females 16	Males 17	Females 12	Males 14	Females 18
Average age (range)						
	51(31-66)	43(30-60)	45(30-60)	50(33-66)	42(30-67)	51(30-70)
BMI kg/m^2	Normal body mass					

18.5 - 24.9	2	4	3	1	0	**1**
Increase in body mass						
25 - 29.9	7	6	5	2	4	**9**
≥ 30	4	6	9	9	10	**8**
Total percentage % when 25 < BMI < 51						
%	(11) 84	(12) 70	(14) 82	(11) 91	(14) 100	**(17) 94**
***BMI :- Body mass index , Con :- Healthy group , D1:- Patients with mild hepatic steatosis, D2:- Patients with moderate to severe fatty liver.**						

4. DISCUSSION OF THE RESULTS: -

1.4 Interpretation of analytical results:

In this experimental study, the measurements were in different units in the two analytical methods, so the detection limit and quantitative estimation were used in two units (ng/mL), (nmol/mL) and on the other hand to give a clear vision when comparing them. The results of the detection comparison showed differences and a large statistical significance indicating the difference in analytical performance between the two analytical methods, where the results of the comparison by empty solution by reaction with thiobarbituric acid showed that it is less accurate and sensitive in detecting very low concentrations and with a very small quantitative estimate , and with a variation coefficient CV% is less than (2% < 1.3) compared to the method of immunoassay (ELIZA), due to the difference in reaction conditions (temperature and pH) and also to the quality of chemical materials and reagents used in both methods. The characteristics of the method of interaction with thiobarbituric acid were characterized by very simple methods, easy to analyze, with accurate selectivity and acceptable sensitivity, but need more model size and longer time to make measurements, compared to the enzyme-linked immunoassay method (ELIZA), which is characterized by simplicity, ease and speed in analysis, but inaccurate selectivity, sensitivity, high accuracy and less model size. When comparing the results of MDA detection, the two methods were statistically significant ($p< 0.05$), but interestingly, the statistical significance ($p=0.029$) in the method of interaction with thiobarbituric acid was stronger than the ELIZA immunoassay method ($p = 0.042$) and this can be explained by the method of interaction with thiobarbituric acid is of precise selectivity and is due to many reasons, including, including an effective reaction (chemical force) between MDA With thiobarbituric acid as a result of condensation of two molecules of acid with a molecule of MDA and the formation of a reddish-pink complex whose absorption is measured at two wavelengths in order to correct the color interactions of other compounds and give steady and stable products that reflect the concentration of MDA in the sample [31]. Since this reaction requires a high temperature (90-100 °C), this prevents interference with other compounds such as proteins and nucleic acids that denature at high temperatures [32] , previous studies have shown the effectiveness of MDA increases as the acidity of the medium increases, and since thiobarbituric acid has a high pH (pH=3.6), this increases the direct reaction [33]. As for the method of immunoassay associated with the enzyme ELIZA, there are many reasons for the lack of selectivity due to multiple interactions, first the interaction of MDA with primary antibodies, second the interaction with secondary antibodies associated with an enzyme, third the interaction with the reagents of the enzyme to give a complex with high color intensity, then its absorption is measured at a certain wavelength that reflects the MDA concentration in the sample . It can be explained that the antibodies may be of inaccurate selectivity that give less accurate results, and the reaction needs a normal temperature of 37 ° C as proteins and nucleic acids are in their normal state and this leads to interference. The (pH = 7.4) of the reagents used reduces the effectiveness of MDA activity as mentioned earlier, which reduces its selective reaction, or the signals may be inaccurate due to the method of washing wells may include residues of unrelated substances or undesirable reactions. In other cases, the MDA concentration level may be very low in the samples and cannot be detected by the ELIZA method .

2.4 Interpretation of biochemical results: -

Many studies are still looking at the extent of the effect of fat on the liver, and the main reason is eating foods rich in fats that cause liver function dysfunction and damage them by a process called oxidative stress, which in turn is associated with many chronic diseases such as heart, atherosclerosis, type II diabetes and obesity [34]. Many studies have shown that the oxidative stress process is the main factor for non-alcoholic fatty liver disease and the catalyst for the cytokines causing lipid peroxidation and the production of MDA as a final product, and that its higher levels in serum than antioxidants indicate the extent of disease progression. In the course of our results, it was found that the concentration of MDA was associated with non-alcoholic fatty liver disease and was consistent with previous studies [35] . In addition to the positive association between high MDA concentration and high concentration of enzymes ALT and AST for both sexes, but especially in males. It may be due to the sex hormones estrogen in women protect them from lipid peroxidation, it has been found that mice removed ovaries are more susceptible to lipid peroxidation [36]. Another study found that men produce more superoxide radical in mitochondria with lower levels of enzymatic antioxidants than women, leading to more oxidative damage [37] . It may affect liver cell damage and there is a sign to examine this damage mainly which is the enzyme ALT, our results showed that the relationship is very positive and strong between high ALT concentration and non-alcoholic fatty liver disease, as it tended to be slightly higher than normal levels in patients compared to healthy people, this can be explained that some of them may suffer from fatty accumulation in the early stages of non-alcoholic fatty liver disease without The occurrence of major infections, which may be related to a metabolic imbalance, as stated in a previous study[38] , In addition, high blood pressure and type II diabetes raise blood sugar levels, turning into accumulated fat in hepatocytes and leading to a slight rise in the level of ALT, while in the exceptional case signs of non-alcoholic steatohepatitis may appear. As a study by Gohel et al [39] . In the current study, the healthy people were eating foods low in fat and sugars and less than 4 meals a day, but few of them were enjoying a normal weight, although the majority did not have their weight within the normal range, due to the fact that this increase is not due to fat, but rather an increase in muscle mass of the body, as stated in the study of Gangopadhyay when he injected mice with a substance that helped increase muscle fibers in the absence of fat, which led to an increase in body mass and found that the inverse association between the increase Muscle mass and accumulation of body fat [40] However, patients in this study were overweight (above 25 kg/m2) at a higher rate than healthy people, as the dietary pattern was high in fat and sugars and ate more than 4 meals a day, which led to an increase in their body mass. A recent study concluded that diet control has a greater impact than exercise in weight loss, as this helps early intervention to prevent non-alcoholic fatty liver disease and reduce its complications [41] . There have been recent reports that fatty liver accumulation and weight gain increase with age, especially in the elderly [42] , as a result of the decrease in muscle mass in the body due to the loss of a number of muscle fibers and capillaries associated with muscles due to the replacement of stem cells with fat cells and not muscle cells, the body loses the ability to practice physical activities, so the process of burning high calories decreases [43] , another study confirmed a close relationship between muscle loss, non-alcoholic fatty liver disease, cardiovascular disease and type II diabetes [44] , another study reported that weight gain in women with age is due to menopause as a result of a decrease in the level of estrogen due to the accumulation of fat in the abdominal tissue [45].The current study also showed that the relationship is strong between increased body mass (obesity) and non-alcoholic fatty liver disease, as it is one of the main factors for the severity of the disease, where when fats and sugars are broken down in the body, they produce free fatty acids that play in contributing to the production of triglycerides in adipose tissue/liver cells. In this case, hepatocytes and fat cells are activated and damaged, and it leads to an increase in the secretion of inflammatory cytokines by phagocytes in the liver, thus enhancing insulin resistance, which disrupts the process of decomposition and formation of fat in fat cells [46]. The results of the current study that linked body mass and ALT to NAFLD disease are consistent with the Klisic study [47] , through our results, there was a strong association between the enzymes ALT, AST and ALP, which indicates a problem in liver health, although AST is present in the liver, muscles, heart and brain, but when associated with the enzyme ALT,

the injury can be in cells and mitochondria in the liver mainly, as stated in the Mohammadi study [48].The results of the current study showed that high AST concentration is largely associated with non-alcoholic fatty liver disease, as it came in agreement with a study conducted in 2022, which found a relationship between an increase in the level of liver enzymes and the level of lipids in the blood in diabetics and high blood pressure as a result of the increase in the level of blood lipids led to an imbalance in normal liver function [49] , and when comparing the increased concentration of enzymes ALT and AST in patients, we find that it is higher in men than in females, and many studies have proven that the reason behind this difference is due to a difference in the chemical composition of the plasma of liver enzymes and also to the difference in the metabolic rate in both sexes, as the male hormone androgen contributes to the rise of the enzymes ALT, AST, The female hormone estrogen contributes to a decrease in the enzymes ALT, AST, which plays a protective role in female[10] .. The current study showed no association between the enzyme alkaline phosphate ALP and non-alcoholic fatty liver disease, as it came in agreement with a 2022 study [50] . The results of another study confirmed that the enzyme ALP is produced from adipose tissue cells and liver cells [51] , it is located in many vital organs such as the pancreas and in the bile duct in the liver [52] in the bones, intestines, but the rise in the concentration of ALP in the blood may be due to type II diabetes, blood lipid disorder, high blood pressure or cardiovascular disease [53]. A recent study in 2020 indicated that the high concentration of ALP in the blood is due to cholestasis and yellow fluid retention in the bile duct in the majority of patients with biliary cellular liver disease [54]. The results of the current study showed that albumin is not associated with non-alcoholic fatty liver disease independently, but in a 2022 study, the concentration of albumin in serum showed a large statistical score ($p<0.05$) in patients with non-alcoholic fatty liver [55],
but when analyzing the associations, the relationship between albumin and the ALT enzyme and the ALP enzyme is a strong negative relationship in patients with light fatty accumulation, and many studies have reported that albumin is produced by hepatocytes, and is released into the bloodstream at low levels in the event of damage to its constituent cells, which affects its normal vital functions such as maintaining body fluids and transporting many compounds, including fatty acids [56] , bile acids, total bilirubin, antioxidant and anti-inflammatory drugs [57]. Our results also showed that the level of yellow total bilirubin in women is lower than in male [58] , and that its level decreases as the accumulated fat in the liver increases, as its levels were higher in the healthy group, as indicated by a recent study in 2021 that this is related to body weight within the normal range. In this study, although total bilirubin concentration is not correlated with body mass, the decrease in bilirubin concentration appears in those with impaired liver enzyme function and metabolic disorder, and both factors appear in obese patients, non-alcoholic fatty liver patients, type II diabetics, and cardiovascular patients [59]. Previous studies have indicated that in cases of obesity caused by oxidative stress, adipose tissue is stimulated to increase the secretion of inflammatory cytokines that have an inverse association with bilirubin level [60], as we mentioned earlier, the secretion of inflammatory cytokines from hepatocytes leads to an increase in the secretion of enzymes ALP, AST, ALT , where our results also proved that the relationship is inverse between the concentration of the enzyme ALP and total bilirubin. In this case, the secretion of the enzyme clocornil (responsible for the metabolism of bilirubin) increases in the liver and the likelihood of its association with bilirubin decreases, which disrupts the process of filtering it from the blood, and the results were confirmed by a study that found that the relationship between them was inverse. In addition bilirubin is associated with the receptor of fat burning, when the activity of the enzyme generating bilirubin is weakened, its production decreases, which stimulates the process of oxidative stress and thus increases fat in the liver and adipose tissue [61] , the results of the current study were consistent with a study conducted in 2025 [62] . The results of the current study showed no relationship between vitamin D deficiency and non-alcoholic fatty liver disease and were consistent with a previous study [63] , and When observing the concentrations of vitamin "D" in patients and healthy people was somewhat similar in that it is less than normal limits, when interviewing the participants in this study, most of them suffered from bone pain and fatigue without exerting any muscular effort, and this may be due to vitamin D deficiency because it is associated with the health and safety of bones, heart and blood vessels and there are many reasons for vitamin D deficiency It seems that because of the lack of exposure to sunlight, which is the main source

of vitamin D formation, or lack of Eating foods that provide vitamin D, but in small quantities, such as fish or weak vitamin D receptors in the cells of body tissues, and also to a weakening of the immune system in chronic diseases such as the heart and blood vessels. However, its deficiency may be significantly affected in those who suffer from body mass outside the normal limits (obesity), as it is found in the form of stock in adipose tissue [64]. The obesity associated with metabolic syndrome usually have a disorder in the files of fat and sugar in the blood, where a study found that the decrease in the level of vitamin "D" is due to the appearance of signs of metabolic syndrome, especially in young people who suffer from overweight, at this stage disturbed their function of fatty tissue as a result of filling them with fat droplets weaken vitamin "D" receptors or perhaps due to the property enjoyed by vitamin "D" in its solubility in fat and its spread in Fat, liver and muscle tissue, Which reduces its presence in blood serum [65] . Vitamin D concentrations are decreasing, especially in the elderly, because at this stage they have impaired skin cell regeneration, which affects the skin's response to vitamin D even after exposure to sunlight. As it appears Vitamin D deficiency is also significantly seen in patients with type II diabetes [66] , due to the weakness beta cells in pancreatic responsible for reception vitamin D in the event of oxidative stress, which in turn secrete insulin to regulate blood sugar levels, so the increased stress of the endoplasmic reticulum leads to the inability to tolerate excessive amounts of glucose and the emergence of insulin resistance, which is the beginning of type II diabetes [67].

5. Conclusion and future directions :-

Analytical methods in interaction with thiobarbituric acid were less accurate and sensitive compared to the Eliza immunoassay method in detecting MDA, but they are more selective and the best standard method in this study . Therefore, it is recommended to make such comparisons between analytical methods to confirm and repeat the method or compare it with other analytical methods in different fields to obtain the most accurate results, in addition to improving the protocol used to conduct tests with an increase in the amount of reagents and chemicals for the possibility of repeating the experiment more than once. This study also found that high levels of MDA and liver enzymes AST , ALT, total bilirubin and body mass increase the incidence of NAFLD disease, but the enzyme ALP, albumin and vitamin " D " did not show any relationship with NAFLD disease In the future, it may be necessary to study indicators indicative of the symptoms of this disease associated with fatigue and body aches, or study indicators associated with vitamin D deficiency in NAFLD patients.

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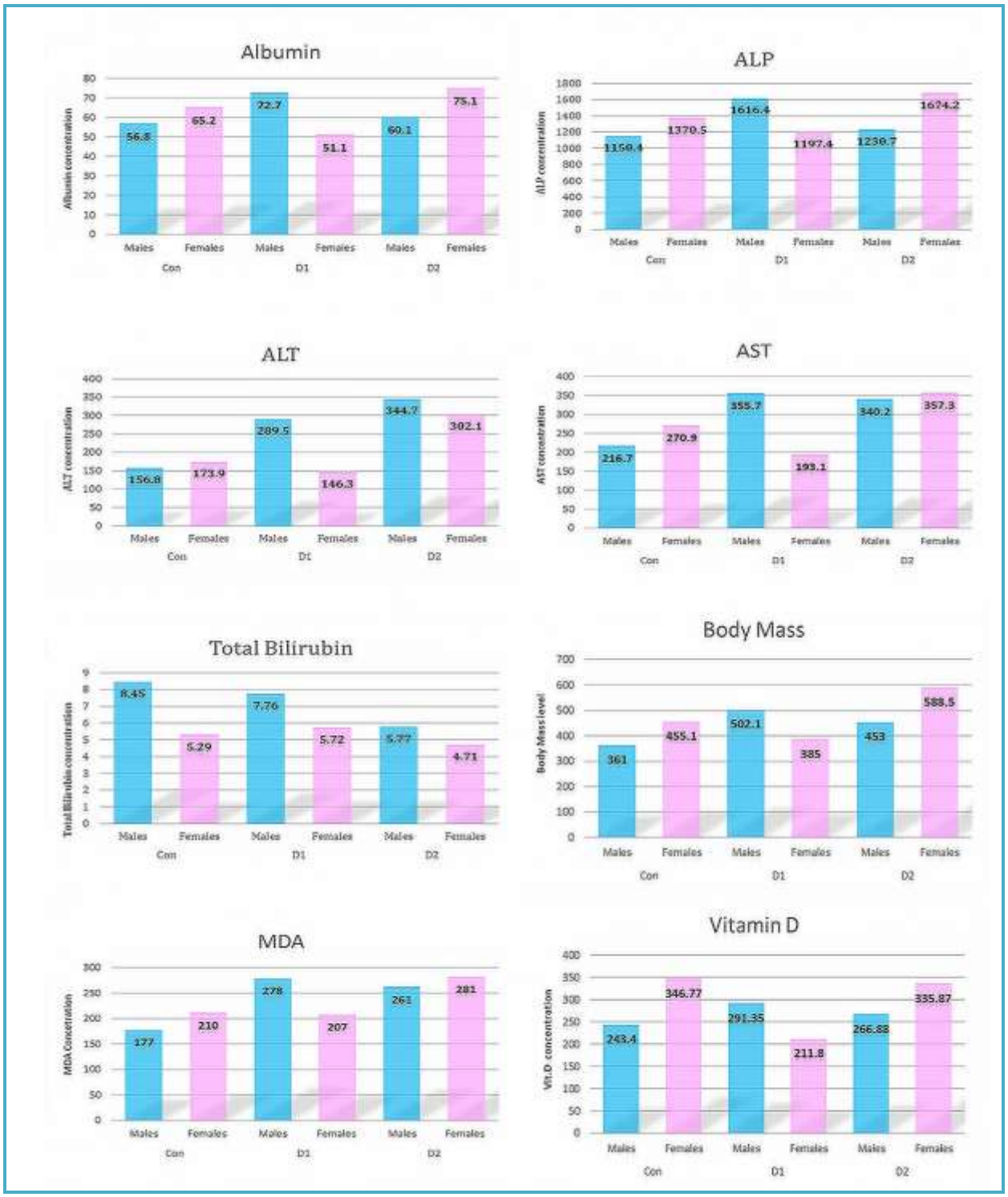

Chart (2):- Shows a summary of the comparison of data for males and females in the healthy group Con, the group of patients with mild fatty accumulation D1, and the group of patients with moderate to severe fatty accumulation D2. The data includes the concentration levels of malondialdehyde MDA, alanine aminotransferase ALT, aspartate aminotransferase AST, alkaline phosphate ALP, albumin, total bilirubin, vitamin D, and body mass index BMI.