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Association between CD3 as a Sensitive T Cells Lineage Marker and NAFLD

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Abstract

CD3 antigen represents the most specific as well the most sensitive T cells lineage marker, including NK and NKT. Aim and methods: our study was to evaluate CD-3 in nonalcoholic steatohepatitis patients as a diagnostic and prognostic marker in such patients. Serum samples were collected from 41 NAFLD patients, in addition to 14 healthy subjects. They were recruited from the internal medicine department - Cairo University. All patients and controls were subjected to clinical assessment, abdominal ultrasound examination, laboratory assessment including liver function and enzymes, kidney function, and lipid profile. Fib-4 and NAFLD fibrosis score were calculated for all patients. Also, serum levels of CD-3 were detected by ELISA technique. Results: The NAFLD patients group was divided into three groups: fifteen obese patients, fourteen diabetic patients, twelve obese patients with T2DM. BMI, HbA1C, and cholesterol levels were significantly elevated in the NAFLD cases compared with healthy controls ($p<0.05$). The mean serum level of CD3 was 64.53±84 ng/mlin NAFLD cases vs. 4.76±2.94 ng/ml in controls. Overall, there was a statistically significant increase in serum CD3 levels in NAFLD cases ($p<0.001$). Additionally, serum level of CD3 was significantly higher in the diabetic non-obese subjects and obese non-diabetic subjects when compared with the lean subjects (89.4±113.53ng/ml and 60.91±73.76 vs. 4.76±2.94 ng/ml, P = 0. 003& 0.05; respectively. Conclusion: Serum level of CD3 can be used as a prognostic marker in cases with NAFLD as they increase with the progression of the disease and is correlated with fibrosis stage.

Keywords: NAFLD - CD3 – NAFLD fibrosis score –Fib-4.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a widely prevalent hepatic metabolic disorder affecting 24% of the adult population globally [1]. Nonalcoholic fatty liver disease (NAFLD) ranges from simple steatosis, defined by fat accumulation exceeding 5% of liver weight in the absence of alcohol abuse, to the progressive form namely nonalcoholic steatohepatitis (NASH), which is characterized by lobular inflammation and hepatocellular ballooning, and to hepatic fibrosis [2]. NASH may progress to cirrhosis and, in a small percentage of patients, to hepatocellular carcinoma (HCC) [3], and increased liver-related and cardiovascular mortality [4]. Therefore, identifying patients with NASH is a key clinical issue. Precise histological diagnosis, including disease stages (SS and NASH), is commonly based on liver biopsy. Nevertheless, because this method imposes certain limitations, including potential complications such as bleeding and some pain, and needs to be performed in a special setting, noninvasive approaches now more acceptable and have gained considerable attention [5]. Therefore, new noninvasive and simple,

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serologic based tests are required to detect hepatic inflammation, and distinguish simple steatosis seen in NAFLD from NASH non-invasively. CD3 antigen represents the most specific as well the most sensitive T cells lineage marker, including NK and NKT [6]. CD3 is required for T cell activation. The CD3 T cell receptor complex play a central role in the T cell mediated immune-response as it is involved in the recognition of antigens and activation of T lymphocytes. T cell activation requires a T cell receptor (TCR) to recognize its cognate peptide in the context of an MHC molecule. In addition, the association of CD3 with the TCR–peptide–MHC complex transmits the activation signal to intracellular signalling molecules to initiate a signalling cascade in the T cell. The mechanism by which CD3 signals that the TCR has recognized antigen is not known [7]. Therefore, the aim of this study was to investigate the relationship between CD3 antigen and the severity of NAFLD as well as correlating it with hepatic inflammation and fibrosis. Also, the objective of this study was an assessment of sensitive novel diagnostic and prognostic markers in the serum of nonalcoholic steatohepatitis patients who are likely to have fibrosis.

2. Experimental: This study was conducted on 55 individuals, age range from 18-65 years, and diagnosed by ultrasonography to confirm whether they have NAFLD or not. They were recruited from inpatients and outpatients clinics of the internal medicine department-Cairo University and were divided into 4 groups: Healthy control, obese non-diabetic, obese diabetic, Non-obese diabetic

Patients with history of viral hepatitis, autoimmune hepatitis, or other forms of chronic liver disease, those with self-reported acute infection within 2 weeks, and those with body mass index less than 18.0 kg/m2 were excluded from the study.

The study protocol was approved by the Medical Research Ethical Committee - National research centre, Cairo, Egypt (Approval No.16-118). All persons provided their informed consent prior to their inclusion in the study.

Methods

All patients and controls were subjected to:

1- Detailed history taking including the history of smoking, alcohol intake, and occupational exposure to chemicals, drug intake, previous diseases etc.

2- Thorough clinical assessment and physical examination with calculating BMI.

3- Abdominal Ultrasonography.

NAFLD was diagnosed sonographically, and the severity of the disease was categorized according to the NAFLD fibrosis score [8] and FIB-4 score [9].

4- Blood Sampling:

5 ml of venous blood samples were collected from patients and control cases. Within 30 minutes, the sera were separated by centrifugation at 3000 rpm for 10 min after a minimum time span of 30 min and serum was removed, aliquoted, and stored at –80ºC until further processing. Biochemical analysis including aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, serum albumin, creatinine, urea, CBC, cholesterol, triglyceride, HDL, LDL and random blood sugar were done according to the manufacturer's instructions. The reagents were purchased from Spectrum Company, Cairo, Egypt.

Quantitation ofCD3 by (ELISA):

Serum CD3 was measured in all enrolled subjects using ELISA kit supplied by (NOVA, No. 18, Keyuan Road, DaXing Industry Zone, Beijing, China). The assay is based on a double-antibody sandwich ELISA technique for the quantitative assay of human CD3 in samples. The assay was performed according to the manufacturer’s instructions and values were reported as ng/ml.

Statistical analysis:

SPSS was used to analyze and examine the data (version 16; IBM Corp.).For categorical data, (count) frequency are shown along with the mean± standard division or median (Range) of the quantitative data. The T-test and Mann-Whitney U test was used to compare quantitative variables. The Chi-square test was used to compare categorical data. When the anticipated frequency was less than five, Fisher's exact test was applied instead. Pearson correlation coefficient was used to analyze correlations between quantitative variables. We assessed the diagnostic performance of serum CD3 levels by analyzing the receiver operating characteristic (ROC) curves. The ROC curve is a plot of sensitivity versus 1–specificity for all possible cutoff values. The most commonly used index of accuracy is the area under the ROC curve (AUROC), with values close to 1.0 indicating high diagnostic accuracy. The accuracy of serum CD3 levels for discriminating cases with NAFLD and severity of NAFLD was determined by calculating the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), Statistically significant differences were judged to exist when $P<0.05$ was used.

3. Results: The present study included Forty-one individuals diagnosed by ultrasonography to have NAFLD. They were recruited from inpatients and outpatients clinics of the internal medicine department - Cairo University and were divided into 3 groups: fifteen obese patients, fourteen diabetic patients, twelve obese patients with T2DM. Also, fourteen age-matched lean healthy control subjects were included in this study.

Cases were diagnosed between the period of December 2016 and January 2019. The detailed characteristics of the studied cases were summarized in **Table (1&2).**

In this study, BMI, NAFLD fibrosis score (NFS), uric acid, cholesterol, and triglyceride (TG) levels were significantly elevated in the NAFLD cases compared with healthy controls ($p<0.05$) (**Table 1**).

The obese subjects had significantly higher BMI than the lean subjects ($P< 0.001$). Furthermore, the level HbA1C was significantly elevated in the diabetic patients compared with euglycemic subjects ($P<0.01$).

In addition, diabetic patients showed increase levels of cholesterol and triglyceride compared to healthy subjects (**Table 2**).

Table (1): Demographic, clinical and biochemical parameters of the control and NAFLD patients:

Parameters	Healthy control group (N=14)	NAFLD patients (N=41)	P-value
Age (Years)	48.86±10.08	47.66±11.56	0.731
Sex (male/female) Female %	5/9 64.3%	2/39 95.1%	0.009**
BMI (kg/m^2)	23.8±1.5	31.94±7.73	<0.001**
HbA1C (%)	5.76±1.16	6.42±1.1	0.085
ALT (U/L)	23.07 ±5.43	23.74±8.93	0.747
AST(U/L)	21.36±4.25	22.5±6.44	0.542
GGT(U/L)	41.62±12.29	47.08±40.82	0.639
ALP(U/L)	100.39±29.97	106±46.08	0.685
ALB(g/dl)	3.94±0.33	3.71±0.50	0.122
TP(g/dl)	7.35±0.21	7.28±0.42	0.47
Total bilirubin(mg/dl)	0.55±0.19	0.59±0.39	0.717
Direct bilirubin (mg/dl)	0.054±0.05	0.16±0.36	0.304
Urea (mg/dl)	22 ±3.97	36.14±49.07	0.308
Creatinine (mg/dl)	0.746±0.11	1.26±1.84	0.091
Na^+ (mmol/l)	135±3.24	137.2±4.23	0.093
K^+ (mmol/l)	4.19±0.37	4.36±0.59	0.344
Uric acid (mg/dl)	4.41±0.87	5.51±2.69	0.038*
Cholesterol(mg/dl)	150.9±17.83	194.15±51.48	0.004**
Triglycerides (mg/dl)	99.43±21.62	225.47±313.2	0.026*
LDL	93.5±13.63	101.1±35.57	0.298
HDL	49.2±7.49	49.76±14.76	0.868
RBCs ($\chi 10^6/\mu L$)	4.076±0.29	4.3±0.547	0.057
Hb(g/dl)	12.11±0.99	11.93±1.76	0.639
PLT ($\chi 10^3/\mu L$)s	264.7143±50.185	261.5744±210.62	0.956
WBCs ($\chi 10^3/\mu L$)	10.103±2.438	7.657±4.05	0.038*
NAFLD fibrosis score (NFS)	-2.733±0.887	-1.04±2.87	0.036*
FIB-4 score	0.55±0.39	1.896±3.58	0.169
CD-3	4.76±2.94	64.53±84	<0.001**

- *: $P \leq 0.05$, **: $P \leq 0.01$

Table 2: Demographic, clinical and biochemical parameters of the control and patient population:

Parameters	Control group (N=14)	Obese diabetic group (N=15)	Diabetic Non-obese group (N=14)	Obese Non-diabetic group (N=12)
Age (Years)	48.86±10.08	47.4±13.69	47.29±13.78	48.42±4.69
Sex (male/female) Female %	5/9 64.3%	0/15 a** 100%	1/13 92.9%	1/11 91.7%
BMI (kg/m^2)	23.8±1.5	34.13±4.1 a**	24.13±1.08 b**	38.9±7.86 ,ac**
HbA1C (%)	5.76±1.16	6.7±0.76	7.17±1.26 a**	5.7±0.62 c**
ALT (U/L)	23.1 ±5.43	23.5±10.75	21.92±6.42	25.8±9.06
AST(U/L)	21.36±4.25	19.7±6.69	22.75±6.03	25.5±5.52 b*
GGT(U/L)	41.62±12.29	57.6±63.34	37.27±11.1	42.92±11.05
ALP(U/L)	100.4±29.97	99.5±52.2	103.5±36.12	115.5±50.2
ALB(g/dl)	3.9±0.33	3.66±0.62	3.8±0.54	3.68±0.28
TP(g/dl)	7.35±0.21	7.41±0.44	7.32±0.5	7.09±0.22 b*
Total bilirubin(mg/dl)	0.55±0.19	0.66±0.59	0.49±0.15	0.59±0.19
Direct bilirubin (mg/dl)	0.054±0.05	0.33±0.55 a*	0.09±0.075 b*	0.03±0.045 b*
Urea (mg/dl)	22 ±3.98	60.38±77.3 a*	20.36±3.96 b*	24.3±5.5 b*
Creatinine (mg/dl)	0.75±0.11	1.85±2.55	1.12±1.57	0.7±0.17
Na^+ (mmol/l)	135±3.24	136.1±4.98	137.8±2.88	137.9±4.39
K^+ (mmol/l)	4.19±0.37	4.59±0.83	4.28±0.38	4.2±0.25
Uric acid (mg/dl)	4.4±0.87	5.84±3.69	4.97±1.98	5.45±1.49
Cholesterol (mg/dl)	150.9±17.84	175.4±17.46	209.33±76.7 a**	194.58±35.22 a*
Triglycerides (mg/dl)	99.4±21.6	192.2±138.3	323.1±504.4 a*	155.58±86.3
LDL (mg/dl)	93.5±13.6	103±24.1	84.5±43.17	114.6±32.21
HDL (mg/dl)	49.2±7.49	44.3±19.8	57±9.44	47.7±12.1
WBCs ($\chi 10^3/\mu L$)	10.1±2.4	10.2±5.08	5.89±2.4 ab**	6.56±2.46 ab**
RBCs ($\chi 10^6/\mu L$)	4.08±0.29	4.06±0.55	4.43±0.5	4.5±0.53 ab*
Hb(g/dl)	12.1±0.99	11.56±1.65	12.34±2	11.9±1.6
PLT ($\chi 10^3/\mu L$)s	264.714± 59.185	341.333±317.116	177.254± 77.44 b*	260.250± 92.283
NAFLD fibrosis score (NFS)	-2.73±0.89	-1.38±4.1	-0.35±1.54 a*	-1.32±1.98
FIB-4 score	0.55±0.047	1.55±1.69	3.11±6.15 a*	1.1±0.58
CD-3 (ng/ml)	4.76±2.94	39.1±39.4	89.4±113.53 a**	60.91±73.76 a*

ALT: Alanine Aminotransferase, AST: aspartate aminotransferase; LDL: low density lipoprotein; HDL: High density lipoprotein; WBCs: White blood Cells; RBCs: Red blood cells; Hb: Hemoglobin
- [a]: t-test of each group compared to controls group
- [b]: t-test of each group compared to obese diabetic group
-[c]: t-test of each group compared to diabetic non-obese group - *: P ≤0.05, **: P ≤0.01

According to the serum level of CD3, the comparison between NAFLD patients group and healthy control group indicated that the mean serum level of CD3 was 64.53±84 ng/mlin NAFLD cases vs. 4.76±2.94 ng/ml in controls. Overall, there was a statistically significant increase in serum CD3 levels in NAFLD cases (p<0.001).
Additionally, serum level of CD3 was significantly higher in the diabetic non-obese subjects and obese non diabetic subjects when compared with the lean subjects (89.4±113.53ng/ml and 60.91±73.76 vs. 4.76±2.94 ng/ml, P = 0. 003 & 0.05 respectively.
Furthermore, non-significant increase in the median serum levels of CD3 was detected in fatty liver disease patients with NAFLD fibrosis score (NFS) >0.675 (27.07ng/ml) when compared with those with NFS<0.675 (13.65 ng/ml); P=0.185 **(Table 3)**.

Table 3: CD3 levels (ng/ml) in different NAFLD scores groups:

Parameters	NFS score(<0.675) (N=41)	NFS score(>0.675) (N=9)	P-Value
CD3 (ng/ml) Median (Range)	13.65 (2.3-297.2)	27.07(9.8-268.42)	0.185

Additionally, non-significant increase in the median serum levels of CD3 was detected in fatty liver disease patients with FIB-4 score>1.3 (30.28 ng/ml) when compared with those with FIB-4 score<1.3 (11.27 ng/ml); P=0.096 **(Table 4)**.

Table 4: CD3 levels (ng/ml) in different FIB-4 score groups:

Parameters	FIB-4 score<1.3 (N=36)	FIB-4 score>1.3 (N=14)	P-Value
CD3 (ng/ml) Median (Range)	11.27 (2.34-297.2)	30.28(2.88-268.42)	0.096

Additionally, serum CD3 was positively correlated with total protein, cholesterol, and triglyceride of the participants (r = 0.297, P = 0.041, r = 0.488, P = 0.001, and r = 0.646, P = 0.001; respectively). However, negative correlation was observed between age as well as LDL and CD-3(r =- 0.41, P = 0.003 and r = -0.499, P = 0.001; respectively) (Table 5).

Table (5): Correlation between CD3 and other studied parameters

Parameters	concentration of CD3 (ng/ml)	
	R	P-value
Age(Yrs.)	-0.41	0.003**
BMI (kg/m^2)	0.01	0.944
FIB-4 score	-0.03	0.837
NFS	0.061	0.675
HbA1c (per gm %)	-0.01	0.949
ALT(U/L)	-0.029	0.845
GGT(U/L)	-0.017	0.907
TP(g/dl)	0.297	0.041*
Urea (mg/dl)	-0.084	0.579
Creatinine (mg/dl)	-0.11	0.453
Uric acid (mg/dl)	-0.099	0.519
Cholesterol(mg/dl)	0.488	0.001**
Triglycerides (mg/dl)	0.646	0.001**
LDL	-0.499	0.001**
HDL	0.29	0.056
Hemoglobin(g/dl)	-0.185	0.19
platelet count(10^3/µL)	-0.134	0.343

Receiver operating characteristic curve analysis for the diagnostic accuracy of CD3 for diagnosis NAFLD patients from healthy controls:

Figure (1) illustrates the ROC plots to assess the diagnostic efficiency of serum CD3 distinguish patients with NAFLD in obese and/or diabetic subjects from healthy control. ROC curve analysis showed that serum CD3 had higher significantly diagnostic accuracy in diagnosis of NAFLD (p<0.001).
ROC curve showed the optimum cutoff for CD3 was 7.59 (g/l) for distinguishing patients with NAFLD from healthy control with sensitivity 89.5% and specificity 92.9%; an area under the ROC curves (AUROC) 0.957(95% CI: 0.905-1).

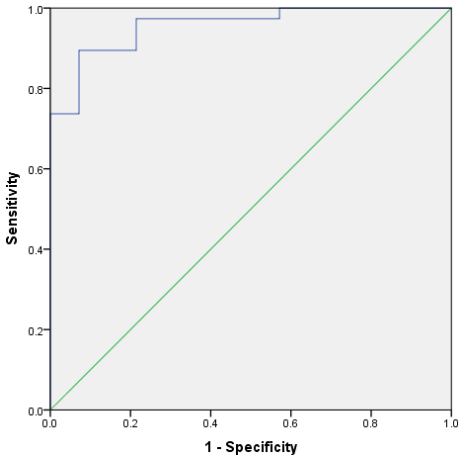

Fig (1): ROC curve of CD3 (ng/ml) to differentiate between NAFLD from health control

The ROC plots to assess the diagnostic efficiency of serum CD3 for early detection of patients with NAFLD fibrosis Score <0.675 from those with NAFLD fibrosis Score ≥ 0.675.

Figure (2) illustrates the ROC plots to assess the diagnostic efficiency of serum CD3 for differentiating patients with NFS<***0.675*** from those with NFS ≥ ***0.675***. ROC curve analysis showed that serum CD3 had **non-significant diagnostic accuracy** in diagnosis of NFS (p=0.756).

ROC curve showed the optimum cutoff for CD3 was 119.6 (g/l) for distinguishing patients with NFS<***0.675*** from those with NFS≥ ***0.675*** with sensitivity 33.3% and specificity 85.2%; an area under the ROC curve (AUROC) 0.465(95% CI: 0.235-0.695).

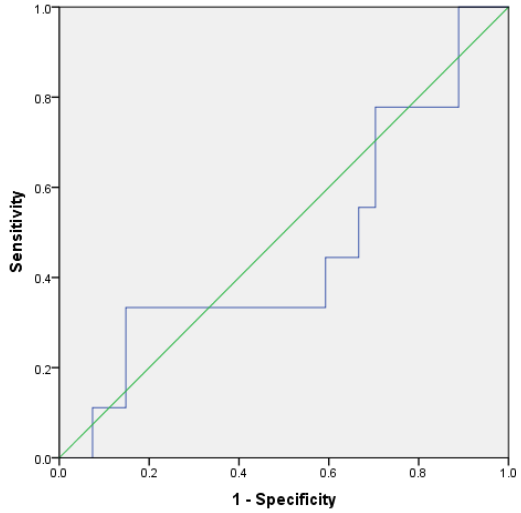

Fig (2): ROC curve of CD3 (ng/ml) to differentiate between NFS<0.675 from those with NFS≥ 0.675

The ROC plots to assess the diagnostic efficiency of serum CD3 for early detection of patients with FIB-4 <1.3 from those with FIB-4 ≥1.3

Figure (3) illustrates the ROC plots to assess the diagnostic efficiency of serum CD3 for differentiating patients with *FIB-4 <1.3 from those with FIB-4 ≥1.3*. ROC curve analysis showed that serum CD3 had non-significant diagnostic accuracy in diagnosis of patients with *FIB-4≥1.3* (p=0.754).

ROC curve showed the optimum cutoff for CD3 was 14.55 (g/l) for distinguishing patients with ***FIB-4 <1.3*** from those with ***FIB-4 ≥1.3*** with sensitivity 92.3% and specificity 34.8%; an area under the ROC curve (AUROC) 0.532(95% CI: 0.334-0.729).

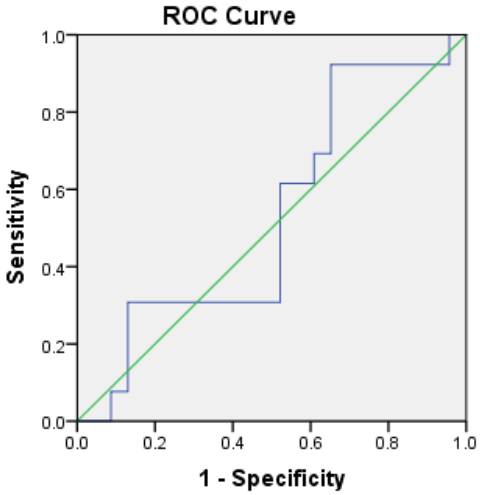

Fig (3): ROC curve of CD3 (ng/ml) to differentiate between patients with FIB-4 <1.3 from those with FIB-4 ≥1.3.

4. Discussion:

In light of increasing NAFLD prevalence, non-invasive markers for the assessment of disease severity are needed for decision making in clinical practice[10]. The progression of NAFLD is driven by inflammatory mechanisms involving apoptosis and necrosis of hepatocytes, which ultimately result in the development of liver fibrosis and cirrhosis [11-14].

This study showed that certain NAFLD cases who suffer from diabetes and/ or obesity had higher urea levels. Previous results recommended that NAFLD may accelerate the development and progression of CKD as it is particularly frequent among NAFLD patients, ranging from 20 to 50%. A meta-analysis study that evaluates the incidence and prevalence of CKD in patients with simple fatty liver, NASH, and advanced fibrosis concluded that the severity of NAFLD was directly associated with CKD [15].

Growing numbers of evidence supports a role for the immune system and inflammatory cells as potential players in the pathogenesis of fatty liver and its progression to NASH [16-18].

NAFLD livers show varied degrees of necroinflammatory changes in addition to hepatocyte degeneration, but the precise role of liver infiltrating cells remains unclear.

Compared with other organs, the liver contains a unique population of resident lymphocytes including innate immune cells, possibly because of the constant exposure to a variety of toxins and antigens from intestinal bacteria by a unique anatomical location being blood supplied directly from the intestines [19]. Natural killer T (NKT) cells have been recently reported to be associated with various lipid disorders however, the role of NKT cells in the hepatic lipid disorder, nonalcoholic fatty liver disease (NAFLD) has not yet been clarified [19]. CD3 antigen represents the most specific as well the most sensitive T cells lineage marker, including NK and NKT. We evaluated CD3 in patients with NAFLD by ELISA. CD3 were found to be significantly higher in patients with NAFLD when compared to controls. Also, this study indicated elevation in the median of CD3 in patients with higher NAFLD fibrosis score and FIB-4 score compared to that in cases with lower scores.

This is in line with earlier observations of Gadd et al., who stated that there is a significant increase in number of CD3 between control subjects and patients with progressive NASH [20]. Moreover, Tajiri et al., concluded that intrahepatic CD3+CD56+ NKT cells are increased in NAFLD as NAFLD activity score (NAS) increased. He expected that these cells may enhance disease activity through cytokine production after the recognition of lipid antigens presented with CD1d in livers of NAFLD patients [19].

Our data are also in accordance with the findings observed by Ali et al (2019) who showed an increase in the frequency of peripheral NKT cells of the phenotype (CD3+/CD56+/CD161+) among patients with either steatosis or concomitant HCV/steatosis compared to normal controls [21]. Additionally, Adler and co-workers who noted that NKT cells increased significantly in both liver and blood of patients with NAFLD [22].

In contrast, other studies reported a depletion in the peripheral blood NKT cells in patients with clinically diagnosed NALFD [23-25]. Whereas Kremer et al (2010) found that NKT cells depletion was associated with severe stages of steatosis [26].

However, Zhao (2021), observed that CD3+ in the diabetic cases either they have NAFLD or not was decreased compared with the control group, and CD3+ in the NAFLD group decreased compared to the Non-NAFLD group. Also, they detected that there was negative correlation between CD3+ and all these parameters FPG, HbA1C, TG, TC, HDL and LDL [27].

Additional studies should be performed to evaluate whether the reduction in CD3+ cells is specific for NKT or more generalized, and whether, on the contrary, a selective increase in intralobular CD3+ cells is associated with the progression of liver disease.

References in the text should be indicated by Arabic numerals in square brackets that run consecutively through the paper. Authors should ensure that all references are cited in the text and vice versa. The reference list should contain only literature references; other information (e.g. experimental details) should be placed either in the body of the text, or as a footnote. Each reference should contain only one literature citation. Authors are expected to check the original source reference for accuracy. Journal titles should be abbreviated according to American Chemical Society guidelines (The ACS Style Guide; Dodd, J. S., Ed.: American Chemical Society: Washington, DC, 1997). See examples for journal articles [1], books [2], multi-author books [3], proceedings [4] and personal communications [5], shown in **References** below.

5. Conclusions

Serum level CD3 can be used as a prognostic marker in cases with NAFLD as they increase with the progression of the disease and is correlated with fibrosis stage. The current study has some limitations, including a relatively small sample size. Nevertheless, the study also has strengths, as it represents the first study on the role of serum CD3 in patients with NAFLD. The extension of the study to a larger cohort of patients, as well as the evaluation of the possible involvement of other and more specific inflammatory markers are certainly required.

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6. Acknowledgements:

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