Bratisl Lek Listy 2011; 112 (11)
626 – 629

CLINICAL STUDY

Alkaline phosphatase: can it be considered as an indicator of liver fibrosis in non-alcoholic steatohepatitis with type 2 diabetes?

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Abstract: *Objective:* While isolated hepatosteatosis is a benign disease, in minority of cases non-alcoholic steatohepatitis (NASH) may even lead to cirrhosis in long-term. In order to find the stage of the disease and determine the prognosis, a liver biopsy is indicated. In this study, we studied the relationship of liver histopathological findings with serum levels of hepatic enzymes.
Methods: We recruited 52 cases of NASH with Type 2 diabetes mellitus. Diagnosis of NASH was made based on biochemical tests, ultrasound images and liver biopsy.
Results: Steatosis was mild in 57.7 %, moderate in 30.8 %, and severe in 11.6 % of patients. While no infiltration was found in 78.8 % of cases, there was a grade-1 infiltration in 15.4 % and a grade-2 infiltration in 5.8 % of cases. Similarly, no fibrosis was found in 42.3 % of patients, but there was a stage-1 fibrosis in 50 %, and a stage-2 fibrosis in 7.7 % of cases. In patients with severe steatosis, serum levels of AST were higher than mild or moderate stage steatosis. Accordingly, in patients with no inflammation, serum levels of ALT were higher than in patients with inflammation. However, in patients with fibrosis, triglycerides levels were significantly lower and ALP was significantly higher than in patients without fibrosis. The correlation analysis indicated a positive association between serum levels of ALP and C-peptide.
Conclusion: In addition to conventional risk factors such as age, presence of diabetes, female sex; higher levels of ALP may be considered as a risk factor linked to hepatic fibrosis in patients with NASH and type 2 diabetes *(Tab. 6, Ref. 8)*. Full Text in free PDF *www.bmj.sk.*
Key words: non-alcoholic steatohepatitis (NASH), alkaline phosphatase (ALP), liver fibrosis, type 2 diabetes mellitus.

Non-alcoholic steatohepatitis (NASH) which is associated with central obesity, insulin resistance (IR) and type 2 diabetes mellitus (T2DM) is an increasing crucial health problem (1). The disease has a broad clinic-pathological spectrum, which changes from a relatively mild and commonly found asymptomatic isolated steatosis to NASH. While isolated hepatosteatosis is a benign disease, NASH might be a slowly progressing disease leading to fibrosis and even to cirrhosis in the minority of patients long term.

"The two hits" hypothesis is considered in the pathogenesis of NASH. In the first hit, IR is obvious and consequently liver steatosis occurs (2, 3). In the second hit, oxidative stress, which is caused by hepatotoxic agents, endotoxins, endogenous alcohol production, cytokines and peripheral toxins and consequently the inflammation of lipid laden hepatocytes occur. In order to find the stage of the disease and categorize the staging and determine the prognosis of disease, a liver biopsy should be done. It is not clear, in which groups of patients a biopsy need to be performed. Although risk factors for fibrosis were defined, we do not have enough knowledge on this.

In this study we aimed to study the relationship between the liver histopathological findings and serum levels of alkaline phosphatase (ALP) in NASH patients with type 2 DM (T2DM).

Study design and methods

In this cross-sectional study we recruited 52 cases of NASH with T2DM. NASH was diagnosed by biochemical tests and ultrasound (US) images. General characteristics of the patients were as follows: F/M 22/30, mean age 53.2±7.3 years, DM duration 7.4±4.7 years. The mean BMI was 33.8±3.9 kg/m^2 in females, and 30.5±3.8 kg/m^2 in males. Diabetes duration was not different between sexes.

The study group included cases of T2DM and NASH without any of the following factors: any hepatotoxic drug (corticosteroid, estrogen, methotrexate, tetracycline, amiodaron); any gastrointestinal surgical operation; diseases such as viral or autoimmune hepatitis, cholestatic liver disease, hemachromatosis,

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Wilson Disease; and history of alcohol consuption higher than 20 g/day. The alcohol issue was verified by patient's relatives. Hypertension was diagnosed as the presence of blood pressure higher than 140/90 mmHg or as known hypertension with regular antihypertensive drugs. Dislipidemia was diagnosed by the presence of serum fasting triglycerides over 150 mg/dl and/or HDL-cholesterol level <40 mg/dl in male, <50 mg/dl in female or being under a regular anti-lipidemic drug. According to these findings, of the 52 cases with NASH, 33 (63.5 %) had hypertension and 35 (67.3 %) had dislipidemia.

A needle liver biopsy was performed in all patients under US and the diagnoses of NASH were confirmed. Histopathological evaluation of the biopsy materials was done by the same pathologist according to the Brunt Criteria, after the specimens were stained by hematoxylen-eosin and mason trichrome stains. Biochemical analyses such as fasting blood glucose (FBG), fasting lipids, serum creatinine, alkaline phosphatase (ALP), alanine and aspartat aminotransferases (AST and ALT), lactate dehydrogenase (LDH) and gamma glutamyl transpeptidase (GGT) were analyzed by the photometric method in DPP module, modular system, Roche Diagnostics; high sensitive C-reactive protein (hs-CRP) was analyzed as mg/l by the immunoturbidometric method, Cobas Integra 800 auto-analyzer, Roche Diagnostics. Glycosylated heamoglobin A_{1c} (A1C) was measured by the turbidometric inhibition immunoassay method in DPP module, modular system, Roche Diagnostics.

Insulin resistance was calculated from the 'Homeostasis Model of Assessment' HOMA-IR formula. In order to increase the sensitivity, a log transform was performed (4). We used the fasting C–peptide levels for the patients under the insulin therapy. Statistical analyses were done via the package program for computers, SPSS 13.0 (2006, SPSS Inc. Chicago, USA). The Pearson correlation test was used for parametric values and the Spearman's rho correlation test was used for non-parametric values. The *p* value <0.05 within 95% confidential interval (CI) was considered as statistically significant. The study was approved by the Clinical Ethical Committee of Istanbul University, Faculty of Istanbul Medicine and a signed informed consent was obtained from each patient.

Results

The biochemical results of the study group are shown in the Table 1. While 69.2 % of patients have used oral anti-diabetic drug (OAD), 7.7 % of patients used only insulin, and 23 % of patients were on OAD plus insulin therapy. There was no difference between the two sexes in terms of all biochemical parameters except that females had a significantly higher FBG and A1C levels than males (p=0.044 and p=0.05 respectively, data not shown).

The histopathological evaluation of the needle biopsy specimens according to the Brunt classification system is shown in the Table 2. According to the morphological steatosis staging, macro-vesicular steatosis was found in 63.5 % of cases (n=33) and a mixed type (micro- plus macro-vesicular) steatosis in 36.5 % (n=19). Steatosis was found in 57.7 % (n=30) in mild stage, 30.8 % (n=16) in moderate stage and 11.5 % (n=6) in severe stage. Inflammatory activity score evaluation revealed that there was no infiltration in 78.8 % of cases (n=41), 15.4 % (n=8) had a grade 1 and 5.8 % (n=3) had a grade 2 infiltration. According to the fibrosis scores, fibrosis was not found in 42.3 % of cases (n=22), but 50 % (n=26) had stage 1 and 7.7 % (n=4) had stage 2 fibrosis.

According to the Brunt's classification system, hepatosteatosis is categorized as (<33 %), moderate (33–66 %) and severe (>66 %) stages. As shown in the Table 3 in patients with severe stage steatosis, the mean AST was higher than in patients with mild and moderate stages (severe; 56.7±20.4 vs. mild; 38.2 ± 11.8, moderate; 38.1±10..0 U/l, p = 0.051).

According to the inflammatory activity, in patients with no inflammation, ALT was higher than in patients with inflammation (p = 0.0001). In addition, although HOMA-IR (p=0.09 and triglycerides (p=0.06) levels did not reach the limit of significance, they tended to be higher in the inflammatory group (Tab. 4).

Tab. 1. Biochemical data of the study population.

	Mean±SD	Interval (Min–Max)
FBG (mg/dl)	162.9±61.4	81–341
A1C (%)	7.6±1.7	5.1–12.8
HOMA-IR	3.0±2.1	0.7–11.4
TG (mg/dl)	174.7±110.3	32–709
LDL-c (mg/dl)	108.3±32.9	34–212
HDL-c (mg/dl)	42.0±9.41	23–74
ALT (U/l)	64.5±16.3	50–110
AST (U/l)	40.3±13.6	22–92
ALP (U/l)	204.3±74.1	89–406
GGT (U/l)	65.3±70.7	17–460
C-peptide (ng/ml)	4.1±1.7	1.6–10.0
hs-CRP (mg/l)	4.0±3.3	0.7–15.0

Min – Minimum, Max – Maximum, SD – Standard deviation, FBG – Fasting blood glucose, A1C – Glycosylated hemoglobin A1C, HOMA-IR – Homeostasis model of assessment, TG – Triglycerides, LDL-c – Low density lipoprotein cholesterol, HDL-c – High density lipoprotein cholesterol, ALT – Alanine aminotransferase, AST – Aspartat aminotransferase, ALP – Alkaline phosphatase, GGT – Gamma-glutamyl transpeptidase, hs-CRP – High sensitive C-reactive protein.

Tab. 2. Brunt's staging of the study population.

Steatosis stage	n	%
Mild (<33 %)	30	57.7
Moderate (33-66 %)	16	30.8
Severe (>66 %)	6	11.5
Inflammatory activity		
Absent	41	78.8
Grade-1	8	15.4
Grade-2	3	5.8
Fibrosis score		
Absent	22	42.3
Stage-1	26	50
Stage-2	4	7.7

Tab. 3. Clinical and biochemical data of the study population according to steatosis staging.

	Mild (n=30)	Moderate (n=6)	Severe (n=6)	Variance*	
	Mean±SD	Mean±SD	Mean± SD	χ^2	p
Age (year)	52.3±7.2	54.2±7.0	54.8±9.0	1.147	0.56
DM duration (year)	7.6±4.9	7.7±4.8	5.3±2.9	0.879	0.64
BMI (kg/m2)	31.9±3.9	32.4±4.9	30.3±3.2	0.495	0.78
A1C (%)	7.3±1.4	7.8±1.9	8.7±2.2	2.471	0.29
FBG (mg/dl)	151.9±55.3	174.6±72.4	186.5±56.3	2.590	0.27
TG (mg/dl)	158.8±59.0	191.5±163.0	209.2±142.6	0.713	0.70
LDL-c (mg/dl)	107.3±28.8	109.3±24.9	111.2±65.6	0.095	0.95
HDL-c (mg/dl)	40.4±9.4	45.3±9.8	41.2±7.6	3.163	0.21
HOMA-IR	2.9±2.0	3.2±2.4	2.9±1.9	1.627	0.44
ALT (U/l)	63.6±17.4	62.4±12.1	74.3±19.8	3.047	0.21
AST (U/l)	38.2±11.0	38.1±10.0	56.7±20.4	5.958	0.051
ALP (U/l)	215.5±8.9	181.6±54.3	208.7±93.7	1.398	0.49
GGT (U/l)**	64.6± 82.2	69.8±60.6	56.5±26.0	1.119	0.57
C-peptide (ng/ml)	4.1±1.9	3.8±1.3	4.6±1.2	2.136	0.34
hs-CRP (mg/l)	3.6±3.4	4.1±2.4	6.0±4.4	3.441	0.18

*Kruskal-Wallis variance analysis, **Mann-Whitney U test was used. DM – Diabetes mellitus, BMI – Body mass index, A1C - Glycosylated hemoglobin A1c, FBG - Fasting blood glucose, TG – Triglycerides, LDL-c – Low-density lipoprotein cholesterol, HDL – High density lipoprotein cholesterol, HOMA-IR – Homeostasis model of assessment-insulin resistance, ALT – Alanine aminotransferase.AST – Aspartat aminotransferase, ALP – Alkaline phosphatase, GGT – Gamma-glutamyl transpeptidase.

Tab. 4. Clinical and biochemical data of the study population according to inflammatory activity.

	No inflammation (n=41)	With inflammation (n=11)	t-test	
	Mean±SD	Mean±SD	t	p
Age (year)	52.6±7.1	55.3±8.0	1.068	0.29
DM duration (year)	7.4±4.9	7.4±3.8	0.017	0.99
BMI (kg/m2)	31.8±4.3	32.0±3.9	0.133	0.89
A1C (%)	7.6±1.7	7.6±1.8	0.068	0.95
FBG (mg/dl)	163.0±59.2	162.4±72.2	0.030	0.98
TG (mg/dl)	160.0±84.2	229.6±171.7	1.908	0.06
LDL-c (mg/dl)	105.8±34.7	117.8±23.7	1.081	0.29
HDL-c (mg/dl)	41.0±9.6	45.7±8.0	1.497	0.14
HOMA-IR	2.6±1.5	4.5±3.2	1.879	0.09
ALT (U/l)	67.1±17.3	54.9±5.8	3.769	0.0001
AST (U/l)	40.6±14.4	39.3±10.5	0.282	0.78
ALP (U/l)	202.1±72.6	212.2±82.8	0.396	0.69
GGT (U/l)*	56.7±44.6	97.2±127.0	1.32	0.19
C-peptide (ng/ml)	3.9±1.5	4.7±2.3	1.154	0.27
hs-CRP+ (mg/l)	3.8±3.4	4.6±3.0	1.27	0.20

*Mann-Whitney U test was used. DM – Diabetes mellitus, BMI – Body mass index, A1C – Glycosylated hemoglobin A1c, FBG – Fasting blood glucose, TG – Triglycerides, LDL-c – Low-density lipoprotein cholesterol, HDL – High density lipoprotein cholesterol, HOMA-IR – Homeostasis model of assessment-insulin resistance, ALT – Alanin aminotransferase, AST – Aspartat aminotransferase, ALP – Alkaline phosphatase, GGT – Gamma-glutamyl transpeptidase.

Tab. 5. Clinical and biochemical data of the study population according to fibrosis staging.

	No Fibrosis (n=22)	Fibrosis present (n=30)	t-test	
	Mean±SD	Mean±SD	t	p
Age (year)	52.9±6.9	53.4±7.6	0.276	0.78
DM duration (year)	7.0±4.6	7. 7±4.8	0.504	0.62
BMI (kg/m2)	31.8±3.7	31.9±4.5	0.121	0.90
A1C (%)	7.9±2.0	7.4±1.4	1.137	0.26
Fasting glucose (mg/dl)	167.6±67.8	159.4±57.2	0.467	0.64
TG (mg/dl)	218.0±142.9	143.0±64.4	2.296	0.03
LDL-c (mg/dl)	112.6±36.1	105.2±30.5	0.799	0.43
HDL-c (mg/dl)	40.2±8.2	43.3±10.2	1.167	0.25
HOMA-IR	2.9±1.6	3.1±2.4	0.368	0.72
ALT (U/l)	64.5±15.2	64.5±17.4	0.010	0.99
AST (U/l)	39.3±11.6	41.1±15.0	0.467	0.64
ALP (U/l)	179.2±36.4	222.6±88.7	2.414	0.02
GGT (U/l)*	48.4±34.2	77.7±87.0	1.42	0.16
C-peptide (ng/ml)	3.9±1.52	4.2±1.8	0.464	0.65
hs-CRP (mg/l)	3.4±3.0	4.4±3.5	1.066	0.29

*Mann-Whitney U test was used. DM – Diabetes mellitus, BMI – Body mass index, A1C – Glycosylated hemoglobin A1c, FBG – Fasting blood glucose, TG – Triglycerides, LDL-c – Low-density lipoprotein cholesterol, HDL – High density lipoprotein cholesterol, HOMA-IR – Homeostasis model of assessment-insulin resistance, ALT – Alanine aminotransferase. AST – Aspartat aminotransferase, ALP – Alkaline phosphatase, GGT – Gamma-glutamyl transpeptidase.

According to the presence of hepatic fibrosis, a classification was performed. The serum level of triglycerides in patients with fibrosis was significantly lower than in patients with no fibrosis (p=0.03). Similarly, in patients with fibrosis, the ALP levels were significantly higher than in patients with no fibrosis (p=0.02) (Tab. 5).

The staging of steatosis was also performed according to the US images, 7.7 % of cases (n=4) was in Stage I, 50 % (n=26) in Stage II and 42.3 % (n=22) in Stage III. The Stage I and Stage II were combined because less patients were present in Stage I. Therefore, comparisons were done between Stage (I+II) and Stage III. In Stage III, ALP (p=0.043) and GGT (p=0.034) levels were found to be significantly higher than in Stage (I+II).

The correlation analyzes are shown in the Table 6. The Pearson correlation analysis revealed a positive correlation between ALP and C-peptide (r=0.35, p=0.012), and hs-CRP (r=0.32, p=0.02) and GGT (r=0.51, p=0.0001). After an adjust-

Tab. 6. Correlation analysis between liver enzymes and other laboratory parameters.

	General correlations	
	r	p
ALP		
GGT*	0.51	0.0001
C-peptide	0.35	0.012
hs-CRP	0.32	0.020
ALT		
AST	0.53	0.0001
AST		
BKI	0.41	0.002
A1C	0.38	0.005
HOMA-IR*	0.28	0.044
hs-CRP	0.35	0.011
GGT		
hs-CRP*	0.34	0.014

*Spearman correlations, DM – Diabetes mellitus, ALP – Alkaline phosphatase, ALT – Alanine aminotransferase, AST – Aspartat aminotransferase, GGT – Gamma glutamyl transpeptidase, hs-CRP – High sensitive C-reactive protein.

ment for age and diabetes, the correlation maintained between ALP and GGT (r=0.48, p=0.0001, data not shown). Similarly, a positive correlation was observed between ALT and AST (r=0.53, p=0.0001), and this was also maintained after an adjustment for age and diabetes duration (r=0.55, p=0.0001).

Discussion

Non-alcoholic steatohepatitis has a broad clinico-pathological spectrum, which changes from a range of commonly seen asymptomatic isolated steatosis to NASH. Although steatosis, which is the first step of this spectrum, is a benign clinico-pathological condition, NASH may rarely progress to more serious problems such as fibrosis and cirrhosis. It is still unclear, which cases will progress and which will stay as an isolated steatosis in future. The clinical and laboratory findings of NASH by non-invasive methods are not definitive, imaging procedures may show steatosis, but they cannot reveal inflammation and staging of fibrosis. The needle biopsy is a reliable method, which excludes many causes of chronic hepatitis and is capable to show steatosis, inflammation and hepatic fibrosis. It is also useful to define the stage of the disease. Although there is no consensus, patients over the age of 45, with type 2 diabetes and/or hypertension and central obesisty, those with AST/ALT >1 and with increased triglycerides levels carry a higher risk of NASH. Biopsy is commonly recommended in this group of patients. These conditions also indirectly indicate the presence of liver fibrosis (1, 2, 6).

In this study, serum levels of ALP were found to be higher in cases with liver fibrosis than without fibrosis (p=0.02). Pantsari et al in their study showed that isolated ALP increase was found in 10% of patients with NASH, especially in elderly women. Further investigations revealed that NASH was present in 50 % of cases and 71 % of these were in severe stage (5). In our study, which is different from that research, we observed the same condition in younger age group and without sex discrimination. In addition to this, staging according to US images, compared to mild-moderate stage ALP and GGT levels, were found to be higher in severe cases (stage III; p=0.043 vs. stage [I+II]; p=0.034). On the other hand, after adjustment for age, ALP revealed a positive correlation with C-peptide (an indirect marker of IR) and hs-CRP, which are considered as risk factors for liver fibrosis (r=0.35 and p=0.028 and r=0.32 and p=0.02, respectively) (7).

Although there was a positive correlation between ALP and GGT, we did not find any correlation between the stage of liver fibrosis and serum GGT levels. This might be due to fact that our study group was composed of cases with early fibrosis, as it is well known that GGT levels increase in severe stage fibrosis due to destruction of bile ducts. No one of our cases in the study population had cholelithiasis or choledocolithiasis. These findings showed that increased ALP levels, especially in the early phase, may play an important role in addition to conventional risk factors such as age, female sex, presence of obesity, diabetes, and the ratio of AST to ALT in NASH. The current study by Singh et al. found that alkaline phosphatase level was an independent predictor of fibrosis stage as supported our findings (8).

The results of this study suggested a link between the histopathological liver findings and serum ALP levels. We conclude that in addition to classical risk factors, increased serum levels of ALP but not GGT may also be used as a risk marker indicating fibrosis during the natural course of NASH. However, in order to evaluate the disease stage more precisely, the needle biopsy may be required.

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Received November 11, 2009.
Accepted June 26, 2011.