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Clinical study

ALKALINE PHOSPHATASE IS INDEPENDENTLY ASSOCIATED WITH RENAL FUNCTION IN NORMOALBUMINURIC TYPE 1 DIABETIC PATIENTS

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Running head: alkaline phosphatase and renal function

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is associated with an increased prevalence of chronic kidney disease in patients with type 1 diabetes. The aim of this study was to explore the relationship between markers of NAFLD, namely concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALK), γ-glutamyltransferase (GGT), bilirubin and renal function in type 1 diabetic patients. Study included 313 normoalbuminuric type 1 diabetic patients with estimated glomerular filtration rate (eGFR) > 60 $mlmin^{-1}1.73m^{-2}$, without clinical evidence of cirrhosis or other causes of chronic liver disease and before any interventions with statins, ACE inhibitors or angiotensin II receptor blockers. ALT, GGT, and bilirubin levels were significantly higher in subjects in the highest quartile of serum creatinine compared to those in lowest quartile (21 vs 20 U/L, 18 vs 14 U/L, and 14 vs 10 μmol/L, respectively, for all $p<0.05$). ALK levels were significantly higher in subjects in the highest quartile of urinary albumin excretion rate compared to those in lowest quartile (71 vs 69 U/L, $p=0.03$), as well as in hyperfiltrating subjects compared to those with normal or mildly impaired eGFR (81 vs 68 and 64 U/L, $p<0.001$). In a multiple logistic regression model adjusted for age, sex, duration of diabetes, HbA1c and BMI, only ALK levels were significantly associated with disturbances in serum creatinine and eGFR in our subjects ($p\leq0.007$), with odds ratios of 0.98 to 1.02. NAFLD associated markers, particularly ALK, are associated with renal function in normoalbuminuric type 1 diabetic patients.

Keywords: alkaline phosphatase, type 1 diabetes, renal function, nonalcoholic fatty liver disease

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by accumulation of fat in the liver and refers to a spectrum of disorders ranging from simple hepatic steatosis to more severe manifestations, including nonalcoholic steatohepatitis (NASH), in the absence of substantial alcohol consumption or other causes of liver disease such as viral hepatitis (1, 2). NAFLD is usually clinically silent, and most patients seek care because of an incidental finding of elevated aminotransferase levels or radiographic studies suggesting the liver is fat (3). Mildly to moderate elevated serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT) are the most common abnormality found in patients with NAFLD (1, 4, 5). Serum alkaline phosphatase (ALK) exceed referent interval in many patients, although their degree of elevation is smaller than one seen in alcoholic hepatitis (6, 7). Hyperbilirubinemia can be also found in patients with NAFLD (8, 9). Previously mentioned markers of liver injury may be surrogate measures of NAFLD and related conditions for larger studies (10, 11).

Patients with type 1 diabetes have a 20-50% probability of developing end-stage renal disease (12), and identification of the determinants of the onset of early diabetic nephropathy is essential for reducing the morbidity and mortality associated with diabetes. In recent years, the possible link between NAFLD and chronic kidney disease (CKD) has attracted scientific interest, because NAFLD and CKD share many important risk factors and both are linked to an increased risk of cardiovascular diseases (13-15). In patients with type 1 and type 2 diabetes it was found that ultrasound-diagnosed NAFLD was associated with CKD (defined as estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73m^2 or overt proteinuria), independently of traditional risk factors (16, 17). It was also found that NAFLD, diagnosed by ultrasound or liver biopsy, was associated with microalbuminuria in subjects with or without diabetes, independently of several potential confounders (18, 19). Moreover, it was found that

elevated serum liver enzyme levels, as surrogate markers for NAFLD, were independently associated with an increased incidence of CKD (20-22).

Little is known about the relationship between NAFLD markers and change in renal function among individuals with normal or mildly impaired renal function, because most studies have focused on the progression of established renal disease. The objective of this study was to investigate relationship between NAFLD associated markers and renal function parameters in normoalbuminuric type 1 diabetic patients with normal or mildly impaired renal function (eGFR > 60 ml/min/1.73m^2).

SUBJECTS, MATERIALS AND METHODS

This study included 313 patients with diabetes mellitus type 1. Type 1 diabetes was defined as an onset of diabetes before the age of 35 years, positive autoantibodies and permanent insulin treatment initiated within 1 year of diagnosis. The study included patients with following characteristics: age of 18-65 years, minimum duration of type 1 diabetes for 1 year, no medical history of liver, renal and cardiovascular diseases, absence of any systemic disease, and absence of any infections in the previous month. Patients who had reported daily alcohol intake exceeding 20 g/day were excluded from the study. None of patients had serological and clinical evidence of viral hepatitis, hemochromatosis, Wilson disease, autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis or biliary obstruction, hepatitis B or C virus infections, and none of them were intravenous drug addicts. Patients were excluded from the study if they took any of the following: lipid-lowering therapy, antihypertensive therapy including ACE inhibitor or angiotensin II receptor blockers, medications that might affect liver function (amiodarone, glucocorticoids, methotrexate, tamoxifen and other drugs), and medications that might affect glucose metabolism such as glucocorticoids. Acute and chronic inflammation was excluded on the basis of medical history, physical examination, and routine laboratory tests, including measurement of temperature and urinalysis.

All subjects were studied in the morning after an overnight fast. Basic anthropometric measurements were performed on all study subjects. Blood pressure was measured twice in the sitting position with a mercury sphygmomanometer after a resting period of 10 minutes and expressed in mmHg. Urinary albumin excretion rate (UAE) was measured from at least two 24-h urine samples and determined as the mean of 24-h urine collections. Patients performed collections on two consecutive days to minimize variability. Normoalbuminuria was defined as a UAE<30 mg/24h. Those with microalbuminuria (UAE≥30<300 mg/24h) and

macroalbuminuria (UAE≥300 mg/24h) were excluded from the study. Serum creatinine was measured in fasting blood sample. Data on serum creatinine levels, age, sex and race were used to calculate the estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula (23), which has been shown to be accurate in determining renal function in diabetic patients with normal renal function. Those with chronic kidney disease, defined as the presence of impaired eGFR (less than 60 ml min^{-1} $1.73m^{-2}$), were excluded from the study.

Fasting venous blood samples were collected in the morning between 08:00 and 09:30 hours after an overnight fast for the determination of HbA1c, AST, ALT, GGT, ALK, total bilirubin, total, LDL, HDL cholesterol, and triglycerides. Microalbumin and HbA1c was measured spectrophotometrically by turbidimetric immuno-inhibition (Olympus AU600, Beckman-Coulter, USA). Glucose, cholesterol and triglycerides in serum were measured by an enzymatic colorimetric method. Complete blood count was determined on an automatic blood counter (Advia 120, Siemens Diagnostic Solutions, USA). AST, ALT, GGT, ALK, and total bilirubin were measured using standard laboratory methods.

The study protocol complies with the Declaration of Helsinki as well as with local institutional guidelines, and was approved by the local ethics committees.

Data are expressed as means ± SD for normally distributed values, as median with range for non-normally distributed values, and percentage. Pearson's correlation coefficients were used to calculate correlations between normally distributed values and Spearman's rank correlation coefficients were used for non-normally distributed values. To investigate the relation between NAFLD markers with renal function parameters data were also stratified in quartiles depending on UAE and serum creatinine and in different groups of eGFR. Kruskal-Wallis test was used for calculating the significance of the trend for each variable among the different groups. Separate multivariate logistic regression models were used to assess

associations of NAFLD markers and risk of progression of renal disease, taking in account of potential confounders. Two models were constructed for each marker: in model A, adjustments were made for age and sex; model B included further adjustment for duration of diabetes, HbA1c and BMI. Level of statistical significance was chosen to be $\alpha=0.05$. Statistical analysis was performed by statistical package STATA/IC ver.11.1.

RESULTS:

The characteristics of the study subjects are listed in Table 1. Mean/median values of BMI, waist to hip ratio (WHR), HDL cholesterol, triglycerides, AST, ALT, ALK, GGT, bilirubin, serum creatinine, UAE, eGFR as well as blood pressure were within the normal range for patients with diabetes, with slightly elevated HbA1c and LDL cholesterol levels. Association of NAFLD associated markers with parameters of renal function are presented in Table 2. Serum creatinine was significantly associated with ALT, GGT and bilirubin, with bilirubin showing the strongest correlation ($r=0.29$, $p<0.001$). eGFR was significantly associated with ALK and bilirubin, with ALK showing the strongest correlation ($r=0.23$, $p<0.001$). Finally, UAE was significantly associated only with GGT ($r=0.11$, $p=0.04$). In addition, NAFLD associated markers and renal parameters significantly correlated with various metabolic variables, mainly with parameters included in diagnosis of metabolic syndrome (WHR, HbA1c, HDL cholesterol, triglycerides and blood pressure). The mentioned correlations were most significant for WHR.

Relationship between NAFLD markers among those in the 2^{nd}, 3^{rd} and 4^{th} quartiles of serum creatinine compared to those in quartile 1 are presented in table 3. Stratifying NAFLD associated markers for the degree of serum creatinine, trends across quartiles of serum creatinine for ALT, GGT and bilirubin were statistically significant (all $p<0.05$). Subjects in the 4^{th} quartile of serum creatinine had significantly elevated ALT, GGT and bilirubin levels compared to subjects in 1^{st}, 2^{nd}, and 3^{rd} quartiles. We also explore relationship between NAFLD markers among those in the 2^{nd}, 3^{rd} and 4^{th} quartiles of UAE compared to those in quartile 1 (<6.8 mg/24h). Stratifying NAFLD markers for degree of UAE, trends across quartiles was statistically significant only for ALK ($p=0.03$). Subjects in the 4^{th} quartile of UAE (≥16.6 mg/24h) had elevated levels of ALK (71 units/L (42-202)) compared to subjects in 1^{st} quartile (69 units/L (34-229)) (data not shown).

Relationship between NAFLD markers among those subjects with normal, mild decreased renal function or with renal hyperfiltration are displayed in Table 4. Stratifying NAFLD associated markers for the degree of eGFR, trends across different groups for AST, ALK and bilirubin were statistically significant (all $p<0.05$). Subjects with eGFR $\geq$ 125 ml min^{-1} $1.73m^{-2}$ had significantly higher levels of AST and ALK than subjects with an estimated GFR below 125 ml min^{-1} $1.73m^{-2}$. In contrast, hyperfiltrating subjects had significantly lower levels of bilirubin than subjects with normal or mild decreased renal function.

In multivariate logistic regression analysis, after adjustment for age and sex, ALK was significantly associated with risk of progression of renal function measured with serum creatinine and eGFR in our subjects ($p\leq0.008$), with odds ratios of 0.97 to 1.03 (Table 4, Model A). Odds ratio for ALK were attenuated slightly but remained significant after further adjustment for duration of diabetes, HbA1c and BMI (Table 4, Model B).

DISCUSSION

Previous studies documented that NAFLD is associated with an increased prevalence and incidence of CKD in diabetic subjects (13, 16, 17, 24). In the present study we found significant associations of NAFLD associated markers, including AST, ALT, ALK, GGT and bilirubin with parameters of renal function in normoalbuminuric type 1 diabetic patients with normal or mild decreased renal function. Furthermore, we demonstrated that concentrations of ALT, GGT and bilirubin worsened in parallel with increased in quartiles of serum creatinine and ALK with increased in quartiles of UAE. In addition, concentrations of AST and ALK were higher in hyperfiltrating subjects. Finally, in multivariate logistic regression analysis only ALK was significantly associated with renal function parameters, after adjustment for covariates.

The most obvious explanation for our findings is that higher NAFLD markers may reflect fatty change in the liver and the coexistence of metabolic syndrome, an atherogenic condition closely associated with NAFLD and development of renal disease. In correlation analysis majority of NAFLD markers and renal parameters significantly correlated with components of metabolic syndrome, most notably with WHR, a measure of obesity and better predictor of cardiovascular diseases than waist circumference and BMI (25). In addition, metabolic syndrome is an independent risk factor for CKD and NAFLD (2, 26). Insulin resistance is the central pathophysiological phenomenon of metabolic syndrome, and it has been shown that NAFLD markers are associated with insulin resistance in type 1 diabetes and that insulin resistance contributes to the progression of nephropathy in type 1 diabetes (27, 28). It has been suggested that NAFLD may be involved in the pathogenesis of CKD through the systemic release of various proinflammatory and procoagulant mediators from steatotic liver including CRP, TNF-alfa, TGF-beta 1, PAI-1, interleukin-6, advanced glycated end-products, and other pro-inflammatory cytokines (13). However, it has been shown that

NAFLD is associated with an increased prevalence of CKD in type 1 diabetes independently of metabolic syndrome (17, 24). Moreover, it has been demonstrated that patients with NAFLD and biopsy-proven NASH have decreased eGFR and abnormal albuminuria independently of insulin resistance and components of metabolic syndrome (29, 30).

Although AST, ALT and GGT are the most commonly elevated enzymes found in patients with NAFLD (1, 4, 8), some patients with biopsy proven NAFLD may present with an isolated elevation of ALK instead of the more typical liver enzymes (7). In our study we observed that ALK levels were significantly higher in subjects in the highest quartile of UAE compared to those in lowest quartile, and that only ALK was associated with disturbances of renal function parameters, after adjustment for covariates. It is possible that ALK is associated with albuminuria in type 1 diabetes through pathogenic mediators from the liver, including proinflammatory cytokines, who are documented to disrupt the glomerular endothelial glycocalyx leading to microalbuminuria (31). In addition, it has been shown that urinary ALK has a potential value in the diagnosis of nephropathy in diabetes (32), and that ALK was an independent risk factor for cardiac death in subjects with advanced CKD (33). Moreover, we have previously observed that insulin resistance, the most important antecedent of microalbuminuria, was independently associated with ALK in type 1 diabetes (27).

In multivariate logistic regression analysis we found that ALK levels tend to be higher in patients with lower serum creatinine levels and in those with higher eGFR, which seems to be contradictory to the previous studies indicating that higher ALK levels are not associated with worsening of renal function. However, the levels of ALK was especially increased in hyperfiltrating subjects. Hyperfiltration in type 1 diabetes is associated with ultrastructural changes and increased risk of developing diabetic nephropathy and usually precedes changes in albuminuria by several years (34, 35). Moreover, kidney lesion in obesity, condition associated with NAFLD, is characterized by hyperfiltration and cosequent glomerulomegalia,

podocyte hypertrophy and intracellular accumulation (36). In addition, fat not only accumulate in the liver, but also in the kidney and may play a role in glomerular and tubular changes leading to hyperfiltration (36, 37). It was also suggested that glomerular hyperfiltration may be considered as a new marker of metabolic risk (38). It is obvious that hyperfiltration is associated with the higher risk of developing diabetic nephropathy and may mask a pathological decline in glomerular function.

The present study has a number of potential limitations. First, our study was cross-sectional, which limited our ability to infer a causal relation between NAFLD markers and risk for the progression of renal disease in type 1 diabetes. Second, our study is limited by the unavailability of performing liver biopsy, the "gold standard" procedure for diagnosis of NAFLD. However, most of our patients with the higher serum markers of liver damage, including ALT, AST and ALK, had ultrasound-proven fatty liver that may be reasonable noninvasive surrogate measures (11, 39), although full histological spectrum of NAFLD may be present in subjects with normal liver enzymes (40). Third, we used estimated GFR rather than more precise measures of kidney function. Fourth, our analyses were based on measurement of UAE, serum creatinine and eGFR on two consecutive days that may not reflect the relation over time. According to these limitations, our results can probably be considered as conservative estimates of the relationship between NAFLD markers and renal function in normoalbuminuric type 1 diabetic patients.

In conclusion, we have shown that NAFLD associated markers, including ALT, AST, ALK, GGT and bilirubin are associated with renal function parameters in normoalbuminuric type 1 diabetic patients. Elevated liver markers might reflect liver fatty and consequent pathophysiological changes predating the development of renal disease in the context of the metabolic syndrome, an atherogenic condition closely associated with both NAFLD and kidney disease. In fact, we found that majority of NAFLD and renal markers significantly

correlated with metabolic parameters included in the diagnosis of metabolic syndrome (WHR, HbA1c, HDL cholesterol, triglycerides and blood pressure). After adjustment for covariates only ALK was associated with progression of renal disease measured with serum creatinine and eGFR. Whether the detection of elevated ALK levels in normoalbuminuric type 1 diabetic patients has predictive value for development of renal disease needs to be assessed in further follow-up studies.

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Table 1: Clinical and metabolic characteristics of all patients

VARIABLE	VALUE
Age (years)	34 (18-65)
Duration of diabetes (years)	12 (1-42)
Body mass index (kg/m^2)	24 (15-37)
Waist to hip ratio	0.81±0.07
Fasting glucose (mmol/L)	5.4 (2.7-10.2)
Hemoglobin A1c (%)	7.43±1.63
Systolic blood pressure (mmHg)	120 (79-180)
Diastolic blood pressure (mmHg)	80 (50-100)
Total cholesterol (mmol/L)	5.0±0.8
LDL cholesterol (mmol/L)	2.8±0.7
HDL cholesterol (mmol/L)	1.7±0.4
Triglycerides (mmol/L)	0.91 (0.3-4.1)
AST (units/L)	20 (10-146)
ALT (units/L)	19 (7-171)
ALK (units/L)	69 (11-229)
GGT (units/L)	16 (7-553)
Bilirubin (µmol/L)	12 (5-103)
Ferritin (µg/L)	55 (5-697)
Serum creatinine (µmol/L)	71±14
eGFR (ml min^{-1} $1.73m^{-2}$)	106±16
Urinary albumin excretion (mg/24h)	11.0 (1.7-29.8)

eGFR, estimated glomerular filtration rate.

Table 2: Spearman correlation analysis of associations of NAFLD markers and renal function parameters with metabolic parameters

Variable	AST	ALT	ALK	GGT	bilirubin	creatinine	UAE	eGFR
Duration of diab.	0.04	0.03	-0.06	0.14*	-0.04	0.00	0.14*	-0.29*
BMI	0.07	0.05	0.13*	0.01	0.13*	0.15*	-0.02	-0.10
WHR	0.14*	0.25*	0.25*	0.37*	0.16*	0.39*	0.01	-0.02
HBA1c	0.06	0.11*	0.14*	0.11*	-0.13*	-0.14*	0.07	0.15*
Total cholesterol	0.01	0.05	0.02	0.16*	-0.14*	-0.01	0.02	-0.21*
LDL cholesterol	-0.08	0.07	0.05	0.14*	-0.81	0.08	0.03	-0.18*
HDL cholesterol	0.01	-0.11*	-0.13*	-0.03	-0.09	-0.19*	-0.13*	-0.17*
Triglycerides	0.02	0.18*	0.19*	0.23*	-0.03	0.07	0.11*	0.06
Systolic BP	0.06	0.09	0.12*	0.13*	0.08	0.11*	0.09	-0.08
Diastolic BP	-0.05	0.02	0.16*	0.11*	0.03	0.08	0.23*	-0.01
Serum creatinine	0.01	0.16*	0.01	0.18*	0.29*			
eGFR	0.05	0.01	0.23*	-0.01	-0.12*			
UAE	-0.05	0.04	0.06	0.11*	0.03			

BMI, body mass index; WHR, waist to hip ratio; HbA1c, hemoglobin A1c; GFR, estimated glomerular filtration rate; UAE, urinary albumin excretion rate

*P<0.05

Table 3: Quartiles of serum creatinine

	1st quartile (<63 μmol/L)	2nd quartile (≥63<71)	3rd quartile (≥71<80)	4th quartile (≥80 μmol/L)	P for trend
AST (units/L)	20 (10-146)	20 (12-113)	19 (12-143)	21 (12-55)	0.3
ALT (units/L)	18 (9-146)	19 (7-69)	18 (8-171)	23 (10-120)	0.007
ALK (units/L)	68 (34-208)	66 (36-125)	69 (25-229)	71 (11-120)	0.7
GGT (units/L)	14 (8-213)	15 (7-117)	17 (7-553)	18 (8-100)	0.006
Bil. (μmol/L)	10 (5-43)	12 (6-31)	13 (5-46)	14 (7-113)	<0.001

Bil, bilirubin.

Table 4: Levels of NAFLD markers depending on level of estimated glomerular filtration rate

Variable	eGFR >60≤90	eGFR >90≤125 ml min^{-1} $1.73m^{-2}$	eGFR >125	P
AST (units/L)	18 (12-42)	20 (10-143)	21 (13-146)	0.02
ALT (units/L)	18 (8-58)	20 (7-171)	20 (10-146)	0.1
ALK (units/L)	64(11-120)	68 (34-202)	81 (38-229)	<0.001
GGT (units/L)	15 (7-48)	16 (7-753)	16 (8-54)	0.5
Bilirubin (µmol/L)	13 (7-46)	13 (5-113)	9 (5-28)	0.02

Table 5: Multivariate logistic regression analysis of NAFLD markers with risk of progression of renal disease in type 1 diabetes

Independent variable	Group	Model A	Model B
AST	1	1.00 (0.98-1.02)	1.00 (0.98-1.02)
	2	0.98 (0.96-1.00)	0.98 (0.96-1.00)
	3	1.00 (0.98-1.02)	0.99 (0.97-1.02)
ALT	1	1.01 (0.99-1.02)	1.01 (0.99-1.02)
	2	0.99 (0.97-0.99)	1.00 (0.98-1.01)
	3	0.98 (0.97-1.00)	0.98 (0.96-0.99)
ALK	1	1.00 (0.99-1.01)	1.00 (0.99-1.01)
	2	0.98 (0.97-0.99)*	0.98 (0.97-0.99)*
	3	1.01 (1.00-1.03)*	1.02 (1.00-1.03)*
GGT	1	1.01 (0.99-1.02)	1.00 (0.99-1.02)
	2	0.99 (0.99-1.00)	0.99 (0.99-1.00)
	3	0.99 (0.99-1.00)	0.99 (0.99-1.00)
Bilirubin	1	0.99 (0.96-1.02)	0.99 (0.97-1.02)
	2	1.03 (0.99-1.07)	1.03 (0.99-1.07)
	3	0.97 (0.93-1.00)	0.97 (0.94-1.01)

Group1: urinary albumin excretion rate; Group 2: serum creatinine; Group 3: estimated glomerular filtration rate.

Data are OR (95% CI) from separate models. Model A adjusted for age and sex; model B adjusted for age, sex, duration of diabetes, BMI and HbA1c.

*P < 0.05.