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Alcohol Dehydrogenase Isoenzymes and Aldehyde Dehydrogenase Activity in the Serum of Patients with Non-alcoholic Fatty Liver Disease

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Abstract. *Background/Aim: Non-alcoholic liver disease (NAFLD) is one of the most common causes of chronic liver disease, and its prevalence and medical importance is increasing worldwide. Changes in enzyme activity in liver cells in various liver diseases are reflected by an increase in serum enzymatic activity. For example, alcohol dehydrogenase activity (ADH) and aldehyde dehydrogenase (ALDH), that occur in the liver in large quantities, correlate with disease severity during cirrhosis. In the current study, the activity of ADH isoenzymes and ALDH in the serum of patients with NAFLD was investigated. Materials and Methods: Serum samples were collected for routine biochemical studies from 55 patients with NAFLD patients and from 50 healthy individuals. Class I and II ADH and ALDH activity were measured by spectrofluorometric method. Photometric methods were used to measure ADH class III, IV and total ADH activity. Results: Total ADH activity was significantly higher in non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) than in healthy individuals (44 and 48.5% activity, respectively). The median total activity of ADH was 1,164 mU/l in patients with NAFLD, 1,258 mU/l in NASH and 648 mU/l in the control group. The increase in ADH class I and II isoenzyme in serum of patients with NAFL and NASH was statistically significant. The activity of ADH I, ADH II, and total ADH significantly increased with increasing disease progression. Conclusion: The activity of isozymes of class I and II alcohol dehydrogenase in patients with NAFLD is enhanced and appears to be due to the release of these isoenzymes from damaged hepatocytes.*

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Liver disease is a significant burden for public health systems worldwide. Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver diseases comprising nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NAFLD is the most common disease of the liver in the Western world, with an estimated median prevalence of 20%, ranging from 6.3-33% depending on the population, ethnic origin, and diagnostic evaluation (1). NAFL, the accumulation of fat in the liver caused by factors other than alcohol, is a frequent manifestation of metabolic syndrome, leading to hypertriglyceridemia and abnormal hepatic fat accumulation, presenting both as simple steatosis (NAFL) and NASH, the latter combined with fibrosis. Most patients have simple 'steatosis' or NAFL without inflammation or fibrosis (2). Liver biopsy is still the gold standard in the final diagnosis, but the high incidence of the disease and the invasive nature of the test mean that the biopsy of the liver is impractical. The development of non-invasive biomarkers for non-alcoholic liver disease has become a major concern.

Some studies show that changes in enzyme activity in the liver cells in the course of liver diseases are reflected by the change of its activity in the serum. Serum alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activity has been noted as an indicator of liver cell injury (3, 4). Moreover, the activity of ADH and its isoenzymes in the serum may primarily be elevated during *e.g.* hepatocellular carcinoma, cirrhosis, viral hepatitis, alcoholism (5). Human ADH comprises a family of enzymes which have been grouped into several classes. Class I is a classical liver ADH, but is also detected in the gastrointestinal tract. Class II ADH

in humans is found only in the liver, whereas class III is present in all tissues examined to date. Class IV isoenzyme of ADH is preferably expressed in the upper part of the digestive tract (6). In humans, liver ALDH exists as several isoenzymes which differ in their kinetic properties, electrophoretic mobility and subcellular location (7).

In the present study, we investigated the effect of liver cell damage by steatosis on serum ADH isoenzymes and ALDH activity. We hypothesized that the changes in activity of ADH and ALDH in hepatocytes in the course of NAFLD are reflected in the plasma and perhaps could be helpful for diagnosing of this disease.

Materials and Methods

Patients. The research protocol was approved by the Human Care Committee of the Medical University in Bialystok, Poland (Approval no. R-I-002/181/2016). All patients gave their informed consent for the examination.

Serum samples were taken for routine biochemical investigations from 55 patients suffering from NAFLD (32 males and 23 females, age range =24-75 years) hospitalized in the Department of Infectious Diseases and Hepatology University Hospital, Medical University of Bialystok (Poland).

All patients were diagnosed with fatty liver by ultrasonography, and were clinically considered to have NAFLD. For the analysis, patients were divided into two groups: 30 patients with NAFL and 25 patients with NASH. Disease activity grade and fibrosis stage were quantitatively scored according to the METAVIR classification (grade 1 was found in 24 patients, grade 2 in 12, grade 3 in 10 and grade 4 was observed in nine) (2). Appropriate exclusion of liver disease of other etiologies, including alcohol-or drug-induced liver disease, autoimmune hepatitis or viral, cholestatic and metabolic liver diseases, was performed using specific clinical, biochemical, histological and radiographic criteria. All patients had a negative history of alcohol abuse as indicated by an average daily consumption of 20 g or less (alcohol consumption was assessed using a validated questionnaire). None of the patients were on treatment with corticosteroids.

Serum samples taken from 50 volunteers (30 men and 20 women, aged 25-66 years) with normal results for all physical and blood examinations. Controls were selected from healthy community residents who attended the hospitals for routine physical check-ups at the Department of Preventive Medicine. Controls were recruited from the same geographical location and ethnic populations as the patients. All persons of the control group drank alcohol occasionally and self-reported an intake of <20 g of ethanol per week.

Determination of total ADH activity. Total ADH activity was estimated by photometric method with p-nitrosodimethylaniline (NDMA) as a substrate (8). The reaction mixture (2 ml) contained 0.1 ml of serum and 1.8 ml of a 26 mM solution of substrate in 0.1 M of sodium phosphate buffer, pH 8.5, and 0.1 ml of a mixture containing 0.25 M *n*-butanol and 5 mM NAD. The reduction of NDMA was monitored at 440 nm on a Shimadzu UV/VIS 1202 spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany) (LOD/LOQ is 10-8 M).

Determination of total ALDH activity. ALDH activity was measured using a fluorogenic method based on the oxidation of 6-methoxy-2-naphthaldehyde to the fluorescent 6-methoxy-2naphthoate (9). The reaction mixture contained 60 ml of serum, 60 ml of substrate, 20 ml of 11.4 mM NAD and 2.8 ml of 50 mM of sodium phosphate buffer, pH 8.5. The mixture also contained 50 ml of a 12 mM solution of 4-methylpyrazole as a specific inhibitor of ADH activity. The fluorescence was read at excitation wavelength 310 and emission wavelength 360 nm (LOD/LOQ is 10-9 M).

Determination of class I and II ADH isoenzymes. Class I and II ADH isoenzyme activity was measured using fluorogenic substrates (4-methoxy-1-naphthaldehyde for class I and 6-methoxy-2-naphthaldehyde for class II) in reduction reaction according to Wierzchowski *et al*. (10). The assays were performed in a reaction mixture containing serum (60 ml), substrate (150 ml of 300 mM), NADH (100 ml of 1 mM) and 0.1 M of sodium phosphate buffer, pH 7.6 (2.69 ml) under conditions previously described (11). The measurements were performed on a Shimadzu RF-5301 spectrofluorophotometer (Shimadzu Europa GmbH, Duisburg, Germany) at excitation wavelenghth 316 nm for both substrates and emission of 370 nm for class I and 360 nm for class II isoenzymes.

Determination of class III ADH isoenzyme. The assay mixture for class III ADH activity contained serum (100 ml), n–octanol as a substrate (31 ml of 1 mM), NAD (240 ml of 1.2 mM) in 0.1 M NaOH-glycine buffer pH of 9.6 (12). The reduction of NAD was monitored at 340 nm and 25˚C on a Shimadzu UV/VIS 1202 spectrophotometer (LOD/LOQ is 10-8 M).

Determination of class IV ADH isoenzyme. The assay mixture for class IV ADH activity contained serum (50 ml), m–nitrobenzaldehyde as a substrate of (132 ml of 80 mM), NADH (172 ml of 86 mM) in 0.1 M sodium phosphate buffer pH 7.5 (13). The oxidation of NADH was monitored at 340 nm and 25˚C on a Shimadzu UV/VIS 1202 spectrophotometer (LOD/LOQ is 10-8 M).

Statistical analysis. A preliminary statistical analysis (chi-square test) revealed that the distribution of ADH and ALDH activities did not follow a normal distribution. Consequently, the Wilcoxon test was used for statistical analysis. Statistical significant differences were defined as comparisons resulting in $p<0.05$.

Results

The activity of ADH, its isoenzymes and ALDH in serum is shown in Table I. Total ADH activity was significantly higher in patients with NAFL and NASH than in healthy individuals (44 and 48.5% of activity, respectively). The median total activity of ADH was 1,164 mU/l in patients with NAFL, 1,258 mU/l in those with NASH and 648 mU/l in healthy individuals. Analysis of ALDH activity did not show any significant difference between the study groups and healthy individuals.

Comparison of ADH isoenzyme activity showed that the median activity of ADH I in the NAFL and NASH group was about 45% and 51% higher, respectively, in the comparison to the control level (2.26 mU/l). This increase was statistically significant ($p<0.01$). The median activity of ADH II was significantly higher in patients with NAFL and

Table I. *Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activity in the serum of patients with non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) compared to healthy controls.*

Group	ALDH (mU/l)		ADH (mU/l) I		II		III		IV		Total	
	Mean	Median (range)	Mean	Median (range)	Mean	Median (range)	Mean	Median (range)	Mean	Median (range)	Mean	Median (range)
Control (n=50)	2.49	2.60 (1.22-6.27)	2.14	2.26 (0.54-5.28)	13.77	14.42 (5.35-23.48)	10.16	10.68 (6.14-18.63)	4.51	4.98 (2.14-10.74)	612	648 (241-1216)
NAFL (n=30)	2.55	2.78 (1.25-6.46)	4.02	4.11 (1.04-6.86)[a]	21.43	22.38 (10.41-46.75)[a]	11.09	11.47 (6.84-19.68)	5.14	5.36 (2.28-11.68)	1138	1164 (373-2977)[a]
NASH (n=25)	2.76	2.94 (1.30-6.88)	4.22	4.62 (1.21-7.03)[ab]	22.97	23.54 (10.86-49.42)[a]	11.36	11.96 (7.04-20.25)	5.43	5.62 (2.43-12.06)	1197	1258 (412-3642)[ab]

Significantly different at $p<0.01$ *vs*. [a]control, and [b]NAFL.

Table II. *Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activity in the serum of patients with non-alcoholic fatty liver disease (NAFLD) according to the grade of fibrosis.*

Group	ALDH (mU/l)		ADH (mU/l) I		II		III		IV		Total	
	Mean	Median (range)	Mean	Median (range)	Mean	Median (range)	Mean	Median (range)	Mean	Median (range)	Mean	Median (range)
Control (n=50)	2.49	2.60 (1.22-6.27)	2.14	2.26 (0.54-5.28)	13.77	14.42 (5.35-23.48)	10.16	10.68 (6.14-18.63)	4.51	4.98 (2.14-10.74)	612	648 (241-1216)
Grade 1 (n=24)	2.61	2.77 (1.25-6.16)	3.75	4.03 (1.04-6.25)[a]	21.85	22.11 (10.41-42.66)[a]	11.03	11.12 (6.84-18.53)	5.08	5.18 (2.28-11.36)	1124	1150 (373-2704)[a]
Grade 2 (n=12)	2.75	2.82 (1.58-6.42)	4.10	4.17 (1.36-6.68)[a]	22.56	22.89 (10.81-44.17)[a]	11.20	11.26 (7.06-18.69)	5.28	5.39 (2.75-11.56)	1187	1204 (444-2943)[a]
Grade 3 (n=10)	2.86	2.90 (1.64-6.73)	4.31	4.47 (1.88-6.95)[a]	23.26	23.48 (11.34-47-55)[a]	11.44	11.74 (7.31-18.90)	5.62	5.69 (2.99-11.87)	1217	1240 (503-3234)[a]
Grade 4 (n=9)	2.94	3.01 (1.78-6.88)	4.67	4.75 (2.01-7.03)[a]	23.70	23.92 (11.98-49.42)[a]	11.83	11.98 (8.12-20.25)	5.70	5.81 (3.17-12.06)	1246	1294 (605-3642)[a]

Significantly different at $p<0.01$ *vs*. [a]control.

NASH than in controls. The other tested classes of ADH isoenzymes had higher activities in the patient groups than in controls, but the differences were not statistically significant ($p>0.01$).

The activity of specific ADH isoenzymes depending on the progression of fibrosis was analyzed. ADH I and ADH II activity tended to increase with disease progression (Table II). Significantly higher class I and II ADH activity was found in the patients with NAFLD regardless of stage in comparison to the control group ($p<0.01$). The other isoenzymes did not exhibit any marked differences of activity among patients with different advancing stages of the fibrosis. The serum level of total ADH activity was significantly higher in both NAFL and NASH groups for each grade compared with the control group.

Discussion

NAFLD is characterized by hepatic steatosis, without a history of excessive alcohol use, in the absence of other known liver disease. NAFL can be seen in any setting in which there is an excess of energy intake and increased hepatic lipid storage in the form of triglycerides. Steatosis may be worsened by *de novo* lipogenesis in the liver and

decreased export of triglycerides from the liver in the form of very-low density lipoproteins (14). Whereas NAFL has negligible risk of progression, patients with NASH often develop cirrhosis or hepatocellular carcinoma. The prevalence of NAFL in the global population is around 25% and in the presence of obesity, as high as 51%. The overall global prevalence of NASH is estimated to be between 1.5% and 6.45% (15). NAFLD is most commonly asymptomatic, although some patients have non-specific complaints, such as fatigue, right upper quadrant discomfort or epigastric fullness.

Greater effort is needed in screening for early diagnosis to improve the effectiveness of treatment of patients with NAFLD. It is important to distinguish people with advanced disease or who are at risk of developing advanced disease from those who have a simple disease and with disease that does not develop further. Liver biopsy is not a practical tool in population studies, although the disease is still being diagnosed histologically (16). Diagnosis of NAFLD at an early stage is problematic because the disease is usually asymptomatic. The development of modern non-invasive imaging, biochemical and genetic studies will undoubtedly provide future clinicians with a wealth of information and ways to better understand pathogenesis and targeted treatment.

All harmful factors acting on the liver and damaging the liver cells can eventually cause fibrosis. Clinical precursors for advanced fibrosis in NAFLD include: diabetes mellitus, obesity and increased aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (17). AST is a better predictor for advanced fibrosis than ALT. Angulo *et al*. showed that an AST/ALT ratio greater than 1 is associated with advanced fibrosis (18). Other laboratory parameters related to fibrosis are serum ferritin and the AST:platelet ratio index (APRI) (19, 20). These simple markers are neither sensitive nor specific enough in isolation. Better understanding of the pathophysiology of the disease has allowed us to explore more specific, biomarker-based mechanisms. More complex evaluations include markers related to matrix turnover. Guha *et al*. developed the Enhanced Liver Fibrosis Panel, a panel comprising tissue inhibitor of matrix metalloproteinase 1, aminoterminal peptide of pro-collagen III and hyaluronic acid (21). Although different markers in non-alcoholic liver disease have been studied, no diagnostic significance was found for ADH isoenzymes and ALDH activity. The present study demonstrated that the total serum ADH activity during NAFLD changed. The increase in total ADH activity was positively correlated with ADH I and ADH II, and thus the increase in total ADH in NAFL and NASH is increased by ADH class I and II isoenzymes. Over 95% of ADH is present in liver cells. Class II ADH occurs only in hepatocytes. Thus, elevated serum activity of ADH I and ADH II in patients with NAFLD appears to be due to isoenzymes released from altered hepatocytes. Changes in the serum activity of other ADH isoenzymes were not significant in patients with NAFLD. ALDH occurs in hepatocytes, although ALDH activity appears to be disproportionately low in relation to ADH activity. The serum ALDH level was not significantly different in patients with NAFLD compared to the control group.

NAFLD (especially NASH) is also a cause of progressive fibrosis of liver tissue. NAFLD accompanied by cellular injury and necrosis leads to the release of cytoplasmic enzymes such as ADH. There are several noninvasive scoring systems designed to increase the detection of NAFLD and advanced fibrosis, but the best way to incorporate these into a screening model is not clear. Our research showed that the activity of class I and II ADH in NAFLD increased in parallel with disease progression. We found a tendency for overall activity of ADH to increase with the progression of the disease. Other markers of liver damage (bilirubin, aminotransferase levels), also increased significantly. The activity of both aminotransferase and ADH correlates with severity and regular monitoring may be a diagnostic marker of liver cell damage during NAFLD.

In most cases, non-alcoholic liver disease is a random diagnosis caused by changes in the chemical profile or when imaging for other purposes shows steatosis in the liver. In the absence of accidental discovery, patients are often asymptomatic until decompensation of liver function occurs. There is an urgent need to find a less invasive screening method for the general population, stratifying the severity of the disease and disease progression. Our research indicates that increased isoenzyme levels of ADH class I and II in patients with NAFL appear to be due to the release of this isoenzyme from damaged hepatocytes, and serum ADH I and II assays may be helpful in NAFLD diagnostics.

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