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## The activity of N-acetyl- $\beta$ -D-hexosaminidase in serum and urine of parenterally fed patients

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### Summary

**Background:**

The aim of the study was evaluation of the influence of parenteral nutrition on the catabolism of glycoconjugates reflected by changes in the activity of N-acetyl- $\beta$ -D-hexosaminidase (HEX) in serum and urine.

**Material/Methods:**

Blood from the cubital vein and daily collections of urine were obtained from 23 patients fed parenterally. Specimens from each patient were collected three times: before the beginning of parenteral nutrition and subsequently on the fifth and tenth day of intravenous alimentation. HEX activity in serum and urine was determined by the colorimetric method of Zwierz et al., as modified by Marciniak et al.

**Results:**

During the parenteral nutrition, the serum concentration of the HEX activity significantly decreased on the fifth day ( $p < 0.0439$ ), and then significantly increased on the tenth day ( $p < 0.0214$ ) of the parenteral feeding. The parenteral nutrition did not affect significantly the concentration of the HEX activity in the urine.

**Conclusions:**

The early suppressive effect of parenteral feeding on the catabolism of glycoconjugates, is reflected by the reduced HEX activity in the serum and urine. The return of the HEX activity after 10 days of parenteral nutrition, to the high level before the nutrition, may indicate on the remodeling and the adaptation of human organism (including liver), to the intravenous alimentation. The concentration the serum activity of HEX may facilitate the evaluation of the influence of parenteral nutrition on the glycoconjugates catabolism.

**Key words:**

**serum • urine • N-acetyl- $\beta$ -D-hexosaminidase • parenteral nutrition**

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## BACKGROUND

The intravenous application of all necessary nutrients in the form allowing immediate absorption has been named parenteral nutrition [1]. Parenteral nutrition is an aggressive form of treatment. It may provoke number of metabolic disorders such as: hepatic disturbances, hyper- and hypoglycemia, lactic acidosis, uremia, respiratory failure and deficiencies in the fatty acids resulting from bypassing of gastrointestinal tract and liver [1,2]. In order to avoid complications of the parenteral nutrition, a proper evaluation of the acid-base homeostasis and water-electrolyte balance tests, as well as protein, fat and carbohydrates balance examination is necessary [1]. Periodical evaluation of appropriate organs and systems, particularly liver, by the determination of aspartate and alanine aminotransferases,  $\gamma$ -glutamyltransferase, alkaline phosphatase, bilirubin, albumin, prealbumin and lipoprotein X (LpX) concentrations, is also recommended [1]. However up to now, no appropriate method allowing for early recognition and efficient prophylaxis of complications connected with parenteral nutrition has been found [1,3].

The enzyme which may reflect liver glycoconjugates (glycoproteins, proteoglycans, glycolipids) catabolism during parenteral nutrition, is N-acetyl- $\beta$ -D-hexosaminidase (HEX). HEX is the most active among lysosomal exoglycosidases [4]. HEX catalyses the release of N-acetylglucosamine and N-acetylgalactosamine residues from the non-reducing oligosaccharide chain of glycoconjugates. The main substrates of HEX are glycosaminoglycans and glycolipids [5].

The aim of our research was evaluation the influence of parenteral nutrition on the glycoconjugate's catabolism, reflected by the activity of N-acetyl- $\beta$ -D-hexosaminidase (HEX) in the serum and urine of intravenously fed patients. Our aim was also to establish catabolism of glycoconjugates as a potential parameter evaluating the condition of intravenously fed patients.

## MATERIAL AND METHODS

The consent of Bioethics Committee, Medical University of Białystok no. R-I-003/320/2006 has been obtained in order to conduct the study. Examined group included 23 patients (8 women, 15 men, at age of 22–82; average age –  $57.1 \pm 19.37$ ) fed parentally, hospitalized at 1<sup>st</sup> Department of General and Endocrinological Surgery, Medical University of Białystok. Diseases with recognized influence on HEX activity such as: diabetes, obesity, alcoholism, renal and hepatic insufficiencies as well as septic complications connected with introduction of cannula, were the main criteria for exclusion from the examined group.

The nutritional mixtures were provided in all-in-one system with 24 hours injection using infusion pump which allows to maintain constant speed of inflow. Nutritional mixtures have been prepared by Hospital Pharmacy, Medical University of Białystok. Patients were fed with 4 types of mixtures: a) 15% Aminoplasmal E (1000 ml), 10% Intralipid (500 ml), 20% Glucose (1000 ml), Gensulin R (36j), 15% KCl (40 ml), 20%  $MgSO_4$  (10 ml), Addamel (1 bottle), Addiphos (1 bottle), Cernevit (1 bottle); b) 10% Aminoplasmal hepa (1000 ml), 10% Intralipid (500 ml), 10% Glucose (500 ml), Gensulin

R (36j), 15% KCl (40 ml), 20%  $MgSO_4$  (10 ml), Addamel (1 bottle), Addiphos (1 bottle), Cernevit (1 bottle); c) 15% Aminoplasmal E (500 ml), 20% Clinoleic (100 ml), 20% Glucose (1000 ml), Gensulin R (36j), 15% KCl (40 ml), 20%  $MgSO_4$  (10 ml), Addamel (1 bottle), Addiphos (1 bottle), Cernevit (1 bottle); d) 10% Aminosteril KE (500 ml), 20% Clinoleic (100 ml), 20% Glucose (1000 ml), Gensulin R (40j), Vit. B<sub>1</sub> (100 mg), 20%  $MgSO_4$  (10 ml), Addamel (1 bottle), Addiphos (1 bottle), Cernevit (1 bottle).

The examined material consisted of blood from cubital vein and urine from daily collection. The samples from each patient fed parenterally were obtained three times: before the parenteral nutrition, after 5 and 10 days of the intravenous feeding. Collection of the material to enzymatic tests from patients before the parenteral nutrition, after 5 and 10 days of feeding, enabled elimination of the influence of basic disease (which was main reason for parenteral nutrition) on the activity of HEX. Serum (after coagulation) and urine were centrifuged (20 minutes, 4°C;  $4,000 \times g$ ). The supernatants were kept in –80°C for further examination.

Examination of HEX activity was performed in duplicates by Zwierz et al. method [6], modified by Marciniak et al. [7] as follow: 0.01 ml of serum or urine, 0.04 ml of 0,1 M phosphate-citrate buffer at pH 4.7 and 0.03 ml of 20 mM substrate solution (p-Nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide, Sigma, St. Louis, Mo., USA) were pipetted into well on microplate (NUNC). Microplates were mixed in microplates incubator (Varishacker Incubator, Dynatech) at 37°C for 60 minutes. The reaction was stopped by addition of 0.2 ml of 0.2 M borate buffer at pH 9.8. Absorbance of released p-nitrophenol was measured at wavelength 405 nm and read from calibration graph using microplates reader (Elx 800™, Bio-Tek Instruments, Inc. Vermont, USA).

The creatinine concentration in urine was assayed with Jaffe's method without deproteinization, using Cobas Mira plus analyzer Roche.

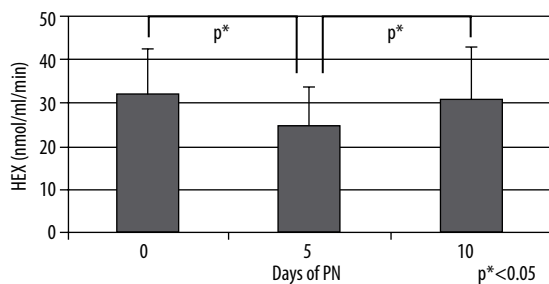
## Statistical analysis

Anova test was used for the statistical analysis. Results were expressed as the mean and SD. A level of  $p < 0.05$  was considered to be significant.

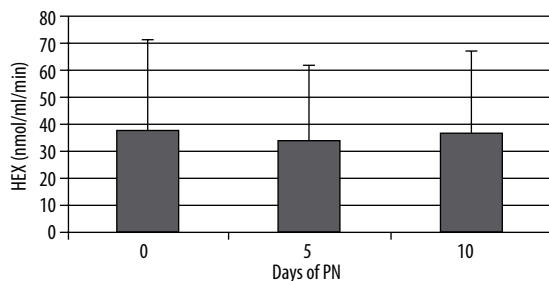
## RESULTS

The mean concentration of the HEX activity in serum of patients before the parenteral nutrition, was  $32.101 \pm 10.647$  nmol/ml/min and  $24.624$  nmol/ml/min, and  $30.836 \pm 12.087$  nmol/ml/min after 5 and 10 days respectively (Figure 1). The concentration of the HEX activity in serum during the parenteral nutrition, decreased significantly ( $p < 0.0439$ ) after 5 days, in comparison to the concentration before parenteral feeding, and significantly increased ( $p < 0.0214$ ) after ten days, in comparison to the 5th day of parenteral feeding (Figure 1).

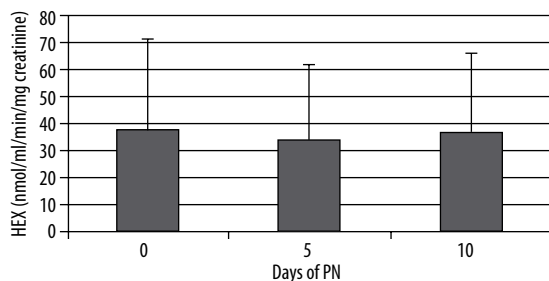
In the urine of patients fed parenterally, the mean concentration of HEX activity before intravenous nutrition was  $38.157 \pm 32.882$  nmol/ml/min; after 5 days –  $33.572 \pm 28.104$  nmol/ml/min, and after 10 days it was  $36.354 \pm 29.013$  nmol/ml/min (Figure 2). The mean HEX activity in urine



**Figure 1.** The concentration of HEX activity in serum of patients fed parenterally. PN – parenteral nutrition.



**Figure 2.** The concentration of HEX activity in urine of patients fed parenterally. PN – parenteral nutrition.



**Figure 3.** The activity of HEX in urine per 1 mg of creatinine. PN – parenteral nutrition.

per 1 g of creatinine before application of parenteral nutrition was  $78.569 \pm 58.323$  nmol/ml/mg of creatinine; after 5 days –  $72.004 \pm 66.652$  nmol/ml/mg of creatinine, and after 10 days it was  $67.822 \pm 34.525$  nmol/ml/mg of creatinine (Figure 3). There was no significant influence of the parenteral nutrition on the HEX activity in the urine (Figures 2 and 3).

## Discussion

During an oral nutrition, liver and intestine are the main organs responsible for preliminary digestion, storage and processing of nutrients. It is the reason why bypassing omission of intestines and liver causes metabolic complications in 15–40% and even 90% of patients fed parenterally [8,9]. Therefore, a basic knowledge about the disturbances of biochemical mechanisms is necessary for sufficient prophylaxis, early recognition and treatment of complications of the parenteral nutrition [1,3].

According to Szajda et al. [10], the activity of lysosomal exoglycosidases may be a predictor of liver glycoconjugates catabolism. Of lysosomal exoglycosidases, they proposed HEX as a predictor of the liver glycoconjugate catabolism, applied as an indicator for cholestasis, fibrosis and hepatocyte damage [10]. The changes in the activity of lysosomal exoglycosidases in serum were found to be due to the metabolic disturbances in tissues and organs in the course of parenteral nutrition. In the liver of rats fed intravenously vascular stasis, increased concentration of leukocytes in capillaries, increase in the number of lysosomes, deposition of fat in macrophages, and the increase in the activity of HEX, GAL, GLU and cathepsin D in hepatic homogenates were observed [11–14]. Parenteral nutrition may be the reason for Kupffer's cells activation and increased release of lysosomal exoglycosidases. According to Poriadkova and Vasilev [11,14], the increase in the hydrolase activity may be the result of an adaptive reaction of organism to the nutritional deficit and reveals reconstructive changes in the lysosomal exoglycosidases structure.

Recently, the activity of HEX has been investigated in many clinical conditions. This is due to presence of HEX in most tissues and body fluids [4,15–19]. The increase in the HEX activity in urine is the most striking abnormality among other biochemical changes in several renal disorders [20]. Results of our research suggest that self-burning of tissues in patients before introduction of parenteral nutrition is reflected by a high activity of the HEX. Our results agree with the report on limitation of the tissue self-catabolism after application of the parenteral nutrition [3]. The significant decrease in the HEX activity in serum, observed at 5<sup>th</sup> day of parenteral feeding (Figure 1), may indicate on the limitation of the self-catabolism of tissues, which gives the possibility for organism to survive in the critical condition. It may be assumed that at the initial stage of the parenteral nutrition, supply of nutrients fulfilled the demand for them that resulted in the decrease of glycoconjugates degradation. After 10 days of parenteral nutrition, the normalization of the catabolic process occurred, resulting in the increase in the glycoconjugates catabolism and activity of HEX in serum, approaching the level before the application of parenteral nutrition. The significant increase in the activity of HEX (Figure 1) in serum after 10 days of parenteral nutrition, in comparison to the activity at 5<sup>th</sup> day of parenteral nutrition, may result from adaptation of human organism to parenteral feeding and may reflect the increase in the glycoconjugate catabolism, resulted from removal of an old and creation of a new tissue by the set of exoglycosidases. The concentration of the HEX activity in urine and the urinary HEX activity calculated per 1g of creatinine, are less convincing, as at the initial stage of the parenteral feeding they had only a tendency to decrease, and after longer period of parenteral nutrition a tendency to increase (Figures 2 and 3).

## CONCLUSIONS

1. The parenteral nutrition significantly decreases the serum HEX activity at 5<sup>th</sup> day and increases at 10<sup>th</sup> day, in comparison to the level before the nutrition.
2. The restoration of the serum HEX activity at 10<sup>th</sup> day of parenteral nutrition may suggest an adaptation of the human organism to the intravenous alimentation or/and rebuilding of the tissue.

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## Serum and urinary $\alpha$ -mannosidase in acute alcohol intoxication

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### Summary

**Background:**

The lysosomal exoglycosidase,  $\alpha$ -mannosidase (MAN) is involved in the cleavage of the alpha form of mannose of oligosaccharides. It also catalyzes the first committed step in the biosynthesis of complex N-glycans. Increased activities of MAN have been reported in the serum of alcohol dependent patients after a heavy drinking period. The objective of this study was to determine the activity serum and urinary  $\alpha$ -mannosidase, after a single but a large dose of alcohol intoxication (binge drinking).

**Material/Methods:**

The serum and urine of eight healthy binge drinkers were collected before binge drinking, and 2 and 5 days after the drinking session. The activity of MAN was determined by the colorimetric method.

**Results:**

Binge drinking session induced the increase in the activity of MAN in serum two days after drinking; whereas the urinary activity of MAN decreased significantly at second day after the drinking. Five days after drinking the serum activity of MAN started to decrease, however remained still higher when compared to the preconsumption level. We have found a positive correlation between urinary MAN and serum aspartate aminotransferase at 2nd day after the drinking. The cut-off value at 81 pKat/ml for the serum MAN activity showed good sensitivity (87.5%) and fair specificity (75%).

**Conclusions:**

Binge drinking-induced increase in the activity of serum MAN is associated with alcohol-induced temporal liver dysfunction, and can be detected two days after the drinking.

**Key words:**

$\alpha$ -mannosidase • serum • urine • aminotransferase

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## BACKGROUND

The lysosomal exoglycosidase,  $\alpha$ -mannosidase (MAN) is involved in the cleavage of the alpha form of mannose from the oligosaccharides. It also catalyzes the first committed step in the biosynthesis of complex N-glycans [1]. Earlier studies found the increased activity of MAN in the serum of alcohol dependent patients after a heavy drinking period [2,3]. It was even reported that serum MAN activity might be a marker of alcohol abuse in persons dependent on alcohol [3]. Other serum exoglycosidases such as  $\beta$ -hexosaminidase and  $\alpha$ -fucosidase, have also been reported in the sera of patients suffering from alcohol dependence, when chronic alcohol intoxication coincided with the liver damage [1–3]. On the other hand, in the saliva of binge drinkers, no any changes in the MAN activity were found [4]. In other exoglycosidase studies, there was found an increase in the serum activity of  $\beta$ -hexosaminidase but not  $\beta$ -glucuronidase and  $\alpha$ -fucosidase, after acute alcohol intoxication [5–8]. *Binge drinkers* are characterized as persons that consume alcohol till intoxication [4]. It is also known that the ethanol administration can inhibit the activity of mannosidase II, most likely by acetaldehyde adduct formation, resulting in the synthesis of cell surface glycoproteins with high mannose structures [1]. As most lysosomal enzymes are glycoproteins, which have carbohydrate moieties consisting of asparagine-linked complex and high-mannose oligosaccharides [1], we can speculate that the same mechanisms which are involved in the decreased synthesis, modification, transport, clearance and degradation of glycoproteins in the liver during alcohol drinking, may also potentially contribute to the degradation of some of these lysosomal enzymes including MAN. Therefore, the increase in the MAN activity might not be observed.

On the other hand, earlier studies reported that alcohol mainly affects cell surface glycoconjugates containing terminal non-reduced mannose [7]. Hence, it is probably that even single heavy drinking occasion can rise the activity of MAN. Therefore, the aim of the study was to examine, whether the single heavy binge drinking session can change the activity of  $\alpha$ -mannosidase.

## MATERIAL AND METHODS

Eight healthy male volunteers aged  $27.2 \pm 2.5$  (mean  $\pm$ SD; range 22 to 31 years), who anticipated heavy drinking on Friday evening, took part in the study. The mean  $\pm$ SD body weight was  $79.1 \pm 9.7$  kg (range 71 to 98 kg). Prior to the experiment, all volunteers were verified clinically to be in good general health. None of the participants were taking medication. All of them have a history of social drinking or occasional drinking, but they did not meet any alcohol abuse criteria. All men were infrequent binge drinkers (reported bingeing 1–11 times per year and/or 1–2 episodes in the past month), who had abstained from alcoholic beverages and drugs for 10 days, before the experiment. The participants stayed at home during the drinking session, under the supervision of sober friends and a physician, who helped verify quantities and the time when drinking stopped. During the alcohol session (7 p.m. to 1 a.m.), participants drank 120–160 g of ethanol (12–16 standard drinks) as 40% vodka (2.0 g/kg of body weight; ranging from 1.42 to 2.5 g/kg), together with light meals and fruit juice (excluding grapefruit

juice). Such amounts of alcohol are common in spirit-drinking countries, including Poland, provoking a tolerable but severe intoxication [8]. The study was approved by the local Bioethical Committee of the Medical University of Białystok, Poland. Informed written consent was obtained from all participants after the explanation of the nature, purpose and potential risks of the study. The subjects were deprived of food and beverages, except water, for 2 h before sample collection. The sets blood and urine samples were collected (before drinking -BD, on second day after drinking, and on fifth day after drinking), and then centrifuged to remove cells. The supernatants were divided into 200- $\mu$ L portions, frozen and kept, until analyzed.

Activity of MAN in supernatants of serum and urine, were determined in duplicates by the colorimetric determination of p-nitrophenol released from p-nitrophenyl- $\alpha$ -D-mannopyranoside (Sigma, USA) [9].

The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined according to standard laboratory techniques (EMAPOL reagents, Poland).

Statistical analysis was performed using Statistica 10.0 PL (Statsoft, Tulsa, OK, USA). Changes of the MAN activity across time were analysed by Friedman's analysis of variance (ANOVA) and Kendall's concordance. For comparison between aminotransferases levels, Wilcoxon matched pair test was used. Spearman's rank correlation coefficient was used to measure the statistical dependence between two variables. Statistical significance was defined as  $p < 0.05$ . Calculations of specificity and sensitivity were performed using STATISTICA's Rapid Deployment of Predictive Models. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

After the binge drinking session, the activity of MAN (pKat/ml) in serum significantly increased at 2<sup>nd</sup> day ( $p = 0.008^{***}$ ) and remained higher at 5<sup>th</sup> day ( $p = 0.032^*$ ), as compared to the level before drinking (Figure 1A). In the urine, the activity of MAN significantly decreased at second day after the binge drinking session ( $p = 0.03^*$ ) (Figure 1B).

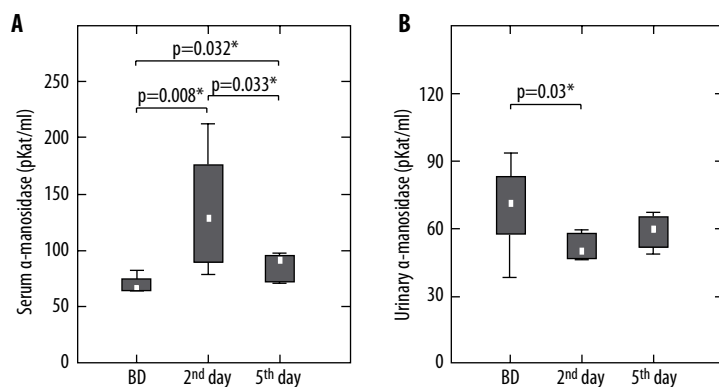
The AST activity was significantly lower at fifth day after binge drinking session than at second day ( $p = 0.021^*$ ) (Table 1). There were no significant differences in the ALT activity between second and fifth day after binge drinking ( $p = 0.402$ ).

We did not find any correlation between amounts of alcohol and: serum and urinary MAN, serum AST and ALT, at any time point after drinking. We have found a positive correlation between urinary MAN and serum AST at 2<sup>nd</sup> day after the drinking ( $r = 0.771$ ,  $p = 0.042^*$ ).

The cut-off value at 81 pKat/ml for the serum MAN activity showed good sensitivity (87.5%) and fair specificity (75%).

## DISCUSSION

An increasing number of young adults prefer alcohol as a recreational drug, tending to concentrate their drinking at weekends [10]. Binge drinking is characterized by the consumption of alcohol leading to intoxication (drinking to get



**Figure 1.** The activity of serum (A) and urinary (B)  $\alpha$ -mannosidase before drinking (BD), on second day after drinking (2<sup>nd</sup> day), and on fifth day after drinking (5<sup>th</sup> day).

**Table 1.** The effect of binge drinking on the serum activity of aspartate (AST) and alanine (ALT) aminotransferases at second day after drinking (2<sup>nd</sup> day), and at fifth day after drinking (5<sup>th</sup> day AD).

Variable	2 <sup>nd</sup> day	5 <sup>th</sup> day	p value
AST	31±10	26±10	0.021
ALT	38±11	36±9	0.402

Values are expressed as mean  $\pm$ SD; n=8; p value <0.05 considered statistically significant; p Value: 2<sup>nd</sup> day to 5<sup>th</sup> day.

drunk), often measured as having more than four (women) or five (men) number of drinks on one occasion. On more than 30% of occasions when men go out to drink, they consume binge amounts of alcohol [10,11].

The increased MAN activity in the serum may be due to the lysosomal membrane permeability in the liver cells and leakage of the enzyme to the cell, intercellular space, and to the serum and finally to the urine [1]. Other potential mechanisms of increased serum MAN activity may include impaired glycosylation and trafficking of MAN to organelles, enhanced synthesis of the enzyme by activated leucocytes, or leakage from damaged cells [8–11]. Inadequately protected intestinal mucosal surface, due to the alcohol-induced failure in glycoprotein synthesis and secretion, becomes considerably more permeable than normally protected mucosa for gut endotoxin (LPS-lipopolysaccharide) [1]. LPS binding to endotoxin receptor CD14, activates Kupffer cells to produce various mediators such as prostaglandin D2 and E2 (PGD2, PGE2), reactive nitrogen and oxygen species (RNS, ROS), endothelin-1 (ET-1), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin 1 and 6 (IL-1, IL-6). Subsequent hepatic hypermetabolic state with toxic metabolites production such as acetaldehyde, reactive oxygen species (ROS), fatty acid ethyl esters (FAEEs), may result in the liver cell damage/death and additional hydrolase releasing [1]. An increased synthesis of lysosomal enzymes during reticuloendothelial cell activation may enhance degradation of linked oligosaccharides with subsequent tissue damage [1,10–13]. Damaged cells as well as the regeneration processes induced by ethanol metabolites (as those released by activated reticuloendothelial cells e.g. Kupffer cells), may be in turn the source of increased lysosomal enzyme activities in serum. The decreased clearance of lysosomal enzymes from the blood has also been proposed to be an explanation for

their increase in the blood. However, a higher level of precursor forms with higher molecular weight than intracellularly localized enzyme forms, seems to be due to increased production and secretion, rather than to decreased elimination or leakage from damaged cells [1]. Therefore, increased serum MAN activity in our study seems to be due to its increased production and secretion in the liver. Observed by us the decrease in the urinary MAN (at second day after drinking) might be due to the temporal renal dysfunction, since heavy alcohol use may cause renal dysfunction/damage [14]. As we noticed the association of urinary MAN with serum AST after binge drinking, it supports the hypothesis that changes in the MAN activity are due to the temporal liver dysfunction. The activity of AST is generally a better and more specific marker of the alcohol-induced liver damage than ALT [15].

As the serum MAN activity showed good sensitivity, it may be applicable to the binge drinking detection. However, the usefulness of serum MAN activity as a laboratory marker of excessive alcohol drinking needs confirmatory further research, based on a relatively large sample to be sufficiently representative of a vast population.

## CONCLUSIONS

Single binge drinking session increases the serum activity of  $\alpha$ -mannosidase, which is associated with the alcohol-induced temporal liver damage. The rise in the serum MAN activity can be detectable two days after a single heavy drinking session.

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## Binge drinking episode increases activity of a senescence marker $\beta$ -galactosidase in serum

### Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
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**Source of support:** None

### Summary

#### Background:

Beta-galactosidase (GAL) is a lysosomal glycohydrolase that catalyzes the hydrolysis of  $\beta$ -galactosides into monosaccharides and is a marker of the senescence process. Increased activities of GAL have been earlier reported in the serum of alcohol dependent patients after a chronic heavy drinking period but not after acute intoxication (called binge drinking). The binge drinking phenomenon is an accelerating and alarming public health issue that requires better prevention.

#### Objectives:

The objective of this study was to determine the activity serum and urinary GAL, after an acute, single, and a large dose of alcohol intoxication in binge drinkers.

#### Material/Methods:

The serum and urine of eight healthy binge drinkers were collected before binge drinking, and 2 and 5 days after the drinking session. The activity of GAL was determined by the colorimetric method.

#### Results:

Binge drinking induced the increase in the serum GAL activity 2 days after drinking and remained still in the rise 5 days after the drinking. The urinary activity of GAL was not changed after drinking. The cut-off value at 77 pKat/ml for the serum GAL activity showed good sensitivity (87.5%) and fair specificity (75%).

#### Conclusions:

Binge drinking-induced increase in the serum GAL activity was detectable even 5 days after drinking, which might be due to the alcohol-induced liver dysfunction. As a single binge drinking session increases serum GAL activity, heavy acute drinking frequent events may potentially be related to the accelerated senescence process of hepatocytes.

#### Key words:

**$\beta$ -galactosidase • binge drinking • acute alcohol intoxication • serum • urine • aminotransferase**

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## BACKGROUND

Binge drinking problem is increasing worldwide. It occurs on a third of drinking occasions. In Poland, it was estimated that 38.5% males and 8.5% females drink alcohol in a binge drink manner [1,2]. Binge drinking increases the risk for numerous acute adverse health and social events including alcohol poisoning, acute myocardial infarction, injuries, accidents, suicide, interpersonal violence, etc [3,4]. Binge drinking is characterized by the consumption of alcohol leading to intoxication (drinking to get drunk), often measured as having more than four (women) or five (men) number of drinks on one occasion (1 standard drink contains 10 g of ethanol) [5,6].

Beta-galactosidase (GAL) is a lysosomal glycohydrolase that catalyzes the hydrolysis of  $\beta$ -galactosides to monosaccharides. In acute alcohol intoxication, the tendency to increase in the salivary GAL activity was noted earlier after a single large ethanol dose [7]. In chronic alcohol intoxication, it was found no significant changes in the serum GAL activity in alcoholics [8], whereas rats chronically exposed to the ethanol, had increased liver GAL activity [9], as compared to the controls. It was also found that hyposialylated forms of transferrin with terminal galactose residues are less eliminated by the hepatocytes, and become senescent glycoproteins [10–12]. GAL expression was also shown to be a reliable indicator of the switch mechanism used by cells to enter the senescence, being a marker of the senescence process [13,14]. The activity of other serum and urinary exoglycosidases e.g.  $\beta$ -hexosaminidase,  $\alpha$ -mannosidase, or  $\alpha$ -fucosidase, have also been reported in alcohol-dependent patients after chronic drinking [11,12,15]; whereas acute alcohol intoxication increased salivary and/or serum and/or urinary activities of  $\beta$ -hexosaminidase,  $\alpha$ -fucosidase,  $\beta$ -glucuronidase and  $\alpha$ -mannosidase [5,16–18].

The aim of the study was to examine, whether the single binge drinking session can change the activity of a senescence marker  $\beta$ -galactosidase in serum and urine.

## MATERIAL AND METHODS

### Participants and procedure

Eight healthy male volunteers aged 27.2 $\pm$ 2.5 (mean  $\pm$ SD; range 22 to 31 years), who anticipated heavy drinking on Friday evening, took part in the study. The mean  $\pm$ SD body weight was 79.1 $\pm$ 9.7 kg (range 71 to 98 kg). Prior to the experiment, all volunteers were verified clinically to be in good general health. None of the participants were taking medication. All of them gave a history of social drinking or occasional drinking, but they did not meet any alcohol abuse criteria. All men were infrequent binge drinkers (reported bingeing 1–11 times per year and/or 1–2 episodes in the past month), who had abstained from alcoholic beverages and drugs for 10 days, before the experiment. The participants stayed at home during the drinking session, under the supervision of sober friends and a physician, who helped verify quantities and the time when drinking stopped. During the alcohol session (7 p.m. to 1 a.m.), participants drank 120–160 g of ethanol (12–16 standard drinks) as a 40% vodka (2.0 g/kg of body weight; ranging from 1.42 to 2.5 g/kg), together with light meals and a fruit juice (excluding grapefruit juice). Such amounts of alcohol are common

in spirit-drinking countries, including Poland, provoking a tolerable but severe intoxication [5]. The study was approved by the local Bioethical Committee of the Medical University of Bialystok, Poland. Informed written consent was obtained from all participants after the explanation of the nature, purpose and potential risks of the study. The sets blood and urine samples were collected [before drinking (BD), on second day after drinking (2<sup>nd</sup> day AD), and on fifth day after drinking (5<sup>th</sup> day AD)], and then centrifuged to remove cells. The supernatants were divided into 200- $\mu$ L portions, frozen and kept, until analyzed.

### $\beta$ -galactosidase assay

Activity of GAL in supernatants of serum and urine, was determined in duplicates by the colorimetric determination of p-nitrophenol released from p-nitrophenyl- $\beta$ -D-galactopyranoside (Sigma, USA) [19].

### Aminotransferases assay

The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined according to standard laboratory techniques (EMAPOL reagents, Poland).

### Statistical analysis

Statistical analysis was performed using Statistica 10.0 (Statsoft, Cracov, Poland) software. Changes in GAL activity across time were analyzed by Friedman analysis of variance (ANOVA) and Kendall concordance. For comparison between aminotransferase levels, Wilcoxon matched pair test was used. Spearman's rank correlation coefficient was used to measure the statistical dependence between two variables. Calculations of specificity and sensitivity were performed using STATISTICA's Rapid Deployment of Predictive Models software. Statistical significance was defined as  $P < 0.05$ .

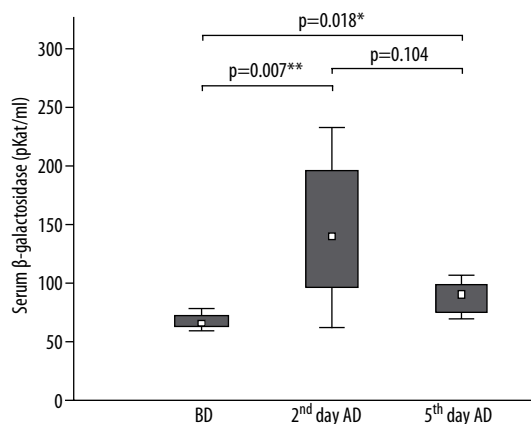
## RESULTS

After the binge drinking session, the activity of GAL (pkat/ml) in serum significantly increased at 2<sup>nd</sup> day (147 $\pm$ 59) ( $p = 0.007^{**}$ ) and remained still in the rise at 5<sup>th</sup> day (91 $\pm$ 12) ( $p = 0.018^*$ ), as compared to the level before drinking (71 $\pm$ 6) (Figure 1). No statistical changes were noted in the GAL activity between 2<sup>nd</sup> and 5<sup>th</sup> day after drinking ( $p = 0.104$ ). In the urine, there were no significant changes in the activity of GAL between 2<sup>nd</sup> day (130 $\pm$ 78) and 5<sup>th</sup> day (128 $\pm$ 29) after the binge drinking session, as compared to the preconsumption activity (122 $\pm$ 61) (Figure 2).

The AST activity (U/L) was significantly lower at fifth day after the binge drinking session (26 $\pm$ 10) than at second day (31 $\pm$ 10) ( $p = 0.021^*$ ). There were no significant differences in the ALT activity between second and fifth day after binge drinking ( $p = 0.402$ ).

We did not find any correlations between amounts of alcohol and: serum and urinary GAL activity, serum AST and ALT, at any time point after drinking.

The cut-off value at 77 pKat/ml for the serum GAL activity at 2<sup>nd</sup> day showed good sensitivity (87.5%) and fair specificity (75.0%).

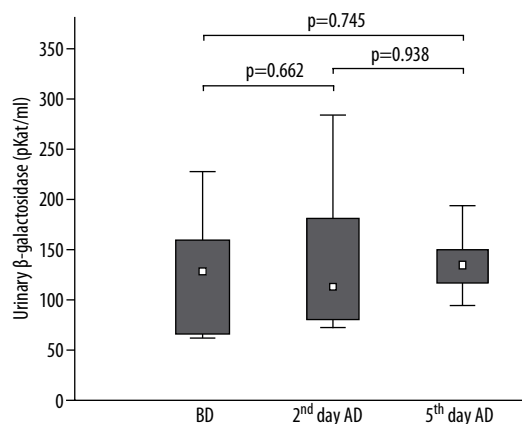


**Figure 1.** The activity of serum  $\beta$ -galactosidase before drinking (BD), on second day after drinking (2<sup>nd</sup> day AD), and on fifth day after drinking (5<sup>th</sup> day AD).

## DISCUSSION

The metabolism of liver glycoconjugates (glycoproteins, glycolipids, and proteoglycans) is seriously affected during alcohol drinking due to the alterations in glycoconjugate synthesis, transport, glycosylation, secretion, degradation, and elimination processes [1,12]. The alcohol-induced pathomechanism of the liver damage is complicated by the fact that the effect of alcohol intoxication on the glycoconjugate metabolism depends not only on the duration of ethanol exposure, but also demonstrates dose-sensitivity [12]. Liver tissue damage is induced by the action of: ethanol (ethanol-water competition mechanism), acetaldehyde, reactive oxygen species (ROS), and nonoxidative metabolites of alcohol – fatty acid ethyl esters (FAEEs) [12].

Various mechanisms have been proposed to increase the activity of serum exoglycosidases (e.g. GAL) during alcohol drinking such as changes in the lysosomal membrane permeability and leakage of the enzyme from lysosomes and subsequently from cells into body fluids including serum [3]. Lysosomal and cellular membranes are more permeable while drinking, which is due to the ethanol action and its metabolite [12]. Acetaldehyde, the main metabolite of ethanol, may modify amino acid residues of the membrane proteins by reacting with their free amino and sulfhydryl groups. Reactive oxygen species, by inducing lipid peroxidation, lipid hydroperoxide formation, and protein disruption, may additively destroy lysosomal and cellular membranes. Also decreased nicotinamide adenine dinucleotide-oxidized/reduced ratio ( $NAD^+/NADH$ ) and decrease in the ATP synthesis, may impair membrane ion channel functions ( $Na^+$ ,  $K^+$ ,  $H^+$ ). Finally, formed FAEEs, may increase the membrane fluidity, hence increasing the lysosomal fragility. Membranes serve as a proximate site of alcohol action [1]. Ethanol and water compete with each other on a target membrane molecules. Glycoproteins attract a large volume of water (up to 95%). Thus displacement of water by ethanol from hydrogen-bonded sites creates the opportunity for allosteric changes that lead to the conformational changes of membrane glycoconjugates [12]. All the abovementioned mechanisms are involved, directly or indirectly, in the destabilization of lysosomal and cellular



**Figure 2.** The activity of urinary  $\beta$ -galactosidase before drinking (BD), on second day after drinking (2<sup>nd</sup> day AD), and on fifth day after drinking (5<sup>th</sup> day AD).

membranes, releasing lysosomal enzymes including GAL. The rise in the serum lysosomal enzyme may also be due to the delayed removal of the enzyme by alcohol-impaired liver, or may result from the hypermetabolic liver state during the drinking with concomitant hydrolase releasing from the activated Kupffer cells [1].

Observed by us the increase in the serum GAL (at second day after drinking) might be due to the liver dysfunction, since increased AST activity was observed earlier in acute alcohol intoxication [1,6]. We found also that the activity of AST, which is generally a better and more specific marker of the alcohol-induced liver damage than ALT [6], decreased at 5<sup>th</sup> day after the drinking, which suggest remitting liver dysfunction.

The serum GAL activity showed good sensitivity and fair specificity, suggesting its applicability to the binge drinking detection, and potentially to the binge drinking prevention in the future. However, the usefulness of serum GAL activity as a laboratory marker of binge drinking needs confirmatory further research, based on a relatively large sample.

Evidence shows that chronic alcohol consumption causes both accelerated (or premature) aging (symptoms of aging appear earlier than normal) and exaggerated aging (symptoms appear at the appropriate time but in a more exaggerated form) [20]. The production of toxic metabolites such as ROS which follows aerobic metabolism is highly enhanced in aging and alcohol consumption. It was also found that the GAL expression was an indicator of the switch mechanism used by cells to enter the senescence process, thus being a marker of the senescence [12–14,20]. Therefore we may speculate that the repetitive binge drinking sessions may accelerate the senescence process in binge drinker's tissues including liver.

## CONCLUSIONS

1. Binge drinking increase of the serum activity of  $\beta$ -galactosidase is due to the acute liver injury.
2. The rise in the serum GAL activity has good sensitivity and can be detectable two days after the binge drinking session.

### 3. Binge drinking frequent events may potentially accelerate the senescence process of liver cells.

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- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

## Is spontaneous recovery from HCV infection more often?

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<b>Background:</b>	Prevalence of anti-HCV in Poland is estimated for about 2%. HCV infection should be confirmed by HCV RNA detection. The aim of this study was confirmation of HCV infection among patient with suspicion of chronic hepatitis C admitted to the Wards of Liver Diseases (WLD).
<b>Material/Methods:</b>	83 patients (47 women, 36 men) in age 20–77 years (mean age 45.77 years) with anti-HCV positive were checked for HCV RNA presence in their blood EDTA plasma. Anti-HCV antibodies were analyzed using Elisa Murex HCV v.4.0 assay. EDTA plasma HCV RNA qualitatively was determined by PCR Cobas Amplicor v.2.0 Roche (the lowest detection 10 IU/ml) and quantitatively by real time PCR Cobas TaqMan v.2.0 Roche (15–1×10 <sup>8</sup> IU/ml).
<b>Results:</b>	HCV infection was confirmed in 48 cases (57.83%), in 27 women and 21 men. Mean age in this group (46.25 years) was not significantly different from the patient with unconfirmed HCV RNA (45.3 years). This is preliminary report.
<b>Conclusions:</b>	It seems that spontaneous elimination of HCV infection is more frequent than it is previously assumed. Further researches in this field are necessary.
<b>Key words:</b>	<b>HCV • anti-HCV • diagnostic procedures</b>

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## BACKGROUND

According to WHO about 180 million people are infected by HCV (3% of the world population) [1]. About 20–30% with chronic hepatitis C will develop end-stage liver disease and hepatocellular carcinoma [2]. Most of them have no knowledge of their disease because it may remain asymptomatic and symptoms of cirrhosis are the first sign of infection. This advanced liver disease caused by HCV is the single largest indication for liver transplantation of adults in the USA and Western Europe. In Poland prevalence of anti-HCV is estimated for about 2% [3]. HCV infection thus represents a significant healthcare burden. It is important to diagnose the disease as early as possible. Tests to confirm HCV infection include anti-HCV and HCV RNA detection.

The aim of this retrospective study was assessment of confirmation number of HCV infection among patients with suspicion of chronic hepatitis C admitted to the Ward of Liver Diseases (WLD) during one year.

## MATERIAL AND METHODS

83 patients (47 women and 36 men) at the age of 20-77 (mean age 45.77 years) were admitted to the WLD in 2012 with suspicion of HCV infection. Anti-HCV were detected in each person. HCV RNA presence was checked in their blood EDTA plasma in order to confirm active hepatitis C virus infection.

Each patient was checked for presence of risk factors in his/her past. Anti-HCV antibodies were analyzed using Elisa Murex HCV v.4.0 assay. Plasma HCV RNA qualitatively was determined by PCR Cobas Amplicor v.2.0 Roche with the lowest detection limit 10 IU/ml and quantitatively by real time PCR Cobas TaqMan v.2.0 Roche with dynamic range from 15 to  $1 \times 10^8$  IU/ml.

## RESULTS

HCV infection was confirmed in 48 cases (57.83%), in 27 women and 21 men. The mean age in this group (46.25 years) was not significantly different from the patient with unconfirmed HCV RNA (45.3 years). Risk factors of HCV infection were not found in 15/83 cases (in 8/48 patients with confirmed hepatitis C). In those patients anti-HCV antibodies were investigated due to aminotransferases (ALT/AST) elevation. The main possibility to transmit HCV was medical treatment (both small medical and surgery procedures, dialysis, blood transfusion before 1993). One person, from the group with unconfirmed HCV RNA, had sexual partner with HCV infection.

## DISCUSSION

The optimal path to diagnose HCV infection is to recognize patients with risk factors of exposure to the virus and screen them using diagnostic procedure [4,5]. In our study the main risk factor was health-care procedures in the past and ALT/AST elevation. It is consistent with other Polish publications where surgery and hospitalization in a non-operative ward were related to HCV infection [6,7]. In another study Chlabicz indicates that blood transfusion before 1993 is the most frequent risk factor which appears in

26.8% from 250 HCV-infected patients [8]. In our research there was only one person in that condition in the past. The large Polish trial including 17930 individuals indicated three independent risk factors: more than three hospitalization, blood transfusion before 1992 and intravenous drug using [9]. Another potential route of HCV transmission is sexual exposure. This problem mainly touches people with multiple sexual partners. In monogamous couples sexual transmission is uncommon [4,5].

Next step of HCV infection diagnosis is laboratory tests. They are divided into two categories: serological and molecular. The first one is using as screening test. Anti-HCV antibodies are detected by enzyme-linked immunosorbent assays, now in the third generation. They contain core, NS3, NS4 and NS5 antigens. Sensitivity and specificity is about 99% [10,11]. False negative results can appear in patients with severe immunosuppression [5,11]. To confirm active infection it is necessary to find HCV RNA. The main method of its detection is polymerase chain reaction (PCR). There are three categories of the molecular assays for hepatitis C: 1) qualitative which detects presence or absence of the HCV genome, 2) quantitative HCV RNA which determines viral load and 3) HCV genotype. The most recent tests are based on real time polymerase chain reaction. This kind of assays has high sensitivity (10-50IU/ml) and specificity (98–99%) [4,5,11]. In our study we used appropriate methods of anti-HCV antibodies detection (the third generation of Elisa test) and HCV RNA determination (very sensitive qualitative and quantitative assays).

Previously it was estimated that about 15% of HCV infected people spontaneously eradicate this virus but nowadays according to the analysis of prospective and retrospective reports it is believed that the range is wider: from 15 to 45% [4,5,10]. Our one-year data consistent with these reports.

## CONCLUSIONS

It seems that spontaneous elimination of HCV infection is more frequent than it was previously assumed. Further researches in this field are necessary.

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## Drug – induced hepatitis secondary to chemotherapy for nasopharyngeal carcinoma – case report

### Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
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### Summary

#### Background:

Several substances like drugs, toxins and herbs have been described for their potential hepatotoxic effects. Among them, hepatotoxicity induced by drugs represents an important cause of liver injury and encompasses both a hepatocellular and a cholestatic pattern. The degree of the hepatic disfunction varies between an asymptomatic presentation with biological disturbances and a severe form with fulminant hepatic failure, depending on drug type, dose or preexisting liver damage. The hepatotoxicity of antineoplastic agents plays an important part the more so as an oncologic patient carries on a poor clinical condition and other comorbidities.

#### Case Report:

We report a case of cholestatic hepatitis induced by cisplatin, when used as chemotherapy for a nasopharyngeal carcinoma along with docetaxel in a patient with previous normal hepatic status.

#### Conclusions:

Liver dysfunction develops after administration of the regimen based on standard doses of cisplatin and was reversible with drug discontinuation.

#### Key words:

**cholestatic hepatitis • CISPLATIN • nasopharyngeal carcinoma**

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## BACKGROUND

Toxic hepatitis represents a distinct clinical entity with certain characteristics and important medical consequences as it is considered a possible cause of acute liver failure [1]. Hepatic toxicity is induced by variable toxins like drugs, herbal extracts, alcohol, toxic substances, chemical agents. Among drugs, the most common one that could be responsible for liver injury and acute liver failure when used in high doses is acetaminophen [2]. Drug-induced hepatotoxicity is well recognized as an important cause of morbidity and mortality, leading to 50% of all acute hepatic failures [3]. Several drugs are known for their hepatotoxic potential, the most commonly pharmacological substances reported to cause liver impairment are summarised in Table 1 [4].

The clinical picture in drug-induced liver injury is variable, ranging from an asymptomatic presentation accompanied by biological abnormalities to an acute form of hepatitis with or without cholestasis that can progress to fulminant liver failure. Establishing the diagnosis of hepatitis due to drug ingestion is challenging. A high index of suspicion is always needed because it can mimic any form of acute or chronic hepatitis [4]. Therefore, toxic hepatitis is a diagnosis of exclusion after other causes of liver disease are ruled out and a comprehensive anamnesis focused on drugs or chemicals ingestion was taken. Regarding the mechanisms responsible for liver toxicity, there have been described two main pathways: on one hand there is dose-related toxicity which appears shortly after the drug ingestion and on the other hand idiosyncratic reactions, not necessarily influenced by the dose, occurring at an unpredictable interval after drug administration [5]. Drug-induced liver injury may show either a hepatic pattern characterized by aminotransferases elevation, a cholestatic pattern with increased level of alkaline phosphatase or a mixed pattern of acute hepatitis and cholestasis. Antineoplastic drugs represent a special category of medication, through the significant symptom burden that an oncologic patient carries on. Assessing the potential hepatic adverse effects of chemotherapy is difficult and should take into account the patient's comorbidities, the pre-existing biological hepatic status, the clinical condition and others like paraneoplastic syndromes, poly-medication [6]. Cisplatin, a platinum derivate used in different therapeutical regimens for multiple tumor types is rarely associated with liver injury. Hepatotoxicity induced by cisplatin is rare at standard doses, the severity of liver damage is usually mild and expressed either by asymptomatic elevation of aminotransferases level, cholestasis or cholestatic hepatitis [7,8]. There are very few cases of liver injury due to cisplatin reported in the literature. Acute hepatitis has found to be dose-related occurring only when high doses are administered, but even so the outcome is benign and results in recovery of hepatic function [8].

## CASE REPORT

A 63-years old man came to our attention for intense skin and mucosal jaundice occurring 3 weeks after a chemotherapy regimen including cisplatin and docetaxel. The patient presented with a complex medical history consisting in a malignant tumor localized in the head and neck region. Seven months before the admission, he accused neurological symptoms and was diagnosed through magnetic

**Table 1.** Drugs associated with liver injury.

Class of substance	Drugs
Paracetamol	
Non-steroidal anti-inflammatory drugs	Diclofenac Ibuprofen Naproxen
Antibiotics	Amoxicillin/ clavulanate (augmentin) Flucloxacillin Erythromycin Ciprofloxacin Anti-tuberculosis drugs (Isoniazid, rifampicin, pyrazinamide)
Anti-epileptics	Phenytoin Carbamazepine Valproic acid
Immunosuppressants	Azathioprine Cyclophosphamide
Anti-arrhythmia drugs	Amiodarone
Psychiatric drugs	Chlorpromazine Paroxetine

resonance imaging with a skull base tumour developed in both ethmoidal and sphenoidal sinuses with retropharyngeal extension. Through transnasal approach, a tumor reduction was attempted but without total mass removal. The histological examination completed with immunohistochemistry tests revealed a non-keratinized nasopharyngeal carcinoma. Regarding his past medical conditions, no history of liver disease, viral hepatitis or alcohol abuse were identified. The patient associated diabetes mellitus, arterial hypertension controlled with medication and gastroesophageal reflux disease. He denied cigarette smoking and illicit drug use. His family history was unremarkable for malignant diseases. Postoperatively, the patient was put in a treatment protocol consisting of cisplatin 70 mg/m<sup>2</sup> and docetaxel 80 mg/m<sup>2</sup>, following to be referred after that to radiotherapy. He received four cycles of chemotherapy based on this regimen, with liver enzymes concentrations within normal limits prior each cycle of therapy. At the moment of presentation in our clinic, three weeks after the last infusion, the physical examination revealed an icteric patient, afebrile, with stable vital signs and a normal neurological status, without rash or lymphadenopathy. The abdominal examination revealed a nontender, nondistended abdomen, unpainful, with no clinically detectable ascites. No hepatomegaly or splenomegaly were detected. The laboratory studies showed a normal leukocyte count of 6.8×10<sup>3</sup>/mm<sup>3</sup>, a mild normochrome normocytic anemia with a hemoglobin level of 11.7 g/dl and a platelet count of 203000/mm<sup>3</sup>. An important cytolytic syndrome expressed by a marked increase in aminotransferases level (AST 1418U/L, ALT 1540U/L) was found in addition with a significant cholestasis – alkaline phosphatase was 187 mg/dl, gamma-glutamyl transpeptidase 422 mg/dl and total bilirubin 15.2 mg/dl. Viral markers for hepatitis B, C and HIV were negative, as well as antinuclear and anti-mitochondrial antibodies. Abdominal ultrasonography was

performed and showed a normal sized liver with diffuse hyperogeneity and no focal lesions, no dilatation of intrahepatic biliary tree or common biliary duct, no ascites, a normal gall bladder, no mass lesion in the head of pancreas. The biological picture correlated with the information provided by abdominal imaging which excluded a mechanical cause of jaundice stated for a cholestatic hepatitis. Besides the obstructive causes, other causes of cholestatic hepatitis were ruled out: viral hepatitis, decompensated liver cirrhosis, granulomatous hepatitis, alcoholic hepatitis, primary biliary cirrhosis or autoimmune hepatitis. Given the clinical context, the onset of jaundice after chemotherapy with hepatotoxic potential, we considered the liver injury to be drug-related. Considering the two main drugs used during the cycles and knowing that docetaxel expressed hepatotoxicity in very rare cases, especially in immunodepressed people HIV positive, the most probable cause of cholestatic hepatitis in our patient is cisplatin toxicity. This idea is supported by the fact that liver damage induced by cisplatin is dose-related. Although dose escalating was not performed in this case, the jaundice occurred after the fourth cycle, therefore we could take into account a cumulative toxicity of the drug. Subsequently, the chemotherapy was interrupted and the patient was started on corticotherapy at a dose of 0.75 mg/kg, tapering it progressively. Liver protection with silymarin at a dose of 300 mg/day was added. The clinical course was favourable, the jaundice disappeared gradually, and the biological markers of cytolysis and cholestasis started to decline, with normalization within one month.

## DISCUSSION

Cisplatin is one of the most widely platinum derivate used to treat multiple types of tumors, including head and neck, colon, breast, lung. Generally, platinum compounds do not exhibit hepatotoxicity, excepting cisplatin and carboplatin. Cisplatin appears to induce usually a mild liver-injury, self-limited and with a favourable outcome [8]. It was suggested that hepatotoxicity of cisplatin is dose-related, the severity of liver injury expressed by histological changes-steatosis and cholestasis became evident when used high doses of drug [8]. According to data from literature, there are very few cases of cisplatin hepatotoxicity reported. Drug-induced liver injury represents an important public health issue through its medical consequences: high morbidity and mortality rates, accounting for one-half of the cases of acute liver failure one hand and on the other hand, hepatic adverse events are the most common reason for withdrawal a drug from the clinical use [9–11]. The mortality rate from drug-induced liver injury is around 5% [12]. Given that the liver is the principal target for multiple toxins, including drugs, this entity called “drug-induced liver injury” needs better understanding and characterization. There are described two mechanisms responsible for liver damage: overdose and idiosyncrasy [4,9,10]. An idiosyncratic reaction is an unexpected adverse event, occurring with variable latency from the drug ingestion, while a dose-related reaction is predictable, the most common example for this type is acetaminophen [4]. The intrinsic mechanism of liver damage is considered to be mitochondrial dysfunction which is in turn responsible for the oxidative stress and liver damage [11,13]. Regarding the clinical presentation, there are no pathognomonic features of drug-induced liver injury, in fact the clinical picture is variable, ranging from asymptomatic elevations of

functional hepatic tests, acute hepatitis and even acute liver failure [4]. The clue of establishing the diagnosis is a high index of suspicion. Liver biopsy is not necessary because both clinical and histological findings are not specific, mimicking any type of acute or chronic hepatitis [4]. As for cisplatin, the evidence from the literature stands for a hepatic toxicity that is dose-related, although mild elevations in liver aminotransferases have been reported with standard doses. Considering our patient case, we suggest that the toxicity of cisplatin was due probably to a cumulative dose of drug, because the cholestatic hepatitis developed after the fourth cycle of chemotherapy, and other etiologies and potentially hepatic toxins were excluded. Another fact that supports our hypothesis is normalization of hepatic parameters after discontinuing the drug. The mechanism of cisplatin hepatotoxicity is not fully understood, but it seems that the oxidative stress plays an important part in inducing hepatic injury [14]. As for its counterpart docetaxel, hepatic adverse reactions are not well defined, slight increase in aminotransferases level was reported. Therefore we cannot take into account its role in causing the cholestatic hepatitis. The practical approach in such situation of suspected liver injury induced by medication is to withdraw the drug with the highest likelihood of hepatocellular or cholestatic injury followed by closely monitoring of the liver hepatic tests. We proceeded the same, chemotherapy protocol was stopped and the patient received hepatic protection and a short course of corticotherapy for the component of intrahepatic cholestasis, with favourable outcome and total clinical and biological recovery within one month.

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## Vertical HBV infections among patients at the Provincial Hospital of Infectious Diseases in Bydgoszcz

### Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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**Source of support:** None

### Summary

#### Background:

The introduction of compulsory vaccination and passive immunoprophylaxis recommendation minimized the risk of HBV infections. Observation of patients with congenital hepatitis B can trace the course of the infection for over 20 years. The aim of the study was the evaluation of the course of liver disease in patients with vertical HBV infections.

#### Material/Methods:

We retrospectively analyzed the medical records of 6 patients with vertical HBV infections treated at the Provincial Hospital of Infectious Diseases in Bydgoszcz

#### Results:

The study group consisted of 6 men. The mean age of patients was 24 years. The average age at diagnosis was 6 years old. At the time of diagnosis in 3 patients ALT activity was within normal limits, in 3 ALT activity was increased with the highest activity 151 U/L. In all patients HBeAg was present. Patients administered multiple antiviral treatment. During the follow-up, in 3 patients elimination of HBeAg occurred, on average in the 18. year of life. In 4 patients we observed the progression of histopathological changes in the liver. In 3 patients during the treatment transient exacerbation of the inflammatory process with a maximum ALT activity 1304 U/L was observed, not related with the HBeAg/ anti-HBe seroconversion. One patient eliminated HBsAg in the 27. year of life, with the development of cirrhosis and varices of the esophagus II° presence.

#### Conclusions:

1. In all patients vertically infected with HBV histopathological progression of the disease was observed. 2. HBsAg elimination does not protect against the development of liver cirrhosis.

#### Key words:

**vertical HBV infection • mother-to-infant infection • perinatal infection • HBV vaccination**

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## BACKGROUND

Viral hepatitis is one of the most common liver infection diseases. There are ca. 350 million people infected with HBV in the world [1]. In Poland, before immunization was introduced, about 2% of the population were infected with HBsAg and in 1985 the incidence of HBV infections in Poland was 45/100 000 people. In 1989 the vaccination of newborns and infants of HBV-infected mothers was implemented with a recommendation of a specific immunoglobulin application. From 1994 to 1996 vaccination of all newborns against the viral hepatitis of type B was included in the program of compulsory immunization. This procedure minimized the risk of appearance of new mother-to-infants infections caused by the HBV and protected this group of children from the development of chronic viral hepatitis, which earlier concerned 90% newborns born by infected mothers.

### The aim of the work

The aim of this work was to analyse the process of mother-to-infant viral hepatitis B in the period of over 20 years taking into consideration the activity of inflammation process, the progression of liver histological changes and the effects of anti-viral treatment.

## MATERIAL AND METHODS

The subject of a retrospective analysis was the medical documentation of 6 patients born in years 1983–1992 suffering from mother-to-infant HBV infection treated in years 1993–2013 in the Provincial Hospital of Infectious Diseases in Bydgoszcz. The results of biochemical, serological, viral and histological studies were analysed. At a patient with ultrasonographically diagnosed advanced liver hyperechogenicity an endoscope examination was conducted aiming to examine the presence of portal hypertension symptoms.

## RESULTS

The examined group is comprised of 6 men, including 2 pairs of brothers. When it comes to the rest of the group, a brother of one of the patients eradicated HBsAg in 1994 at the age of 12 and stopped turning up for hepatological check-ups. Average patients' age in the group examined is currently 24 years, the eldest patient is 30 years old, the youngest is 20. The average age at the moment of the diagnosis was 6, with the age of patients varying from 6 months to 13 years. Half of the patients was observed with an elevated ALT activity at the moment of the diagnosis, with the highest rate of 151 U/L (Figure 1).

HBeAg was present in all the patients at the moment of the diagnosis of the illness. Patients were subjects of a repeated anti-viral treatment (Table 1).

During the observation HBeAg/anti-HBe seroconversion, occurring on average in the 18. year of life, was reported at half of the group examined. The youngest patient at whom the seroconversion was observed was 12 years old, a year and a half after finishing a recombinant IFN therapy. The second patient eradicated HBeAg in the 17. year of life, a year after one year long lamivudine treatment and two earlier IFN therapies. The third patient came through

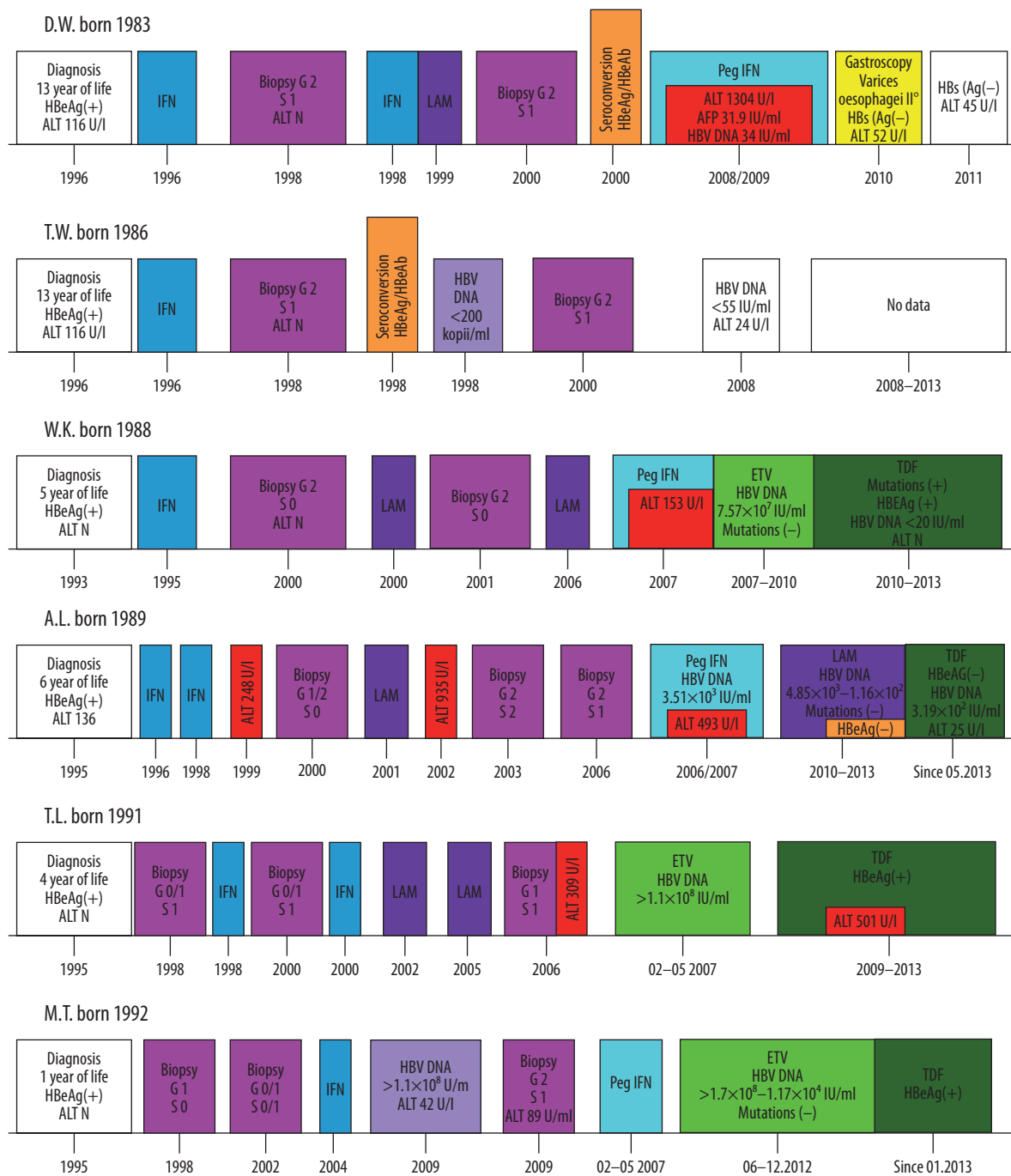
the seroconversion during a 2-year lamivudine therapy in the 23. year of life.

Liver biopsy was conducted on all the patients. The first biopsy was conducted on average in the 9. year of life with a maximum rate of grading equal 2 and staging equal 1 according to the modified Scheuer range. In four of the patients histopathological changes progression was confirmed in a control biopsy with the highest fibrosis rate of 2. In 2 among 6 patients splenomegaly was observed and cirrhosis was diagnosed in 1 patient. This patient during a PegIFN alfa-2a treatment at the age of 25 years demonstrated ALT activity increase up to 1304 U/L, with AFP concentration increase up to 31.9 IU/ml and HBV viremia  $3.4 \times 10^1$  IU/ml and after 2 years eradicated HBsAg with a concomitant development of cirrhosis, esophageal varices II° and flow disturbances of the hepatic portal vein basin. Also in two other patients a temporary (non-HBeAg-seroconversion-related) inflammation process exacerbation was observed. In one of the patients mutants of the HBV polymerase were present. Currently 3/6 of the patients receive tenofovir, 2 of them with HBeAg(+) have an increased ALT activity rate (up to  $2.5 \times N$ ). Three patients do not stay under anti-viral treatment, one of them eradicated HBsAg, the second after the eradication of HBeAg stopped turning up for the checkups, the third is waiting for the treatment qualifying study results.

## DISCUSSION

The introduction of the anti-HBV vaccination into the Polish compulsory immunization calendar played the main role in the spectacular decrease of incidence of this disease from 45/100 000 in 1985 to 4,11/100 000 in 2004 [2]. In our Department the last pediatric patient suffering from acute hepatitis B was treated in 2006, the youngest patient infected with HBV is currently 12. Before the introduction of the active-passive prevention hepatitis B made a serious clinical problem. The HBV mother-to-infant infection usually takes place during the delivery or just after it, occasionally via the placenta. A chronic hepatitis develops in over 85% of the infected newborns, causing a risk of the occurrence of cirrhosis or hepatocellular carcinoma [3]. A proof of the effectiveness of immunization as an anti-hepatitis B prevention method is the decrease of incidence of hepatitis B in Taiwan, where vaccination was introduced in 1984. In 1984 the incidence rate was 10%, in 2009 0.9% [4]. The application anti-HBs immunoglobuline (HBIG) in addition to the active protection lessens the probability of HBV infection in children, especially taking into consideration the fact that the probability of infection in the child is dependent on the viremia of the pregnant mother [5]. Nevertheless, the introduction of the anti-hepatitis B prevention did not bring about an utter elimination of the new cases of HBV transmission, not only because lack of the post-vaccination response. Two patients from the examined group, currently 22 and 20 years old, were born in the period of compulsory anti-HBV vaccination, did not receive the vaccine due to the mistakes of the medical staff. Despite obligatory diagnosis of pregnant women in the field of HBV infection, the possibility of newborns getting infected cannot be eliminated, especially that the studies are not personalized [6,7].

Another way to diminish the risk of mother-to-child HBV transmission is a pharmacological reduction of the mother's



**Figure 1.** The treatment scheme of patients with mother-to-infant HBV infection.

HBV viremia during pregnancy. Despite the lack of registration nucleotide analogues recommendation there is quite a big number of clinical studies describing the treatment of this group of patients with lamivudine, tenofovir and telbivudine in the third trimester of pregnancy [8–13]. These medicines considerably decrease HBV viremia rate, notably diminishing the risk of transmitting the infection to the newborn. However, it is to remember that in the FDA classification lamivudine is a C-category medicine, telbivudine and tenofovir belong to category B[1]. One more method of preventing a mother-to-child infection is a planned

Caesarean section. This way brings a much smaller risk of mother-to-infant HBV infection than a natural delivery or an abrupt Caesarean section [14].

Predictably, the number of new HBV infections in Poland is to diminish systematically due to a 17-years-long newborns active prevention period and entering the age of procreation by the people born in that time. Although, the population of people infected with HBV is getting old and the length of the disease is growing. In the examined material liver cirrhosis was diagnosed in a 27 years old patient. Taking

**Table 1.** Current results of viral and biochemical studies of the patients with an mother-to-infant HBV infection taking into consideration past anti-viral therapies.

Patient	Therapies	Current serological status	Current viremia	ALT activity
DW	2×IFN, LAM, PegIFN	HBsAg(-)	–	1.5×N
TW	IFN	HBeAg(-)	<55 IU/ml	N
WK	LAM, PegIFN, ETV, TDF	HBeAg(+)	<20 IU/mL	N
AL	2×IFN, LAM, PegIFN, TDF (since 05.2013)	HBeAg(-)	3.19×10 <sup>2</sup> IU/mL	N
TL	2×IFN, 2×LAM, TDF	HBeAg(+)	>1.1×10 <sup>8</sup>	2.5×N
MT	IFN, PegIFN, ETV, TDF	HBeAg(+)	8.29×10 <sup>3</sup>	2×N

into consideration the lack of effective anti-viral treatment it is to be expected that in the patients infected with HBV in the perinatal period the complications of the infection are to still manifest in the early adulthood.

## CONCLUSIONS

1. Despite some cases of normal ALT activity, in all of the patients histopathological changes was observed.
2. HBsAg elimination does not prevent from the development of liver cirrhosis.

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- A** Study Design
- B** Data Collection
- C** Statistical Analysis
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## Tissue localization of NS3 protein and NK cells in HCV infected liver biopsies

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### Summary

**Background:**

Mutual links between HCV non-structural proteins and NK cells are relevant for the understanding HCV pathogenesis.

The aim of this study was to find out their topographical relationship *in situ* within liver parenchyma.

**Material/Methods:**

Small fragments of liver biopsies collected during routine diagnostic procedures of 61 HCV-infected patients and 2 uninfected ones. Fresh frozen liver tissue sections were subjected to indirect immunofluorescence using monoclonal primary antibodies *vs.* HCV NS3 protein, CD56 and NKG2D antigens of NK cells.

**Results:**

NS3 was demonstrated in 44% of biopsies as cytoplasmic fluorescence in single dispersed hepatocytes. CD56+ cells were present in inflammatory cellular infiltrates in almost 74% of cases, and usually accompanied NS3+ cells. NKG2D+ ones had similar distribution to CD56+ cells and were detected in 76% of biopsies.

**Conclusions:**

The results of this study show, that NS3+ in hepatocytes is not universally expressed, perhaps due to its enzymatic activity. Presence of NK cells in their vicinity suggests some mutual relationship between them.

**Key words:**

**hepatitis C virus (HCV) • liver biopsy • HCV NS3 protein • NK cells • immunofluorescence**

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## BACKGROUND

Hepatitis C virus (HCV) responsible for at least 170 million infected people worldwide, has been thoroughly examined by several authors and apparently its protein and genetic structure is well known. Transcription of viral mRNA and subsequent translation to polyprotein leads to the formation of non-structural HCV proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B). Some of them have enzymatic activity, including NS3 [1]. The latter, serine protease and RNA-helicase have multiple functions. Serine protease cleaves the boundaries of proteins resulting in their release from polyprotein [2]. Moreover, NS3 C-terminal fragment possess a nucleotide triphosphatase/helicase activity necessary for the replication of viral RNA [3]. Another HCV protein, NS4A forms a complex with NS3 functioning as a co-factor for the protease activity [4]. NS4A-NS3 complex hampers the production of type I interferons by the inhibition of interferon regulatory factor-3 (IRF-3) [5]. This may be of paramount importance for the behaviour of host innate immune response and NK cells in particular. It is possible that NS3 and remaining non-structural HCV proteins have other not yet known functions *in vivo*, because most of our knowledge about their activity have been shown in artificial *in vitro* culture systems.

Natural killer (NK) cells are known to play an important role in early stage of HCV infection. They are even able to contribute in the resolution of acute HCV infection in some cases, owing to their inhibitory receptors and down-regulation of HLA antigens on hepatocytes [6]. In chronic stage of viral hepatitis their activity is severely hampered or lost due to inhibitory action of several viral non-structural proteins, including NS3 [7]. It seemed of interest to see topography and frequency of both, NK cells and NS3+ hepatocytes in fresh liver biopsies of HCV+ patients, tested prior the initiation of anti-viral therapy. It might provide some clue, how often and where NK cell inhibition takes place.

## MATERIAL AND METHODS

### Tissues

Small fragments of liver (0.5 cm length) obtained by diagnostic percutaneous liver biopsy from patients with confirmed HCV RNA viremia, awaiting for anti-viral therapy. Two liver samples were from HCV – negative patients biopsied for diagnostic purposes. All patients provided informed written consent for this procedure.

**Table 1.** Antibodies used.

Specificity	Type	Lot/clone	Label	Source
HCV-NS3	Mouse monoclonal	6010622	-----	Leica Microsystems
HCV-NS3	Mouse monoclonal	MMM33	-----	Novocastra
CD56	Mouse monoclonal	6008458	-----	Leica Microsystems
NKG2D/CD314	Mouse monoclonal	1A5	-----	Leica Microsystems
Mouse IgG	Goat polyclonal F(ab') <sub>2</sub>	1075233	Alexa Fluor 488	Life Technology
Mouse IgG	Goat polyclonal F(ab') <sub>2</sub>	1037267	Alexa Fluor 594	Life Technology

**Table 2.** Prevalence of positive cells in examined tissues.

Specificity	No positive/no examined	Per cent
NS3	27/61	44,2
CD56	45/61	73,7
NKG2D	23/30	76,0

Tissue blocks, wrapped in an aluminium foil, were snap frozen in dry ice-acetone slurry and stored in a deep freeze (–70°C) until used. In the day of experiment they were cut in a cryostat on 4 µm thick sections and fixed in a cold acetone prior use.

### Antibodies

The list of antibodies used in indirect immunofluorescence (IMF) reaction is shown in a Table 1.

### Immunofluorescence reaction

Liver sections were incubated overnight in 4°C with appropriate dilution of primary antibody. After extensive washing in PBS, sections were submitted to the reaction with labelled anti mouse IgG fluorescent reagent for 30 min at ambient temperature in the dark. Following washing as above, sections were mounted in PBS-glycerol mixture (V/v).

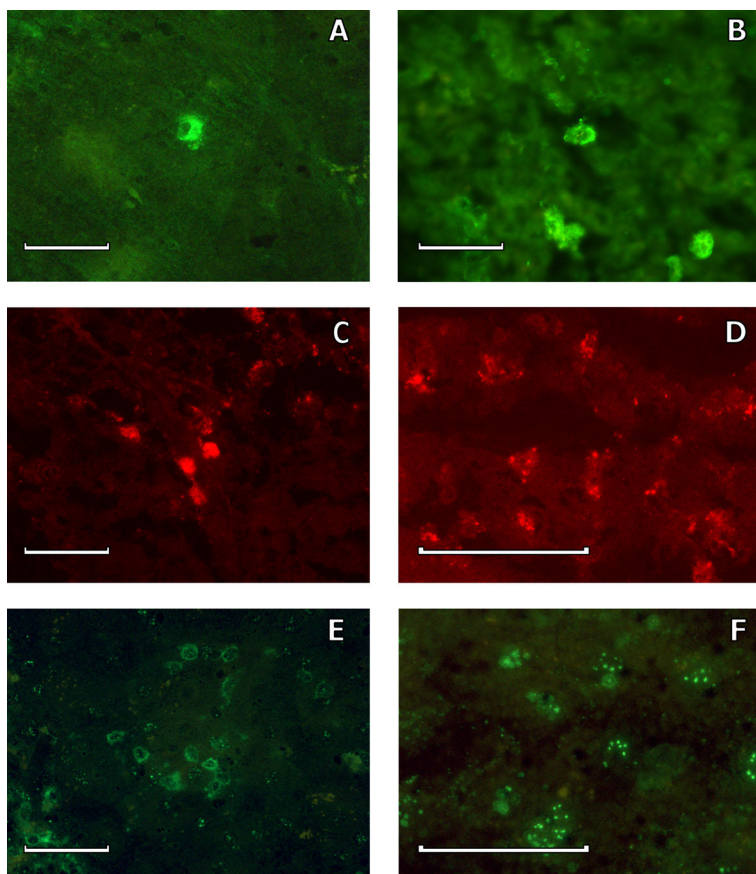
Control reactions included replacement of primary antibody with PBS and the reaction on NS3-negative liver tissue. All of them came out unanimously negative.

Specimens were searched and photographed in Olympus research microscope (BX41) connected with digital graphical acquisition system – analysis^B. Positive cells were recorded on the Yes/No basis.

## RESULTS

In general, only single positive of all three specificities tested (NS3 CD56, NKG2D) could be seen. In the case of NS3 the fluorescence was cytoplasmic only, while CD56+ and NKG2D+ ones manifested both, cytoplasmic and membrane bound staining. At high magnification both, CD56+ and NKG2D+ cells expressed occasionally cytoplasmic granules. Prevalence of positive cells in relation to cases examined is shown in the Table 2.





**Figure 1.** Immunofluorescent detection of NS3 and NK cell antigens in HCV infected liver biopsies. (A) patient (pnt) GK. Positive NS3 staining in cytoplasm of single hepatocyte. Anti NS3 MoAb and Alexa 488 Fluor anti mouse IgG. (B) pnt PP. Several NS3 positive hepatocytes. The reaction as above. (C) pnt GK. Several CD56 positive cells. Anti CD56 MoAb and Alexa Fluor 594 anti mouse IgG. (D) pnt. PT. CD56 positive cells showing granules in cytoplasm. The reaction as above. (E) pnt KW. Several NKG2D positive cells. Anti NKG2D MoAb and Alexa 488 Fluor anti mouse IgG. (F) pnt MT. Several NKG2D positive cells showing granules in cytoplasm. The reaction as above. Bar – 50  $\mu$ m.

Examples of positive cells are depicted in the Figure 1A–F).

No positive cells were found in 2 HCV-negative liver specimens.

There were 7 biopsies showing diffuse steatosis of hepatocytes. None of these biopsies have shown single NS3 positive cell. On the other hand, in 5 of 7 samples CD56+ cells could be traced (Results not shown).

In 15 biopsies abundant inflammatory infiltrates were evident while searching hematoxylin + eosin stained sections. In 14 out of 15 CD56+ cells were relatively the most frequent. NKG2D+ cells seemed to be the most numerous, but they were tested in only half of biopsies. There was no topographical proximity between NS3+ cells and CD56+ ones. However, NS3+ biopsies were always accompanied by CD56+ cells (Results not shown).

## DISCUSSION

NS3 as an enzyme of double specificity cannot be considered as a protein filling the space of cell interior. Apparently, its expression is probably linked to enzymatic activity. Its importance is underscored by recent findings, that this enzyme cleaves T cell protein phosphatase (TCPTP), what certainly compromises T cell anti viral function. Moreover, intrahepatic NS3 and TCPTP levels were shown to correlate with viral load and severity of viral hepatitis [8]. It suggests, that precise intrahepatic determination of NS3 may have clinical value. We deliberately have used fresh frozen tissue specimens of liver to avoid protein degradation

by routine processing and paraffin embedding. In spite of it the number of NS3 positive hepatocytes was scarce. The application another anti NS3 primary antibody did not increase substantially the number of positive cells. It suggests that NS3 protein exposure is strictly dependent on its enzymatic activity. On the other hand, Kasprzak et al. [9] using immunohistochemistry have obtained NS3 reaction in several hepatocytes, but applied ImmunoMax technology (Perkin Elmer) based on the amplification of the reaction product by means of biotinylated tyramine. This artificial enhancement of the reaction is impressive, but apparently does not correspond to the real situation *in vivo*.

NK cells appear to have crucial role in anti-viral activity, especially in early stage of infection. Their presence was tested using anti CD56 and anti-NKG2D antibodies. The latter marker expression correspond to activated/cytotoxic stage of NK cell. We could not demonstrate accumulation of NK cells in direct proximity of NS3+ hepatocytes, presumably because of inhibitory action of viral proteins, known to suppress NK cells mainly in chronic hepatitis C. Nevertheless, in each biopsy positive for NS3+ cells, relatively frequent CD56+ ones were visible, what was not the case in NS3- biopsies. It hints for possible interactions between them.

NKG2D+ cells came out slightly more frequently than CD56+ cells in the current study. This may be due to the fact, that activated T cells may also express this marker [10]. It was also seen in our previous study [11]. The number of NKG2D+ cells nevertheless came out low, perhaps because of downregulation of NKG2D ligands by HCV NS3/4A complex, as it has been shown previously [12]. Nevertheless, the cytoplasmic

granules shown in this study in both, CD56+ and NKG2D+ cells suggest their initial cytotoxic potential. Moreover, all lymphocyte subsets were shown to be suppressed and even eliminated by apoptosis by NS3 triggering of oxygen radical production in mononuclear phagocytes [13]. It is of interest that NS3 protein has been demonstrated in CD14+ monocytes and CD19+ B cells but not in CD3+ T cells [14]. It suggests that HCV is not only able to infect other cells apart from hepatocytes, but takes advantage of residing in monocytes to use its cytotoxic machinery against potentially anti-viral T and NK cells.

## CONCLUSIONS

Results of this study show that NS3 antigen in HCV-infected hepatocytes is not universally expressed. Our data hint for the mutual interactions between expression of NS3 protein and prevalence of NK cells in HCV infected liver tissue.

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