Original paper

Vaspin mRNA levels in the liver of morbidly obese women with nonalcoholic fatty liver disease

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The aim of this study was to evaluate hepatic vaspin mRNA in morbidly obese women with nonalcoholic fatty liver disease (NAFLD) and to look for its relationships with metabolic and histopathological features.

The study included 56 severely obese women who underwent intraoperative wedge liver biopsy during bariatric surgery. Hepatic vaspin mRNA was assessed by quantitative real-time PCR.

Vaspin mRNA found in all included patients was markedly higher in patients with body mass index (BMI) ≥ 40 kg/m^2 (4.59 ±3.09 vs. 0.44 ±0.33; p = 0.05). An evident but statistically insignificant difference in vaspin mRNA levels was observed between patients with and without hepatocyte ballooning (4.77 ±4.23 vs. 0.45 ±0.29, respectively), with and without steatosis (4.80 ±4.20 vs. 0.41 ±0.29, respectively), without and with fibrosis (0.25 ±0.80 vs. 6.23 ±7.2, respectively), and those without and with lobular inflammation (0.27 ±1.0 vs. 5.55 ±10.1, respectively). There was marked difference in vaspin mRNA between patients with simple steatosis/borderline nonalcoholic steatohepatitis (NASH) compared to those with definite NASH (0.24 ±0.96 vs. 10.5 ±10.4).

Adiposity is an undoubted confounding factor influencing vaspin levels. Hepatic vaspin mRNA seems to be markedly elevated in morbidly obese patients with more advanced NAFLD and when hallmarks of NASH were observed. Pointing to non-linear mRNA levels within the NAFLD spectrum and an evident increase in patients with fibrosis and definite NASH, the detrimental action of vaspin cannot be excluded.

Key words: vaspin, adipokine, liver, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, obesity.

Introduction

An alarming increase in the rate of obesity and nonalcoholic fatty liver disease (NAFLD) has been observed recently, particularly in developed countries. The prevalence of NAFLD is estimated in the general population of western countries to be greater than 30%, achieving 67%-75% in obese [1] and 75-95% in morbidly obese patients [2]. The prevalence of nonalcoholic steatohepatitis (NASH) reaches 20-54% [3, 4]. NASH-related cirrhosis has become the second-leading disease among liver transplant waitlist registrants in the United States [5]. Increased triglyceride accumulation in hepatocytes leads to hepatic steatosis, which may progress to NASH, cirrhosis, or hepatocellular carcinoma (HCC) [6, 7]. The rate of progression from simple steatosis to more advanced stages of NAFLD has not been well defined. It has been known that 7-37% of patients with NASH have concomitant advanced fibrosis and the tendency for progression to cirrhosis [8, 9]. Pathogenesis of NAFLD and NASH is multifactorial and multiple. Various factors have been suggested to lead to disease development and progression. Essential for NAFLD occurrence are dietary factors, insulin resistance (IR), and adipokines [10, 11, 12]. Genetic, immune, and endocrine factors, alongside gut microbiota and *Helicobacter pylori*, should also be considered [13, 14, 15]. Consequently, the recognition of a phenomenon leading to progression from benign fatty liver to NASH with severe fibrosis is of crucial importance. The search for markers of this progression has substantial meaning for clinical proceeding. Many factors like cytokines, adipokines, and adhesive molecules are considered as the factors participating in the progression of liver injury in NAFLD [12, 16, 17, 18]. However, the role of these factors in the development of liver damage, as well as the practical utility as diagnostic markers, has not been fully examined, but this issue is being intensively explored nowadays.

Vaspin (visceral adipose tissue-derived serine protease inhibitor) is a novel adipokine, which has been isolated from both the visceral and subcutaneous white adipose tissues of obese and impaired glucose tolerant subjects [19]. The percentage of body fat appears to be the strongest determinant of subcutaneous vaspin expression. Vaspin, which improves insulin sensitivity and glucose tolerance, seems to be a compensatory mechanism switching in the obesity associated with insulin resistance [19, 20]. Additionally, vaspin has been found to down-regulate the expression of profibrogenic and proinflammatory agents such as leptin, tumour necrosis factor α (TNF-α), and resistin [21]. On the other hand, serum vaspin in HCC patients was positively associated with intensity of hepatic angiogenesis. Angiogenesis is a phenomenon which aggravates chronic liver disease progression and is observed in the very early stages of NAFLD independently of NASH [22, 23].

The aim of this study was to assess vaspin mRNA levels in the liver tissue and its association with laboratory parameters, histopathological features, and metabolic abnormalities in morbidly obese women with NAFLD.

Material and methods

Patient selection and serological assays

Fifty-six consecutive severely obese women who underwent bariatric surgery in the time period between 2015 and 2016 were included. All the female patients were qualified for the study in cooperation with the Gynaecological Department, where the patients had been assessed in an epidemiological study regarding the prevalence of obesity in the region of Upper Silesia and had then been proposed bariatric surgery. The assessed clinical and laboratory parameters are presented in Table I. Patients with a history of excessive alcohol consumption (more than 20 g/day), drug abuse, autoimmune or infectious hepatitis, thyroid diseases, and human immunodeficiency virus (HIV) infection were excluded. Patients of the study group had their systolic and diastolic blood pressure and waist circumference measured. For further analysis, we defined two subgroups: body mass index (BMI) < 40 and BMI $\geq 40\,kg/m^2$. On the day of liver biopsy during bariatric procedure, a single blood sample was drawn in the morning from all patients subjected to fasting. The samples were centrifuged, and serum was aliquoted and frozen at –70°C until further processing. All the study participants underwent oral glucose tolerance test for diagnosis of diabetes mellitus or impaired glucose tolerance. Laboratory data were measured routinely, using standard methods. The upper limit of alanine aminotransferase (ALT) activity was set at 38 UI/l and aspartate aminotransferase (AST) at 40 UI/L. Non-invasive markers of liver tissue alterations were calculated as originally described – AAR = AST/ALT; APRI = [(AST/ULN)/platelet count ($\times 10^9$)] × 100.

Insulin resistance (IR) was calculated according to the homeostasis model assessment for IR (HOMA-IR) by the formula: fasting insulin level (mUI/l) × fasting glucose level (mg/dl)/405. Because HOMA-IR was up-regulated in all analysed patients, for further analysis patients were divided into two subgroups with HOMA-IR value below four and equal to or above four.

The study was approved by the Ethical Committee of the Medical University of Silesia in Katowice and conformed to the ethical guidelines of the Declaration of Helsinki (KNW/0022/KB1/95/13). Informed consent was obtained for the whole study series.

Table I. Detailed comparison of demographic and laboratory parameters between morbidly obese patients with different BMI

Parameter	Entire group (n = 56)	BMI < 40 kg/m^2 (n = 18)	BMI > 40 kg/m^2 (n = 38)	p
Age (years)	39.4 ±6.0	41.1 ±6.2	39.6 ±7.1	NS
Height (cm)	170.0 ±8.7	168.6 ±8.4	170.9 ±8.9	NS
Weight (kg)	129.5 ±27.4	102.3 ±12.1	141.5 ±23.3	< 0.001
BMI kg/m^2	44.5 ±8.1	35.8 ±2.3	46.2 ±6.6	< 0.001
WC (cm)	128.5 ±17.0	114.5 ±10.8	135.8 ±15.0	NS
HGB (g/l)	13.8 ±1.2	13.7 ±0.9	13.9 ±1.2	NS
WBC (G/l)	7.8 ±1.6	7.6 ±1.8	7.9 ±1.6	NS
PLT (G/l)	269.9 ±55.3	292.8 ±63.4	263.8 ±50.7	NS
Bilirubin (mg/dl)	0.6 ±0.2	0.5 ±0.2	0.6 ±0.2	NS
ALT (U/l)	39.3 ±25.4	28.4 ±16.6	43.9 ±27.3	0.04
AST (U/l)	31.1 ±18.7	23.3 ±10.5	33.3 ±19.2	0.04
GGTP (U/l)	42.5 ±29.5	19.7 ±8.5	51.1 ±29.9	0.001
ALP (U/l)	76.8 ±18.3	76.2 ±20.8	76.4 ±17.6	NS
TP (g/l)	74.7 ±5.1	73.9 ±5.4	75.4 ±5.2	NS
Albumin (g/l)	55.1 ±9.5	53.2 ±17.5	55.8 ±3.0	NS
CRP (mg/l)	9.4 ±9.8	8.2 ±6.8	10.1 ±10.9	NS
Creatinine (mg/dl)	0.8 ±0.1	0.7 ±0.1	0.8 ±0.1	0.04
TSH (mlU/l)	2.3 ±1.4	2.1 ±1.1	2.3 ±1.5	NS
FT_4 (ulU/l)	1.6 ±2.5	2.4 ±4.4	1.3 ±0.3	NS
Protein C (mg/l)	9.7 ±17.3	6.1 ±2.5	11.7 ±21.9	NS
Glucose (mg/dl)	142.9 ±44.1	140.4 ±21.3	142.8 ±49.2	NS
Insulin (μU/l)	23.9 ±10.1	19 ±8.0	24.9 ±9.9	0.04
HOMA IR	8.4 ±4.6	6.6 ±3.2	8.9 ±5.2	NS
Vaspin mRNA	2.87 ±2.43	0.44 ±0.33	4.59 ±3.09	0.05

BMI – body mass index; WC – waist circumference; HGB – haemoglobin; WBC – white blood cells; PLT – platelets; ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGTP – gamma-glutamyl transpeptidase; TP – total protein; CRP – C-reactive protein; TSH – thyroid-stimulating hormone; fT_4 – thyroxine; HOMA-IR – homeostatic model assessment for insulin resistance; NS – non-significant

Liver histology

All included patients underwent a wedge liver biopsy, from the left liver lobe, performed during bariatric procedure. It is well known that assessing liver fibrosis at a distance of < 5 mm from the capsule may give inadequate results, overestimating fibrosis. Therefore, all liver specimens for the histology were taken repetitively with the following procedure – with laparoscopic scissors (no energy usage) a liver fragment with its capsule were resected to 1 cm depth. Then from the exposed parenchyma a 7 × 7-mm liver fragment was excised (the same tools were used). Every bariatric procedure was started by receiving the liver biopsy. There were two reasons for the above routine: the first one, liver cells inflammatory factor exclusion caused by the surgery itself ("surgical hepatitis"); and the second one, having the ability to evaluate the haemostasis grade in place of biopsy towards the end of the bariatric procedure. Tissue samples were immediately divided into two parts: the first one for routine histopathological examination; and second one was stabilised in RNA*later* (Sigma-Aldrich, St. Louis, USA) and frozen at –80°C for further molecular procedures. The liver biopsy samples for routine histological examination were stained with haematoxylin and eosin, the azan method was used for collagen fibres, and they were examined by two independent experienced pathologists.

Histopathological evaluation was carried out according to Kleiner's scoring scale [24].

mRNA vaspin expression in liver tissue

Total RNA was isolated from biopsy specimens using the RNeasy Mini Kit (Qiagen, Hilden, Germany). In addition to the standard procedure, RNase Free DNase Set (Qiagen, Hilden, Germany) was used to remove trace amounts of genomic DNA. RNA was quantified by measuring the absorbance at 260 and 280 nm (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, USA), and the integrity was assessed by electrophoresis in 1.2% agarose gel ethidium bromide stained. RNA isolates were used for cDNA synthesis with reverse transcription method using High Capacity RNA – to cDNA Kit (Applied Biosystems, Foster City, USA) according to the manufacturers' instructions. Received cDNA was used to determine vaspin gene expression level by real-time quantitative PCR (RT-Q-PCR) assay (TaqMan system). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. TaqMan primers and probe for vaspin and GAPDH were bought as ready-to-use assays: vaspin and human GAPD endogenous control (FAM/MGB Probe, Nonprimer Limited) for GAPDH (Applied Biosystems, Foster City, USA). RT-Q-PCRs were performed in duplicate on an ABI PRISM 7300 Real Time PCR Detection System (Applied Biosystems, Foster City, USA), including negative control in all amplification reactions. Thermal cycling for both analysed genes and GAPDH was initiated with an incubation step at 50°C for 2 min, followed by a first denaturation step at 95°C for 10 min, and continued with 40 cycles of 95°C for 15 s and 60°C for 1 min. The standard curves for a housekeeping gene GAPDH and the target genes were generated by serial dilutions of the control cDNA (equivalent to 1 μg of total RNA) in four two-fold dilution steps. The vaspin expression levels were determined in every sample from the respective standard curve and divided by the GAPDH gene expression to obtain a normalised target value (relative expression level).

Statistical analysis

The results were presented as the mean values with standard deviation (±SD). The distribution of the values was assessed using Shapiro-Wilk test. Due to abnormal distribution of the values, nonparametric methods were used for calculation. Differences between groups were tested using U Mann-Whitney and ANOVA rang Kruskal-Wallis tests for independent groups. The Spearman rank correlation coefficient was used to calculate the correlation between different values. $P < 0.05$ was considered to be statistically significant. The software STATISTICA 12.5 from StatSoft was used to perform the analysis.

Results

Demographic and clinical data

Demographic and clinical data of all subjects are presented in Table I. The study included 18 women with BMI < 40 kg/m^2 and 38 with BMI ≥ 40 kg/m^2. There were significantly higher ALT, AST, and gamma-glutamyl transpeptidase (GGTP) activities ($p = 0.04$; $p = 0.04$; $p = 0.001$, respectively) and increased insulin concentration ($p = 0.04$) in patients with higher BMI. There were no differences in glucose concentration and HOMA-IR level between patients with various BMIs. However, HOMA-IR level was above 3 in all analysed patients, and only in 15 patients it was less than 4. The detailed comparison between patients with different BMI is shown also in Table I.

Histological examination

The results of histopathological examination are shown in Table II. Patients with BMI ≥ 40 kg/m^2 had significantly more advanced fibrosis stage compared to those with BMI < 40 kg/m^2. There was no significant difference in grade of necroinflammatory activity, grade of steatosis, and hepatocyte ballooning between both analysed subgroups. Fibrosis was observed in 29 of the analysed patients. There was no advanced (stage 3 or 4) fibrosis in the analysed group. Stage 1 fibrosis was found in 20 patients, whereas stage 2 (perisinusoidal and portal/periportal) fibrosis was observed just in nine patients.

Lobular inflammation was found in 31 women. However, inflammatory activity of grade 2 was diagnosed only in nine patients.

Table II. Histopathological examination of the liver tissue in morbidly obese women.

Histopathological features	Patients (n = 56)	
	Number	%
Fibrosis (stage 0/1/2)	27/20/9	48/36/16
Lobular inflammation (grade 0/1/2)	25/22/9	45/39/16
Steatosis (grade 0/1/2/3)	17/10/14/15	30/18/25/27
Hepatocyte ballooning (grade 0/1/2)	19/17/20	34/30/36
NAS score (1-2/3-4/5-8)	25/14/17	45/25/30
NASH/noNASH*	24/32	43/57

*NAS – NAFLD score; * NASH diagnosed on the basis of histological patterns*

Macrovesicular or mixed steatosis was observed in all analysed patients. However, in 17 patients steatosis affected less than 5% of hepatocytes. Steatosis grade 1 was found in 10, grade 2 in 14, and grade 3 in 15 patients.

Hepatocyte ballooning, which is a hallmark of NASH, was also assessed and observed in 27 patients. It reached grade 2 in 20 females.

NAFLD Activity Score (NAS) was assessed in all analysed patients. Definite NASH (NAS ≥ 5) was found in 17 (30%), NAS 3-4 in 14 (25%), and simple steatosis (NAS ≤ 2) in 25 (45%) morbidly obese women.

The diagnosis of definite NASH or the absence of NASH based on evaluation of characteristic lesions on liver biopsy does not always correlate with threshold values of the semiquantitative NAS. Therefore, we decided to analyse and compare the results in the group of patients with NASH recognised on the basis of histological patterns (steatosis, lobular inflammation, and hepatocyte ballooning) and NAS. On the basis of histological hallmarks, we found that not all biopsies with NAS ≥ 5 had findings that meet the diagnostic criteria for definite NASH. The criteria were met in 16 out of 17 patients. On the other hand, eight cases of NAS ≤ 4 fulfilled the histological criteria of NASH. NASH according to NAS was diagnosed in 17 patients, whereas in 24 patients it was diagnosed on the basis of histological hallmarks. The detailed results are shown in Table III.

Vaspin mRNA levels in the liver tissue and its association with analysed parameters

Vaspin mRNA was found in all included patients. The mean vaspin mRNA level reached 2.87 ±2.43 and was markedly higher in patients with BMI ≥ 40 kg/m^2, but the difference was on the threshold of statistical significance (4.59 ±3.09 vs. 0.44 ±0.33; p = 0.05). Similarly, evidently up-regulated vaspin mRNA was observed between patients with hepatocyte ballooning (4.77 ±4.23 vs. 0.45 ±0.29; p = 0.27) and those with steatosis (4.80 ±4.20 vs. 0.41 ±0.29; p = 0.42). Also, additional analysis, which compared the subgroup of patients with grade 0 and 1 and those with grade 2 and 3 of steatosis, revealed an evident difference. However, the difference did not reach statistical significance (7.04 ±6.28 vs. 0.28 ±0.18, respectively; p = 0.60). There was also a noticeable difference in vaspin mRNA levels between patients with and without fibrosis (6.23 ±7.20 vs 0.25 ±0.80) and those without and with lobular inflammation (5.55 ±10.10 vs. 0.27 ±1.0, respectively). All the results are summarised in Table IV.

There was no statistically significant difference in vaspin mRNA levels between patients with definite NASH and those without, assessed according to NAS. Nevertheless, vaspin mRNA levels were markedly higher in patients with definite NASH when compared to those with simple steatosis or borderline NASH (10.50 ±10.40 vs. 0.24 ±0.96, respectively). Similar results regarding vaspin mRNA levels were found when compared patients with NASH and without NASH assessed on the basis of histological hallmarks (8.80 ±11.10 vs. 0.26 ±0.90, respectively). A comparison of vaspin mRNA levels and parameters of glucose metabolism in patients with and without NASH assessed according to NAS and histological arrangement is shown in Table V.

Association between other assessed parameters

There were significantly higher levels of glucose, insulin, and HOMA-IR when comparing patients with and without fibrosis (p = 0.007; p = 0.04; p = 0.004, respectively) (Table IV). Higher glucose levels were found in patients with lobular inflammation (p = 0.006). Higher levels of insulin and HOMA-IR were revealed in patients with steatosis (p = 0.003; p = 0.005, respectively). APRI seemed to be a surrogate marker to differentiate severely obese patients with and without fibrosis (0.21 ±0.02 vs. 0.37 ±0.04, respectively, p = 0.005), despite the fact that our analysed group did not included patients with advanced fibrosis.

Hepatocyte ballooning was positively associated with lobular inflammation (r = 0.54, p < 0.001) and steatosis grade (r = 0.57, p < 0.001).

Body weight was positively associated with ALT (r = 0.38, p = 0.03) and GGTP activity (r = 0.59, p = 0.002), whereas waist circumference was positively associated with ALT (r = 0.56, p = 0.001) and AST activity (r = 0.46, p = 0.008) and fibrosis stage (r = 0.37, p = 0.04). Fibrosis was positively associated with inflammatory grade (r = 0.45, p = 0.001), glucose concentration (r = 0.32, p = 0.02), HOMA-IR levels (0.36, p = 0.01), and APRI (r = 0.50, p < 0.001). A negative relationship was found between fibrosis stage and platelet count (r = –0.35, p = 0.01). Steatosis grade was found to be

Table III. The relationship between diagnosis of NASH based on semiquantitative NAS and histopathological patterns

NAS ≥ 5 17 patients	**NAS ≤ 4 39 patients**
NASH according to histological patterns 16 patients (94%)	NASH according to histological patterns 8 patients (20%)
noNASH according to histological patterns 1 patient (6%)	noNASH according to histological patterns 31 patient (80%)

NAS – NAFLD score

Table IV. Association between histopathological features and hepatic vaspin mRNA levels and parameters of glucose metabolism in morbidly obese women with NAFLD

Steatosis grade			
	Grade 0/1	Grade 2/3	p
Vaspin mRNA	0.28 ±0.18;	7.04 ±6.28	NS
Glucose (mg/dl)	137.7 ±40.6	148.8 ±50.9	NS
Fasting insulin (μU/l)	21.3 ±8.8	26.9 ±10.5	**0.04**
HOMA-IR	7.2 ±2.9	9.9 ±5.8	**0.04**
Steatosis grade			
	Grade 0	Grade 1/2/3	p
Vaspin mRNA	4.80 ±4.20	0.41 ±0.29	NS
Glucose (mg/dl)	134.4 ±50.8	146.9 ±42.9	NS
Fasting insulin (μU/l)	18.7 ±6.6	26.3 ±10.3	**0.003**
HOMA-IR	6.2 ±2.53	9.5 ±5.0	**0.005**
Hepatocyte ballooning			
	Grade 0	Grade 1/2	p
Vaspin mRNA	4.77 ±4.23	0.45 ±0.29	NS
Glucose (mg/dl)	144.2 ±59.0	142.1 ±37.2	NS
Fasting insulin (μU/l)	23.7 ±9.4	23.9 ±10.3	NS
HOMA-IR	8.4 ±3.3	8.4 ±5.3	NS
Lobular inflammation			
	Grade 0	Grade 1/2	P
Vaspin mRNA	0.27 ±1.00	5.55 ±10.10	NS
Glucose (mg/dl)	127.3 ±44.1	154.8 ±43.6	**0.006**
Fasting insulin (μU/l)	23.6 ±10.6	24.1 ±9.6	NS
HOMA-IR	7.4 ±3.7	9.2 ±5.1	NS
Fibrosis			
	Stage 0	Stage 1/2	p
Vaspin mRNA	0.25 ±0.80	6.23 ±7.20	NS
Glucose (mg/dl)	125.9 ±31.4	159.6 ±51.5	**0.007**
Fasting insulin (μU/l)	22.0 ±10.6	25.5 ±9.0	NS
HOMA-IR	6.8 ±3.2	10.0 ±5.3	**0.006**

HOMA-IR – homeostatic model assessment for insulin resistance; NAS – NAFLD score; NS – not significant

positively associated with insulin ($r = 0.30$, $p = 0.04$) and HOMA-IR levels ($r = 0.34$, $p = 0.02$), hepatocyte ballooning ($r = 0.57$, $p < 0.001$), inflammatory grade ($r = 0.44$, $p = 0.002$), and fibrosis stage ($r = 0.40$, $p = 0.006$).

Discussion

Generally, adipokines can be classified, according to their impact on chronic liver disease, into those having positive and negative influence. The data regarding the role of some novel adipokines in NAFLD is scarce, especially in morbidly obese patients. It was suggested that different adipokines are in balance when adipose tissue is in normal range. However, when adipose tissue expands, the fragile balance tends to be disrupted. As far as we are concerned, this is the first study regarding hepatic vaspin mRNA in morbidly obese patients. The role of vaspin in the development and progression of NAFLD and NASH

Table V. Comparison of hepatic mRNA levels and parameters of glucose metabolism in morbidly obese women with NAFLD assessed according to NAS and on the basis of histological patterns

	NAFLD score (NAS)		
	Simple steatosis/ borderline NASH (n = 39)	Definite NASH (n = 17)	p
Vaspin mRNA	0.24 ±0.96	10.50 ±10.40	NS
Glucose (mg/dl)	136.6 ±45.0	153.0 ±46.3	NS
Fasting insulin (μU/l)	23.0 ±9.3	24.9 ±11.9	NS
HOMA-IR	7.7 ±3.1	9.4 ±3.8	NS
	NASH diagnosed on the basis of histological patterns		
	noNASH (n = 32)	NASH (n = 24)	
Vaspin mRNA	0.26 ±0.90	8.80 ±11.10	NS
Glucose (mg/dl)	133.3 ±49.6	154.2 ±37.3	0.02
Fasting insulin (μU/l)	22.8 ±10.2	24.8 ±11.5	NS
HOMA-IR	7.5 ±3.3	9.4 ±3.3	NS

HOMA-IR – homeostatic model assessment for insulin resistance; NS – not significant

is still unclear. Currently, we did not find correlation between mRNA vaspin level and lipid parameters. On the other hand, we observed the strong tendency to increase values of hepatic vaspin mRNA in patients with more severe obesity reflected by higher BMI.

In our previous study including NAFLD patients without severe obesity [25], vaspin serum concentration tended to be decreased in subjects with NAFLD compared to healthy controls. On the other hand, vaspin serum levels increased in patients with NASH when compared to those with simple steatosis. However, Genc *et al*. did not show any significant difference in serum concentration of vaspin between patients with NAFLD and healthy controls. Moreover, Genc *et al*. found serum vaspin concentration to be positively correlated with HDL cholesterol and negatively with LDL cholesterol, total cholesterol, and triglycerides levels and suggested that vaspin take part in lipid metabolism [26]. The study by Aktas *et al*. revealed serum vaspin levels to be raised in patients with NAFLD, regardless of potential confounders, and to represent an independent predictor of liver fibrosis [27]. However, these differences did not remain robust after adjustment for BMI or waist circumference [27]. The most recent study by Polyzos *et a*l. revealed lower circulating vaspin in NASH patients than in controls [28].

Our study revealed that vaspin mRNA was found in liver specimens of all analysed patients. Some authors suggest that the induction of vaspin mRNA in human adipose tissue could serve as a compensatory mechanism associated with obesity and severe IR [26, 29]. The data with respect to hepatic vaspin mRNA and its relationship with IR is lacking. Our study did not disclose a significant relationship between hepatic vaspin expression and HOMA-IR. However, all the patients included in our study had abnormal HOMA-IR levels. None of the patients had HOMA-IR less than 3, suggesting the occurrence of IR in all morbidly obese patients included in our study. To better analyse the relationship between insulin sensitivity and hepatic vaspin mRNA levels, patients were divided into two subgroups: with HOMA-IR less than and above 4, and subsequently less than and above 5. Unfortunately, there was no difference in hepatic vaspin mRNA. The explanation of obtained results may be due to the fact, as described previously, that IR induces vaspin expression only in adipose tissue. The other explanation is the possibility that vaspin mRNA synthesis is evidently up-regulated when IR appears, and its increase is not proportional to further IR up-regulation. As mentioned above, all the patients analysed in our study had IR.

Steatosis is a hallmark of NAFLD. Macrovesicular or mixed steatosis was observed in all included patients. However, in 17 patients it affected less than 5% of hepatocytes. Steatosis was positively related to fibrosis stage and IR. HOMA-IR was significantly higher in patients with grade 0 than in the rest of the patients. Moreover, the difference was still significant comparing patients with grade 0 and 1 to those with grade 2 and 3 of steatosis. Similarly, analysis of mRNA vaspin levels in the liver tissue showed a marked difference with the higher intensity in patients with lipid storage affecting more than 5% of hepatocytes. Additional analysis revealed further increase of vaspin mRNA in patients with greater extent of steatosis, but the difference did not reach statistical significance. These results are in accordance with findings of our previous studies regarding vaspin serum lev-

els in CHC and NAFLD. All the studies showed increased serum vaspin concentration in patients with higher steatosis grade [22, 25, 30]. Pointing to the fact that steatosis results from increased IR, we could expect higher hepatic vaspin production in the case of more extensive steatosis. Our study revealed up-regulated vaspin mRNA in patients with more extensive steatosis, but it did not reach statistical significance. The ambiguous results may be due to the small number of patients with extensive steatosis and the small number in the entire analysed group.

The next histopathological feature included into the scoring system for diagnosis of NASH is hepatocyte ballooning [31]. In our study, its grade was associated with grade of lobular inflammation and steatosis. Our previous study in CHC patients showed a negative relationship between hepatocyte ballooning and serum vaspin levels [30]. In contrast, in NAFLD serum vaspin levels were positively associated with hepatocyte ballooning [27]. However, the most recent study did not find such a relationship [28].

Ongoing inflammatory process results in development of NASH and progression of fibrosis. Vaspin has anti-inflammatory activity inhibiting pro-inflammatory cytokines and adipokines. Surprisingly, in our study vaspin was markedly higher in patients with lobular inflammation. Alterations of synthesis of some adipokines and secretion during expansion of adipose tissue are compensatory mechanisms to neutralise the negative effects of up-regulated harmful factors. Lobular inflammation, steatosis, and hepatocyte ballooning are hallmarks of NASH. All three features are components of NAS and must coexist for diagnosis of NASH based on histopathological pattern. Pointing to the obtained results, revealing an evident increase of vaspin mRNA levels in patients with lobular inflammation, hepatocyte ballooning, and greater extent of steatosis, as expected the NAS score was also associated with vaspin mRNA levels. As mentioned above, the diagnosis of definite NASH based on evaluation of histological hallmarks (steatosis, inflammation, and hepatocellular ballooning) on liver biopsies does not always correlate with threshold values of the semiquantitative NAS. Although NAS and the diagnostic system based on histological patterns are closely correlated, they also have distinct clinical and pathological relationships [32]. To avoid the potential influence of different diagnostic procedure on our results, we investigated both diagnostic arrangements. In both cases the most evident up-regulation of vaspin mRNA levels was observed in patients with definite NASH.

NASH development is strictly associated with fibrosis progression. Our study revealed markedly higher vaspin mRNA in patients with fibrosis. Unfortunately, our study did not include patients with advanced fibrosis and cirrhosis and only a small group of patients with significant fibrosis.

Data regarding the relationship between serum vaspin and fibrosis in NAFLD is equivocal. The study by Aktas *et al*. showed positive association between serum vaspin and fibrosis stage. After stepwise linear regression analysis, serum vaspin concentration was the only independent predictor of liver fibrosis in patients with NAFLD. Serum vaspin levels were up-regulated in NAFLD patients regardless of potential confounders [27]. Our previous study and the study by Genc *et al.* did not find any relationship between serum vaspin and fibrosis stage in non-severely obese NAFLD patients [25, 26]. However, our study in CHC patients showed vaspin serum levels to be significantly higher in patients with significant and advanced fibrosis. Nonetheless, in patients without fibrosis and with insignificant fibrosis vaspin serum levels were significantly lower. An evident increase in vaspin levels was observed in patients with at least periportal fibrosis (stage 2), with no further increase in patients with advanced fibrosis. The next intriguing aspect was the fact that serum vaspin levels in patients with advanced fibrosis was similar to those in the control group [33]. Although its improper clearance by impaired liver or/and a compensatory increase having a protective effect directed against further liver injury and fibrosis progression could be possible explanations, a potential negative action of vaspin when NAFLD progresses to more advanced stages with fibrosis may not be excluded. This presumption is based on the fact that vaspin inhibits expression of profibrogenic factors such as leptin, TNF-α, and resistin [21]. Nevertheless, when fibrosis was established vaspin serum levels were not associated with its severity assessed according Child-Pugh and Model of End-Stage Liver Disease (MELD) scores [34]. It is well known that IR is an important risk factor of fibrosis progression in chronic liver diseases, including NAFLD [4, 12, 35, 36, 37]. In our study, HOMA-IR was significantly associated with fibrosis stage supporting earlier findings. In the current study, hepatic vaspin mRNA expression was higher in patients with fibrosis, but the difference did not reach statistical significance. Unfortunately, significant fibrosis (stage 2) was observed only in 16% of patients. Moreover, there were no patients with bridging fibrosis or cirrhosis. This small number of patients with significant and the lack of those with advanced fibrosis in our group may influence the obtained results.

This study has some limitations. First, the analysed group of patients is relatively small. Second, the study included patients without advanced fibrosis and cirrhosis. Third, there was no possibility to compare the obtained results in morbidly obese patients with overweight and obese subjects with NAFLD and healthy controls. Fourth, the study has an observational nature. Fifth, there was no possibility to assess vaspin mRNA synthesis in adipose tissue, which

is suggested to be the main source of systemic vaspin. Sixth, the difference of means between more and less advanced states was evident but statistically insignificant, probably due to the large statistical dispersion of the values of vaspin mRNA levels. This phenomenon indicates that the expression of vaspin gene could be very diversified in different subjects and depends not only on the amount of adipose tissue and BMI. Seventh, the study included only female patients and the results should not be extrapolated to all patients. Finally, eighth, wedge liver biopsy was performed in all analysed patients. As was mentioned above, it is well known that assessing liver fibrosis at a distance of < 5 mm from the capsule, when compared to needle liver biopsy, may give inadequate results, overestimating fibrosis. However, to minimise the potential negative influence of overestimated fibrosis on the obtained results, all the specimens were resected to 1 cm depth, with an excised fragment of 7 × 7 mm for further evaluation.

Most alterations of adipokines during the expansion of adipose tissue and exacerbation of IR seem to compensatory and directed to retain a proper balance or to eliminate a negative effect exerted by other factors. It seems that adipokines are in balance when adipose tissue is in normal range. However, when adipose tissue expands the balance tends to be lost. At the beginning, compensatory mechanisms may be successful and efficient to maintain insulin sensitivity and to dam hepatic steatosis and progression to NASH. Unfortunately, when adipose tissue continues to extend, vaspin alteration may be more harmful than beneficial. These suggestions may be supported by the study by Auguet *et al*., who found vaspin mRNA levels to be significantly higher in both visceral and subcutaneous adipose tissue in morbidly obese compared to healthy lean women, but there was no difference in serum vaspin levels [38]. Additionally, hepatic vaspin expression may not correlate with its systemic levels and may be associated with paracrine or autocrine action in the liver.

In conclusion, we may infer from our study that adiposity is an undoubted confounding factor influencing serum vaspin levels. Also, hepatic vaspin gene expression seems to be associated with adipose tissue extent. Vaspin mRNA levels in the liver seem to be markedly elevated in morbidly obese patients with more advanced NAFLD and when hallmarks of NASH were observed. Vaspin may be a compensatory factor in IR and fibrosis. Pointing to non-linear mRNA expression within the NAFLD spectrum and the evident increase in patients with fibrosis and definite NASH, a detrimental action of vaspin cannot be excluded. Prospective cohort studies in men and women with paired biopsies would facilitate the assessment of the pathophysiologic interplay between vaspin and NAFLD progression over time.

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