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Visfatin and TGF-β1 in primary biliary cirrhosis and two other common liver diseases

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Abstract: The aim of this study is to investigate plasma concentration of visfatin and transforming growth factor β1 (TGF-β1) in three groups of patients: primary biliary cirrhosis (PBC), non-alcoholic fatty liver disease (NAFLD) and toxic cirrhosis (TC). We qualified the patients into the study and assigned them to the appropriate group according to clinical examination, laboratory tests and ultrasound imaging technic (US). We showed that plasma concentrations of visfatin in PBC, NAFLD and TC group were respectively 1.41 ± 1.76 ng/mL, 1.22 ± 1.08 ng/mL and 0.70 ± 1.22 ng/mL. Plasma concentration of visfatin was significantly lower in TC group than in others both ($p < 0.017$). The differences of visfatin concentration between NAFLD and TC group were not statistically significant. The values of TGF-β1 in PBC, NAFLD and TC group were respectively 21031 ± 7822 pg/mL, 21588 ± 12639 pg/mL, and 9678 ± 4757 pg/mL. The statistical analysis showed that the value of cirrhotic group was significantly ($p < 0.017$) lower compared to both others groups. The difference between PBC and NAFLD was insignificant.
In conclusion: Despite the PBC and NAFLD are the diseases of different pathogenesis and origin, plasma concentration of visfatin and TGF-β1 were similar in these both groups but significantly lower in TC probably due to decreased activity as well as number of cells producing these cytokines in the cirrhotic liver.

Key words: PBC, NAFLD, cirrhosis, visfatin, TGF-β1.

Introduction

PBC is the chronic disease of autoimmunologic etiology with cholestasis caused by the damage of intrahepatic small ducts. This process leads to progressive fibrosis and consequently end-stage liver disease. PBC was first described by Addison and Gull in 1857 [1]. Recently, some investigators suggest that primary biliary cholangitis would be the better name for this disease. The reason for the specific aggression of immune system by antymitochondrial antibodies against E2 complex of pyruvate dehydrogenase on the internal mitochondrial membrane and destruction of the epithelium of the bile ducts is uncertain. Many conceptions of the pathogenesis of this disease such as: an appropriate but increased response to infected cells (PBC as a kind of infectious disease), an undesirable responses to normal cells (PBC as an autoimmune disease), responses by specific autoantibodies and autoreactive T-cells are considered [1]. Some recent studies emphasize the significance of transmembrane G coupled receptor (TGR5), nuclear receptors, mainly farnesoid X receptor (FXR) [2]. It is uncertain whether TGF-β1 has the pathogenic value in the development of PBC.

The importance of cytokines is also considered because they are involved in the interactions between the cells via receptors, amplification of the inflammatory response, regulation of autoimmune processes and fibrogenesis.

Visfatin is an adipokine — hormone of the adipose tissue. It has the proinflammatory and insulin-mimetic properties contributing to whole body glucose and lipid metabolism [3]. Its plasma level is associated with the amount of fat (mainly visceral), type II diabetes mellitus (T2DM) and metabolic syndrome. Secretion of visfatin is elevated after glucose administration [4, 5]. However, the exact role of visfatin in NAFLD and non-alcoholic steatohepatitis (NASH) is not clear. Decreased level of visfatin was found in NASH when compared to NAFLD patients and healthy controls [6]. The level of visfatin was positively correlated with portal inflammation [7]. The importance of visfatin in fibrinogenesis is uncertain. The median visfatin level significantly increased from Child–Pugh A to Child–Pugh C [8]. Genc *et al.* found that plasma levels were not altered in the early stages of NAFLD and were inversely associated with the concentration of tumor necrosis factor-alpha (TNF-α) [3]. These authors suggested that visfatin was crucial in the protection against liver injury in NAFLD. The significance of visfatin in PBC was not explored.

TGF-β1 is an important antiproliferative and profibrogenic cytokine. It signals through TGF-β1 receptor II (TβRR II) and TGF-β1 receptor I (TβRR I) that phosphorylate Smads at the mad homology 2 domain [9, 10]. Disturbance of TGF-β1signaling contributes to the development of several disorders and diseases including autoimmune diseases, fibrosis and cancer [9, 11–13]. A dominant negative form of TβRR II in transgenic mice, under the CD4 promoter lacking the CD8 silencer [9, 14] leads to the features characteristic for PBC [9, 15]. A compromised cytoarchitecture and polarized

trafficking of TGF-β1signaling molecules including embryonic liver fodrin and Smad3 are probably included in the pathogenesis of PBC as well [9, 16]. Some studies indicated that TGF-β1 could be a marker of fibrosis and could be even the sign of disease's severity in patients with PBC [17, 18]. Liu *et al.* showed in the experimental study that TGF-β1 played a dual role in the development of PBC because it led to enhance fibrogenesis but suppressed inflammatory response [9].

However, the indicators of fibrosis and inflammation in the liver diseases are insufficient up today. Liver biopsy is still the gold standard for the determination of the steatosis, inflammation and fibrosis. The exploration of less invasive methods to define of the grade of these important pathological features is carried out.

The aim of the present study is to compare plasma concentrations of visfatin and TGF-β1 in three groups of patients with the liver diseases: PBC, NAFLD and TX and to explore the relationship between these concentrations and the stage of the disease determined on the basis of histopathological, clinical and laboratory data.

Material and methods

Patients and study design

This examination was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and was approved by the Ethics Committee of the Medical University of Silesia (resolution number KNW/0022/KB1/8/13, 12.02.2013.). Subjects provided the written informed consent before enrollment to the study. Individuals with diagnosed PBC, NAFLD or TC with an age range of 20 to 75 were included to the study. Exclusion criteria included the presence of any inflammatory, systemic, endocrine, or metabolic disease, pregnancy, or lack of participant agreement.

A total of 61 patients participated in the study: 17 with PBC, 21 with NAFLD and 23 with TC. Demographic data of the patients are presented in the Table 1. Participants

Table 1. Demographic of patients participating in the study.

	PBC	NAFLD	TC
Number of subjects	17	21	23
Women	17	11	5
Men	0	10	18
Age [years]	58.24 ± 9.09 (41–75)	32.67 ± 12.06 (20–60)	54.57 ± 10.38 (36–70)
BMI [kg/m^2]	25.88 ± 3.76 (20.81–32.41)	31.8 ± 7.73 (18.4–51.9)	26.99 ± 5.09 (17.40–36.33)

PBC — primary biliary cirrhosis
NAFLD — non-alcoholic fatty liver disease
TC — toxic cirrhosis

of all three groups were comparable regarding age. BMI was significantly ($p < 0.05$) higher in NAFLD group comparing to other ones. In the PBC group were only women, but in TC group the number of men was much higher than women. The dominance of the number of women or men in the groups is similar to normal population and arises from the etiology of the diseases. In NAFLD group the number of participants of both sexes was comparable.

Clinical diagnosis of PBC was based on laboratory tests including increased activity of cholestatic enzymes — alkaline phosphatase (ALP) and gamma-glutamylotranspeptidase (GGT), presence of antimitochondrial antibodies (AMA), US imaging technic where the normal size and appearance of bile ducts was showed. Clinical diagnosis of NAFLD was based on the ultrasound imaging technic (US) and the results of laboratory tests. If the laboratory results are in normal range we diagnosed simple steatosis. If the values of aminotransferases (AST and ALT) activity were above the normal range, we suspected non-alcoholic steatohepatitis (NASH) and classified the patients to liver biopsy. Clinical diagnosis of TC was based on the anamnesis (consumption of alcohol >20 g/day in women and >60 g/day in men), physical examination with the characteristic traits, low concentration of protein (<6 g/dL) and albumin (<3.5 g/dL), high level of INR (>1.2), characteristic appearance of liver in US, symptoms of portal hypertension.

We performed liver biopsy on all patients with PBC and on these ones from the NAFLD group that had the activity of aminotransferases above the normal range (11 of 21 patients with suspection of NASH). We determined the degree of fibrosis and inflammation by histopathologic evaluation using Ludwig scale [19] for PBC patients and Kleiner scale [20] for the NAFLD group. Table 2 presents the number of patients in each grade of fibrosis and inflammation in both groups.

Table 2. Severity of fibrosis and inflammation in the PBC and NAFLD group.

	Number of patients	Fibrosis (grade)						Inflammation (grade)					
	Total	0	I	II	III	IV	Not deter -mined	0	I	II	III	IV	Not deter -mined
PBC	17	0	7	4	5	1	0	0	5	6	4	2	0
NAFLD	21	7	1	2	1	0	10	5	2	0	3	1	10

PBC — primary biliary cirrhosis
NAFLD — non-alcoholic fatty liver disease
Fibrosis and inflammation — calculated in Ludwig scale for PBC (according to reference [19]).
Fibrosis and inflammation — calculated in Kleiner scale for NAFLD (according to reference [20]).

In the TC group the biopsy was not performed. The severity of the disease was determined on the basis of Child-Pugh [21, 22] as well as of Model for End-stage Liver Disease (MELD) scale [23]. The numbers of patients of each grade of both scale are presented in the Table 3.

Table 3. Severity of the disease in the cirrhosis group according to Child-Pugh and MELD.

	Number of patients	Child-Pugh (grade)				MELD		
	Total	Mean (points)	A	B	C	Mean (points)	<12 points	≥12 points
TC	23	7.70 ± 1.96 (5.00–12.00)	6	13	4	14.30 ± 5.32 (7.00–26.00)	10	13

TC — toxic cirrhosis
Child-Pugh — scale of severity of cirrhosis (according to references [21], [22])
MELD (Model for End-stage Liver Disease) — scale of severity of cirrhosis (according to reference [23]).

Patients from the group with PBC were treated with UDCA — 750 mg per day (250 mg in the morning and 500 mg in the evening). They had not any other diseases and did not received other medicaments. The patients included to the NAFLD group did not received any medications. The patients from the TC group received diuretic therapy due to ascites or edema of the lower limbs, or both of them, (spironolakton — 9 patients, furosemid and spironolakton — 10 patients). The drugs decreasing the portal hypertension and the risk of bleeding from the esophageal varices (propranolol — 14 patients and carvedilol — 6 patients) were administered, as well. The doses of spironolacton were 100–200 mg per day, furosemid 40–80 mg per day. The doses of propranolol were 20–60 mg per day and carvedilol 12.5–25 mg per day.

Blood samples were collected from the patients, under fasting conditions. Blood was centrifugated 10 min after collection and serum samples were immediately frozen at –80°C. Enzyme-linked immunosorbent assays (ELISA) were used to determine serum concentration of visfatin using Visfatin (NAMPT) Human ELISA KIT, BioVendor, Brno, Czech Republik and TGF-β1, using Human TGF-β1 ELISA KIT, Diaclone SAS, France.

Statistical analysis

All analysis was performed using Statistica PL version 10.0 (StatSoft Inc, Tulsa, OK, USA). Intergroup differences for each cytokine were analyzed using the Kruskal-Wallis test (nonparametric ANOVA) for global comparison between PBC, NAFLD and TC subjects. Visfatin and TGF-β1 concentrations were compared between the groups using the post-hoc Mann-Whitney U test with Bonferroni correction for multiple comparisons. Linear regression analysis was performed to assess the associations between the severity of the disease and the concentrations of visfatin and TGF-β1. Data are expressed as mean ± standard deviation, and a p value of 0.05 or less was considered as statistically significant. Rang correlations test of Spearman was used for evaluation of dependency between two measured parameters.

Results

The results of visfatin and TGF-β1 plasma concentration are presented in Table 4.

Table 4. Comparison of plasma concentration of visfatin and TGF-β1 in patients with PBC, NAFLD and TC.

	PBC	NAFLD	TC	H	p
Visfatin	1.41 ± 1.76 *	1.22 ± 1.08 ^	0.70 ± 1.22	12.19	0.002
TGF	21031 ± 7822 *	21588 ± 12639 ^	9678 ± 4756	22.56	0.000

mean ± standard deviation (SD); test H of Kruskal-Wallis (nonparametric ANOVA)
* p <0.017 PBC vs. C; test U of Mann-Whitney with Bonferroni correction
^ p <0.017 NAFLD vs. C; test U of Mann-Whitney with Bonferroni correction
PBC — primary biliary cirrhosis
NAFLD — non-alcoholic fatty liver disease
TC — toxic cirrhosis

Baseline serum concentration of visfatin was significantly lower in TC group if compared to PBC or NAFLD group. However, the concentrations of visfatin concentration in PBC and NAFLD were similar and the difference did not reach statistical significance.

Baseline serum concentration of TGF-β1was also significantly the lowest in TC group and it was similar in PBC and NAFLD group without significant difference between them.

The regression analysis showed that there was no correlation between the concentration of visfatin or TGF-β1 and the grade of fibrosis as well as the grade of inflammation in the PBC group.

We did not find the correlation between the concentration of visfatin or TGF-β1 and the grade of fibrosis or inflammation in the NASH subgroup of NAFLD group.

Our results showed that the concentrations of visfatin and TGF-β1 in the group TC were not dependent on the grade of Child-Pugh classification and on the number of points in MELD classification.

We classified the patients of cirrhosis group according to Child-Pugh classification, into three subgroups: A, B and C. We did not find the differences of visfatin and TGF-β1 concentration between these subgroups.

We also classified the patients of TC group dependently on points number according to MELD classification into two subgroups: <12 points and ≥12 points and we also did not find any differences of visfatin as well as TGF-β1 concentration between these subgroups.

Discussion

Although the etiology and physiopathology of PBC remains unclear, the most prevalent hypothesis is that the disease develops due to dysfunction of the immunologic system. NAFLD has quite different origin and many metabolic disturbances were taken into

consideration in the field of the pathogenesis of this disease. However, both these diseases lead to the same end-stage liver disease — cirrhosis.

We explored the concentrations of two cytokines — TGF-β1 and visfatin in three groups of patients with liver diseases: PBC, NAFLD and TC in this study. We compared plasma levels of these cytokines with the stage of the liver disease determined on the basis of histopathological, clinical and laboratory data.

It was found that visfatin plasma level did not differ between two groups of patients: PBC and NAFLD but it was higher than in TC group in both cases.

There are many conflicting data about the importance of visfatin in liver diseases. It is produced by hepatocytes, adipocytes, lymphocytes, monocytes, neutrophils, and pneumocytes [3]. This protein has many immunomodulating and proinflammatory properties. Visfatin enhances production of proinflammatory cytokines, synthesis of adhesion molecules and activation of leukocytes [3]. The up-regulation of visfatin expression was observed in a variety of acute and chronic inflammatory diseases [3, 24–26]. Since many years visfatin was an object of interest in terms of liver diseases, particularly NAFLD. It was shown that plasma visfatin levels were significantly increased in subjects with NAFLD comparing to both lean and obese healthy control subjects. Moreover, the level of visfatin was lower in patients with NASH [6]. In other study, serum visfatin level was not related to the grade of steatosis in overweight and obese patients with NAFLD [7]. However, the level of visfatin was related to the severity of portal inflammation. Nevertheless, the correspondence between the level of visfatin and the grade of lobular inflammation in NAFLD was not showed [7]. Other authors showed that the amount of visfatin in the visceral tissue was significantly lower in patients with NASH than in patients with simply steatosis and was higher in non-NAFLD subjects. Moreover, low level of visfatin in the visceral tissue was independent of body mass index (BMI) and insulinoresistance (IR). The authors of these observations indicate that visfatin could have protective importance in NAFLD [27]. In the other interesting study expression of visfatin in the liver tissue and the serum level of this protein were decreased in patients with NAFLD, but the difference between simply steatosis and NASH was not found [28]. Other authors did not found the difference between plasma level of visfatin in NAFLD patients comparing to healthy controls [3].

The number of studies about the relations between visfatin and advanced cirrhosis is not large. The significance of this protein in the development of PBC was almost not investigated. The studies about the importance of visfatin in liver cirrhosis are contrary. De Boer *et al.* showed that plasma level of visfatin was 78% lower in cirrhotics and it decreased with worsening of the clinical stage of liver disease. It also decreased according to reduced liver function [29]. In correspondence with these finding, hepatic visfatin mRNA expression was significantly lower in cirrhotic livers. The level of visfatin in plasma was correlated with body cell mass and with body fat mass. The authors concluded that plasma level of visfatin is significantly decreased in

liver cirrhosis and it is presumably attributable to decreased hepatic expression and production [29].

These results and interpretation are in agreement with our finding because we also showed that plasma concentration of visfatin was the lowest in TC group. Similarly as in De Boer *et al.* studies, we can suggest that the visfatin level could decrease accordingly not only with the decreasing body cell mass but also with the diminishing number of active hepatic cells. However, in contrary to de Boer study we did not prove that plasma level of visfatin is dependent on the stage of the disease in any of the explored groups of patients. We did not find the correlation between plasma concentration of visfatin and the grade of fibrosis or inflammation in PBC and NAFLD group (NASH subgroup) as well as the number of points in Child-Pugh and MELD scale in TC group. However it should be emphasized that we had a small number of patients with the most advanced disease — only 4 patients were classified into C grade and 13 patients into B one in Child-Pugh scale. It could be the reason why the results of regression analysis were not significant. The larger number of patients in each subgroup is necessary during the continuation of the study.

The versatile effect of TGF-β1 on other cells includes the control of the maturation, differentiation and activity of various T cells subsets. It actuates or prevents infections, immune diseases, graft-versus-host reactions and cancer formation [30]. TGF-β1 is the very important regulator of the hepatic fibrosis and probably it is of high importance in the development of PBC [31, 32]. The abnormality of the TGF-β1 signaling pathway in T lymphocytes may play an essential role in the pathogenesis of PBC and seems a key phenomenon, leading to the development of mechanisms such as: peripheral intolerance and, consequently, autoimmunologic diseases [15]. The considerable increase in frequency of CD8+ T cells in peripheral blood of PBC patients and correlation of it with damage of the biliary ducts was showed in PBC patients [33–35]. One study showed the decrease of the circulating precursors of CD4+ CD25+ regulatory T cells (Treg) number in PBC patients [36]. However, the data are contrary because the other — experimental study — showed meaningful increase in the number and percentage of CD4+ CD25+ FOXP3+ lymphocytes in experimental PBC compared to control mice [9]. TGF-β1 is the key modulator of FOXP3+ expression in Treg cells [37]. TGF-β1 regulation of FOXP3+ Tregs can be involved in the maintenance of chronic inflammation in PBC [9]. TGF-β1 could down-regulate harmful inflammatory responses in the liver, regardless increasing the possibility of scar formation [38]. TGF-β1 induces the phosphorylation of Smad2 and Smad3. These factors could thereafter be translocated into the nucleus, leading to regulation of expression of target genes — α1collagen and α-SMA [39].

TGF-β1 could be involved in the fibrogenesis in PBC, including many complicated phenomena. Liver fibrosis occurs as a result of the differentiation of hepatic stellate cells (HSCs) into myofibroblasts. This process is regulated by TGF-β1 [40]. The signal pathway of TGF-β1 is involved in myofibroblast differentiation and, consequently, liver fibrosis [9].

However, it is highly probable that TGF-β1 could play a double role in the development of PBC, because it suppresses inflammatory response but leads to enhance fibrogenesis.

Our results showed that TGF-β1 has comparable concentration in the group of patients with PBC and NAFLD and significantly higher one than in TC group. The interpretation of decrease plasma concentration of TGF-β1 in TC patients compared to PBC and NAFLD group is unclear. Other studies showed that the level of TGF-β1 is increased in hepatic cirrhosis and this increment could serve as a biomarker of the stage of fibrosis and functional impairment of the liver [30, 41]. The explanation of our results can be following: it is possible that although the fibrosis in liver cirrhosis is the most advanced, the "*de novo*" fibrogenesis is not so active as in "precirrhotic" diseases like PBC and NAFLD. On the other hand the inflammatory processes as well as fibrogenesis in PBC and in NASH could be similarly active despite different pathogenesis and mechanisms leading to advanced stage of the disease — liver cirrhosis. In other words, the immunologic and inflammatory processes could be more active in PBC and NASH than in cirrhosis, where they are slower. TGF-β1 is produced in the liver by multitude of non-parenchymal liver cells including hepatic stellate cells (HSCs), liver sinusoidal endothelial cells (LSECs), Kupfer cells (KCs), dendritic cells (DCs) and natural killer (NK) cells [30]. It's worth emphasizing that the number of all liver cells in advanced cirrhosis is decreased. Consequently, the number of cells that produce TGF-β1 is decreased, as well. To sum up, this attempt to explain decrease plasma concentration of TGF-β1 in cirrhotic compared to PBC and NASH patients in our results is similar to presented above regarding visfatin. Similarly, we did not found the correlation between plasma concentration of TGF-β1 and the degree of severity of the disease, probably for the same reasons as in regard to visfatin.

In conclusion, our results showed that visfatin and TGF-β1 could be the essential factors in the pathogenesis and, probably, in the development of liver diseases. These results are partially in agreement with some, but not all examinations in the available references. However, very complicated pathways of TGF-β1 action are the reason why the exact importance of it in the development on liver diseases is yet far from explanation. Our results showing that plasma concentrations of visfatin and TGF-β are lower in TC compared to PBC and NAFLD indicate that not only fibrotic stage but also the activity of fibrogenesis, as well as, other phenomena like inflammation or immunologic activity could be of great importance. Many contrary data about the role of visfatin and TGF-β1 in liver diseases require further research.

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Conflict of interest

None declared.

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