

ORIGINAL PAPER

EXPRESSION OF RESISTIN IN THE LIVER OF PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

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Adipokines are cytokines that presumably connect the pathologies of metabolic syndrome. One of the adipokines is resistin, the role of which in insulin resistance, obesity, and non-alcoholic fatty liver disease (NAFLD) needs to be determined. Liver biopsy specimens were obtained intraoperatively from 214 obese patients. Histological assessment was based on NAFLD activity score according to Kleiner. Statistical analysis involved semi-quantitative immunohistochemistry assessment of resistin staining and: NAFLD status in obese patients compared with a non-obese control group, selected clinical data (age, sex, body mass index – BMI), selected biochemical data, comorbidities (hypertension, type 2 diabetes mellitus, dyslipidaemia), and metformin treatment in patients with type 2 diabetes mellitus. Resistin expression was observed in the histiocytes of inflammatory infiltrate, Kupffer cells, and histiocytes surrounding the hepatocytes with steatosis. There was a positive correlation between the total expression of resistin and: (1) NAFLD advancement (NAFLD Activity Score- NAS), (2) AST, ALT, BMI, glucose, insulin, Homeostasis Model Assessment (HOMA), LDH, GGT, triglycerides (TG), and glycated haemoglobin (HbA_{1c}). Resistin expression was more intense in patients with type 2 diabetes mellitus and dyslipidaemia and less intense in the control group. Resistin probably plays a role in the pathogenesis of hepatic insulin resistance and aggravates pathologic changes in the liver of patients with NAFLD.

Key words: adipokines, insulin resistance, steatohepatitis, obesity.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as a lipid accumulation exceeding 5% of hepatocytes. It is estimated that NAFLD is present in 17-33% of the population in developed countries [1]. However, its prevalence among obese people may reach 70-80% [1]. NAFLD is related to increased mortality risk, with primary causes of death being advanced liver disease, cardiovascular disease, and diabetes mellitus [2]. Cytokines released from the adipose tissue called adipokines

seem to be important contributing factors in the development of NAFLD (ranging from steatosis to cirrhosis). Adipokines are believed to constitute an important link between obesity, insulin resistance, and NAFLD. One of the adipokines is resistin. In animals, it is mainly released from adipose tissue, while in humans it is released from macrophages [3]. The exact role of resistin in the pathogenesis of NAFLD is ambiguous. Its correlation with liver insulin resistance, steatosis, liver inflammation, and obesity has been suggested [1, 3, 4].

Table I. Study group characteristics based on NAFLD activity score according to Kleiner [5]

| NAS scale total score | 0 | 1-2 | 3-4 | 5-8 |
|---|-------------|------------|------------|-------------|
| No. of patients | 9 (4.2%) | 27 (12.6%) | 66 (30.8%) | 112 (52.4%) |
| Steatosis score in NAS scale | 0 | 1 | 2 | 3 |
| No. of patients | 37 (17.3%) | 73 (34.1%) | 79 (36.9%) | 25 (11.7%) |
| Inflammation score in NAS scale | 0 | 1 | 2 | 3 |
| No. of patients | 14 (6.5%) | 52 (24.3%) | 89 (41.6%) | 59 (27.6%) |
| Liver cell injury (ballooning) score in NAS scale | | 0 | 1 | 2 |
| No. of patients | | 51 (23.8%) | 96 (44.9%) | 67 (31.3%) |
| Fibrosis stage | 0 | 1 | 2 | 3 |
| No. of patients | 123 (57.5%) | 74 (34.6%) | 14 (6.5%) | 3 (1.4%) |

The aims of the study included the following:

1. Immunohistochemical assessment of resistin expression in the liver of morbidly obese patients undergoing bariatric surgery.
2. Evaluation of the correlation between resistin expression and NAFLD advancement in obese patients in comparison with an obese non-NAFLD group and non-obese control group.
3. Assessment of resistin expression correlation with selected clinical and biochemical data, comorbidities, and metformin treatment in diabetic type 2 patients.

Material and methods

Materials

The study is a retrospective analysis. Liver biopsy specimens were obtained routinely via intraoperative incisional biopsy from 214 morbidly obese patients operated on at the Chair and Department of General, Transplant, and Liver Surgery, Medical University of Warsaw during the period from 2005 to 2013. The control group was made up of specimens taken from 17 patients with normal weight operated due to haemangiomas of the liver between 2002 and 2013. The specimens were embedded in paraffin blocks. They were evaluated by one experienced pathologist from the Chair and Department of Pathology of the University Hospital.

The patients were qualified for bariatric surgery if their BMI was ≥ 40 kg/m² or if their BMI was ≥ 35 with at least one comorbidity, such as hypertension, type 2 diabetes mellitus, or dyslipidaemia. The mean age of patients was 42.3 years (range 19-65) and mean BMI was 46.8 (range 33.5-71.6). Diabetes was diagnosed in 42.1%, hypertension in 60.7% and dyslipidaemia in 42.9% of patients. Women accounted for 63.1% of the patients.

Histology

Liver specimens were fixed in 4% formalin, and standard haematoxylin and eosin (HE) staining was performed. The antigen retrieval was performed in PT Link (DAKO) in Targent Retrieval Solution high-pH buffer (DAKO K8004). For immunohistochemical staining, primary anti-resistin monoclonal mouse antibody was used (Acris, AM01373PU-N, 1:500 dilution in Antibody Diluent – DAKO En Vision Flex K8006, visualization DAB). Immunohistochemistry was also performed with CD68 antibody (Flex Monoclonal Mouse Anti-Human CD68, Clone KP1, Ready-to-Use, DAKO GA609). To evaluate the fibrosis, Silver Impregnation for reticulum 04-040801 was used (Bio-Optica special stains kit).

Histological assessment was based on NAFLD activity score according to Kleiner [5]; non-alcoholic steatohepatitis (NASH) was recognised if the score exceeded four points. Histological group characteristics are shown in Table I.

The assessment of resistin expression was semi-quantitative in:

- the histiocytes (macrophages) of inflammatory infiltrate (0-3 pts),
- sinusoidal Kupffer cells (0-3 pts),
- histiocytes surrounding the hepatocytes with steatosis (0-3 pts).

The total expression of resistin was the sum of all points.

Expression was scored as the following: 0 – if there was no expression, 1 – if there was expression in 1-10% cells, 2 – if there was expression in 11-30% cells, 3 – if there was expression in $> 30\%$ cells.

Statistical analysis

Statistical analysis included the correlations/differences between:

1. Immunohistochemistry results of resistin expression and NAFLD advancement (NAS scale, inflammation, steatosis, hepatocytes ballooning, and fibrosis) in obese patients compared with a non-obese control group.
2. Immunohistochemistry results of resistin expression and:
 - selected clinical data (patient's age, sex, BMI),
 - the levels of selected biochemical parameters (leucocytes, lymphocytes, monocytes, eosinophils, neutrophils, haemoglobin, MCV, platelet count, protein, albumin, C-reactive protein [CRP], bilirubin, AST, ALT, de Ritis index [AST/ALT ratio], LDH, ALD, GGT, total cholesterol [TC], HDL, LDL, TG, atherogenicity index (TC/HDL-C ratio), glucose, uric acid, creatinine, HbA_{1c}, INR, fibrinogen, vitamin B₁₂, C-peptide, insulin, Homeostasis Model Assessment [HOMA index = fasting insulin { μ IU/ml} \times fasting glucose {mmol/l} / 22.5], iron, Total Iron Binding Capacity [TIBC], transferrin, ferritin, folic acid),
 - comorbidities of metabolic syndrome (hypertension, type 2 diabetes mellitus, dyslipidaemia),
 - metformin treatment in patients with type 2 diabetes mellitus.

U Mann-Whitney test was used to compare the differences in total resistin expression between the study group and controls, while the chi-square test was employed to compare the differences in resistin distribution in macrophages, Kupffer cells, and histiocytes surrounding the hepatocytes with steatosis between the study group and the control group.

Chi-square test was used to investigate possible dependence of resistin expression in macrophages, Kupffer cells, and histiocytes surrounding the hepatocytes with steatosis upon the levels of the analysed binary variables (sex, dyslipidaemia, diabetes mellitus). U Mann-Whitney test was used to check if total resistin levels differed depending on the category of binary variables (sex, dyslipidaemia, diabetes). ANOVA (analysis of variance) was performed to determine whether mean levels of continuous variables depended on the level of resistin expression. The correlation between total resistin expression and continuous variables was also investigated by calculating the Spearman correlation coefficient. P values <0.05 were considered to be statistically significant.

Results

The assessment of resistin location in the liver showed:

1. Lack of resistin expression in hepatocytes.
2. In each case, resistin expression was noted in histiocytes of the infiltrate (Fig. 1).
3. Expression of resistin in the Kupffer cells (Fig. 2), correlated with NAS scale.

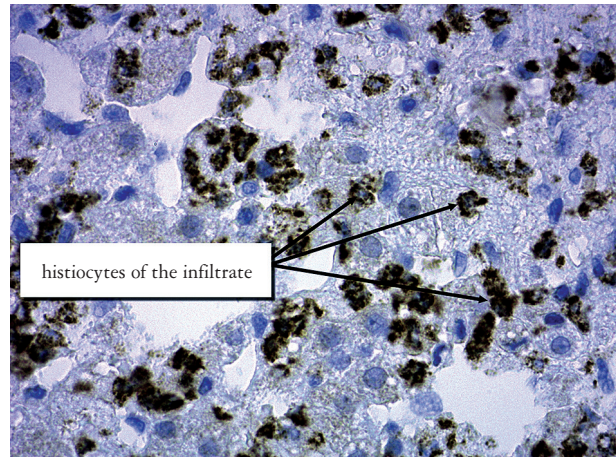


Fig. 1. Resistin expression in the histiocytes of inflammatory infiltrate (the objective lens magnification is 40 \times)

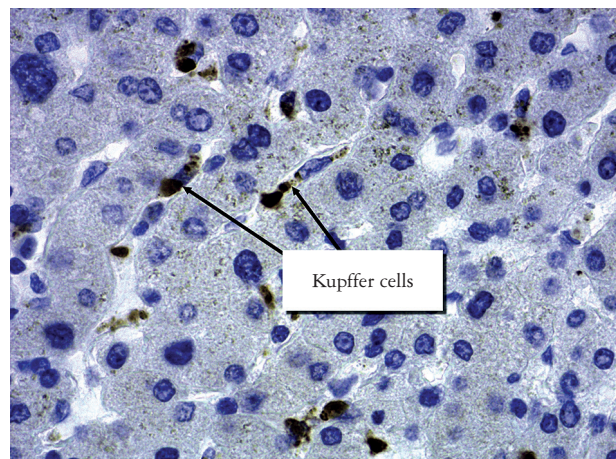


Fig. 2. Resistin expression in sinusoidal Kupffer cells (the objective lens magnification is 40 \times)

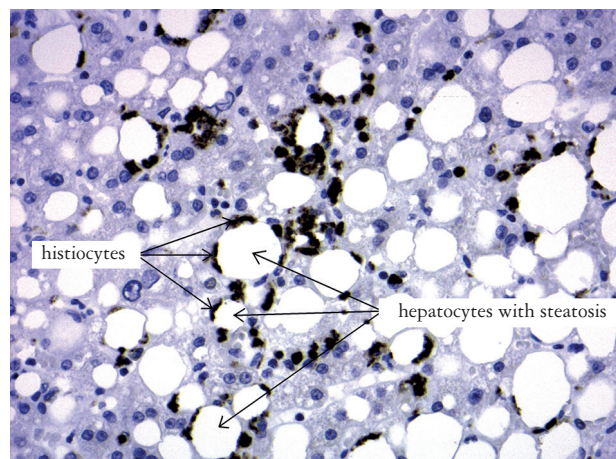


Fig. 3. Resistin expression in histiocytes surrounding the hepatocytes with steatosis (the objective lens magnification is 40 \times)

4. Resistin expression in histiocytes (CD68+) surrounding the steatotic hepatocytes (Fig. 3), correlated positively with NAFLD advancement.

There was a positive correlation of resistin expression with Kleiner NAFLD status in all basic param-

Table II. Results for resistin expression according to location and NAFLD Activity Score scale parameters

| LOCATION OF RESISTIN EXPRESSION | | HISTIOCYTES OF THE INFILTRATE | KUPFFER CELLS | HISTIOCYTES SURROUNDING THE STEATOTIC HEPATOCYTES | TOTAL RESISTIN EXPRESSION |
|--------------------------------------|-------------------------|-------------------------------|---------------|---|---------------------------|
| NAS score | Correlation coefficient | 0.502 | 0.164 | 0.562 | 0.478 |
| | p value | 0.000 | 0.019 | 0.000 | 0.000 |
| Steatosis score | Correlation coefficient | 0.401 | 0.188 | 0.507 | 0.435 |
| | p value | 0.000 | 0.006 | 0.000 | 0.000 |
| Inflammation score | Correlation coefficient | 0.566 | 0.272 | 0.604 | 0.571 |
| | p value | 0.000 | 0.000 | 0.000 | 0.000 |
| Liver cell injury (ballooning) score | Correlation coefficient | 0.272 | 0.192 | 0.288 | 0.275 |
| | p value | 0.000 | 0.005 | 0.000 | 0.000 |

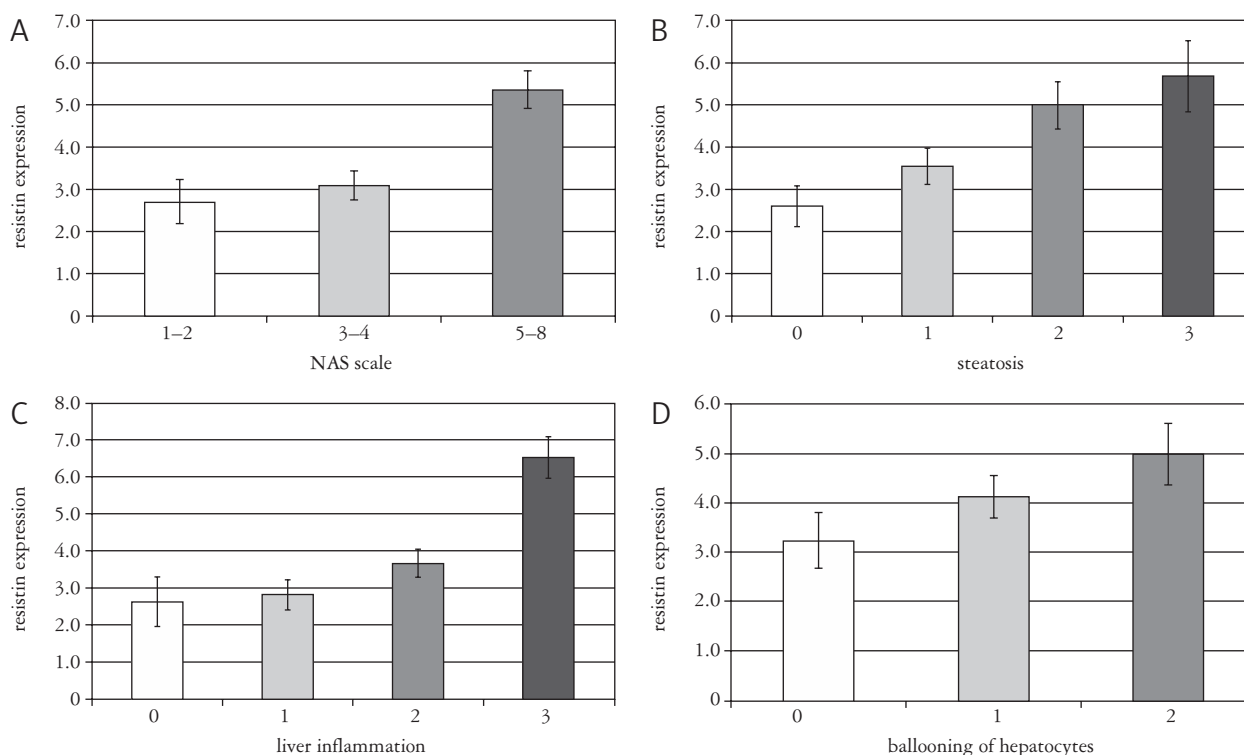


Fig. 4. Mean total resistin expression depending on: A) NAS scale, B) steatosis, C) liver inflammation, D) ballooning of hepatocytes

eters: total NAS score, the level of hepatocyte steatosis, the severity of inflammation, and ballooning of hepatocytes (Table II). This correlation was observed at all locations of analysed resistin expression: in the histiocytes of the inflammatory infiltrate, in the Kupffer cells, in histiocytes surrounding the hepatocytes with steatosis, as well as in total resistin expression. No such correlation was observed with the severity of organ fibrosis at either of the locations analysed, including the total assessment. Graphi-

cal interpretation for the total resistin expression is shown in Fig. 4.

Analysis of resistin expression in the histiocytes of inflammatory infiltrate

There was a statistically significant correlation with the levels of glucose, HbA_{1c}, insulin, triglycerides, HOMA index, and vitamin B₁₂ (Table III). For other biochemical parameters, statistical significance was

not reached (in the case of: AST: $p = 0.081$, ALT: $p = 0.074$, and C-peptide: $p = 0.082$). No differences were observed between the study group and the control group. No statistically significant correlation was found with patient's age and BMI. The difference in resistin expression was not noted with sex, hypertension, or metformin treatment. Also in the case of dyslipidaemia the difference was not statistically significant ($p = 0.089$).

Analysis of resistin expression in Kupffer cells

There was a statistically significant association with the levels of albumin, vitamin B₁₂, and HDL cholesterol (Table III). For other biochemical parameters, statistical significance was not reached (in the case of: de Ritis index $p = 0.057$, total protein $p = 0.064$, and HbA_{1c} $p = 0.092$). No statistically significant correlation was observed with patient's age or BMI, and no difference in resistin expression was noted with sex, hypertension, metformin treatment, diabetes mellitus, or dyslipidaemia. A significant difference was demonstrated in resistin expression in Kupffer cells between the control group and the study group.

Analysis of resistin expression in histiocytes surrounding the hepatocytes with steatosis

There was a statistically significant positive correlation with the level of: AST, ALT, glucose, HbA_{1c}, insulin, triglycerides, and HOMA index (Table III). For other biochemical parameters, statistical significance was not reached (in the case of: AST/ALT $p = 0.095$ and TC/HDL $p = 0.089$). No statistically significant correlation was observed with patient's age or BMI, and no difference was noted with sex, hypertension, or metformin treatment. In the case of diabetes mellitus and dyslipidaemia the difference in resistin expression was close to statistical significance (Table III). A significant difference was demonstrated in resistin expression between the control group and the study group.

Analysis of total resistin expression (sum of expression sites in macrophages of the inflammatory infiltration, in Kupffer cells and in histiocytes surrounding the hepatocytes with steatosis).

There was a statistically significant positive correlation with the levels of: triglycerides, glucose, HbA_{1c}, insulin, AST, ALT, LDH, GGTP, and HOMA index (Table III). For other biochemical parameters,

Table III. Analysis of resistin expression with statistically significant parameters according to location

| PARAMETER | LOCATION | | | | |
|-------------------------|---|-------------------------|---|---|--------------------|
| | HISTIOCYTES OF THE INFILTRATE (P VALUE) | KUPFFER CELLS (P VALUE) | HISTIOCYTES SURROUNDING THE STEATOTIC HEPATOCYTES (P VALUE) | TOTAL RESISTIN EXPRESSION CORRELATION COEFFICIENT | (P VALUE) |
| Glucose | 0.034 | NS | 0.026 | 0.227 | 0.001 |
| HbA1c | 0.007 | NS | 0.002 | 0.281 | < 0.001 |
| Insulin | 0.001 | NS | 0.004 | 0.264 | 0.001 |
| HOMA index | 0.001 | NS | < 0.001 | 0.272 | 0.001 |
| Triglycerides | 0.028 | NS | 0.002 | 0.179 | 0.009 |
| Vitamin B ₁₂ | 0.021 | 0.021 | NS | | NS ($p = 0.054$) |
| Albumin | NS | 0.029 | NS | | NS |
| AST | NS | NS | 0.021 | 0.188 | 0.006 |
| ALT | NS | NS | 0.018 | 0.224 | 0.001 |
| LDH | NS | NS | NS | 0.148 | 0.040 |
| GGTP | NS | NS | NS | 0.142 | 0.040 |
| HDL cholesterol | NS | 0.043 | NS | | NS |
| BMI | NS | NS | NS | 0.170 | 0.013 |
| Control vs. study group | NS | 0.018 | 0.002 | | 0.002 |
| Dyslipidemia | NS | NS | NS ($p = 0.055$) | | 0.022 |
| Diabetes | 0.036 | NS | NS ($p = 0.059$) | | 0.026 |

NS – not significant

statistical significance was not reached (in the case of: TC/HDL $p = 0.065$, creatinine $p = 0.059$, C-peptide $p = 0.082$, and vitamin B₁₂ $p = 0.054$).

Statistically significant correlation was not observed with patient's age. No difference in resistin expression was demonstrated with sex, hypertension, or metformin treatment. A statistically significant correlation was noted with BMI. A statistically significant difference in resistin expression was observed with diabetes mellitus and dyslipidaemia, and between the control group and the study group (Table III).

Discussion

The study is focused on diseases of high epidemiological importance (NAFLD, obesity). Adipokines have been the subject of great interest recently, but the results of published studies have been contradictory. There are various observations even regarding resistin location. Like only a few others [4, 5, 6, 7], our study is unique because resistin expression was examined directly in the liver. In our study, we confirm that hepatocytes are not the source of resistin in the liver. Shen *et al.* [4] showed that the immunoreactivity for resistin was primarily located in perisinusoidal cells of the liver lobules, rarely in periportal space, and more marked in inflammatory spaces. Others [6] also point to Kupffer cells and hepatic stellate cells as the main place of resistin expression. Szalowska *et al.* [7] indicated that besides Kupffer cells, endothelium cells and rarely fibroblasts are also the place of resistin expression. In our observation, resistin expression was not limited to perisinusoidal location (in Kupffer cells), but it was distinct in the macrophages in other parts of liver lobules. This observation indicates that immune system cells are the main source of liver resistin. Additionally, we observed marked expression of resistin in histiocytes (CD68+) surrounding the steatotic hepatocytes (the more steatosis, the more marked resistin expression). It may suggest the induction of resistin secretion by lipids in hepatocytes or, conversely, the induction of hepatocyte steatosis by resistin – constitute the “first hit” in NASH development theory.

The results of most studies show higher resistin level in the serum of patients with liver steatosis vs. non-obese controls [8, 9, 10]. Higher levels of resistin in patients with NAFLD and a positive correlation with inflammation were reported in several studies [8, 11]. However, in other studies the correlation between resistin and NAFLD was not reported [12, 13, 14], or even a negative correlation was found [15]. These studies were based on serum level of resistin. There are a limited number of studies evaluating resistin expression directly in the liver. Shen *et al.* [4] compared patients with NAFLD (simple steatosis and NASH) vs. control group. In that study, the serum resistin level was examined (ELISA test) as well

as resistin mRNA and immunohistochemistry in the liver. The serum resistin level was higher in patients with NAFLD, but there was no difference between simple steatosis and NASH. Resistin expression in the liver correlated with NAFLD stage (inflammation, hepatocyte ballooning, fibrosis). Similar results were reported by Huang *et al.* [6]. The authors suggest that serum resistin level reflects the whole-body fat stores, and resistin is accumulated in the liver and may play a role in aggravation of the inflammatory process. However, diverse results from different studies based on the serum level of resistin also suggest that the main source of resistin is not adipose tissue (or macrophages from adipose tissue) but the liver. We confirm a strong correlation between liver resistin expression in each analysed location and NAFLD (steatosis, hepatocytes ballooning, inflammation) with the exception of fibrosis.

Resistin is regarded as a proinflammatory cytokine. Despite a positive correlation with NAFLD stage, we found no correlation with non-specific markers of inflammation such as CRP, leucocytosis, or any white blood cells. There are studies with similar observations as ours [8, 16]; in some of them, CRP was a weak predictive factor for NAFLD [17]. Elevated AST and ALT levels are often the indicator of liver injury. We observed a correlation between resistin levels and these parameters; additionally, we also found a correlation between total resistin expression and LDH and GGTP. However, the de Ritis index was not significant. And again, the results from the literature (usually available only for ALT) are confusing, ranging from similar in NAFLD patients [18], through no correlation at all [19], to negative correlation in obese children [20].

Resistin is a cytokine that is frequently mentioned as a potential pathogenic factor for insulin resistance, secondary to obesity. This relationship was reported in several studies on animal models (mouse or rat) [3, 21, 22, 23, 24, 26]. In humans, however, the results are diverse. In healthy subjects with elevated resistin levels, a higher risk of type 2 diabetes mellitus was noted, especially in subjects with concomitant obesity or inflammatory process [27]. In a group of patients with type 2 diabetes mellitus, Kaplon-Cieslicka *et al.* [28] did not show a correlation between serum resistin level and BMI or HOMA index, regardless of the patient's sex. Pagano *et al.* [8] did not find a difference between serum resistin level and insulin resistance in NAFLD group. A decrease in resistin level was reported after bariatric surgery in a few studies [29, 30, 31, 32], usually based on serum level. In a study Moschen by *et al.* [29] this decrease in serum resistin levels was seen 12 months after the surgery (while six months after the surgery an increase in serum resistin levels was recorded). Moschen *et al.* also examined resistin levels directly in the liver, and in

this location the decrease was seen earlier – after 6 months. In their prospective study of obese patients with NAFLD and a healthy control group, Shen *et al.* [4] reported a correlation between resistin expression (serum and liver) and obesity but not insulin resistance. Most studies, however, are based on serum resistin levels, and their results are confusing. A positive correlation with obesity was reported in many other studies [6, 9, 33, 34, 35, 36, 37, 38, 39], but still in many others no such correlation was found [8, 13, 40, 41]. Similarly, many studies show a positive correlation with insulin resistance [9, 33, 34, 35, 39, 41], while others report conflicting observations [8, 10, 11, 13, 36, 37, 40]. In obese teenagers with initially higher level of resistin, the decrease in resistin levels was observed six months after changing bad habits [6]. Our results additionally support the claim that there is a link between resistin and glucose metabolism. In most locations (especially for total resistin expression), there was a correlation with glucose, HbA_{1c}, insulin, and HOMA index. Only in Kupffer cells was there no correlation with insulin resistance. Generally, when analysing the results of our study, we had an impression that this location was the most constant and changeless of all parts of the liver resistin expression. In most studies comparing obese patients with non-obese controls, a significant difference was found. However, when a comparison was made inside the obese group, most authors did not report correlation with BMI. We think that the statistically significant correlation with BMI inside the obese group (for total resistin expression) in our study results from the size of our group. The observation of patients following bariatric surgery [29, 30, 31, 32], where all studies report the decrease in resistin level after one year, also supports our results. Obviously, currently it is not possible to verify whether resistin can be responsible for weight gain, or whether it is only its consequence. We assume that there must be a connection with hepatic insulin resistance. Perhaps, the liver is the target organ for resistin. HOMA is one of the most commonly applied indices of insulin resistance; however, it is an index of peripheral and hepatic insulin resistance. In a study by Bajaj *et al.* [10] serum resistin level correlated with fat accumulation in the liver and hepatic insulin resistance (not peripheral) in patients with type 2 diabetes mellitus. An additional observation made during our study is the analysis of the diabetic group, revealing that there was no difference with metformin intake. We did not find any similar analysis in the literature. A positive role of metformin in NAFLD treatment has been postulated, but (if so) it is not a process involving resistin.

Other pathologies, probably related to resistin, are disturbances in the lipid metabolism. The results of our study generally support this theory. We noted a re-

lationship between liver resistin expression and serum triglycerides level and dyslipidaemia (with the exception of Kupffer cells). In Kupffer cells, higher resistin expression was observed at lower HDL-C levels. These observations can also be attributed to insulin resistance – when insulin resistance increases, HDL levels decrease and triglyceride levels rise. Several population studies showed that cholesterol level (total and all fractions) was lower at higher resistin concentration (in obese, NAFLD, and diabetic patients) [39, 42]. Similar findings were reported in studies on rats, where excessive resistin expression led to a decrease in total cholesterol and HDL-C [43]. The authors supposed that this effect could be related to the accumulation and sequestration of cholesterol in macrophages. However, also in lipid metabolism, the results of other studies are conflicting, showing no correlation or a positive correlation with total cholesterol and a negative correlation with HDL-C [44, 45, 46, 47, 48].

Although some studies suggest a role of iron in liver fibrosis and insulin resistance, we found no correlation with the parameters related to iron metabolism – Hgb, MCV, Fe, TIBC, transferrin, or ferritin. No such studies have been reported in the literature.

Vitamin B₁₂ is responsible for DNA methylation and plays different roles in humans. Among metformin-treated patients with type 2 diabetes mellitus, a decrease in vitamin B₁₂ level was observed [49, 50]. In patients with low vitamin B₁₂ level, unfavourable changes in lipids also take place, with an increase in total cholesterol and LDL-C and a decrease in HDL-C [49]. In one of study [51] the authors suggest a potential protective role of vitamin B₁₂ – in rats fed with NDMA (N-nitrosodimethylamine), vitamin B₁₂ improved liver parameters. In our study there was a correlation between serum vitamin B₁₂ level and resistin expression in macrophages and Kupffer cells (statistically significant). No such analyses are available in the literature.

In conclusion, we presume that resistin aggravates pathologic changes in the liver of patients with NAFLD. We also confirm that resistin plays a role in the pathogenesis of hepatic insulin resistance. There are still many question marks regarding the role of resistin and its metabolism. We believe that the results of our study will be of value for further analyses since the group of our patients is one of the largest reported in literature.

The authors declare no conflict of interest.

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