Original paper

Hepatic chemerin mRNA in morbidly obese patients with nonalcoholic fatty liver disease

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The aim of this study was to investigate hepatic chemerin mRNA, serum chemerin concentration, and immunohistochemical staining for chemerin and and chemokine receptor-like 1 (CMKLR1) in hepatic tissue in 56 morbidly obese women with nonalcoholic fatty liver disease (NAFLD) and to search for a relationship with metabolic and histopathological features.

Chemerin mRNA was assessed by quantitative real-time PCR, chemerin, and CMKLR1 immunohistochemical expression with specific antibodies, while serum chemerin concentration was assessed with commercially available enzyme-linked immunosorbent assays.

Serum chemerin concentration reached 874.1 ±234.6 ng/ml. There was no difference in serum chemerin levels between patients with BMI < 40 kg/m^2 and ≥ 40 kg/m^2. Serum chemerin concentration tended to be higher in patients with hepatocyte ballooning, greater extent of steatosis, and definite nonalcoholic steatohepatitis (NASH). Liver chemerin mRNA was observed in all included patients and was markedly, but insignificantly, higher in those with BMI ≥ 40 kg/m^2, hepatocyte ballooning, greater extent of steatosis, and definite NASH.

Hepatic chemerin mRNA might be a predictor of hepatic steatosis, hepatocyte ballooning, and NAFLD activity score (NAS) but seemed not to be a primary driver regulating liver necroinflammatory activity and fibrosis. The lack of association between serum chemerin and hepatic chemerin mRNA may suggest that adipose tissue but not the liver is the main source of chemerin in morbidly obese women.

Key words: chemerin, chemokine receptor-like 1, adipokine, liver, nonalcoholic fatty liver disease.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver injury with multifactorial and multiply aetiology. It encompasses a broad spectrum of abnormalities from benign, simple steatosis, through progressive nonalcoholic steatohepatitis (NASH) with different stages of fibrosis, to cirrhosis and hepatocellular carcinoma (HCC) [1, 2, 3]. It is suggested that NAFLD is a hepatic manifestation of metabolic syndrome characterised by high prevalence of arterial hypertension, type 2 diabetes (T2DM), and dyslipidaemia [4]. Adipokines, adipose tissue derived hormones, play an essential role in the regulation of metabolic processes, including insulin sensitivity and inflammatory response. Some adipokines were found to be pivotal players in the development and progression of liver fibrosis and carcinogenesis [5]. The alteration of the profile of adipokines observed in obesity and insulin resistance (IR) may contribute to hepatic steatosis, inflammation, and fibrosis [5].

Chemerin, a member of the adipokine family, is also known as tazarotene-induced gene 2 (TIG2) or retinoic acid receptor responder protein 2 (RARRES2) [6, 7], discovered as a chemotactic peptide directing macrophages and dendritic cells toward sites of inflammation, being involved in both adaptive and innate immunity [8, 9, 10]. It is involved in autocrine/paracrine signalling for adipocyte differentiation and maturation, glucose uptake, and lipolysis stimulation [11, 12, 13]. Chemerin secreted as inactive prochemerin is activated by inflammatory and coagulation serine proteases [14]. Hence, local and systemic levels of bioactive chemerin result from proteolytic processing activity and are not strictly related to chemerin protein concentration [15, 16]. Chemerin levels in humans are associated with body mass index (BMI), lipids concentration, blood pressure, and insulin sensitivity [11, 17, 18]. Several studies indicated altered chemerin levels or hepatic chemerin expression in chronic liver diseases of different origin, including NAFLD and chronic hepatitis C (CHC) [5, 19, 20, 21, 22, 23, 24, 25]. Chemerin acts by binding the G protein-coupled receptor, chemokine receptor-like 1 (CMKLR1) (also known as chemerin receptor 23 (ChemR23)) [7, 26], expressed by a variety of cells [26, 27, 28, 29]. CMKLR1 was highly abundant in the liver and is expressed by primary human hepatocytes, hepatic stellate cells, Kupffer cells, and bile duct cells [30]. The data with respect to chemerin and CMKLR1 mRNA is very limited and equivocal. As far as we are concerned there is no study regarding hepatic chemerin and CMKLR1 mRNA in morbidly obese patients with NAFLD. Here, hepatic chemerin mRNA and immunohistochemical staining for chemerin and CMKLR1 were quantified in a relatively large group of morbidly obese women with histologically proven NAFLD.

We decided to investigate chemerin hepatic mRNA together with serum chemerin concentrations and immunohistochemical staining for chemerin and CMKLR1 in hepatic tissue in morbidly obese women with NAFLD and search for their relationships with metabolic disorders and histopathological features.

Table I. Details on IHC staining of primary antibodies for chemerin and CMKLR1

Antibody	Chemerin	CMKLR1
Host	Rabbit	Rabbit
Clone	Polyclonal, PAA945Hu01	Polyclonal, NB100-92428
Vendor	Cloud-Clone	Novus Biologicals
Dilution	1 : 100	1 : 100
Antigen retrieval	Target Retrieval Solution, Low pH, Dako; 20 min, 97°C	Target Retrieval Solution, Low pH, Dako; 20 min, 97°C
Time of incubation with primary antibody	60 min	60 min
Detection system	Envision, Dako	Envision, Dako

Material and methods

Patient selection and serological assays

Fifty-six severely obese women who underwent bariatric surgery were included. 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: 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 ($\times 10^9$)] $\times$ 100. Chemerin serum concentration was assessed in duplicate by immunoenzymatic method with the commercially available Human Chemerin ELISA Kit, Catalogue number E0945h; Wuhan Uscn Sciences Co. Ltd., China. The study evaluated the full-length form of chemerin. Insulin concentration was measured by Diametra Insulin EIA Kit, Catalogue number DKO076; Diametra S.r.l headquarters: via Garibaldi, Foligno (PG), Italy.

The degree of IR was calculated according to the homeostasis model assessment for IR (HOMA-IR) by the formula fasting insulin level (mUI/l) $\times$ 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: HOMA-IR value below 4; and HOMA-IR value equal to or above 4.

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.

Liver histology

All included patients had wedge liver biopsies performed during bariatric procedure. Tissue samples were immediately divided into two parts: the first one for routine histopathological examination, and the second one was stabilised in RNA*later* (Sigma-Aldrich, St. Louis, USA) and subsequently 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 for collagen fibres, and examined by two independent experienced pathologists. Histopathological evaluation was carried out according to Kleiner's scoring scale [31].

Chemerin mRNA expression in liver tissue

Total RNA was isolated from liver biopsy specimens of NAFLD patients 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 *chemerin* and *CMKLR1* gene expression levels 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 *chemerin* and *GAPDH* were bought as ready-to-use assays: Hs 01123775_m1 was used for chemerin 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 chemerin mRNA 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).

Chemerin and chemokine-like receptor 1 (CMKLR1) immunostaining

Immunohistochemical stains were performed using automated machine (Autostainer Link 48, Dako, Carpinteria, USA). Four-μm-thick sections were placed on adhesive glass slides (Menzel Gläser, Thermo Fisher, Braunschweig, Germany). Heat-mediated antigen retrieval was performed using PT Link module (Dako). For stain visualisation, Envision or Envision FLEX (Dako) detection systems was used, with haematoxylin as a counterstain (Dako). Appropriate positive controls were utilised. For negative control, primary antibodies were omitted. Details on IHC technique are presented in a Table I.

Statistical analysis

The results were presented as the mean values with standard deviation ($\pm$SD). The distribution of the values was assessed using Shapiro-Wilk test. Due to abnormal distribution of the values, nonparametric methods were used for the 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. STATISTICA 12.5 software from StatSoft Corporation was used to perform the analysis.

Results

Demographic and clinical data

Demographic and clinical data of all subjects are presented in Table I. The analysed group was divided according to BMI. Body mass index < 40 kg/m^2 was found in 18 patients whereas BMI ≥ 40 kg/m^2 was observed in 38 females. There were significantly higher ALT, AST, and γ-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 BMI. 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 II.

Histological examination

The results of histopathological examination are collected in Table III. 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 found in 29 patients. There was no advanced (bridging) fibrosis and cirrhosis in the analysed groups. Stage 2 (perisinusoidal and portal/periportal) fibrosis was observed in nine patients.

Table II. 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
Chemerin (ng/ml)	874.1 ±234.6	853.3 ±253.1	866.1 ±233.8	NS
Chemerin mRNA	0.33 ±0.22	0.21 ±0.16	0.40 ±0.21	0.05

BMI – body mass index; WC – waist circumference; HGB – haemoglobin; WBC – white blood cells; PLT – platelets; ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGTP – γ-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

Lobular inflammation was found in 31 females, and reached grade 2 only in 9 patients.

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 found in 37 of the analysed patients, and in 20 patients it reached grade 2.

NAFLD activity score (NAS) was assessed in all analysed patients. Definite NASH (NAS ≥ 5) was found in 17 (30%), undefined/borderline NASH (NAS 3-4) in 14 (25%), and simple steatosis in 25 (45%) morbidly obese women.

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

Histopathological features	Patients (n = 56)	
	n	%
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 (1-2/3-4/5-8)	25/14/17	45/25/30

NAS – NAFLD score

Serum levels of chemerin and its association with liver histology and laboratory parameters

The mean serum chemerin concentration was 874.1 ±234.6 ng/ml.

There was no difference in serum chemerin levels between patients when comparing those with BMI < 40 kg/m^2 and ≥40 kg/m^2 (871.6 ±225.2 vs. 875.0 ±228.9 ng/nl; p = 0.50). An evident difference, but without statistical significance, in serum chemerin concentration was observed between patients with and without hepatocyte ballooning (926.1 ±265.3 vs. 826.9 ±135.8 ng/ml; p = 0.35) and in those with and without steatosis (903.8 ±236.7 vs. 865.3 ±225.3 ng/ml; p = 0.60). Additional analysis, which compared subgroup of patients with grade 0 and 1 and those with grade 2 and 3 of steatosis, revealed again an evident, but statistically insignificant, difference in chemerin serum levels (853.5 ±234.2 vs. 935.1 ±225.1 ng/ml; p = 0.21). There was no difference in serum chemerin concentration in patients with different fibrosis stage and lobular inflammation grade. NAS score was not associated with chemerin serum concentration. However, the comparison between patients with definite and borderline/no NASH revealed a tendency toward lower serum chemerin levels in the latter, but the difference did not reach statistical significance (863.0 ± 227.9 vs. 951.6 ± 233.0 ng/ml; p = 0.50). All the results regarding the relationship between chemerin serum concentration and histopathological features are summarised in Table IV.

Serum chemerin was positively associated with HOMA-IR value (r = 0.33, p = 0.04).

Chemerin mRNA in liver tissue and its association with analysed parameters

Chemerin mRNA was found in the liver of all patients. The mean level was 0.32 ±0.06.

Chemerin mRNA level in the liver was markedly higher in patients with BMI ≥ 40 kg/m^2, but the difference was on the threshold of statistical significance (0.40 ±0.10 vs. 0.21 ±0.06; p = 0.054). Similarly, an evident difference in chemerin mRNA was observed between patients with and without hepatocyte ballooning (0.37 ±0.10 vs. 0.22 ±0.07; p = 0.32) and in those with and without steatosis (0.38 ±0.11 vs. 0.20 ±0.07; p = 0.13). Additional analysis, which compared the subgroups of patients with grade 0 and 1 and those with grade 2 and 3 of steatosis, revealed a significant difference between chemerin mRNA in the liver tissue (0.21 ±0.04 vs. 0.46 ±0.15; p = 0.048). There was no evident difference in chemerin mRNA between patients with different fibrosis stage and lobular inflammation grade. NAS score was also not associated with chemerin mRNA. Nevertheless, the comparison between patients with defined and borderline/no NASH revealed markedly lower chemerin mRNA levels in the latter, but the difference did not reach statistical significance (0.48 ±0.16 vs. 0.25 ±0.07; p = 0.50).

There was no difference in hepatic chemerin mRNA and HOMA-IR between patients with different NAS score. All the results regarding the relationship between chemerin mRNA and histopathological features are summarised in Table IV.

Chemerin and CMKLR1 immunohistochemical analysis

Immunohistochemical chemerin expression in hepatic lobules was observed in 29 out of 56 analysed patients, whereas CMKLR1 expression was seen in 39 subjects. Semiquantitative analysis revealed that chemerin expression ranged from "0" to ++, while CMKLR1 from "0" to +++. Immunohistochemical expression of analysed parameters was not associated with metabolic abnormalities and histopathological features. There was also no mutual association between intensity of immunohistochemical chemerin and CMKLR1 expression in the liver. Immunohistochemical analysis of hepatic expression of chemerin and CMKLR1 was shown in Figures 1-6.

Table IV. Results regarding the relationship between histopathological features and chemerin serum concentration, chemerin mRNA in the liver tissue and parameters of glucose metabolism

	Steatosis grade		
	Grade 0/1	**Grade 2/3**	**p**
Serum chemerin (ng/ml)	853.5 ±234.2	935.1 ±225.1	NS
Chemerin mRNA	0.21 ±0.20	0.46 ±0.31	0.05
Glucose (mg/dl)	137.7 ±40.6	148.8 ±50.9	NS
Fasting insulin (μU/l)	21.3 ±8.8	26.8 ±10.5	0.04
HOMA-IR	7.0 ±2.9	9.9 ±5.8	0.04
	Steatosis grade		
	Grade 0	**Grade 1/2/3**	**p**
Serum chemerin (ng/ml)	865.3 ±225.3	903.8 ±236.7	NS
Chemerin mRNA	0.20 ±0.17	0.38 ±0.30	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	6.0 ±2.53	9.5 ±5.0	0.005
	Hepatocyte ballooning		
	Grade 0	**Grade 1/2**	**p**
Serum chemerin (ng/ml)	826.9 ±135.8	926.1 ±265.3	NS
Chemerin mRNA	0.22 ±0.21	0.37 ±0.31	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	8.0 ±3.3	8.6 ±5.3	NS
	Lobular inflammation		
	Grade 0	**Grade 1/2**	**p**
Serum chemerin (ng/ml)	888.1 ±231.7	893.1 ±235.3	NS
Chemerin mRNA	0.31 ±0.25	0.33 ±0.26	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	7.3 ±3.7	9.2 ±5.1	NS
	Fibrosis		
	Stage 0	**Stage 1/2**	**p**
Serum chemerin (ng/ml)	884.4 ±241.3	897.5 ±225.8	NS
Chemerin mRNA	0.25 ±0.21	0.48 ±0.29	NS
Glucose (mg/dl)	125.9 ±31.4	159.6 ±51.5	0.007
Fasting insulin (μU/l)	22.1 ±10.6	25.5 ±9.0	NS
HOMA-IR	6.7 ±3.2	10.0 ±5.3	0.006
	NAS		
	Simple steatosis/ borderline NASH	**Definite NASH**	**p**
Serum chemerin (ng/ml)	863.0 ±227.9	951.6 ±233.0	NS
Chemerin mRNA	0.25 ±0.07	0.48 ±0.16	NS
Glucose (mg/dl)	136.6 ±45.0	153 ±46.3	NS
Fasting insulin (μU/l)	23.0 ±9.3	25.9 ±11.9	NS
HOMA-IR	7.6 ±3.1	8.6 ±3.8	NS

HOMA-IR – homeostatic model assessment for insulin resistance; NAS – NAFLD score

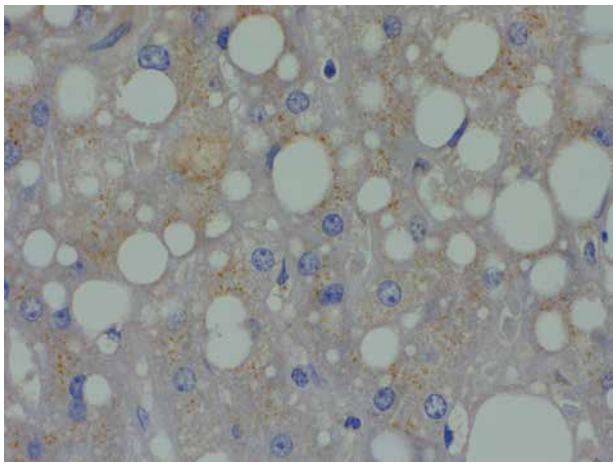

Fig. 1. Extensive hepatic steatosis with low intensity (+), small granular, cytoplasmatic expression of chemerin (magnification 400×)

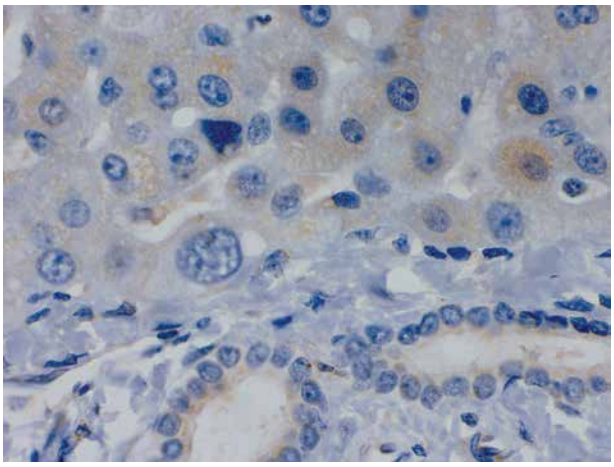

Fig. 2. Stage 2 fibrosis with low intensity (+), small granular, cytoplasmatic expression of chemerin (magnification 400×)

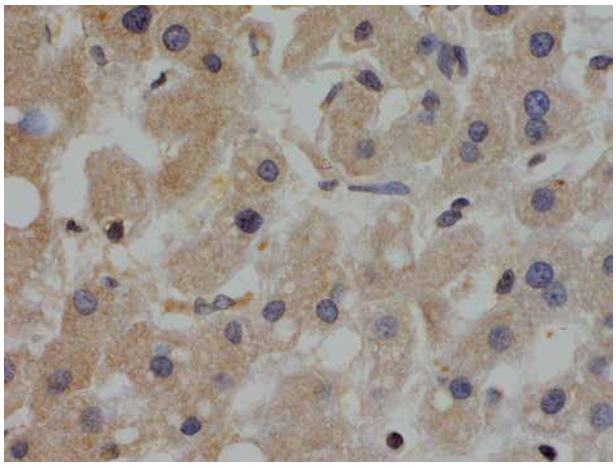

Fig. 3. Hepatocyte ballooning with high intensity (+++), small granular, cytoplasmatic expression of chemerin (magnification 400×)

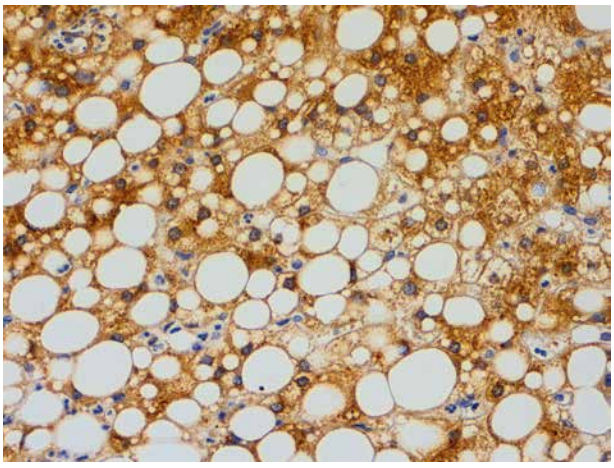

Fig. 4. Extensive hepatic steatosis with high intensity (+++), small granular, cytoplasmatic expression of CMKLR1 (magnification 200×)

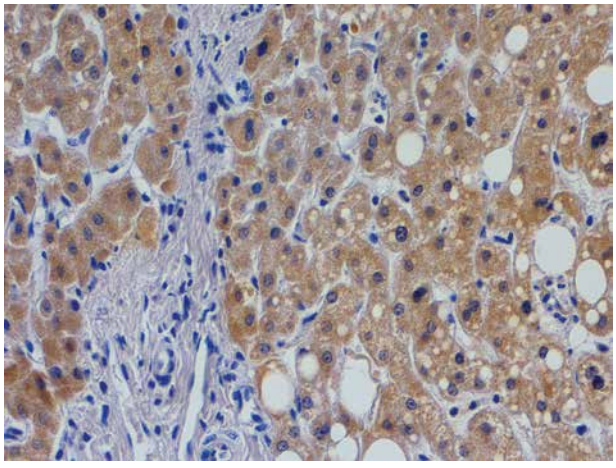

Fig. 5. Stage 2 fibrosis with high intensity (+++), small granular, cytoplasmatic expression of CMKLR1 (magnification 200×)

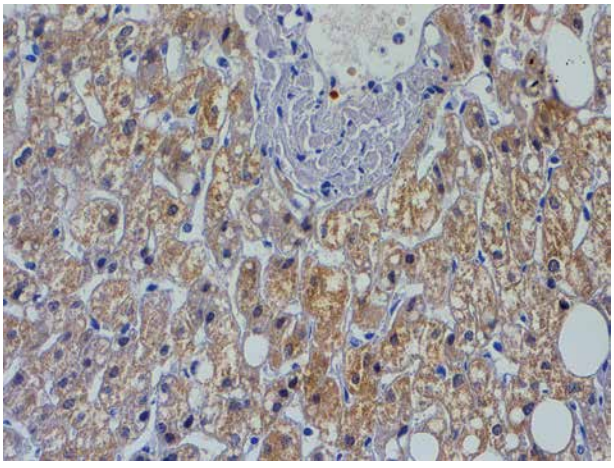

Fig. 6. Hepatocyte ballooning with medium intensity (++), small granular, cytoplasmatic expression of CMKLR1 (magnification 200×)

Association between other assessed parameters

There was significantly higher level 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, $p = 0.005$), despite the fact that our analysed group did not include 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 also with ALT ($r = 0.56$, $p = 0.001$), 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 ($r = 0.32$, $p = 0.02$) and 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 positively related to 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

Chemerin mRNA was found in all analysed morbidly obese patients in our study. A previous report indicated the liver as an important source of chemerin. Higher levels of chemerin in hepatic vein blood samples than in systemic circulation in cirrhotic patients indicated that it was produced and secreted not only by adipose tissue but also by the liver [30]. In our previous study in CHC patients, serum chemerin concentration was higher when compared to controls [32]. Some other studies in patients with histologically confirmed NAFLD found higher circulating chemerin levels in NAFLD patients than in controls [19, 20, 21]. On the other hand, when chemerin serum levels were compared between patient with NASH and simple steatosis the results were not so explicit. Some authors showed higher [19], and others similar, serum chemerin levels between these patients [24]. Hepatic chemerin mRNA was found in CHC patients and was inversely related to serum chemerin levels [23]. Importantly, up-regulated chemerin mRNA levels were observed in definite NASH, unchanged in borderline NASH, and even reduced in simple steatosis [24, 25]. Hepatic CMKLR1 mRNA was found in CHC patients and was significantly reduced in women with advanced liver fibrosis [23]. Another study revealed also enhanced hepatic CMKLR1 mRNA in NASH when compared to simple steatosis [24]. In CHC patients, chemerin mRNA levels were associated with CMKLR1 mRNA, but not with serum chemerin concentration [23].

As far as we are concerned, this is the first study that assessed substantial expression of chemerin in the liver of morbidly obese patients. Obesity with accompanying metabolic syndrome with insulin resistance may directly influence levels of adipokines. A high amount of visceral adipose tissue may become an abundant source of adipokines, and therefore the results of some studies may be unequivocal. Our study investigated chemerin serum levels in morbidly obese NAFLD women, but did not compare them with controls. The comparison between patients with BMI below and equal to or above 40 kg/m^2 clearly showed an evident tendency of increased serum chemerin levels in patients with higher BMI. One of the previous studies by Sell *et al*. [33] showed chemerin serum levels to be elevated in morbidly obese patients compared with non-obese ones, and positively associated with BMI. The lack of statistical significance, in our study, may result from the small study group, especially of patients with NASH. Moreover, all the patients were morbidly obese, with very high amounts of visceral adipose tissue. Therefore, comparison with overweight patients should be performed to better understand relationship between BMI and serum chemerin in NAFLD. Additionally, the subtle difference between our and Sell *et al*.'s study [33] may result from differing statistical analysis regarding to NAS. Our study compared patients with definite NASH to those with borderline NASH and simple steatosis, whereas Sell *et al*. [33] compared patients with defined/borderline NASH to those with simple steatosis. Similarly to serum levels, our study showed hepatic mRNA chemerin to be enhanced in patients with higher BMI, and the difference did not reach statistical significance.

Our study did not show any relationship between serum chemerin and hepatic chemerin mRNA. These results were similar to those obtained by Döcke *et al*. in NAFLD, who did not find any association between serum chemerin and hepatic chemerin mRNA [24]. However, when circulating chemerin levels were adjusted for body fat percentage, there was a trend to correlation with hepatic chemerin mRNA. These observations point to adipose tissue as a main, plentiful source of serum chemerin. On the other hand, the study by Döcke *et al.* included only overweight but not severely obese patients [24].

Defined histopathological features are used to evaluate NAS score including the grades of hepatic steatosis, hepatocyte ballooning, and lobular inflammation [3, 31]. Pointing to the well-described physiological functions of chemerin [5], we hypothesised that some of the categories of Kleiner's scoring system could be related to our targets of interest.

Therefore, we analysed the association of serum chemerin and hepatic chemerin mRNA expression with steatosis extent. However, there was no relationship between both serum chemerin concentration and hepatic chemerin expression and steatosis grade in our study. This observation is in contrast to the results found by Döcke *et al*., who described the expression of both chemerin and CMKLR1 mRNAs to be significantly associated with hepatic steatosis. That study showed chemerin mRNA levels significantly elevated in patients having more than 10% steatotic hepatocytes and when assessed according to Kleiner's scale in those with higher steatosis grade [24]. Moreover, stepwise reverse linear regression analysis indicated hepatic chemerin mRNA expression to be a significant predictor of hepatic steatosis [24].

Hepatocyte ballooning is a component of NAS score and a characteristic feature of NASH, which is strictly associated with lobular inflammation and steatosis grade. It is postulated that it is related with fat droplets accumulation and concomitant cytoskeletal injury. Our study revealed a tendency for higher hepatic chemerin mRNA and serum chemerin levels in patients with compared to those without hepatocyte ballooning, but the difference did not reach statistical significance. Our findings are in agreement with those obtained by Döcke *et al*., who found both hepatic chemerin mRNA expressions to be significant predictors of hepatocyte ballooning [24].

Lobular inflammation of different grade is a hallmark of NASH [3, 31]. Due to the propagated pro- and/or anti-inflammatory action of chemerin in terms of tissue injury [15, 16, 18], we decided to evaluate the potential relationship between hepatic chemerin mRNA and necroinflammatory activity grade. Inflammation contributes to higher adipocyte chemerin synthesis but seems not to upregulate hepatocyte chemerin production. Proinflammatory agents such as interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α), which are significantly increased in NASH [5], had no effect on chemerin mRNA and cellular and soluble protein in primary human hepatocytes (PHH) [24, 25]. In PHH TNF-α even lowered chemerin in cell supernatants without changing cellular levels [25]. The chemerin-CMKLR1 system seems to be involved in tissue inflammation. Our study showed a clear tendency for higher hepatic chemerin mRNA levels in patients with definite NASH (NAS score > 5), but the difference did not reach statistical significance. This is in accordance with the study by Döcke *et al*. [24], which reported that chemerin mRNA in the liver was significantly associated with NAFLD activity score (NAS). Patients with definite NASH revealed markedly elevated hepatic chemerin mRNA compared to those with undefined or no NASH patients [24]. However, that study included only three patients with definite and 10 patients with borderline NASH. As mentioned above, all those patients were overweight, but not obese, therefore comparison is difficult. Our study did not find any relationship between hepatic chemerin mRNA and inflammatory activity grade. Similarly, hepatic chemerin mRNA was not associated with inflammatory activity in CHC [23].

Contrary to our previous findings, which revealed a tendency for lower serum chemerin in patients with more advanced inflammatory activity grade [19], the recent study did not show such a tendency. Our results are also in contrast to the observation by Sell *et al*., who found serum chemerin levels to be positively correlated with portal inflammation [33].

Despite the fact that liver fibrosis represents a recognised histopathological feature of the advanced stages of NAFLD, it is determined separately and is not included in calculation of NAS score. However, fibrosis assessment definitely improves diagnostic specificity and represents a primary prognostic factor. Chemerin was observed to induce synthesis of a potent fibrogenic agent – transforming growth factor β (TGF-β) in macrophages [34]. Higher levels of chemerin in hepatic venous serum compared to portal venous serum of patients with liver cirrhosis indicate that chemerin is released by the cirrhotic liver [30]. However, the question is whether this is the result of higher hepatic release or inappropriate clearance of circulating protein. The limitation of our study is the lack of patients with advanced fibrosis and cirrhosis. Our study did not detect a significant difference with respect to hepatic chemerin mRNA levels in relation to various fibrosis stage. This is in opposite to Sell *et al*.'s results, which showed a positive relationship between fibrosis stage and serum chemerin [33]. Döcke *et al.* [24] found significantly higher hepatic chemerin mRNA in patients with significant fibrosis compared to those without or with insignificant fibrosis. Probably, the main reason for the discrepancy in the obtained results is the relatively small number of patients with significant fibrosis (stage 2 – perisinusoidal and portal/periportal), which was found just in 9 out of 56 (16%) patients in our study. Therefore, further studies with a higher number of patients with advanced fibrosis are necessary to establish the exact expression of chemerin mRNA in these cases.

Insulin upregulates adipocyte chemerin whereas mRNA expression is not enhanced in PHH [4, 35, 36]. Chemerin was reported to enhance insulin-stimulated glucose uptake and insulin receptor

substrate-1 tyrosine phosphorylation, suggesting that chemerin increases insulin sensitivity [13]. Contrary, to our previous study in NAFLD patients [19], the current study found positive correlation between HOMA-IR and serum chemerin levels. However, hepatic chemerin mRNA was not related to IR. The difference may result from the different study group. Moreover, the majority of patients included in the current study had elevated HOMA-IR values and all were morbidly obese. These observations suggest that not hepatic but adipose tissue-derived chemerin may interplay with insulin sensitivity.

Chemerin and CMKLR1 represent a specific signalling system, where CMKLR1 displays the unique combination of high affinity chemerin binding and efficient intracellular signalling [25]. Wanninger *et al*. [30] were the first to study CMKLR1 in the human liver and show the expression of CMKLR1 by different cell populations, i.e. PHHs and Kupffer cells. According to their study, CMKLR1 protein expression was reduced in the hepatocytes of subjects suffering from hepatic steatosis when compared with non-steatotic controls [30]. The study by Döcke *et al*. found hepatic CMKLR1 mRNA to be significantly up-regulated in patients with NASH [24]. Neumann *et al*. found hepatic CMKLR1 mRNA to be up-regulated in patients with NASH, when compared to those with simple steatosis, and was weakly associated with histopathological findings, but only in males [37]. Evident CMKLR1 mRNA expression was found in CHC patients' livers, but was not related to the inflammatory activity grade and was significantly reduced in women with advanced liver fibrosis [23]. The present study did not find any difference in immunohistochemical CMKLR1 expression in patients with different grades of steatosis, lobular inflammation, hepatocyte ballooning, and NAS score and stage of fibrosis. It has to be mentioned that our study measured expression immunohistochemically, whereas the studies by Döcke *et al*. [24] and Neumann *et al.* [37] assessed mRNA levels, which do not necessarily reflect hepatic protein levels. On the other hand, Wanninger *et al.* investigated a very small number of patients and did not assess the grade of steatosis or NAS score in their study. These discrepancies do not allow a proper and precise comparison between the studies. Moreover, all the studies included male and female patients, whereas ours included only females. Pointing to the results obtained by Neumann *et al*., gender may be an additional, important factor influencing the obtained results and complicating comparison between the studies.

There was also no association between immunohistochemical expression of chemerin and CMKLR1. This observation additionally points to adipose tissue as a main source of chemerin, which may be supplied into the liver tissue and exert its activity by binding to CMKLR1.

As far as we know, similar investigations in morbidly obese patients with NAFLD have not been reported so far. Circulating chemerin acts through its receptor, but it is still unknown if higher serum levels lead to lower receptor gene expression in liver and if the adipose tissue-derived chemerin is the main stimulator of its hepatic receptors.

There are some limitations of the study. The first limitation is that the study was carried out using the homogenates of human liver tissue. Therefore, on the basis of the obtained results, it is not possible to define whether hepatocytes or other cell types, which are abundantly present in the liver, constitute the main source of chemerin mRNA. Second, chemerin is activated by proteolytic processing. Therefore, serum levels may not reflect its activity, and assays to measure its local bioactivity have to be carried out. Third, the study included only morbidly obese patients, and there is no comparison with overweight and non-obese patients. Fourth, there are a relatively small number of patients with significant fibrosis, and a lack of patients with advanced fibrosis or cirrhosis.

Conclusions

Our study, which focused on chemerin mRNA expression, confirmed a marked expression of chemerin in the liver of morbidly obese patients with NAFLD. Immunohistochemical analysis confirmed both chemerin and its receptor, CMKLR1 expression, in the liver of NAFLD patients. Chemerin mRNA in the liver tissue may be a predictor of steatosis and hepatocyte ballooning. The results indicate that chemerin may play pivotal role in regulation of some pathogenetic pathways. Despite its documented role in inflammation, hepatic chemerin mRNA expression seemed not to be a primary driver regulating liver necroinflammatory activity. The lack of association between serum chemerin and hepatic chemerin mRNA may suggest that not liver but adipose tissue is the main source of this adipokine. Further research is necessary to clarify hepatic expression of chemerin and CMKLR1 in NAFLD and the function of finally synthesised proteins.

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The authors declare no conflict of interest.

References

1. Buechler C, Wanninger J, Neumeier M. Adiponectin, a key adipokine in obesity related liver diseases. World J Gastroenterol 2011; 17: 2801-2811.

2. Yeh MM, Brunt EM. Pathological features of fatty liver disease. Gastroenterology 2014; 147: 754-764.
3. Ziółkowski A, Wyleżoł M, Kukla M, et al. The Comparison of scoring scales for liver biopsy assessment in morbidly obese patients undergoing bariatric surgery. Obes Surg 2005; 15: 1309-1314.
4. Friis-Liby I, Aldenborg F, Jerlstad P, et al. High prevalence of metabolic complications in patients with non-alcoholic fatty liver disease. Scand J Gastroenterol 2004; 39: 864-869.
5. Kukla M, Mazur W, Bułdak RJ, Mazur W. Potential role of leptin, adiponectin and three novel adipokines visfatin, chemerin and vaspin in chronic hepatitis. Mol Med 2011; 17: 1397-1410.
6. MacDougald OA, Burant CF. The rapidly expanding family of adipokines. Cell Metab 2007; 6: 159-161.
7. Roh S, Song S, Choi K, et al. Chemerin – a new adipokine that modulates adipogenesis via its own receptor. Biochem Biophys Res Commun 2007; 362: 1013-1018.
8. Wittamer V, Franssen J, Vulcano M, et al. Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. J Exp Med 2003; 198: 977-985.
9. Rabe K, Lehrke M, Parhofer KG, et al. Adipokines and insulin resistance. Mol Med 2008; 14: 741-751.
10. Moretta A, Marcenaro E, Parolini S, et al. NK cells at the interface between innate and adaptive immunity. Cell Death Differ 2008; 15: 226-233.
11. Bozaoglu K, Bolton K, McMillan J, et al. Chemerin is a novel adipokine associated with obesity and metabolic syndrome. Endocrinol 2007; 148: 4687-4694.
12. Goralski KB, McCarthy TC, Hanniman EA et al. Chemerin a novel adipokine that regulates adipogenesis and adipocyte metabolizm. J Biol Chem 2007; 282: 28175-28188.
13. Takahashi M, Takahashi Y, Takahashi K, et al. Chemerin enhances insulin signaling and potentiates insulin-stimulated glucose uptake in 3T3-L1 adipocytes. FEBS Lett 2008; 582: 573-578.
14. Zabel BA, Allen SJ, Kulig P, et al. Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. J Biol Chem 2005; 280: 34661-34666.
15. Weigert J, Neumeier M, Wanninger J, et al. Systemic chemerin is related to inflammation rather than obesity in type 2 diabetes. Clin Endocrinol 2010; 72: 342-348.
16. Yoshimura T, Oppenheim JJ. Chemerin reveals its chimeric nature. J Exp Med 2008; 205: 2187-2190.
17. Stejskal D, Karpisek M, Hanulova Z, et al. Chemerin is an independent marker of the metabolic syndrome in a Caucasian population a pilot study. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2008; 152: 217-221.
18. Ernst MC, Sinal CJ Chemerin: At the crossroads of inflammation and obesity. Trends Endocrinol Metab 2010; 21: 660-667.
19. Kukla M, Żwirska-Korczala K, Hartleb M, et al. Serum chemerin and vaspin in non-alcoholic fatty liver disease. Scand J Gastroenterol 2010; 45: 235-242.
20. Yilmaz Y, Yonal O, Kurt R, et al. Serum levels of omentin, chemerin and adipsin in patients with biopsy-proven nonalcoholic fatty liver disease. Scand J Gastroenterol 2011; 46: 91-97.
21. Yilmaz Y, Kurt R, Gurdal A, et al. Circulating vaspin levels and epicardial adipose tissue thickness are associated with impaired coronary flow reserve in patients with nonalcoholic fatty liver disease. Atherosclerosis 2011; 217: 125-129.
22. Kukla M, Berdowska A, Gabriel A, et al. Association between hepatic angiogenesis and serum adipokine profile in non-obese chronic hepatitis C patients. Pol J Pathol 2011; 62: 218-228.
23. Kukla M, Adamek B, Waluga M, et al. Hepatic chemerin and chemokine-like receptor 1 expression in patients with chronic hepatitis C. Biomed Res Int 2014; 2014: 517820.
24. Döcke S, Lock JF, Birkenfeld AL, et al. Elevated hepatic chemerin mRNA expression in human non-alcoholic fatty liver disease. Eur J Endocrinol 2013; 169: 547-557.
25. Krautbauer S, Wanninger J, Eisinger K, et al. Chemerin is highly expressed in hepatocytes and is induced in non-alcoholic steatohepatitis liver. Exp Mol Pathol 2013; 95: 199-205.
26. Bondue B, Wittamer V, Parmentier M. Chemerin and its receptors in leukocyte trafficking, inflammation and metabolizm. Cytokine Growth Factor Rev 2011; 22: 331-338.
27. Vermi W, Riboldi E, Wittamer V, et al. Role of ChemR23 in directing the migration of myeloid and plasmacytoid dendritic cells to lymphoid organs and inflamed skin. J Exp Med 2005; 201: 509-515.
28. Muruganandan S, Parlee SD, Rourke JL, et al. Chemerin, a novel peroxisome proliferator-activated receptor (PPAR) target gene that promotes mesenchymal stem cell adipogenesis. J Biol Chem 2011; 286: 23982-23995.
29. Sell H, Laurencikiene J, Taube A, et al. Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells. Diabetes 2009; 58: 2731-2740.
30. Wanninger J, Bauer S, Eisinger K, et al. Adiponectin upregulates hepatocyte CMKLR1 which is reduced in human fatty liver. Mol Cell Endocrinol 2012; 349: 248-254.
31. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005; 41: 1313-1321.
32. Kukla M, Żwirska-Korczala K, Gabriel A, et al. Chemerin, vaspin and insulin resistance in chronic hepatitis C. J Viral Hepat 2010; 17: 661-667.
33. Sell H, Divoux A, Poitou C, et al. Chemerin correlates with markers for fatty liver in morbidly obese patients and strongly decreases after weight loss induced by bariatric burgery. J Clin Endocrinol Metab 2010; 95: 2892-2896.
34. Cash JL, Hart R, Russ A, et al. Synthetic chemerin-derived peptides suppress inflammation through ChemR23. J Exp Med 2008; 205: 767-775.
35. Tan BK, Chen J, Farhatullah S, et al. Insulin and adipose tissue chemerin. Diabetes 2009; 58: 1971-1977.
36. Bauer S, Bala M, Kopp A, et al. Adipocyte chemerin release is induced by insulin without being translated to higher levels in vivo. Eur J Clin Invest 2012; 42: 1213-1220.
37. Neumann M, Meier EM, Rein-Fischboeck L, et al. Chemokine-Like Receptor 1 mRNA weakly correlates with non-alcoholic steatohepatitis score in male but not female individuals. Int J Mol Sci 2016; 17 (8) pii: E1335.

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