

CLINICAL STUDY

Elevated hepatic chemerin mRNA expression in human non-alcoholic fatty liver disease

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Abstract

Objective: Adipose tissue-derived factors link non-alcoholic fatty liver disease (NAFLD) with obesity, which has also been reported for circulating chemerin. On the other hand, hepatic chemerin and chemokine-like receptor 1 (CMKLR1) mRNA expression has not yet been studied in an extensively characterized patient collective.

Design: This study was cross-sectional and experimental in design.

Methods: Liver tissue samples were harvested from 47 subjects and histologically examined according to the NAFLD activity score (NAS). The concentrations of chemerin and CMKLR1 were measured using semi-quantitative real-time PCR, and the concentration of serum chemerin was measured using ELISA. To evaluate potential effects of chemerin and CMKLR1, cultured primary human hepatocytes (PHHs) were exposed to selected metabolites known to play a role in NAFLD (insulin, glucagon, palmitic acid, and interleukin-6 (IL6)).

Results: Chemerin and CMKLR1 mRNA levels were elevated in the human liver. Their expression was correlated with the NAS ($R^2=0.543$; $P<0.001$ and $R^2=0.355$; $P=0.014$ respectively) and was significantly elevated in patients with definite non-alcoholic steatohepatitis (NASH) ($P<0.05$ respectively). Linear regression analysis confirmed an independent association of liver fibrosis, steatosis, inflammation, and hepatocyte ballooning with hepatic chemerin mRNA expression ($P<0.05$ respectively). The expression of hepatic chemerin and CMKLR1 was correlated with the measures of obesity ($P<0.05$). The incubation of PHHs with IL6 significantly increased the expression of CMKLR1 mRNA ($P=0.027$), while that of chemerin remained unaffected ($P>0.05$). None of the other metabolites showed an influence ($P>0.05$).

Conclusion: This is the first study to show that chemerin mRNA expression is significantly elevated in the liver of NASH patients and that CMKLR1 expression is upregulated in liver inflammation, whereby IL6 could play a causal role.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is common in obese individuals (1). Obesity itself is associated with the so-called low-grade systemic inflammation, which in turn appears to be involved in the pathogenesis of NAFLD (2). There is growing evidence that endocrine-active proteins secreted by the white adipose tissue (WAT), named adipocytokines, play a significant role in this disease (3). Thereby, the enlargement of WAT results in a dysbalance with rising expression of

proinflammatory and a reduced production of anti-inflammatory cytokines, which are released into the blood stream (4, 5). For instance, interleukin-6 (IL6) concentrations in the portal vein of morbidly obese individuals are ~50% higher than those in their peripheral arterial blood (6), and therefore, IL6 is recognized as a central biomarker of NAFLD including non-alcoholic steatohepatitis (NASH) (7). This indicates especially visceral WAT-derived factors to be an important link between obesity and NAFLD (5). Chemerin is a newly described member of the group of

adipocytokines (8), and is known to be expressed in the WAT of mice, *Psammomys obesus*, and humans (9, 10, 11, 12, 13, 14, 15). Chemerin exerts its effects at least in part (16, 17) via the orphan G-protein-coupled receptor chemokine-like receptor 1 (CMKLR1) (18). CMKLR1 is known to be expressed by a number of cells of the innate immune system, i.e., macrophages and natural killer cells, and the binding of chemerin to this receptor promotes the recruitment of these cells to tissue injury sites (19, 20). CMKLR1 has also been shown to be expressed by a variety of cell populations in the human liver, including Kupffer cells and primary human hepatocytes (PHHs) (21). Interestingly, hepatic CMKLR1 protein expression is reduced in the liver of patients suffering from hepatic steatosis and is upregulated by adiponectin (21), suggesting a protective role of the receptor under conditions of hepatic steatosis. On the other hand, a recent report has indicated decreased serum IL6 levels and reduced hepatic inflammatory cell invasion in *Cmklr1*^{-/-} mice (22). Thus, the chemerin–CMKLR1 system seems to be involved in tissue inflammation, which also represents a hallmark of NASH (1, 23), but whether the chemerin–CMKLR1 system exerts pro- or anti-inflammatory effects is currently under discussion (19, 24, 25).

There are studies indicating that the liver may contribute to circulating chemerin concentrations: chemerin mRNA has been detected in mouse models (9, 10), and transcripts of chemerin and *CMKLR1* have been detected in commercially available tissue systems including the liver (14, 18). Most notably, a human study has revealed similar levels of chemerin in the portal and systemic venous systems of healthy subjects and elevated concentrations of chemerin in the hepatic vein than in the portal venous blood of cirrhotic patients (26). Thus, the available experimental evidence suggests that chemerin is secreted by both the WAT and the liver (24), but the liver has not yet been established as an additional site for the expression of chemerin under conditions of definite NASH in well-characterized human collectives.

Several human studies have investigated the association between chemerin levels in the serum and different metabolic disorders (24): obese subjects (BMI > 30 kg/m²) have higher plasma chemerin levels than normal-weight subjects (BMI < 25 kg/m²) (11, 27), and pronounced weight loss after gastric bypass surgery has been reported to decrease serum chemerin levels (15, 28, 29). An association of circulating chemerin levels with the markers of NAFLD has also been highlighted, but the results have been inconsistent in terms of some of the investigated histological features (28, 30, 31).

We hypothesized that with the progression of pure hepatic steatosis to inflammatory NASH the liver could represent an additional site for the expression of chemerin and significantly contribute to serum chemerin concentrations. Therefore, in this study, we aimed to evaluate hepatic chemerin mRNA expression

in patients histologically proven to suffer from different stages of NAFLD vs control subjects without NASH. We also investigated CMKLR1, given that chemerin and CMKLR1 appear to be part of a specific signaling system that differs from those of other chemokines and their receptors (18). We further aimed to assess whether the expression of hepatic chemerin and *CMKLR1* mRNAs might be modulated by factors known to play a role in NASH. Therefore, we expanded our study by using an experimental approach, in which we exposed PHHs to selected metabolites.

Subjects and methods

Experimental design

This was a cross-sectional study that investigated the hepatic mRNA levels of chemerin by means of a semi-quantitative real-time (qRT) PCR analysis. The expression of chemerin was studied in the liver tissue samples of a set of extensively characterized patients suffering from undefined or definite NASH vs subjects who did not meet the NASH criteria. For a more comprehensive picture, the hepatic mRNA levels of the receptor *CMKLR1* were additionally measured, since CMKLR1 exhibits the unique combination of high-affinity chemerin binding and efficient signaling (25). We further hypothesized that hepatic chemerin and *CMKLR1* mRNA expression could be associated with factors known to be involved in the pathogenesis of NASH (32). To elucidate this hypothesis, we used a supporting experimental approach, in which PHHs were exposed to insulin, palmitoic acid, glucagon, and IL6 at defined concentrations to investigate their potential influence on chemerin and CMKLR1.

Subjects and ethics

The faculty's ethics review board approved the study, and all the subjects gave written informed consent at least 24 h prior to surgery. Forty-seven patients met the inclusion criteria of this study and were enrolled during 2009 and 2010. The inclusion criteria were age ≥ 18 years and a medical indication for hepatic surgery. Exclusion criteria were chemotherapy during the last 8 weeks prior to surgical intervention, a history of hepatic cirrhosis, organ transplantation, any acute or chronic inflammatory disease except NAFLD, long-term therapy with glucocorticoids or antirheumatic/anti-inflammatory drugs, drug abuse, excessive alcohol consumption, pregnancy, or thyroideal or mental dysfunction. Excessive alcohol consumption was considered to be > 20 g/day for women and > 40 g/day for men (33).

Basic characterization

Detailed medical history and physical examination data were obtained from all the subjects. Anthropometric

measurements, BMI, waist circumference, body fat percentage, and non-invasive arterial blood pressure were obtained. Skinfold thicknesses were measured using a Lange caliper (Beta Technology, Cambridge, MA, USA) to the nearest 0.1 mm. In addition, waist circumference was determined using a soft-tape measure to the nearest 0.1 cm. Body fat percentage was determined according to the methods of Garcia *et al.* (34).

Type 2 diabetes mellitus (T2D) and impaired fasting glucose (IFG) levels were defined following the criteria of the American Diabetes Association (35); the presence or absence of the metabolic syndrome was defined according to the NCEP ATP III panel criteria (36).

Blood samples, assays, and calculations

All the subjects fasted overnight, and pre-medication, except metformin, was kept constant for all the subjects. Serum and plasma samples were drawn on the morning of the surgery between 0600 and 0800 h and stored at -80°C until the measurements were taken. Clinical chemistry was assessed using standard methods in the research laboratories of the Charité (37). Serum chemerin concentrations were determined using ELISA (Biovendor, Heidelberg, Germany; intra-assay coefficient of variation (CV): 6.0%; inter-assay CV: <10%). The samples were measured in duplicates and the average was determined. Insulin resistance was estimated by calculating the homeostasis model assessment for insulin resistance (HOMA-IR) according to the following formula: $(\text{fasting glucose (mmol/l)} \times (\text{fasting insulin (pmol/l)} / 6)) / 22.5$ (38).

Tissue sampling and histopathological analysis

Tissue samples were collected through knife extraction from a macroscopically non-pathological liver segment during open abdominal surgical procedures before starting therapeutic intervention. This strategy was followed to exclude hepatic ischemia due to surgical inflow or outflow obstruction by clamping of vena portae, arteria hepatica propria, or vena hepatica (Pringle maneuver) to avoid any hypoxia-induced artifacts. The samples were immediately snap-frozen in liquid nitrogen and stored at -80°C (39) until RNA extraction. An additional liver tissue sample was used for histopathological analysis after fixing the probes in 4% formalin (Histofix, Roth, Germany), embedding in paraffin, and staining with hematoxylin and eosin. The staging and grading of the stained samples were carried out according to the NAFLD activity score (NAS) (23) and the Ishak fibrosis scoring system (40). The unweighted sum of points for the intensity of steatosis, lobular inflammation, and hepatocellular ballooning was used to calculate the NAS (23). The subjects were grouped according to undefined (corresponding to a

NAS of 3–4) or definite (NAS ≥ 5) NASH or considered as controls when they did not meet the NASH criteria (NAS ≤ 2) and did not exhibit fibrosis (23, 40). Before the histopathological NAFLD evaluation, a blinded clinical expert pathologist confirmed all the harvested liver tissue samples to be histologically normal with respect to pathologies except for NAFLD. Thus, only healthy tissue samples were used for analyses.

Cell culture

For the isolation of cells, human liver tissue samples were obtained from the liver resections of five donors undergoing partial hepatectomy. PHHs were isolated using a collagenase-containing perfusion buffer followed by a density gradient step with Percoll (25%). While using the standard isolation protocol, the contaminating cells are mainly Kupffer cells and endothelial cells and are <2% when examined using light microscopy. Hepatocytes were initially cultivated in Williams' E medium supplemented with 10% FCS, 1% penicillin/streptomycin, 0.1 mM dexamethasone, and 0.5 μM insulin. After 4 h, the cultivation medium was changed to serum-free modified Williams' E medium (supplementation described above). After ~ 36 h of cultivation, PHHs were washed twice with PBS, incubated with palmitic acid (0.3 mM), IL6 (50 ng/ml), glucagon (100 nM), or insulin (100 nM) for 24 h.

RNA extraction and RT-PCR

Total RNA was extracted from the frozen liver tissue homogenates using the RNeasy Mini Kit (Qiagen). DNA digestion was carried out using RNase-free DNase (Qiagen). Total RNA was extracted from the cells using a method adapted from the study of Chomczynski & Sacchi (41). The concentration and quality of RNA were determined using an ND-1000 spectrophotometer (Nanodrop, PeqLab, Erlangen, Germany). Single-stranded cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems). Semi-quantitative real-time (RT) PCR was carried out in 384-well plates using the Power SYBR Green PCR Master Mix and an ABI Prism 7900 sequence detection system (Applied Biosystems). Quantities were interpolated from standard curves. Concurrent standard curves constructed using the genes of interest and the housekeeping gene were used to calculate fold differences. Hypoxanthine-guanine phosphoribosyl-transferase (*HPRT* (*HPRT1*)) was used as the housekeeping gene. Our standard curves account for reaction efficiencies. Calculations were done as follows: the quantities of the target gene were divided by those of the housekeeping gene. Healthy samples were considered as references. All the samples were determined as triplicates, and non-template controls

were measured using the following cycling conditions: initial denaturation at 95 °C for 10 min, 47 cycles of denaturation at 95 °C for 15 s, and annealing/extension for 1 min at 60 °C. Melting curve profiles confirmed the amplification of specific transcripts. Primers used were chemerin, *RARRES2*: 5'-GACAAGCTGCCGGAA-GAGG-3' and 5'-TGGAGAAGGCGAACTGTCCA-3' (13); *CMKLR1*: 5'-CCCAATCCATATCACCTATGCC-3' and 5'-GTCCCGAAAACCCAGTGGTA-3'; *IL6*: 5'-CAGCCCT-GAGAAAGGAGACATG-3' and 5'-GCATCCATCTTTTT-CAGCCATC-3'; *CD68*: 5'-GCTACATGGCGGTGGAGT-ACAA-3' and 5'-ATGATGAGAGGCAGCAAGATGG-3'; and *HPRT*: 5'-TGACACTGGCAAAACAATGCA-3' and 5'-GGTCCTTTTACCAGCAAGCT-3'.

Statistical analyses

SPSS 16.0 was used for all the statistical analyses. If not stated otherwise, data are reported as means \pm s.e.m. The presence or absence of a normal distribution was verified using the Kolmogorov–Smirnov test, and natural logarithmic transformation was used if required. Depending on the distribution of data, the following statistical methods were used: Pearson's simple coefficient or Spearman's rank correlation

coefficient method, ANOVA, Student's *t*-test, or Mann–Whitney *U* test. Linear relationships were tested using a least-squares regression analysis for linear models. Significance level was defined as two-sided $P < 0.05$.

Results

Body mass-related expression of hepatic chemerin and CMKLR1

The basic and clinical characteristics of the study subjects are given in Table 1. Circulating chemerin levels are known to be associated with obesity in humans (11, 15, 26, 27, 28, 29). In this study, hepatic chemerin mRNA expression was found to be significantly associated with BMI ($R^2 = 0.296$; $P = 0.039$), waist circumference ($R^2 = 0.397$; $P = 0.005$), and body fat percentage ($R^2 = 0.378$; $P = 0.008$). The expression of *CMKLR1* mRNA was correlated with waist circumference ($R^2 = 0.369$; $P = 0.010$) and body fat percentage ($R^2 = 0.539$; $P < 0.001$). We accordingly found modestly elevated hepatic mRNA levels of chemerin in obese (BMI ≥ 30 kg/m²) vs normal-weight (BMI ≤ 25 kg/m²) subjects ($P = 0.028$; Fig. 1A), which was also true for hepatic *CMKLR1* expression ($P = 0.010$; Fig. 1B).

Table 1 Main basic and clinical characteristics of the study population. Means \pm s.e.m. or absolute numbers of subjects are reported. Statistical differences were determined using ANOVA; Bonferroni correction was used for *post hoc* comparisons.

	No NASH (NAS 0–2)	Undefined NASH (NAS 3–4)	Definite NASH (NAS ≥ 5)	P value (ANOVA)
Number (percentage of males)	34 (41)	10 (40)	3 (33)	–
IFG/MeSy/T2D (<i>n</i>)	8/5/5	1/4/2	0/1/2	–
Age (years)	58 \pm 3	61 \pm 4	65 \pm 7	0.68
BMI (kg/m ²)	24.2 \pm 0.6 ^a	32.5 \pm 2.7 ^b	30.7 \pm 0.7 ^{a,b}	<0.001
Body fat (%)	30.4 \pm 1.3	34.4 \pm 3.2	35.6 \pm 4.2	0.29
Waist circumference (cm)	90.8 \pm 2.4 ^a	107.7 \pm 4.7 ^b	111.3 \pm 6.9 ^{a,b}	<0.001
SBP (mmHg)	132.0 \pm 3.7	136.7 \pm 5.8	130.0 \pm 5.8	0.80
DBP (mmHg)	72.0 \pm 2.3	78.3 \pm 2.9	73.3 \pm 4.4	0.39
AST:ALT ratio	1.44 \pm 0.17	1.61 \pm 0.40	0.75 \pm 0.09	0.45
GLDH (U/l)	9.04 \pm 1.56	9.76 \pm 4.67	14.05 \pm 1.65	0.37
Total bilirubin (μ mol/l)	13.64 \pm 1.71	11.90 \pm 1.88	11.34 \pm 0.58	0.81
NH ₃ (μ mol/l)	33.60 \pm 4.38	29.55 \pm 3.46	26.00 \pm 9.00	0.89
Thrombin time (%)	91.39 \pm 1.54	92.89 \pm 3.00	94.00 \pm 0.10	0.85
Albumin (g/dl)	3.87 \pm 0.07	3.70 \pm 0.13	4.08 \pm 0.09	0.73
Ferritin (ng/ml)	209.41 \pm 43.32	416.06 \pm 95.74	202.60 \pm 110.59	0.10
Creatinine (μ mol/l)	85.15 \pm 5.42	82.78 \pm 8.32	81.60 \pm 9.70	0.96
HOMA-IR	1.80 \pm 0.34	3.45 \pm 1.21	5.78 \pm 3.28	0.26
Fasting glucose (mg/dl)	99.18 \pm 4.72	101.72 \pm 11.46	163.12 \pm 82.29	0.50
LDL (mg/dl)	121.64 \pm 8.06	121.33 \pm 17.92	91.50 \pm 41.50	0.66
HDL (mg/dl)	51.55 \pm 3.28	47.67 \pm 3.95	41.00 \pm 4.00	0.64
TAG (mg/dl)	100.23 \pm 7.64 ^a	116.14 \pm 19.76 ^{a,b}	194.00 \pm 51.00 ^b	0.023
NAS (0–8)	0.94 \pm 0.14 ^a	3.15 \pm 0.15 ^b	6.00 \pm 0.58 ^c	<0.001
Statins (<i>n</i>)	4	1	0	–
Oral antidiabetics/metformin (<i>n/n</i>)	3/2	1/1	2/1	–
ACE and AT ₂ inhibitors (<i>n</i>)	5	3	0	–

Different superscript letters indicate significant differences between the groups. IFG/T2D and MeSy were defined according to the criteria of the American Diabetes Association (35) and the NCEP ATP III panel criteria (36). ALT, alanine transaminase; AST, aspartate transaminase; DBP, diastolic blood pressure; GLDH, glutamate dehydrogenase; IFG, impaired fasting glucose; HOMA-IR, homeostasis model assessment of insulin resistance; MeSy, metabolic syndrome; NAS, non-alcoholic fatty liver disease (NAFLD) score; SBP, systolic blood pressure; TAG, triacylglycerides; T2D, type 2 diabetes mellitus.

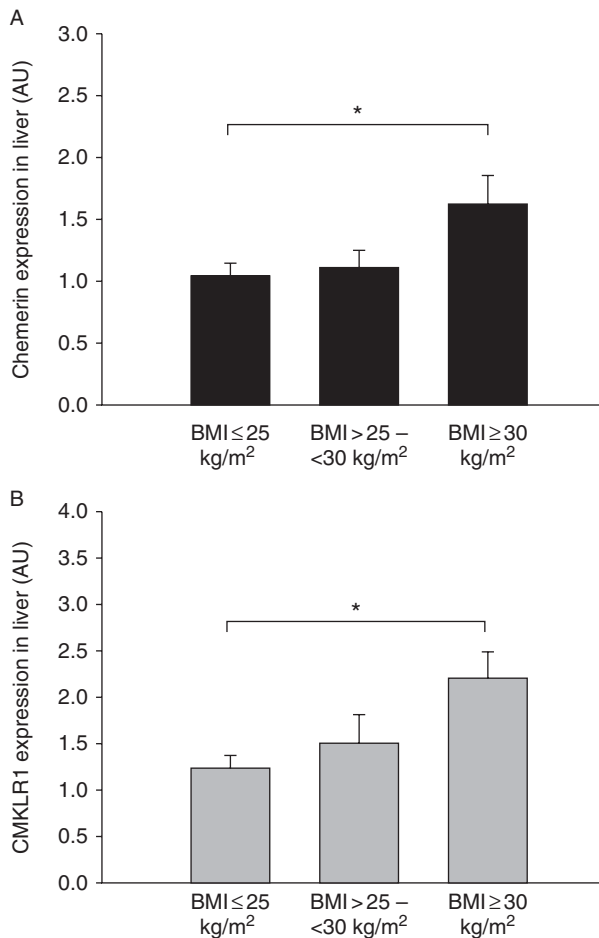


Figure 1 Hepatic (A) chemerin expression and (B) CMKLR1 expression in relation to body mass. Data are means ± s.e.m.; * $P < 0.05$; statistical significance was tested using ANOVA; Bonferroni correction was applied for *post hoc* comparisons.

Hepatic chemerin and CMKLR1 mRNA expression in human NAFLD

In accordance with the literature (42, 43, 44), we found a significant association of BMI with the NAS ($R^2 = 0.516$; $P < 0.001$). Therefore, we hypothesized that the observed association between the hepatic mRNA levels of chemerin or CMKLR1 and the measures of obesity could rather be related to NAFLD than to obesity itself. We accordingly tested for potential associations with the NAS, a recognized histological tool for staging NAFLD (23), and were able to identify a significant correlation with the expression of both hepatic chemerin and CMKLR1 mRNAs ($R^2 = 0.543$; $P < 0.001$ and $R^2 = 0.355$; $P = 0.014$ respectively). In accordance with this finding, we detected significantly lower hepatic mRNA levels of chemerin in the tertiles of subjects having a NAS ≤ 2 and a NAS of 3–4 than in the tertiles of subjects with a NAS ≥ 5 ($P < 0.001$ and $P < 0.001$ respectively; Fig. 2A). Comparably, hepatic

CMKLR1 mRNA expression was significantly increased in patients with a NAS ≥ 5 than in subjects who did not meet the histopathological criteria for NASH ($P = 0.020$; Fig. 2B).

NAS categories and liver fibrosis in relation to hepatic chemerin expression

Defined histopathological features are used to calculate Kleiner’s NAFLD scores, i.e., the intensity of hepatic steatosis, hepatocyte ballooning, and lobular inflammation (23). Based on the supposed physiological functions of chemerin (24), we hypothesized that some of the categories of Kleiner’s system could be predominantly associated with our targets of interest. Therefore, we were interested in studying the association of chemerin and CMKLR1 mRNA expression in the liver with the percentage of steatotic hepatocytes. The expression of both chemerin and CMKLR1 mRNAs was significantly associated with

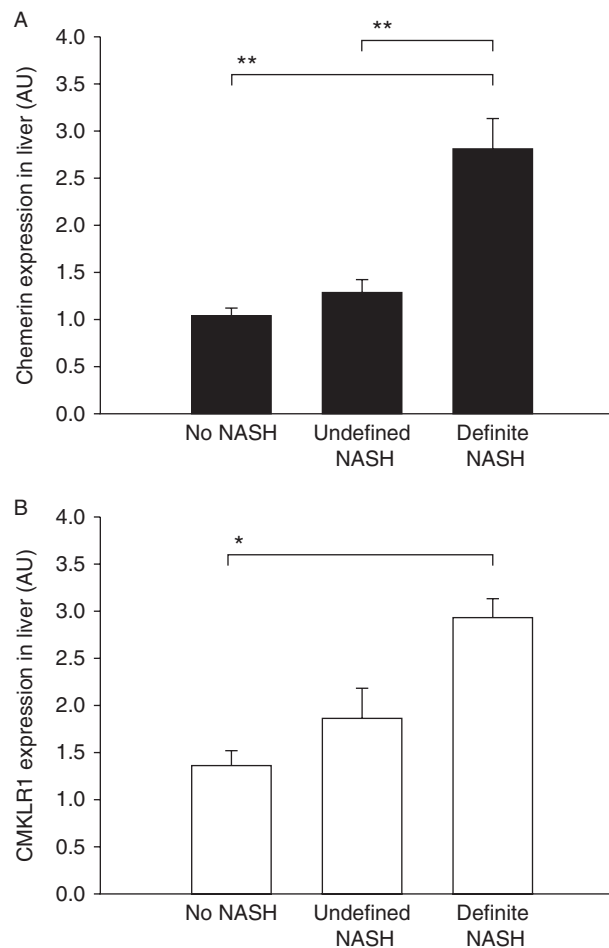
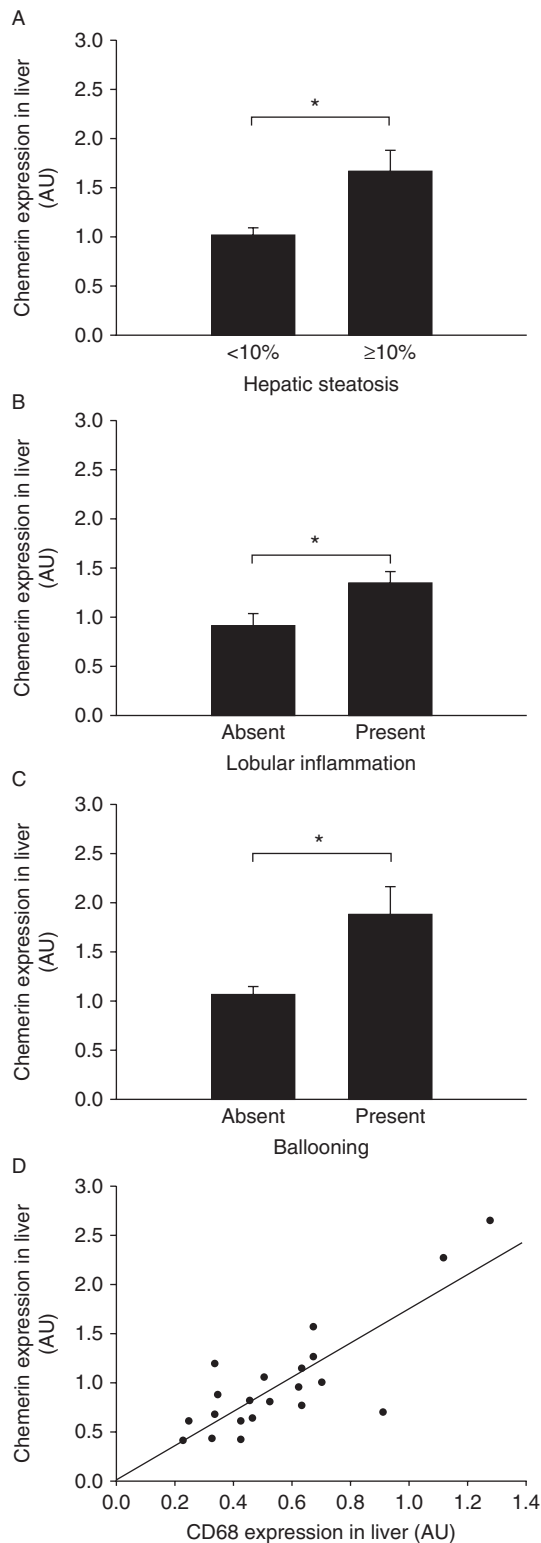


Figure 2 Hepatic (A) chemerin expression and (B) CMKLR1 expression in relation to the NAS; data are means ± s.e.m.; * $P < 0.05$; ** $P < 0.01$; statistical significance was tested using ANOVA; Bonferroni correction was applied for *post hoc* analysis.

hepatic steatosis ($R^2=0.414$; $P=0.003$ and $R^2=0.391$; $P=0.006$ respectively), which was reflected by the significantly elevated levels of chemerin mRNA in subjects having $\geq 10\%$ steatotic hepatocytes



($P=0.013$; Fig. 3A). The latter represented the median value of hepatic steatosis in our study group. However, similar results were obtained when using the quartiles of hepatic steatosis as suggested by Kleiner *et al.* (23). The levels of chemerin mRNA were significantly elevated in subjects having >33 – 66 and $>66\%$ liver fat than in those having <5 and 5 – 33% hepatic steatosis ($P<0.05$ respectively; data not shown). Moreover, the hepatic mRNA levels of *CMKLR1* were also elevated in patients with hepatic steatosis (1.35 ± 0.15 vs 2.12 ± 0.27 AU; $P=0.014$). To assess whether hepatic steatosis would remain independently associated with the hepatic mRNA expression of our targets of interest, we adjusted for several relevant confounders by means of a multivariate linear regression analysis. Thereby, we were surprisingly unable to confirm our findings concerning *CMKLR1* ($P>0.05$; data not shown), indicating that *CMKLR1* mRNA expression in the liver is not independently associated with fatty liver. On the other hand, the degree of histologically determined hepatic steatosis remained to be significantly predicted by BMI and hepatic chemerin mRNA expression (Model A; Table 2). The results continued to be reproducible when further confounders were introduced into the model. In addition, we carried out a stepwise reverse linear regression analysis, where the last model indicated hepatic chemerin mRNA expression to be a significant predictor of hepatic steatosis.

Due to the supposed pro- and/or anti-inflammatory functions of chemerin in terms of tissue injury (24), we aimed to investigate the potential associations between hepatic chemerin and *CMKLR1* mRNA expression and lobular inflammation and hepatocyte ballooning degeneration. Subjects with histopathological signs of hepatocyte ballooning and lobular inflammation exhibited significantly elevated hepatic chemerin expression than their respective controls ($P=0.025$ and $P=0.034$ respectively; Fig. 3B and C). Accordingly, hepatic *CD68* mRNA expression was significantly correlated with either histologically assessed lobular inflammation ($R^2=0.658$; $P=0.002$) and chemerin mRNA expression ($R^2=0.824$; $P<0.001$; Fig. 3D). Moreover, the multivariate linear regression analysis confirmed hepatic chemerin mRNA expression to be the only

Figure 3 (A) Hepatic chemerin expression in subjects with histologically proven hepatic steatosis $<10\%$ vs $\geq 10\%$; data are means \pm S.E.M.; $*P<0.05$; statistical analysis was carried out using Student's *t*-test for unpaired samples. (B) Hepatic chemerin expression in subjects with absent vs present histologically examined lobular inflammation; data are means \pm S.E.M.; $*P<0.05$; statistical analysis was carried out using Student's *t*-test for unpaired samples. (C) Hepatic chemerin expression in absent vs present histologically examined hepatocellular ballooning degeneration; data are means \pm S.E.M.; $*P<0.05$; statistical analysis was carried out using Student's *t*-test for unpaired samples. (D) Correlation between chemerin and *CD68* mRNA expression in human liver tissues ($R^2=0.824$; $P<0.001$). Hepatic *CD68* expression was also correlated with histologically assessed lobular inflammation ($R^2=0.658$; $P=0.002$; data not shown).

Table 2 Results of the multivariate linear regression analysis of histological parameters associated with hepatic chemerin expression.

	Model A Dependent variable: hepatic steatosis β -Coefficient (<i>P</i> value)	Model B Dependent variable: hepatic lobular inflammation β -Coefficient (<i>P</i> value)	Model C Dependent variable: hepatocellular ballooning β -Coefficient (<i>P</i> value)	Model D Dependent variable: liver fibrosis β -Coefficient (<i>P</i> value)
Independent variables	$R=0.67$; $R^2=0.45$; adj. $R^2=0.37$, $P<0.001$	$R=0.53$; $R^2=0.28$; adj. $R^2=0.17$, $P=0.038$	$R=0.74$; $R^2=0.55$; adj. $R^2=0.47$, $P<0.001$	$R=0.65$; $R^2=0.42$; adj. $R^2=0.33$, $P=0.001$
Sex	-0.001 (0.99)	-0.011 (0.94)	-0.021 (0.85)	-0.228 (0.075)
Age	0.093 (0.44)	-0.020 (0.88)	-0.128 (0.28)	0.005 (0.97)
BMI	0.353 (0.005)	0.251 (0.081)	0.278 (0.021)	0.018 (0.89)
Alcohol consumption	-0.020 (0.87)	-0.202 (0.15)	0.114 (0.32)	0.267 (0.038)
Oral antidiabetics	0.191 (0.14)	0.112 (0.46)	0.221 (0.081)	0.428 (0.003)
Hepatic chemerin mRNA	0.371 (0.005)	0.312 (0.040)	0.536 (<0.001)	0.283 (0.038)

Significant correlations are given in bold.

factor to remain independently and significantly associated with the variance of hepatic lobular inflammation (Model B; Table 2), while both hepatic chemerin mRNA expression and BMI were found to be significant predictors of hepatocyte ballooning degeneration (Model C; Table 2). By contrast, the hepatic mRNA levels of *CMKLR1* were comparably high in subjects with histopathological signs of lobular inflammation vs subjects with normal liver (1.75 ± 0.16 vs 1.24 ± 0.29 AU; $P=0.11$) and were also not significantly affected in terms of hepatocyte ballooning (2.17 ± 0.28 vs 1.48 ± 0.18 AU; $P=0.07$).

Liver fibrosis represents a recognized histopathological feature of the advanced stages of NAFLD (1) and is determined in addition to the NAS to improve diagnostic specificity (23). We observed significantly elevated hepatic mRNA levels of chemerin in subjects classified as those suffering from significant liver fibrosis than in those with absent or mild fibrotic liver histology ($P=0.012$ and $P=0.013$ respectively; Fig. 4). With respect to our results concerning the NAS categories, we expected that liver fibrosis would remain significantly associated with the expression of hepatic chemerin even after including potential confounding variables, which was confirmed using a multivariate linear regression analysis (Model D; Table 2).

At this point, it is important to note that nine of our study subjects were suffering from T2D (Table 1), ten were diagnosed as having the metabolic syndrome (Table 1), and a further nine were considered to be suffering from IFG levels (Table 1). All these three conditions are closely associated with insulin resistance, while the latter is known to be associated with NAFLD (1). Thus, insulin resistance represents a confounding variable that could have interfered with the results of this study. However, we introduced the HOMA-IR, as a clinical surrogate of insulin resistance, as an independent variable into each of the above-mentioned models and failed to detect any significant association with our histopathological features of interest. Thus, insulin resistance was not a major factor affecting NAFLD histopathology or liver fibrosis in our study group.

Serum chemerin was not affected by NAFLD

With respect to circulating chemerin levels, we observed no significant difference between the NAS tertiles of our study group (data not shown). Furthermore, we also failed to detect a significant correlation between serum chemerin and hepatic chemerin mRNA levels ($R^2 = -0.104$; $P=0.50$). There was only a trend correlation with hepatic chemerin mRNA expression, when circulating chemerin levels were adjusted for body fat percentage ($R^2 = -0.274$; $P=0.071$).

CMKLR1 and chemerin mRNA expression in PHHs

To elucidate the mechanisms that could be responsible for the observed upregulation of hepatic chemerin and *CMKLR1* levels in NAFLD, we screened the study subjects for associated parameters. Thereby, we found

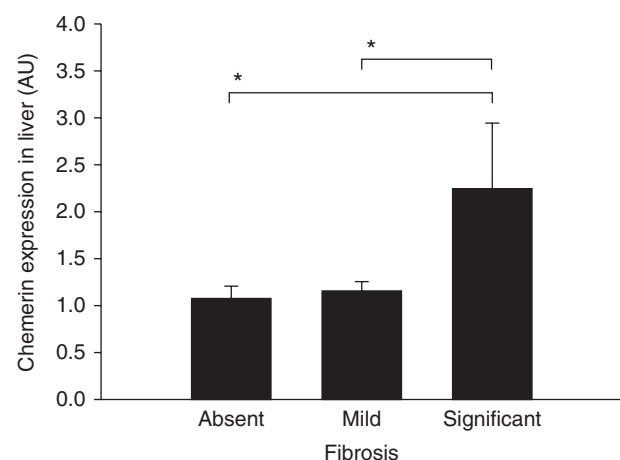


Figure 4 Expression of hepatic chemerin in subjects suffering from histologically assessed absent, mild, and significant liver fibrosis; data are means \pm s.e.m.; * $P<0.05$; statistical significance was tested using ANOVA; Bonferroni correction was applied for *post hoc* analysis.

that the expression of hepatic *IL6* mRNA, which was mainly detectable in subjects having a higher NAS, was significantly correlated with *CMKLR1* levels ($R^2=0.648$; $P=0.017$). Encouraged by the important role of *IL6* in NASH (7), we exposed PHHs to *IL6*. Moreover, we incubated PHHs with palmitoic acid, insulin, and glucagon, since these metabolites are among those believed to impact the microenvironment in NAFLD (7, 32). While all these metabolites did not exhibit a significant influence on the expression of chemerin ($P>0.05$; Fig. 5A), *IL6* significantly increased the expression of *CMKLR1* mRNA in our PHH model ($P=0.027$; Fig. 5B). To confirm these findings, we additionally carried out an *in silico* analysis of the *CMKLR1* promoter DNA using the Genomatix MatInspector software to detect for transcription factor-binding sites within the *CMKLR1* promoter. Using this approach, we were able to identify a STAT-binding site (ATCATTAGTGGAATAATCA) in the proximal promoter.

Discussion

To the best of our knowledge, this is the first study to show that chemerin mRNA is abundantly expressed in the liver of extensively characterized subjects and provides evidence that chemerin mRNA expression is significantly increased in NASH patients. Our data complement and expand the results of studies that have reported significant hepatic chemerin mRNA levels in animal models (9, 11) and increased chemerin concentrations in the hepatic vein of cirrhotic patients (26). NAFLD represents a spectrum of conditions and can be categorized into simple non-alcoholic liver steatosis and NASH (1). NASH is defined by increased liver fat with concomitant inflammatory activity and hepatocellular injury, commonly referred to as ballooning degeneration (23). The risk of suffering from NASH rises with an increasing NAS. The subjects included in our third tertile, who according to their average NAS of 6 points suffered from definite NASH, exhibited about 2.5-fold elevated hepatic chemerin mRNA levels when compared with subjects in the lower tertiles. In addition, after adjustment for defined confounders, hepatic lobular inflammation and ballooning degeneration, two diagnostic hallmarks to distinguish NASH from simple steatosis (23), remained to be associated with hepatic chemerin mRNA expression. These findings were further supported by a significant association of the expression of hepatic chemerin with that of *CD68* mRNA, which represents a recognized marker of mononuclear cells, i.e., Kupffer cells in the human liver (45).

The separation of liver fibrosis from the other features of NASH is an accepted paradigm for the staging and grading of NASH, but the characterization of fibrosis enhances diagnostic specificity (23). We found significantly elevated chemerin mRNA levels under conditions of liver fibrosis, and the linear regression

analysis identified chemerin as one of the independent predictors of liver fibrosis. Taken together, our data provide evidence for a relevant association of hepatic chemerin mRNA expression with NASH. However, a recent study has reported decreased hepatic chemerin mRNA expression in commercially available human fatty liver tissues and in human hepatoma G2 cells after a lipid overload (46). Moreover, using a methionine and choline-deficient diet, the same authors showed reduced hepatic chemerin expression in a rodent NASH model (47). This is at odds with our findings, but could potentially be explained by differences concerning the

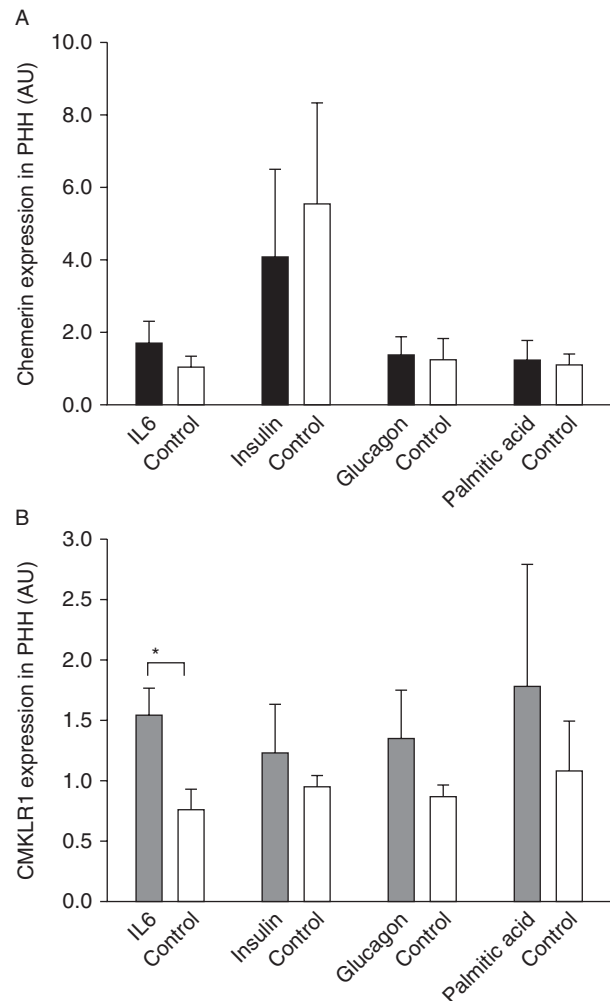


Figure 5 Expression of chemerin and *CMKLR1* mRNAs in primary human hepatocytes (PHH). PHHs were incubated with palmitoic acid (0.3 mM), *IL6* (50 ng/ml), glucagon, or insulin (100 nM) over a period of 24 h. (A) Exposure to *IL6*, insulin, glucagon, and palmitoic acid had no effect on chemerin mRNA expression ($P>0.05$ respectively). (B) Insulin, glucagon, and palmitoic acid had no effect on *CMKLR1* mRNA expression in PHHs. On the other hand, *IL6* at defined concentrations induced a significant increase in *CMKLR1* mRNA expression ($P=0.027$); data are given as means \pm S.E.M. from a set of five independent representative experiments; $*P<0.05$; statistical significance was tested using a *t*-test for unpaired samples.

characteristics of the included patient collectives, which are unfortunately not available for the above-mentioned study (46). In addition, it must be emphasized that significant species differences have been reported concerning the chemerin–CMKLR1 system, especially for rodents and humans (47). Therefore, results obtained from animal studies cannot necessarily be applied to defined clinical human collectives.

Chemerin and CMKLR1 represent a specific signaling system, where CMKLR1 displays the unique combination of high-affinity chemerin binding and efficient intracellular signaling (25). Wanninger *et al.* (21) 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 is reduced in the hepatocytes of subjects suffering from hepatic steatosis when compared with non-steatotic controls (21). Moreover, in their rodent NASH model, hepatic CMKLR1 expression was also reduced (21). In contrast with the former mentioned study, CMKLR1 expression was significantly elevated in the liver of our NASH subjects. However, it has to be mentioned that we studied the mRNA levels, which do not necessarily reflect hepatic protein levels. In addition, we were unable to show an independent association of hepatic *CMKLR1* mRNA expression with hepatic steatosis in our patient collective. Elevated hepatic *CMKLR1* mRNA expression was exclusively detected under conditions of NASH. Unfortunately, Wanninger *et al.* (21) did not report the degree of hepatic steatosis and/or the NAS of their study subjects. This information would be of particular interest, since variances concerning steatosis and hepatic inflammatory activity could explain some differences between the studies. This hypothesis is supported by the suggestion that the activity of the chemerin–CMKLR1 system may depend on the nature of stimuli in the local microenvironment (47). Accordingly, a recent study has reported decreased serum IL6 levels and reduced hepatic inflammatory cell invasion in *Cmklr1*^{-/-} mice (22). Complementary, we found a significant association between the hepatic mRNA levels of *IL6* and those of *CMKLR1* in our NAFLD subjects, and accordingly when exposing the PHHs to IL6, a significant upregulation of *CMKLR1* mRNA expression was observed. This would be in congruence with the observed significantly elevated hepatic *CMKLR1* mRNA expression in the human NASH tertile. IL6 is recognized as an important biomarker of NASH (7), and by carrying out an *in silico* analysis of the *CMKLR1* promoter, we identified a STAT-binding site, supporting the hypothesis that IL6 could play a causal role in the regulation of CMKLR1 expression. On the other hand, according to the study of Wanninger *et al.* (21), IL6 does not significantly influence CMKLR1 expression in PHHs. These contrasting findings can, however, be explained by the differing IL6 concentrations used for the PHH incubation experiment in both the studies. When

compared with those in the peripheral blood, IL6 concentrations in the portal vein of obese subjects are ~50% elevated (6). Thus, it is believed that hepatocytes are exposed to higher local IL6 concentrations in obesity and therefore also in NASH. The expression of *IL6* mRNA detected in the liver tissue samples of our NAFLD subjects further suggests an additional paracrine secretion. Thus, we decided to mimic NASH conditions in our PHH experiments and chose higher IL6 concentrations, which could explain why we observed an effect. In summary, we expanded pre-existing data on humans (21) and showed that the expression of *CMKLR1* seems to be upregulated at the level of mRNA in NASH, whereby IL6 could play a causal role.

Previous reports have indicated that liver injury may be associated with circulating chemerin levels (28, 30, 31) and that the liver may be a contributor to serum levels (9). We observed a trend correlation between hepatic mRNA levels and body fat-adjusted serum chemerin levels, suggesting that in our study the liver was not a major contributor to circulating levels. We also did not detect a difference in our well-characterized tertiles. This contrasts with previous findings that identified elevated serum chemerin levels in subjects with hepatic portal- and fibroinflammation (28). The latter study, however, included morbidly obese subjects and did not adjust for inflammatory conditions (28), which could at least in part explain the observed differences. Another study has reported elevated serum chemerin levels in NASH and simple steatosis (30). The NAFLD subjects included in this study remarkably differed from the controls with respect to HOMA-IR, fasting insulin and fasting glucose levels, and some surrogates of liver function (30). These factors or a combination could have interfered with the reported results and may explain why their data differ from our findings.

The strengths of this study are the extensively characterized study group, the use of non-hypoxic healthy human liver tissue samples, and the general comparability of subjects in terms of age, gender, blood pressure, glucose metabolism, and liver and kidney function. The weaknesses are the limited number of subjects, the inclusion of only Caucasian subjects, and the exclusive investigation of the mRNA levels. Moreover, with our study design, we cannot state whether the chemerin–CMKLR1 system plays a pro- or anti-inflammatory role in human NASH.

We conclude that local inflammation, steatosis, and tissue damage are associated with hepatic chemerin mRNA expression, while IL6 could play a role concerning the regulation of CMKLR1 expression.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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