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# Endogenous formation of $N^{\epsilon}$ -(carboxymethyl)lysine is increased in fatty livers and induces inflammatory markers in an *in vitro* model of hepatic steatosis

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**Background & Aims:** Increased lipid peroxidation and inflammation are major factors in the pathogenesis of nonalcoholic fatty liver disease (NAFLD). A lipoxidation product that could play a role in the induction of hepatic inflammation is  $N^{\epsilon}$ -(carboxymethyl)lysine (CML). The aim of the present study was to investigate the relationship between steatosis and CML and to study the role of CML in hepatic inflammation.

**Methods:** We included 74 obese individuals, which were categorized into 3 groups according to the grade of hepatic steatosis. CML accumulation in liver biopsies was assessed by immunohistochemistry and plasma CML levels were measured by mass spectrometry. Plasma CML levels were also determined in the hepatic artery, portal, and hepatic vein of 22 individuals, and CML fluxes across the liver were calculated. Hepatocyte cell lines were used to study CML formation during intracellular lipid accumulation and the effect of CML on pro-inflammatory cytokine expression. Gene expression levels of the inflammatory markers were determined in liver biopsies of the obese individuals.

**Results:** CML accumulation was significantly associated with the grade of hepatic steatosis, the grade of hepatic inflammation, and gene expression levels of inflammatory markers PAI-1, IL-8, and CRP. Analysis of CML fluxes showed no release/uptake of CML by the liver. Lipid accumulation in hepatocytes, induced by incubation with fatty acids, was associated with increased CML formation and expression of the receptor for advanced glycation endproducts (RAGE), PAI-1, IL-8, IL-6, and CRP. Pyridoxamine

and aminoguanidine inhibited the endogenous CML formation and the increased RAGE, PAI-1, IL-8, IL-6, and CRP expression. Incubation of hepatocytes with CML-albumin increased the expression of RAGE, PAI-1, and IL-6, which was inhibited by an antibody against RAGE.

**Conclusions:** Accumulation of CML and a CML-upregulated RAGE-dependent inflammatory response in steatotic livers may play an important role in hepatic steatosis and in the pathogenesis of NAFLD.

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## Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of abnormal liver function and is characterized by excessive hepatic deposition of fatty acids and triglycerides (i.e. steatosis) [1,2]. The spectrum of NAFLD ranges from fat accumulation in the liver (simple steatosis or fatty liver), to steatosis accompanied by inflammation and necrosis with or without fibrosis (nonalcoholic steatohepatitis or NASH), and ultimately to liver cirrhosis and end-stage liver disease. NAFLD is strongly associated with the presence and severity of obesity and with a high risk of developing insulin resistance, type 2 diabetes, dyslipidaemia, and cardiovascular disease [2-4].

Steatosis in hepatocytes is followed by cell activation induced by oxidative stress, inflammatory cytokines, and increased lipid peroxidation, which are important contributors to NAFLD and its progression [1,5]. Increased levels of oxidative stress and lipid peroxidation have been demonstrated in patients with NAFLD [6-8]. Oxidative stress and lipid peroxidation also stimulate the formation of the advanced glycation/lipoxidation endproduct (AGE/ALE),  $N^{\epsilon}$ -(carboxymethyl)lysine (CML), which is regarded as a major lipid peroxidation product and is one of the best characterized ALEs [9,10].

CML plays an important role in the pathogenesis of many diseases. One mechanism through which CML affects cellular func-

Keywords: Glycation; Inflammation; Cytokines; NAFLD; Obesity.

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Abbreviations: NAFLD, nonalcoholic fatty liver disease; CML,  $N^{\epsilon}$ -(carboxymethyl)lysine; RAGE, receptor for advanced glycation endproducts; UPLC, ultra performance liquid chromatography; MS, mass spectrometry; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; HOMA-IR, homeostasis model assessment for insulin resistance.



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tion is binding to the receptor for AGEs (RAGE) and activation of intracellular signaling pathways like NF- $\kappa$ B [9,11]. Studies have found significant effects of AGEs on hepatocytes, such as increased expression of C-reactive protein (CRP) and induction of insulin resistance [12–14], and suggest a critical role of AGEs in the pathogenesis of NAFLD [15]. AGEs also have effects on hepatic stellate cells, which are involved in liver fibrogenesis [16–18]. Therefore, we hypothesize that CML will accumulate in fatty livers of obese individuals, and that this CML accumulation may lead to increased expression of inflammatory markers, which could contribute to the pathogenesis of NAFLD.

To study whether development of hepatic steatosis is associated with increased formation of CML, we quantified CML in liver biopsies of severely obese individuals and assessed CML accumulation in cultured hepatocytes during development of steatotic morphology. Furthermore, we determined hepatic gene expression of inflammatory markers in relation to hepatic CML accumulation to investigate whether CML plays a role in the induction of hepatic inflammation.

### Materials and methods

#### Liver biopsies

All severely obese individuals ( $n = 74$ ) enrolled in this study underwent a liver biopsy during bariatric surgery at the Department of General Surgery, Maastricht University Medical Center (Maastricht, the Netherlands). None of these individuals had suffered from viral hepatitis or autoimmune-related disorders or reported excessive alcohol consumption ( $>20$  g/day). Details of inclusion and exclusion criteria have recently been described by Rensen *et al.* [19]. This study was approved by the local Ethics Committee and conducted in line with the revised version of the 1975 Declaration of Helsinki guidelines (October 2008, Seoul). All subjects gave written informed consent.

Liver biopsies of obese individuals were fixed in formalin and embedded in paraffin. Biopsies were analyzed for evaluation of histological features by an experienced pathologist who was unaware of the clinical and biochemical data of the individuals. Obese individuals were categorized into 3 groups according to the grade of steatosis; group 1,  $<33\%$  steatotic hepatocytes (low grade of steatosis); group 2, 33–66% steatotic hepatocytes (moderate grade of steatosis); and group 3,  $>66\%$  steatotic hepatocytes (severe grade of steatosis). In addition, histological scoring of liver biopsies regarding lobular (score 0–3) and portal inflammation (score 0–1), hepatocellular ballooning (score 0–2) and fibrosis (0–4) was also performed according to the histological scoring system described by Kleiner *et al.* [20].

#### Immunohistochemistry

Sections of liver biopsies of obese individuals were immunostained with the EnVision + system (Dako, Glostrup, Denmark) according to the manufacturer's instructions. The primary antibodies used in these stainings were directed against CML-modified proteins [21] and RAGE (R&D systems, Minneapolis, USA).

Immunoscore of the CML positivity of the liver biopsies was performed by an independent investigator unaware of the patient's characteristics. CML intensity was scored in four randomly selected fields and classified as score 1 (no or weak CML staining), score 2 (moderate CML staining) and score 3 (strong CML staining). Mean immunohistochemical CML score (IHC CML score) of each liver biopsy was then calculated by averaging the CML intensity scores of the four randomly selected fields. CML staining of liver biopsies of obese individuals was compared with liver sections from normal weight controls who underwent cholecystectomy, and none of these controls had suffered from liver diseases.

#### CML exchange across the liver

To investigate CML exchange across the liver and to determine whether the liver can extract CML from the circulation, protein-bound CML plasma concentrations and net uptake/release of CML across the liver were measured in 22 patients. These patients were studied during major upper abdominal surgery at Maastricht

University Medical Center. Details of the inclusion and exclusion criteria, study protocol and measurements have been described by Bloemen *et al.* [22]. Briefly, blood was sampled from a radial artery (equivalent for hepatic artery), the portal vein, and the hepatic vein, and CML concentrations were measured by ultra performance liquid chromatography–tandem mass spectrometry (UPLC–Tandem MS) as described below. Hepatic artery and portal vein blood flow (BF) were measured using intra-operative Duplex ultrasonography. Next, hepatic artery and portal vein plasma flow (PF) were calculated by correcting BF for hematocrit (Ht) [ $PF = BF \times (1 - Ht)$ ], and were added up to determine total hepatic PF. CML fluxes across the liver were calculated for each patient as follows: CML flux =  $[HV - A] \times \text{hepatic flow} - ([PV - A] \times \text{portal flow})$ . In this equation, [HV], [PV], and [A] indicates CML concentration in hepatic and portal vein, and hepatic artery, respectively. Positive fluxes indicate release from the liver, whereas negative fluxes indicate uptake of CML from the circulation.

#### Expression levels of inflammatory markers in human livers

To investigate whether CML in liver of severely obese individuals may play a role in the induction of hepatic inflammation, gene expression levels of inflammatory markers were measured in liver biopsies taken during bariatric surgery. After isolation, RNA was used for anti-sense RNA synthesis, amplification, and purification by using the Ambion Illumina TotalPrep Amplification kit (Applied Biosystems/Ambion, Austin, USA) according to manufacturer's protocol. Next, this complementary RNA was hybridized to Illumina HumanHT12 Bead-Chips (Illumina, San Diego, USA) and scanned on the Illumina BeadArray Reader. Data were quantile normalized and gene expression of RAGE, PAI-1, IL-1 $\beta$ , IL-6, IL-8, IL-10, and CRP were used for further analysis (Wolfs *et al.*, manuscript in preparation).

#### Hepatocyte cell cultures

Two hepatic cell lines, HepG2 (ATCC, Manassas, USA) and HuH7 cells [23], were used in this study. HepG2 cells were cultured in DMEM (1 g/L glucose) supplemented with 20% FCS, 1% penicillin/streptomycin, and 1% non-essential amino acids, while HuH7 cells were cultured in DMEM (4.5 g/L glucose) supplemented with 10% FCS and 1% penicillin/streptomycin. Steatosis in these cell lines was induced by incubating the hepatocytes with their culture medium with 30 mM mixture of linoleic (18:2) and oleic acid (18:1) (ratio 1:1) during 24 h. These long-chain fatty acids were provided as albumin-conjugated solution (Sigma–Aldrich). As control, hepatocytes were incubated with their culture medium without addition of fatty acids. After incubation, the extent of steatosis in hepatocytes was determined using 0.5% Oil Red O (Sigma–Aldrich) in isopropanol for 15 min and nuclei were counterstained with hematoxylin. In addition, steatotic cells were harvested in 0.1 M sodium phosphate buffer (pH 7.4) with 0.02% Tween and protease inhibitor (Roche, Basel, Switzerland) and cell lysates were used to measure endogenous CML levels by UPLC–Tandem MS (described below). After incubation with the mixture of fatty acids, RNA was also isolated from the hepatocytes to study the effect of endogenous CML on expression levels of pro-inflammatory markers (described below).

#### UPLC–tandem MS

CML levels in plasma of our study population and in hepatocyte lysates after incubation with or without a mixture of fatty acids were quantified by UPLC–tandem MS. Briefly, plasma proteins and cell lysates were reduced with 100 mM sodium borohydride in 0.2 M sodium borate buffer at pH 9.2 for 2 h. The proteins were precipitated with 20% trichloroacetic acid and hydrolyzed with 6 M HCl overnight at 110 °C. HCl was evaporated at 80 °C under nitrogen and residues were resolved in 0.5 mM tridecafluoroheptanoic acid. Analysis was performed using a C18 reverse phase column with a linear gradient of acetonitrile. CML was measured using a positive ionization mode with D4–CML as internal standard.

#### Biological effects of endogenous CML formation in steatotic hepatocytes

To study the effects of endogenous CML formation during intracellular lipid accumulation in hepatocytes, cells were incubated with 30 mM mixture of oleic/linoleic acids during 24 h. After incubation, total RNA was isolated from the cultured hepatocytes using Tri-reagent (Sigma–Aldrich) according to the manufacturer's instructions. RNA (500 ng) was converted to cDNA using the iScript cDNA synthesis kit (Bio–Rad, Hercules, USA). RTq–PCR was performed with Sybr Green Master mix (Quanta, London, UK) and the iQ5 iCycler (Biorad). Expression levels were assessed in triplicate by the  $\Delta\Delta CT$  method after normalization for reference genes [ $\beta$ -actin,  $\beta_2$ -microglobulin, hypoxanthine–guanine phosphoribosyltransferase

**Table 1. Characteristics of obese individuals.**

	Low steatosis (n = 30)	Moderate steatosis (n = 27)	Severe steatosis (n = 17)	p-value
Age (yr)	44.2 (11.3)	42.7 (8.3)	47.5 (8.5)	0.357
Sex (M/F) (%)	30/70	33/67	24/76	0.786
BMI (kg/m <sup>2</sup> )	42.6 [38.5; 50.0]	47.3 [43.2; 51.2]	45.1 [41.1; 56.4]	0.053
Fasting glucose (mmol/L)	5.7 [5.4; 6.4]	6.1 [5.5; 8.9]	6.7 [5.1; 8.3]	0.055
Insulin (mU/ml)	16.0 [11.5; 25.0]	20.0 [12.5; 26.5]	21.0 [8.1; 31.5]	0.708
HOMA-IR	4.2 [2.9; 6.9]	5.8 [3.6; 7.9]	7.0 [2.9; 9.1]	0.145
HbA1C (%)	6.1 [5.7; 6.8]	6.5 [5.7; 7.7]	7.2 [6.2; 8.7]	0.011
Total cholesterol (mmol/L)	4.9 [4.3; 5.4]	5.0 [4.0; 5.5]	5.1 [4.5; 6.2]	0.354
HDL-cholesterol (mmol/L)	1.0 [0.8; 1.2]	0.8 [0.7; 1.0]	1.0 [0.7; 1.1]	0.087
LDL-cholesterol (mmol/L)	3.0 [2.4; 3.7]	3.2 [2.2; 3.9]	3.3 [2.8; 4.0]	0.362
Triglycerides (mmol/L)	1.6 [1.0; 2.4]	2.1 [1.4; 3.1]	2.2 [1.4; 2.7]	0.079
Free fatty acids (mmol/L)	0.5 [0.3; 0.9]	0.6 [0.6; 0.7]	0.7 [0.6; 1.0]	0.161
ALAT (IU)	21.0 [17.3; 29.0]	22.0 [14.8; 33.8]	47.0 [20.0; 56.0]	0.001
ASAT (IU)	18.5 [12.5; 23.0]	24.5 [17.5; 34.3]	33.0 [30.0; 48.0]	<0.001
Creatinine (μmol/L)	75.0 [67.0; 85.0]	72.0 [65.0; 82.0]	78.5 [69.3; 87.3]	0.470
eGFR (ml/min/1.73 m <sup>2</sup> )	85.8 [78.2; 93.4]	89.8 [79.7; 99.9]	80.3 [68.2; 92.3]	0.402
Plasma CML (μM)	1.2 [0.9; 1.3]	1.1 [1.1; 1.3]	1.2 [0.9; 1.2]	0.913

Data are presented as mean (SD), or as percentage, or as mean [interquartile range]. p-Value determined by linear regression of Chi-square analyses.

ase (HRPT)1 and 60S acidic ribosomal protein P0 (RPLP0)]. The expression levels of the genes of interest (RAGE, PAI-1, IL-8, IL-6, CRP, IL1-β, and IL-10) are presented relative to those of the cells cultured in their medium, without addition of fatty acids (controls).

*Effect of pyridoxamine and aminoguanidine on CML formation and inflammatory cytokine expression*

To study the effect of pyridoxamine and aminoguanidine, both inhibitors of the Maillard reaction, on the endogenous CML formation and on the expression of pro-inflammatory cytokines during development of steatosis, hepatocytes were incubated with fatty acids mixture (30 mM), in the presence or absence of pyridoxamine (1.0 mM) (Sigma-Aldrich) or aminoguanidine (250 μM) (Sigma-Aldrich). Cells were harvested in 0.1 M sodium phosphate buffer (pH 7.4) with 0.02% Tween and protease inhibitor (Roche), and CML levels were determined by UPLC-tandem MS as described above. After incubation, RNA was extracted and differences in gene expression of RAGE, PAI-1, IL-8, IL-6, CRP, IL1-β, and IL-10 were determined by RTq-PCR as described above.

*Biological effects of CML incubation on hepatocytes*

In addition to the effects of endogenous CML formation, we studied the effects of exogenous administered CML on pro-inflammatory gene expression in HepG2 hepatocytes.

CML-modified albumin was prepared by incubating human serum albumin, dissolved in 0.5 M phosphate buffer pH8, with and without 3 mM or 300 mM glyoxylic acid and 2 M NaCNBH<sub>3</sub> for 24 h at 37 °C. These reactions were terminated by dialysis against MilliQ. The extent of modification induced by 3 and 300 mM glyoxylic acid will be further addressed as “minimally”- and “highly”-modified CML-albumin, respectively. Albumin incubated without glyoxylic acids served as control-albumin.

HepG2 cells were cultured as described above and incubated with 0.5 μM control-albumin, minimally- or highly-modified CML-albumin during different time periods. After incubation, RNA was extracted and differences in gene expression of RAGE, PAI-1, IL-8, IL-6, CRP, IL1-β, and IL-10 were determined by RTq-PCR as described above. In addition, to assess whether CML affects inflammatory gene expression profiles of HepG2 cells via the RAGE pathway, HepG2 cells were pre-incubated with RAGE antibody (R&D, Minneapolis, USA) (5 μg/ml) to block membranous RAGE, and subsequently incubated with control-albumin, minimally-

and highly-modified CML-albumin. RNA was extracted and differences in gene expression of RAGE, PAI-1, IL-8, IL-6, CRP, IL1-β, and IL-10 were determined by RTq-PCR as described above.

*Statistics*

All analyses were performed with the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) software version 15.0 for Windows. Variables with a skewed distribution (i.e. BMI, fasting glucose, insulin, HOMA-IR, HbA1C, total cholesterol, HDL-, and LDL-cholesterol, triglycerides, free fatty acids, ALAT, ASAT, creatinine, eGFR, and protein-bound CML plasma) were log transformed prior to further analyses.

Comparisons of characteristics between groups with different grades of steatosis were performed with linear regression or Chi-square analyses. Differences in immunohistochemical score of liver biopsies of subjects with different grades of steatosis, lobular and portal inflammation, hepatocellular ballooning and fibrosis were analyzed using One way ANOVA with Bonferroni correction. CML fluxes across the liver were tested versus zero using a one-sample t-test with a theoretical mean of zero (no net release or uptake). The difference in CML flux between patients with a BMI ≤25 kg/m<sup>2</sup> and BMI >25 kg/m<sup>2</sup> was analyzed using a two-sided unpaired Student's t-test. Association between CML fluxes and BMI was determined by partial correlation, adjusted for age and sex. Differences in CML levels and inflammatory gene expression were also investigated using a two-sided unpaired Student's t-test. All data are presented as mean ± SD. p-Values <0.05 were considered statistically significant.

**Results**

*CML accumulates in liver tissue of obese individuals*

Characteristics of the severely obese individuals according to the grade of steatosis are described in Table 1. The majority (41%) of the obese individuals showed a low grade of steatosis, 36% of the individuals had a moderate grade of steatosis, whereas 23% of the individuals had a severe grade of steatosis. We did not find differences in plasma CML levels between

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**Table 2. Analysis of the degree of inflammation, fibrosis and ballooning according to the Kleiner classification of steatosis.**

		Low steatosis (%)	Moderate steatosis (%)	Severe steatosis (%)	X <sup>2</sup>
Lobular inflammation	0	11.3	6.5	3.2	0.006
	1	14.5	25.8	8.1	
	2	4.8	8.1	6.5	
	3	0.0	1.6	9.7	
Portal inflammation	0	25.4	33.3	12.7	0.030
	1	4.8	9.5	14.3	
Fibrosis	0	27.8	31.5	14.8	0.424
	1	1.9	5.6	1.9	
	2	1.9	9.3	3.75	
	3	0.0	0.0	1.9	
	4	0.0	0.0	0.0	
Hepatocellular ballooning	0	15.6	12.5	7.8	0.226
	1	15.6	26.6	14.1	
	2	0.0	3.1	4.7	

the different groups. Further classification of the study group with respect to lobular and portal inflammation, fibrosis and hepatocellular ballooning is summarized in Table 2. Lobular and portal inflammation were significantly associated with the grade of hepatic steatosis, whereas fibrosis and hepatocellular ballooning showed no association. 66% of the study population was diagnosed with NASH, whereas 34% of the obese individuals had only low, moderate or severe grade of steatosis without signs of inflammation, fibrosis and hepatocellular ballooning (simple steatosis). No patients with liver cirrhosis (fibrosis stage 4) were included.

To investigate whether development of steatosis is associated with CML accumulation in the liver, we performed immunohistochemical staining of liver biopsies from severely obese individuals and controls. Liver tissues of controls showed no or a very weak staining for CML, whereas a strong CML staining was observed in liver parenchyma of obese individuals (Fig. 1A). Moreover, the intensity of the CML staining increased with the grade of hepatic steatosis as demonstrated in Fig. 1A. Semi-quantitative analysis of the CML staining of liver biopsies of obese individuals showed that individuals with moderate ( $2.0 \pm 0.7$  vs.  $1.5 \pm 0.6$ ,  $p = 0.010$ ) and severe ( $2.3 \pm 0.8$  vs.  $1.5 \pm 0.6$ ,  $p < 0.001$ ) grade of steatosis had a significantly higher intensity of the CML staining compared with obese individuals with low grade of steatosis (Fig. 1B). In addition, the association between the grade of hepatic steatosis and CML accumulation was similar when our study population was stratified according to diabetic status (data not shown). CML staining was also significantly higher in patients with score 1 and score 2/3 for lobular inflammation compared with patients showing score 0 (Fig. 1C). Higher degree of portal inflammation was also associated with a higher CML staining of liver biopsies (Fig. 1C). On the other hand, fibrosis and hepatocellular ballooning were unrelated to the CML staining (data not shown).

Immunohistochemical staining of liver biopsies for the receptor for CML, i.e. RAGE, demonstrated that RAGE is specifically and exclusively localized at the membrane of steatotic hepatocytes, whereas non-steatotic hepatocytes showed no RAGE staining. This suggests that RAGE levels are increased during development of liver steatosis (Fig. 1D), probably as a result of CML accumulation and the positive feedback mechanism between CML and RAGE.

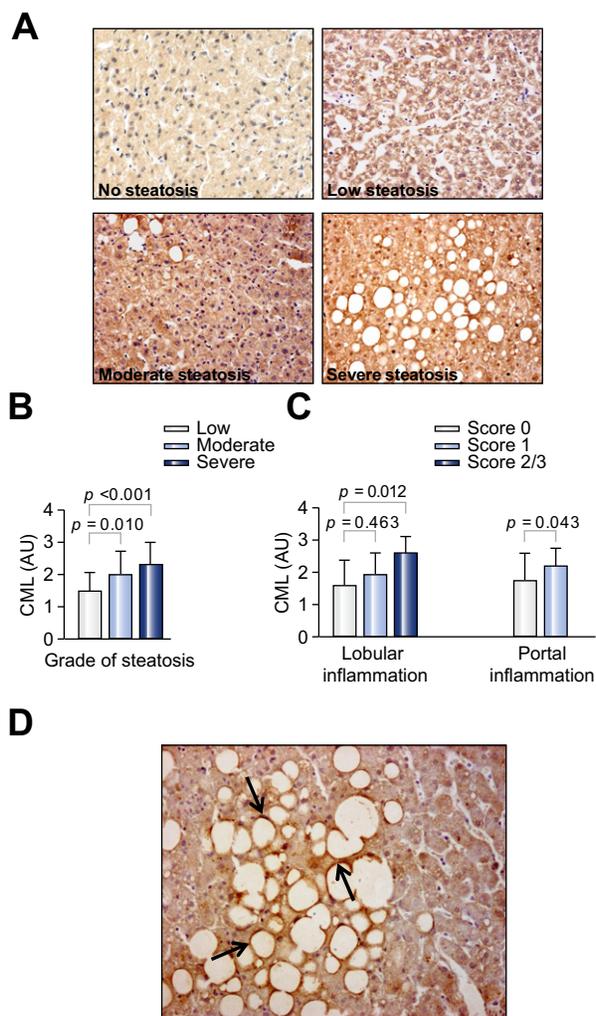
Taken together, these results demonstrate that the development of steatosis is associated with CML accumulation and that this CML accumulation may subsequently play a role in the induction of hepatic inflammation probably via upregulation of RAGE.

### CML exchange across the liver

To investigate whether circulating CML is taken up by the liver, and, as such, contributes to the local accumulation of CML, we investigated CML exchange across the liver in 22 patients (14 men and 8 women, aged  $57 \pm 12$  years and BMI of  $28.9 \pm 5.8$  kg/m<sup>2</sup>). CML concentrations in the hepatic, portal vein, and hepatic artery were  $1.42 \pm 0.89$ ,  $1.42 \pm 0.89$ , and  $1.34 \pm 0.98$  μM, respectively (Fig. 2A). Consequently, the flux of CML across the liver was  $0.007$  [−0.015; 0.038] (mean [interquartile range]) μM × kg bodyweight<sup>−1</sup> × h<sup>−1</sup> ( $p = 0.398$ ), indicating no net release or uptake of CML by the liver in these patients. In addition, a subgroup analysis was conducted to investigate any potential effect of bodyweight. This analysis of patients with a BMI ≤25 kg/m<sup>2</sup> ( $n = 10$ ) compared with patients with a BMI >25 kg/m<sup>2</sup> ( $n = 12$ ) revealed no significant difference in CML flux across the liver ( $0.000 \pm 0.044$  versus  $0.017 \pm 0.059$  μM × kg bodyweight<sup>−1</sup> × h<sup>−1</sup>,  $p = 0.484$ ) (Fig. 2B). Moreover, no correlation between BMI and CML flux across the liver was observed (Fig. 2C). These results suggest that there is no CML uptake/release by the liver, and that the stronger CML staining of steatotic liver biopsies (Fig. 1A) is probably due to increased endogenous CML formation.

### Fat accumulation in hepatocytes is associated with endogenous CML formation, which leads to induction of pro-inflammatory cytokines

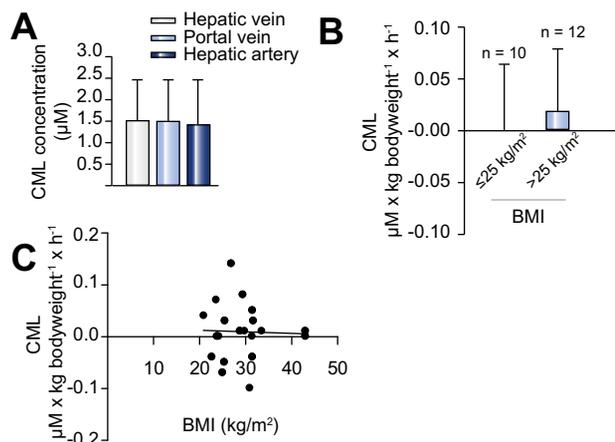
To study endogenous CML formation during lipid accumulation in hepatocytes, we analyzed the intracellular accumulation of lipids and CML in an *in vitro* model of hepatic steatosis. In this model, HepG2 and HuH7 cells were exposed to a mixture of oleic/linoleic acid (30 mM at a 1:1 ratio for 24 h), which was accompanied by a marked accumulation of lipid droplets as indicated by an Oil Red O staining (Fig. 3A). Similar findings were obtained for HepG2 and HuH7 cells. This accumulation of lipid droplets was associated with a 21% and 42% increase of CML



**Fig. 1. CML accumulation in liver tissue of obese individuals.** (A) Representative immunostainings of CML in liver biopsies of control individuals (no steatosis) and different grades of steatosis (low, moderate, and severe steatosis) in obese individuals (magnification  $\times 200$ ). No or a very weak staining was observed in controls, whereas a progressive CML staining was detected in the cytoplasmatic fraction of hepatocytes with increasing grades of hepatic steatosis. (B) Semi-quantitative analysis of CML staining demonstrated that CML accumulation in liver was higher in obese individuals with a moderate and severe grade of steatosis compared with obese individuals with a low grade of steatosis. (C) Higher grades of lobular and portal inflammation were also associated with increased CML staining of liver tissues. (D) Immunostaining of liver biopsies for RAGE showed that RAGE was specifically and exclusively localized at the membrane of steatotic hepatocytes (see arrows).

concentration in HepG2 and HuH7 cells, respectively (Fig. 3B). This *in vitro* experiment demonstrates that intracellular lipid accumulation is associated with increased endogenous formation of CML.

Increased CML formation in hepatocytes was accompanied by a 1.6-, 1.3-, and 2.9-fold increased expression of RAGE, PAI-1, and IL-8 in HepG2 cells, respectively (Fig. 3C). The expression levels of IL-6, IL-1 $\beta$ , IL-10, and CRP were too low in cultured HepG2 cells for reliable detection by RTq-PCR. In HuH7 cells, incubation with fatty acids resulted in a significant upregulation of the gene expression of RAGE (by 1.2-fold), PAI-1 (by 1.3-fold), IL-6 (by 1.3-fold, N.S.  $p = 0.288$ ), IL-8 (by 2.2-fold), and CRP (by 5.4-fold)



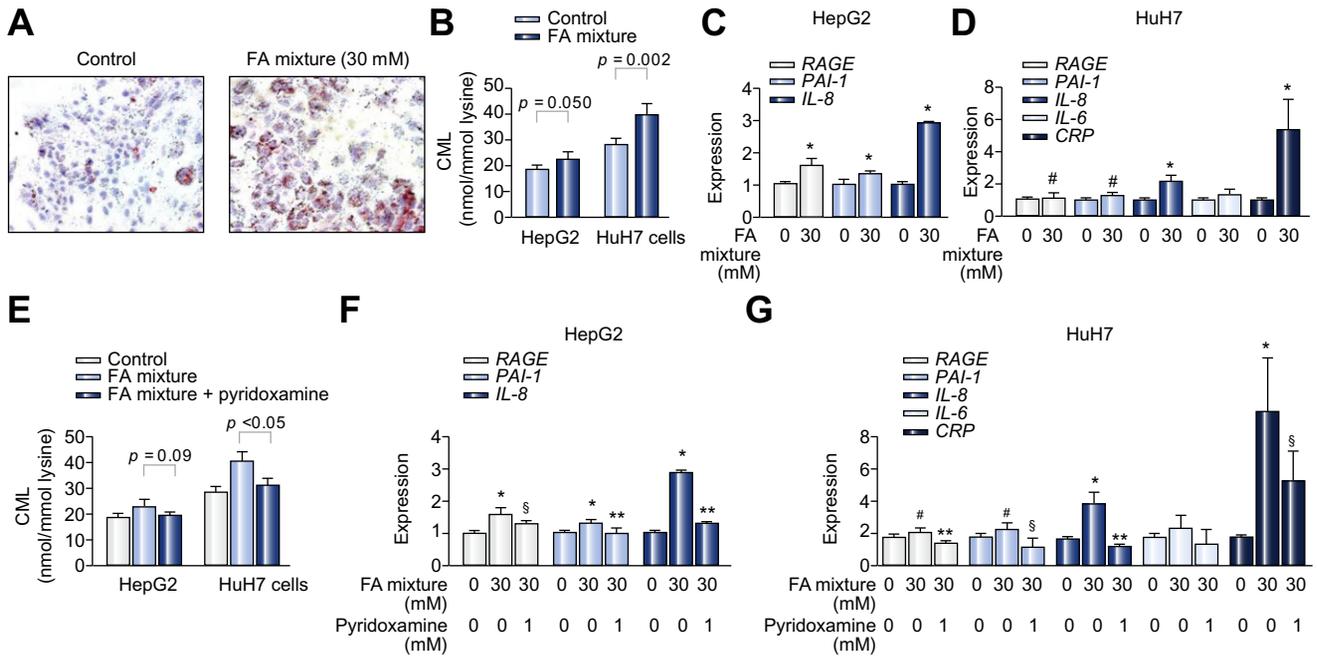
**Fig. 2. CML fluxes across the liver in obese individuals.** (A) CML plasma concentrations were measured by UPLC-tandem MS in the hepatic vein, portal vein and hepatic artery of obese individuals ( $n = 22$ ) and no differences in CML levels were observed. (B) CML fluxes across the liver were not different between individuals with BMI  $\leq 25$  kg/m $^2$  and BMI  $> 25$  kg/m $^2$ . (C) No association between CML fluxes across the liver and BMI was observed.

(Fig. 3D). In accordance with the HepG2 cells, IL-1 $\beta$ , and IL-10 expression levels in cultured HuH7 cells were also too low for detection by RTq-PCR.

To investigate whether CML accumulation in steatotic livers *in vivo* is associated with the induction of hepatic inflammation, gene expression levels of RAGE, PAI-1, IL-8, IL-6, IL-1 $\beta$ , IL-10, and CRP were determined in liver biopsies of severely obese individuals, and the association between these expression levels and mean IHC CML score of the liver biopsies was analyzed. Hepatic CRP expression levels were significantly increased in obese subjects with IHC CML score  $> 1.5$  compared with obese subjects with IHC CML score  $\leq 1.5$  ( $8007 \pm 4093$  vs.  $5989 \pm 2504$  normalized gene expression,  $p = 0.045$ ). Gene expression levels of PAI-1 and IL-8 were also significantly increased in obese subjects with IHC CML score  $> 1.5$  compared with obese subjects with IHC CML score  $\leq 1.5$  (PAI-1:  $1352 \pm 1136$  vs.  $736 \pm 553$  normalized gene expression,  $p = 0.019$ ; IL-8:  $728 \pm 1335$  vs.  $183 \pm 197$  normalized gene expression,  $p = 0.049$ ). RAGE, IL-6, IL-1 $\beta$ , and IL-10 gene expression levels were below the detection limit. Taken together, this demonstrates that higher CML staining in steatotic liver biopsies is associated with increased gene expression levels of inflammatory markers. Therefore, CML accumulation in steatotic liver may play an important role in the induction of hepatic inflammation.

*Effect of pyridoxamine and aminoguanidine on CML formation and induction of pro-inflammatory cytokines*

To demonstrate that increased endogenous CML formation during lipid accumulation is directly involved in the induction of inflammatory cytokines, cells were incubated with or without fatty acids, in the presence or absence of pyridoxamine or aminoguanidine. Pyridoxamine incubation significantly inhibited the increased endogenous CML formation in HepG2 and HuH7 cells (Fig. 3E), without affecting intracellular lipid accumulation (data not shown). In addition to pyridoxamine, HepG2, and HuH7 cells were also incubated with another inhibitor of the Maillard reac-



**Fig. 3. Intracellular lipid accumulation is associated with increased endogenous CML formation and increased hepatic inflammation.** (A) Incubation of hepatocytes with a mixture of oleic/linoleic acids (30 mM) during 24 h resulted in a significant intracellular lipid accumulation as detected by Oil Red O staining. (B) This lipid accumulation was associated with a 21% and 42% increase of endogenous CML levels in HepG2 and HuH7 cells, respectively, compared with hepatocytes not incubated with fatty acid mixture (control). (C and D) Intracellular lipid accumulation-induced CML formation was associated with increased gene expression of inflammatory markers RAGE, PAI-1, IL-8, IL-6, and CRP (the latter two only for HuH7 cells) in HepG2 and HuH7 cells. (E) Pyridoxamine (1 mM) normalizes the fatty acids-induced increase of CML formation in HepG2 and HuH7 cells, respectively. (F and G) The CML-induced upregulation of RAGE, PAI-1, IL-8, IL-6, and CRP gene expression was strongly inhibited by pyridoxamine in HepG2 and HuH7 cells. #p-value <0.05, \*p-value <0.01 vs. control (FA mixture 0 mM); §p-value <0.05, \*\*p-value <0.01 vs. FA mixture 30 mM, pyridoxamine 0 μM.

tion, i.e. aminoguanidine. Aminoguanidine decreased CML formation in a similar trend as pyridoxamine (data not shown).

The increased RAGE, PAI-1, IL-8, and CRP (the latter only in HuH7 cells) gene expression after incubation with fatty acids was strongly inhibited by pyridoxamine in HepG2 and HuH7 cells (Fig. 3G and H). Expression levels of IL-6 were not significantly inhibited by pyridoxamine in HuH7 cells, although the same trend for inhibition was detectable. Similar results were observed after incubation with aminoguanidine (data not shown).

Taken together, these findings demonstrate that development of steatosis in hepatocytes is associated with an increased endogenous formation of CML, which is directly involved in the upregulation of pro-inflammatory cytokines in steatotic hepatocytes.

*Effect of CML incubation on pro-inflammatory gene expression profile of cultured hepatocytes*

To study whether CML released from steatotic hepatocytes influenced gene expression in hepatocytes in a paracrine manner, HepG2 cells were incubated with control-albumin, minimally- and highly-modified CML-albumin. Incubation of HepG2 cells with minimally-modified CML-albumin for 24 h significantly increased the gene expression of RAGE, PAI-1 and IL-8 by 1.9-, 2.8-, and 1.1-fold (N.S.  $p = 0.292$ ), respectively. In addition, incubation of HepG2 cells with highly-modified CML-albumin for 24 h further increased the gene expression of RAGE, PAI-1, and IL-8 by 2.8-, 3.8-, and 1.5-fold, respectively (Fig. 4A–C). Incubation of HepG2 cells with CML-modified albumin during 2, 4,

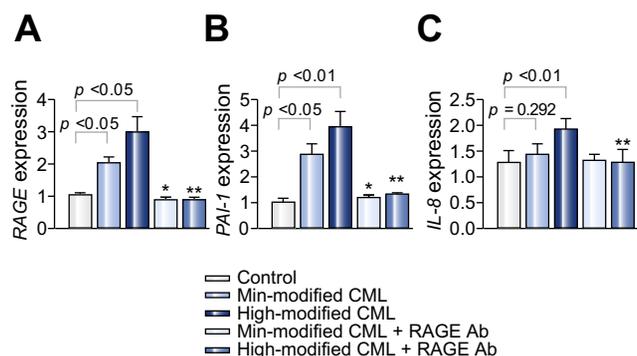
8 h, and longer incubation times (48 and 72 h) had no effect on gene expression of RAGE, PAI-1, and IL-8 (data not shown).

To investigate whether the effects of CML on the gene expression of pro-inflammatory cytokines are mediated via the receptor RAGE, HepG2 cells were pre-incubated with RAGE antibody to block membranous RAGE. Upon blocking of membranous RAGE, incubation of HepG2 cells with CML-albumin had no effect on RAGE, PAI-1, and IL-8 gene expression (Fig. 4A–C).

**Discussion**

In the present study, we demonstrated that hepatic steatosis is associated with CML accumulation in the liver. Analysis of CML fluxes across the liver showed no evidence of hepatic extraction of CML from the circulation. Using an *in vitro* model of steatosis, we demonstrated that intracellular lipid accumulation was associated with increased CML levels, most probably resulting from increased endogenous formation via lipid peroxidation. In addition, we demonstrated in humans and in cultured hepatocytes that CML accumulation in steatosis is associated with increased gene expression of pro-inflammatory cytokines. This study therefore showed that CML accumulation in steatotic livers may play a role in the induction of hepatic inflammation.

Levels of lipid peroxidation products, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), are increased in patients with NAFLD and correlate with the grade of steatosis [6–8]. Weight loss by bariatric surgery significantly reduced lipid



**Fig. 4. Effect of CML incubation on pro-inflammatory cytokine expression.** Incubation of HepG2 cells with control-albumin, minimally- and highly-modified CML-albumin (0.5  $\mu$ M) during 24 h increased the RAGE (A), PAI-1 (B), and IL-8 (C) gene expression in a CML concentration-dependent manner. Pre-incubation with RAGE antibody 5  $\mu$ g/ml, (1 h pre-incubation) inhibited the CML-increased expression of RAGE, PAI-1, and IL-8. \**p*-value <0.05 vs. min-modified CML-albumin, \*\**p*-value <0.05 vs. high-modified CML-albumin.

peroxidation markers [24]. In addition to MDA and HNE, CML is also an important lipid peroxidation product. Some studies showed that in combination with oxidative stress, peroxidation of lipids may lead to the formation of CML [9,10]. Our study indeed demonstrated a strong CML accumulation in fatty liver biopsies, and moreover, CML staining was associated with the grade of steatosis and with the grade of hepatic inflammation. Although our *in vitro* experiments demonstrated that intracellular lipid accumulation leads to increased CML levels, we cannot exclude that other pathways, such as the myeloperoxidase (MPO) pathway, contribute to the CML accumulation in steatotic livers of obese subjects. Recently, it has been demonstrated that MPO expression and activity is increased in liver tissue of obese individuals with NAFLD, and MPO is identified as an important source of CML [19,25].

Our experiments demonstrated a significant inhibition of CML formation by pyridoxamine and aminoguanidine. Pyridoxamine and aminoguanidine are inhibitors of post-Amadori Maillard reactions, most probably due to the trapping of dicarbonyl intermediates [26,27]. Therefore, lipid peroxidation-derived dicarbonyl intermediates, such as glyoxal, seem to be important precursors for the formation of CML in the fatty liver. Although a study has demonstrated that pyridoxamine also influences dyslipidaemia [28], we did not observe an effect of pyridoxamine on the development of a steatotic morphology and lipid accumulation in hepatocytes. Thus, inhibition of CML formation by pyridoxamine is not attributable to a reduction in lipids. Therefore, we conclude that the CML accumulation in steatotic livers of obese individuals and the increased CML levels during intracellular lipid accumulation in cultured hepatocytes result from increased lipid peroxidation.

Studies have suggested that the liver is an important organ for clearance of AGEs, and that clearance and metabolism in the liver may be another determinant for the accumulation of AGEs in the liver [12,29,30]. However, we and others demonstrated that there is no evidence for CML uptake in the liver [31]. Therefore, we conclude that the increased CML accumulation seen in livers from obese individuals results from increased endogenous CML formation, and not from uptake of CML by the liver. In agreement, we and others also found no differences in CML plasma levels

between subjects with different grades of hepatic steatosis [32,33]. However, elevated CML plasma levels were observed in patients with liver cirrhosis compared to controls [30,33,34]. Because CML levels across the liver were also unchanged in patients with liver cirrhosis [31], increased formation of CML favored by increased oxidative stress or impaired kidney function are more plausible explanations for increased CML plasma levels in patients with liver cirrhosis.

Increased inflammation in the fatty liver is a hallmark of the progression of NAFLD [1,2,5]. We demonstrated that CML accumulation in the liver of obese individuals is associated with the grade of hepatic inflammation. Moreover, expression levels of inflammatory markers in liver biopsies were significantly associated with CML staining of the liver biopsies, indicating that increased CML in steatotic livers may directly influence hepatic inflammation. Increased endogenous CML accumulation in hepatocytes was associated with increased RAGE, PAI-1, IL-8, IL-6, and CRP gene expression. Pyridoxamine and aminoguanidine reduced the CML-induced increase of RAGE, PAI-1, IL-8, IL-6, and CRP expression, indicating that endogenous CML is responsible for the upregulation of pro-inflammatory cytokines. In addition, exogenous CML, which may be secreted by steatotic hepatocytes, may also activate nearby hepatocytes and increase the expression of RAGE, PAI-1, and IL-8. Studies have demonstrated that CML-proteins are important ligands for RAGE, and thereby can activate multiple signaling pathways, such as NF- $\kappa$ B, and modulate the expression of several genes [9,11]. We showed that pre-incubation with a RAGE antibody inhibited the CML-induced gene expression of RAGE and PAI-1, indicating that CML released from steatotic hepatocytes could induce inflammation in nearby hepatocytes via a RAGE-dependent pathway. This finding that CML-RAGE plays a role in the induction of hepatic inflammation is confirmed by staining of human liver biopsies for RAGE, which demonstrates that RAGE levels are increased at the membrane of steatotic hepatocytes.

There are several limitations of this study that need to be mentioned. First, we studied severely obese individuals that had a BMI ranging from 35 to 73 kg/m<sup>2</sup>. Therefore, caution is needed in the extrapolation of our findings to other populations, i.e. less severely obese individuals. Second, this study was conducted with Caucasian individuals, so that results cannot be simply extrapolated to populations with different ethnic backgrounds. Third, in this study we did not measure soluble forms of RAGE (including endogenous secretory RAGE, esRAGE), which have been shown to be significantly reduced in liver steatosis and in NASH [35]. As sRAGE and esRAGE may act as decoys and have potential protective effects by scavenging AGEs and preventing AGEs to interact with their receptor RAGE, measuring sRAGE and esRAGE levels may further clarify our findings. Furthermore, other lipid peroxidation products and other ligands for RAGE, such as high-mobility group box-1 (HMGB1), are not assessed in this study.

The proposed mechanism contributing to the pathogenesis of NAFLD fits the two-hit hypothesis [5]. According to this hypothesis, the accumulation of excessive fat in the liver represents the first hit, which increases the vulnerability of the liver to secondary hits that in turn lead to inflammation, injury, and hepatocyte death. Two major pathways of secondary hits are considered to play a role in the pathogenesis of NAFLD, i.e. oxidative stress-induced lipid peroxidation and cytokine-mediated injury. In the present study, we obtained evidence that CML could be one of

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the secondary hits in NAFLD, because (1) intracellular lipid accumulation in hepatocytes leads to increased endogenous formation of CML, probably due to increased lipid peroxidation, and (2) CML accumulation in the liver is associated with increased expression of RAGE and pro-inflammatory cytokines PAI-1 and IL-6, IL-8, and CRP contributing to hepatic inflammation. In conclusion, these results provide new insights into the role of glycation/lipoxidation products in NAFLD and its progression.

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### Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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