C-reactive protein levels in relation to various features of non-alcoholic fatty liver disease among obese patients

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List of abbreviations: NAFLD (Non-alcoholic fatty liver disease); hs-CRP (high sensitivity C-reactive protein); BMI (body mass index); NASH (non-alcoholic steatohepatitis); NAS-score (NAFLD-activity score);

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is a major hepatic consequence of obesity. It has been suggested that high sensitivity C-reactive protein (hs-CRP) is an obesity-independent surrogate marker of severity of NAFLD, especially development of non-alcoholic steato-hepatitis (NASH), but this remains controversial. We aimed to investigate if associations between various features of NAFLD and hs-CRP are independent of body mass index (BMI) in its broad range among obese patients.

Methods: A total of 627 obese adults (80% females) representing three cohorts from France and Belgium, had information on liver histology obtained from liver biopsies and measures of hs-CRP and BMI. We investigated if the different features of NAFLD and BMI, respectively, were associated with hs-CRP, with and without mutual adjustments using linear regression.

Results: BMI and hs-CRP were strongly associated. Per every 10% increase in BMI the hs-CRP-level increased by 19-20% ($P<0.001$), and adjusting for NAFLD-stage (including no-NAFLD) did not influence the association. We found no BMI-independent association between NASH and hs-CRP. However, a positive association between degree of steatosis and hs-CRP was observed ($p<0.05$) and this effect remained significant after adjusting for BMI, lobular inflammation, hepatocyte ballooning, and fibrosis. We found no significant associations between the other features of NAFLD and hs-CRP.

Conclusions: This study indicates that it is the accumulation of fat - both in the adipose tissue and as liver steatosis - that leads to increased hs-CRP levels among obese patients. Thus, hs-CRP may be a marker of steatosis, but not severity of NAFLD, in obese patients.

Key words: Non-alcoholic fatty liver disease, C-reactive protein, Body mass index

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease in the Western world and covers a spectrum of liver disease from steatosis to non-alcoholic steatohepatitis (NASH) with various degrees of fibrosis, that can eventually develop into cirrhosis [1] . Due to the existing correlation between obesity and NAFLD, the prevalence of NAFLD is expected to increase along with the evolvement of the obesity epidemic [2].

In the literature an association between body mass index (BMI) and high sensitivity C-reactive protein (hs-CRP) is well described [3-7]. hs-CRP predominantly originates from the liver, but also seems to be produced in the adipose tissue [8]. hs-CRP has been reported to play an important - although perhaps not a causal [9] - role in the development of atherosclerosis, resulting in a high risk of cardiovascular diseases [10, 11]. Further, studies have reported that hs-CRP level is significantly correlated with liver histology in NAFLD patients, which suggests that NAFLD is associated with low-grade inflammation in the liver [12-18]. It has been suggested that hs-CRP is an obesity-independent marker of NAFLD and also of the severity of NAFLD, since several studies have found that the association persists when BMI has been adjusted for [12-16]. However, in other studies the association disappeared when BMI was accounted for [17, 18]. Thus, it remains uncertain whether hs-CRP is a clinically relevant obesity-independent marker of NAFLD [19].

The aims of the present study were to investigate if there is an association between the different features of NAFLD and hs-CRP in obese patients, and to explore if an association is influenced by BMI within a broad range among obese patients.

Patients and Methods

Three patient cohorts were included in the study. Two of the cohorts were French; one was sampled in Paris in northern France [20], and the other in Nice in the south-eastern part of France. Both cohorts enrolled bariatric surgery patients. The third cohort was sampled in Antwerp in Belgium, and enrolled patients presenting at the obesity clinic for a problem of overweight [21]. In all three cohorts patients were excluded if they reported to consume more than 20 g of alcohol per day. Further, patients were excluded if they had any autoimmune, inflammatory or infectious disease (including viral hepatitis) or cancers. Patients from Paris were moreover excluded if they had any disease of the kidneys, whereas patients from Nice were further excluded due to severe pulmonary or cardiac diseases. Patients from Antwerp were also excluded if they had pre-existing diabetes. In Paris and Nice 33% and 17%, respectively, of the patients had diabetes, whereas 13% of the patients from Antwerp had newly diagnosed diabetes, i.e. their diabetes was discovered when they underwent the clinical examination at the obesity clinic.

Data on NAFLD stage, BMI, hs-CRP, and smoking habits were collected on a total of 526 women and 130 men. The study protocols conform to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institutions human research committee. All participants signed an informed consent before entering the study and allowing for a liver biopsy to be performed. In the two French cohorts the liver biopsy was performed during the bariatric surgery. The Belgian patients underwent a liver-specific program combined to a classical metabolic work-up. In patients who subsequently were referred to bariatric surgery the liver biopsy was performed preoperatively. The remaining patients were proposed for transparietal liver biopsy or transjugular liver vein catheterization and biopsy. In all three cohorts blood

sampling from the bariatric-surgery patients was performed from fasted patients in the morning preoperatively. For the non-operated Belgian patients, blood was sampled in the morning after an overnight 12-hour fast. The cohort from Paris measured hs-CRP using an image automatic immunoassay system (Beckman-Coulter, Fullerton, CA). In the cohort from Nice hs-CRP was measured using nephelometry, and in the Belgian cohort hs-CRP was assayed with nephelometry on BNII (Siemens, Marburg, Germany).

hs-CRP and covariates

hs-CRP (in mg/dL) was used as a continuous variable. As the distribution was highly skewed to the right, it was log-transformed in order to approximate a normal distribution. Body mass index (weight [kg]/ height $[m]^2$) was calculated for each patient on the basis of measured weight and height at the time of blood sampling. BMI was also log-transformed (to base 1.1 for easier interpretation) in the analysis to obtain a better model fit in regard to the linear association with hs-CRP. Data on waist circumference was available from the cohorts from Nice and Antwerp, and were included as a continuous variable.

NAFLD-stages

Slides of liver biopsy were coded and analyzed by a single expert pathologist in Paris, by two expert pathologists in Nice, and by a single expert pathologist in Antwerp. The participating study centers provided information on degree of steatosis, lobular inflammation, and hepatocyte ballooning that were scored according to the categories established by Kleiner and Brunt *et al* [1]. Degree of steatosis, defined as the percentage of hepatocytes with fat droplets, was scored using the following scale: 0 (<5%), 1 (5-33%), 2 (>33-66%), 3 (>66%). Foci of lobular inflammation were defined as two or more inflammatory foci (averaged from 3-4 fields) and scored as: 0 (no foci), 1

(<2 foci), 2 (2-4 foci), 3 (>4 foci). Hepatocyte ballooning was scored according to number of ballooning cells: 0 (none), 1 (few), 2 (many). NAFLD activity score (NAS), which is the unweighted sum of scores of steatosis, lobular inflammation and hepatocellular ballooning, was used to grade activity [1]. Presence of NASH was defined as NAS≥5. Degree of fibrosis was evaluated separately using the Kleiner and Brunt score [1]. Fibrosis was staged as: 0 (none), 1 (zone 3 perisinusoidal or portal fibrosis), 2 (zone 3 perisinusoidal and periportal fibrosis without bridging), 3 (bridging fibrosis), 4 (cirrhosis).

Analytical strategy

Two different analytical approaches were undertaken. *First*, we grouped the patients according to their most severe stage of liver affection according to NAS score [1]. The presence of fibrosis is generally thought to be a result of earlier disease [1], and degree of fibrosis was thus not considered in the first set of analysis. Hence, stage of liver affection was grouped as follows: i) No-NAFLD (i.e. NAS=0); ii) simple steatosis/indefinite NASH (NAS1-4); and iii) NASH (NAS≥5). *Second*, we grouped patients according to degree of steatosis, lobular inflammation, hepatocyte ballooning and degree of fibrosis, where the subjects could appear simultaneously in more than one of the liver affection categories. This approach was taken, as the association with hs-CRP may be dependent on one specific feature of liver affection, but not necessarily with severity of NAFLD.

Confounders and effect modifiers

Serum concentrations of hs-CRP are affected by factors such as age and smoking habits [22, 23], therefore, these were addressed as potential confounders. Age was included as a continuous variable, and smoking was dichotomised as smoker vs. non-smoker. The site of recruitment of the patients was also included as a potential

confounder. Lastly, it was investigated if sex was an effect modifier, since women may have higher CRP-levels than men for a given BMI [24].

Statistical analysis

Nine patients had no data on hs-CRP, eight had no data on lobular inflammation, five had no data on smoking, and one had no data on age and these patients were thus excluded. Further, six patients had a BMI below 30 were also excluded since the focus were on obese patients, leaving 627 patients for the analyses. The likelihood ratio test was used to compare steatosis as a categorical variable with four categories against a dichotomised steatosis variable. As there was no difference the simpler dichotomous variable was used in the analyses. Lobular inflammation, hepatocyte ballooning and fibrosis were tested similarly, and a dichotomised variable for these features was also used for the analyses. First, we investigated the effect of hs-CRP on BMI using linear regression. Then, for the first analytical approach described above, linear regressions of hs-CRP on severity of NAFLD were performed, and repeated with adjustment for BMI. For the second analytical approach linear regressions of hs-CRP on the various features of liver affection were performed, in crude and mutually adjusted analyses, and further with adjustment for BMI. All analyses were adjusted for age, smoking habits, sex and site of recruitment of the patients. Two-way interaction analyses in relation to hs-CRP were performed for sex on BMI, steatosis, inflammation, ballooning, and fibrosis, respectively, and also between the separate NAFLD features and BMI. Likelihood ratio tests assessed whether the model with the product term provided a better fit than the model without the product term.

Results

Study characteristics of the three cohorts are presented in Table 1. The age of the patients ranged from 18 to 69 years. The patients covered the BMI-range from 30 through to 70 kg/m^2, with the majority of the patients being severely obese. The distribution of the different features of liver affection is also presented, and it is seen that the majority of patients have some degree of liver affection, with steatosis/indefinte NASH being the most prevalent condition.

We found no effect modification by sex on the association between BMI and hs-CRP (p=0.12). Neither were there any modification by sex on the associations between the separate NAFLD-stages and hs-CRP. Hence, the subsequent analyses were performed for men and women combined, but with sex maintained in the statistical analyses.

As seen in Figure 1, a clear positive association between BMI and hs-CRP was observed. For every 10% increase in BMI, hs-CRP increased by 20% (β_{crude}: 1.20; 95% CI: 1.13-1.29; P<0.001) in patients with steatosis score 0 and 1, and similarly by 19 % (β_{crude}: 1.19; 95% CI: 1.12-1.26; P<0.001) in patients with steatosis score 2 and 3. The level (intercept) of the two regression lines, representing the two levels of steatosis scores, shown in the figure, was significantly different (P<0.001). The association persisted when lobular inflammation, hepatocyte ballooning and degree of fibrosis were taken into account ($\beta_{adjusted}$: 1.20; 95% CI: 1.12-1.28; P<0.001 (score 0 and 1) and $\beta_{adjusted}$: 1.19; 95% CI: 1.13-1.26; P<0.001 (score 2 and 3)).

We found that waist circumference was not a predictor of hs-CRP when BMI was taken into account in the two cohorts with this information; hence waist circumference was omitted from the remaining analyses.

The circulating levels of hs-CRP by different features of NAFLD are presented as geometric means in Table 2. The mean values seem to increase with increasing scores of steatosis, decrease with increasing scores for lobular inflammation, while there was no pattern in relation to hepatocyte ballooning, total NAS, or stages of fibrosis.

In Table 3 the results of the investigation of the association between the severity of NAFLD and hs-CRP are presented. Here, we observed a positive significant association between NASH and hs-CRP relative to patients with no-NAFLD. However, the association was suppressed when BMI was taken into account.

The results from the linear regression of hs-CRP on degree of steatosis (dichotomised), lobular inflammation (dichotomised), hepatocyte ballooning (dichotomised) and degree of fibrosis (dichotomised) are presented in Table 4. Steatosis score 2 and 3 was positively associated with hs-CRP (β_{crude}: 1.33; 95% CI: 1.16-1.52; P<0.001) relative to score 0 and 1 (reference). Adjustment for BMI reduced the association only slightly, but it remained significant corresponding to the difference in level between the two regression lines in Figure 1. Further adjustment for inflammation, ballooning and fibrosis did not change the association notably. No significant associations between inflammation or fibrosis, respectively, and hs-CRP were observed. A significant association between hepatocyte ballooning and hs-CRP was observed in the crude analyses, but BMI and the other features of NAFLD accounted for the association. Virtually the same results were seen when the

dichotomisation was made as a yes/no variable for steatosis (i.e. score 0 versus 1/2/3), lobular inflammation (i.e. score 0 versus 1/2/3) or fibrosis (i.e. stage 0 versus 1/2/3/4).

Discussion

Results from the present study show a strong positive association between BMI and hs-CRP levels throughout the broad range of obesity. We found no association between NASH and hs-CRP when the patients' BMI was accounted for. However, we found that liver steatosis leads to increased hs-CRP levels, independent of BMI, lobular inflammation, hepatocyte ballooning and fibrosis. It thus seems that it is the accumulation of fat - either in the adipose tissue or as liver steatosis - that leads to increased hs-CRP levels in obese patients. Thus, hs-CRP may be a marker of steatosis, but not of the more severe NASH, in obese patients.

Among the strengths of this study is that all the patients had a liver biopsy taken. Furthermore, by pooling data from three cohorts we obtain a sample size of more than 600 individuals, which is large for a clinical study. The age-ranges in the different cohorts were fairly similar, whereas the BMI of the patients covers the entire range of obesity from 30 through to 70 kg/m^2. This variation in BMI across the entire sample is another strength as it makes comparisons of BMI throughout a broader range possible. However, it should be emphasized that our conclusions apply specifically to the broad range of BMI among obese patients. Further, the study benefits from a presumed considerable diversity in life style, including dietary differences, possibly influencing the NAFLD disease processes. It is a limitation that we do not have a biopsy from the adipose tissue; thus, we cannot take inflammation of the adipose tissue into account in the analysis of hs-CRP on BMI.

Current methods use invasive technologies such as liver biopsy in order to differentiate simple steatosis from NASH [25]. This distinction is of great importance because NASH

is considered to be a disease that can evolve to cirrhosis, whereas steatosis normally does not progress and is considered benign [25]. A specific biomarker that can distinguish between steatosis and NASH would be of great clinical usefulness. Since it is simple, inexpensive and readily available, hs-CRP has been suggested as a surrogate marker of severity of NAFLD, but with conflicting results [12-18]. Our results imply that in obese patients hs-CRP may be a marker of steatosis, but not an obesity-independent marker of the more severe stage of NASH. Large-scale studies in the fields of proteomics and metabolomics are performed in serum of patients and may - in the future - identify novel biomarkers for clinical application [25].

The inflammatory response from hs-CRP that emerges in obesity is, however, not only generated in the liver, but also in the adipose tissue. We found that BMI is a strong predictor of increased hs-CRP-levels in obese patients. This may be due to the inflammatory cytokines produced in the adipose tissue. It is well accepted that the adipose tissue can induce chronic low-grade inflammation by producing proinflammatory cytokines like interleukin-6 [26, 27]. The expression of hs-CRP has been detected in human adipose tissue [28] also in severe obesity [8]. This suggests that the adipose tissue is also a source of hs-CRP production [29]. The hs-CRP expression is relatively larger in the liver than in the adipose tissue. However, the absolute amount of hs-CRP produced in the adipose tissue may be considerable - depending on the absolute amount of adipose tissue of the patient.

In conclusion, the present study of more than 600 European obese patients with simultaneously obtained BMI measures, liver biopsies and hs-CRP measurement confirms an association between BMI and hs-CRP. An association between steatosis and hs-CRP was also observed – independent of lobular inflammation, hepatocyte ballooning, degree of fibrosis or BMI. There was no association between the other

features of NAFLD and hs-CRP. All together this study suggests that it is the accumulation of fat, both in the adipose tissue and in the liver that leads to increased hs-CRP levels among obese patients. hs-CRP may be a marker of steatosis in obese patients, but apparently not a marker of the severity of NAFLD.

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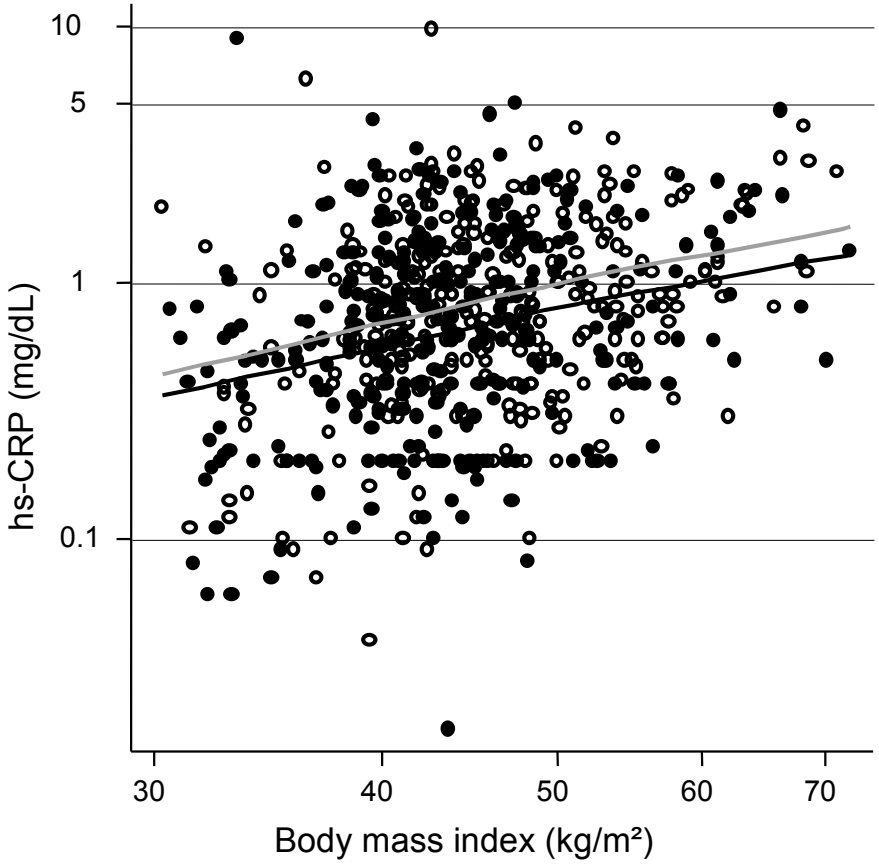
Figure Legends

Figure 1. The association between BMI and hs-CRP among 627 obese patients



The black dots illustrate patients with steatosis stage 0 or 1, whereas the white dots illustrate patients with steatosis stage 2 or 3. The slope of the lines illustrates the association between BMI and hs-CRP. Per every 10% increase in BMI the hs-CRP-level increases by 20% (P<0.001) in patients with steatosis stage 0 or 1 (black line), and by 19% (P<0.001) in patients with steatosis stage 3 or 4 (grey line). There was no significant difference in the slope of the two lines (p=0.67), but the level (intercept) of the lines was significantly different (P<0.001).

Table 1. Characteristics of the three cohorts given as numbers and median (range) and numbers (%)

Cohort and study	Gender	N	Age (yrs)	BMI (Kg/m^2)	Waist circumference (cm)	No-NAFLD	Steatosis/ indefinite NASH	NASH
The POLI cohort	Males	56	46.0 (22.0-63.0)	49.7 (36.0-70.2)	Na	5 (9%)	36 (64%)	15 (27%)
(FR)	Females	234	42.0 (19.0-68.0)	46.5 (32.6-71.1)	Na	54 (23%)	147 (63%)	33 (14%)
	Combined	290	43.0 (19.0-68.0)	47.2 (32.6-71.1)	Na	59 (20%)	183 (63%)	48 (17%)
Nice Obese subjects	Males	33	41.0 (18.0-59.0)	43.4 (38.7-61.1)	132 (115-161)	0 (-)	27 (82%)	6 (18%)
(FR)	Females	190	40.0 (20.0-66.0)	43.0 (35.9-72.3)	114 (93-172)	11 (6%)	162 (85%)	17 (9%)
	Combined	223	40.0 (18.0-66.0)	43.2 (35.9-72.3)	117 (93-172)	11 (5%)	189 (85%)	23 (10%)
The OBBO cohort	Males	36	50.5 (19.0-68.0)	36.9 (31.4-49.9)	121 (104-137)	2 (6%)	22 (61%)	12 (33%)
(BE)	Females	78	43.5 (19.0-69.0)	38.5 (30.3-57.8)	114 (91-160)	5 (6%)	55 (71%)	18 (23%)
	Combined	114	46.0 (19.0-69.0)	37.7 (30.3-57.8)	116 (91-160)	7 (6%)	77 (68%)	30 (26%)

Abbreviations: Na: not analysed
The different NAFLD stages were classified as follows: No-NAFLD: NAS=0; Simple steatosis/indefinite NASH (NAS1-4); NASH (NAS>=5)

Table 2. hs-CRP (Geometric mean with 95% CI) according to the different features of NAFLD in the 627 obese patients

NAFLD features	Score	N	Mean*	95% CI
Steatosis	0	87	0.64	0.52-0.77
	1	216	0.66	0.58-0.74
	2	177	0.76	0.67-0.87
	3	147	0.81	0.71-0.92
Lobular inflammation	0	405	0.76	0.69-0.82
	1	189	0.67	0.59-0.75
	2	29	0.58	0.41-0.81
	3	4	0.42	0.07-2.69
Hepatocyte ballooning	0	420	0.73	0.67-0.79
	1	159	0.73	0.64-0.84
	2	48	0.58	0.43-0.80
Total NAS score	0	77	0.66	0.54-0.81
	1-2	275	0.73	0.65-0.81
	3-4	174	0.74	0.65-0.85
	>=5	101	0.69	0.58-0.82
Fibrosis	0	173	0.61	0.53-0.70
	1	335	0.79	0.72-0.86
	2	99	0.75	0.64-0.87
	3	26	0.53	0.34-0.83
	4	2	0.45	0.00-324.71

*Due to the highly rightwardly skewness of hs-CRP it is presented as the geometric mean (which is the antilog of the arithmetic mean of the logged data) with 95% confidence intervals (CIs).

Table 3. Linear regression of hs-CRP (mg/dL) on severity of NAFLD-stage in the 627 obese patients

Model	N	Estimate†	95% CI
Crude			
No-NAFLD	77	ref	
Simple steatosis/indefinite NASH	449	1.19	0.96-1.47
NASH	101	1.31*	1.01-1.70
Adjusted for BMI			
No-NAFLD	77	ref	
Simple steatosis/indefinite NASH	449	1.13	0.92-1.38
NASH	101	1.20	0.93-1.53

No-NAFLD: NAS=0; Simple steatosis/indefinite NASH (NAS1-4); NASH (NAS>=5)
All models were adjusted for sex, smoking, age and site of recruitment of the patients.
*p<0.05
†Factor of the percentage change in CRP (e.g. an estimate of 1.25 is to be interpreted as a 25% (2-53%) higher CRP-level relative to the reference group)

Table 4. Linear regression of hs-CRP (mg/dL) on steatosis, NASH, or fibrosis in the 627 obese patients‡

Model	Estimate†	95% CI
Steatosis (stage 0/1 (ref) vs. 2/3)		
Crude	1.33*	1.16-1.52
Adjusted for BMI	1.26*	1.11-1.44
Adjusted for inflammation and ballooning	1.31*	1.13-1.53
Adjusted for fibrosis	1.33*	1.15-1.53
Adjusted for inflammation, ballooning, fibrosis and BMI	1.28*	1.11-1.48
Lobular inflammation (0/1 (ref) vs. 2/3)		
Crude	0.99	0.73-1.34
Adjusted for BMI	0.92	0.69-1.22
Adjusted for steatosis and ballooning	0.89	0.65-1.21
Adjusted for fibrosis	0.95	0.69-1.29
Adjusted for steatosis, ballooning, fibrosis and BMI	0.87	0.64-1.17
Hepatocyte ballooning (0 (ref) vs. 1/2)		
Crude	1.17*	1.01-1.36
Adjusted for BMI	1.10	0.96-1.27
Adjusted for steatosis and inflammation	1.05	0.89-1.23
Adjusted for fibrosis	1.16	1.00-1.35
Adjusted for steatosis, inflammation, fibrosis and BMI	1.01	0.86-1.19
Fibrosis (stage 0/1 (ref) vs. 2/3/4)		
Crude	1.10	0.92-1.31
Adjusted for BMI	1.02	0.86-1.20
Adjusted for steatosis, inflammation and ballooning	1.00	0.83-1.20
Adjusted for steatosis, inflammation, ballooning and BMI	0.95	0.80-1.13

All models were adjusted for sex, smoking, age and site of recruitment of the patients.
*$p<0.05$
†Factor of the percentage change in CRP (e.g. an estimate of 1.32 is to be interpreted as a 32% (16-51%) higher CRP-level relative to the reference group)

‡When steatosis was dichotomized as no steatosis (score 0) versus steatosis (scores 1 through 3), lobular inflammation was dichotomized as no inflammation (score 0) versus inflammation (scores 1 through 3), and fibrosis was dichotomized as no fibrosis (stage 0) versus fibrosis (stages 1 through 4) virtually the same results were obtained.