Collagen proportionate area is an independent predictor of long-term outcome in patients with non-alcoholic fatty liver disease

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Keywords: steatohepatitis, prognosis, fibrosis, decompensation, Fibroscan.

Electronic word count: 3,467 words

Number of figures and tables: 6

Conflict of interest statement: None to disclose

Financial support statement: Dr Buzzetti was supported by a fellowship from AIGO-M.I.M.I. (Associazione Italiana Gastroenterologi Ospedalieri - Montecatini Interactive

Medicine) International). Dr Tsochatzis was a recipient of the European Association for the Study of the Liver (EASL) Physician Scientist Award for this project.

Author contributions: EB study design, data acquisition, analysis and interpretation of data, drafting of the article. AH study design, technical support for CPA measurements, drafting of the article (methods part-CPA analysis); ME, SK and EKM selection of patients and data acquisition, critical revision for important intellectual content; TVL central pathology reading; RM, MGM, CC, technical support, critical revision for important intellectual content; GL statistical analysis, critical revision for important intellectual content; MP, APD study design, critical revision for important intellectual content. EAT study concept and design, analysis and interpretation of data, critical revision for important intellectual content. All authors critically revised the manuscript and gave their final approval of the version to be submitted.

Abstract

Background: Collagen proportionate area (CPA) measurement is a technique that quantifies fibrous tissue in liver biopsies by measuring the amount of collagen deposition as a proportion of the total biopsy area. CPA predicts clinical outcomes in patients with HCV and can sub-classify cirrhosis. **Aims:** We tested the ability of CPA to quantify fibrosis and predict clinical outcomes in patients with NAFLD. **Methods:** We assessed consecutive patients with biopsy-proven NAFLD from three European centers. Clinical and laboratory data were collected at baseline and at the time of the last clinical follow-up or death. CPA was performed at two different objective magnifications, whole biopsy macro and x4 objective magnification, named standard (SM) and high (HM) magnification respectively. The correlation between CPA and liver stiffness was assessed in a sub-group of patients. **Results:** Of 437 patients, 32 (7.3%) decompensated and/or died from liver-related causes during a median follow-up of 103 months. CPA correlated with liver stiffness and liver fibrosis stage across the whole spectrum of fibrosis. HM CPA was significantly higher than SM CPA in stages F0-F3 but similar in cirrhosis, reflecting a higher ability to capture pericellular/perisinusoidal fibrosis at early stages. Age at baseline (HR:1.04, 95%CI=1.01-1.08), HM CPA (HR:1.04 per 1% increase, 95%CI=1.01-1.08) and presence of advanced fibrosis (HR:15.4, 95%CI=5.02-47.84) were independent predictors of liver-related clinical outcomes at standard and competing risk multivariate Cox-regression analysis. **Conclusions**: CPA accurately measures fibrosis and is an independent predictor of clinical outcomes in NAFLD; hence it merits further evaluation as a surrogate endpoint in clinical trials.

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome affecting 30% of the population in industrialized countries [1, 2]. NAFLD is a complex pathological entity that develops from multiple factors acting synergistically in genetically and/or epigenetically predisposed individuals [3, 4].

Despite its high prevalence, only a proportion of subjects with NAFLD develop non-alcoholic steatohepatitis (NASH) with potential progression to fibrosis and cirrhosis [1, 5]. Therefore, it is important to stratify patients according to their risk of progression in order to tailor the need for interventions and dedicated specialist follow-up [6]. The gold standard to differentiate NAFLD from NASH and accurately stage fibrosis is a liver biopsy, despite the development of several non-invasive fibrosis assessment tests [7]. This is because the currently available non-invasive techniques have a satisfactory accuracy for the detection of advanced fibrosis but not for lower fibrosis stages [8].

Our group developed the collagen proportionate area (CPA) measurement as a technique to quantify fibrous tissue in liver biopsies by measuring the amount of collagen deposition as a proportion of the total biopsy area. CPA has been validated against hepatic venous pressure gradient (HVPG) measurement and clinical outcomes mainly in patients with chronic hepatitis C. Along these lines, CPA can sub-classify cirrhosis and predict decompensation independently of the model for end-stage liver disease (MELD) score [9-14]. However, NASH, particularly in the early stages of the disease, is characterised by pericellular and perisinusoidal fibrosis in the centrilobular area, which is a pattern of fibrosis progression different from the periportal localization typical of chronic viral hepatitis. Therefore, it is not yet established whether CPA at the standard magnification employed and validated for chronic viral hepatitis, can measure collagen with sufficient accuracy in the early stages of

NAFLD-related fibrosis. Moreover, there are no studies on the utility of CPA in relation to clinical outcomes in NAFLD.

This study had therefore two aims: firstly, to test the optimal CPA magnification to identify liver fibrosis in NAFLD patients; secondly, to assess the association of CPA with clinical outcomes, namely hepatic decompensation and/or liver-related mortality, in a cohort of patients with biopsy-proven NAFLD and longitudinal follow-up.

Materials and methods

This was a multicenter, retrospective study including patients from three centers in the United Kingdom, Sweden and Greece. All cases with biopsy-proven NAFLD with available clinical and laboratory data who were seen at least once after the baseline liver biopsy during a period of 30 years were selected by a systematic search of the hospital histology registers.

Patients with alcohol overconsumption (defined as alcohol intake >20 g/day in women and >30 g/day in men, as confirmed by patient clinical history), secondary causes of steatosis (such as steatogenic medication or previous gastro-intestinal by-pass) or coexistent liver disease were excluded.

Clinical information including body mass index (BMI), presence of hypertension, diabetes mellitus or impaired fasting glucose, dyslipidemia (diagnosed according to the latest Adult Treatment Panel III criteria) [15], cardiovascular disease and previous cardiovascular events were obtained from clinical documentation recorded at the time of liver biopsy or within a range of 6 months. Routine laboratory parameters were collected.

The development of hepatic decompensation (defined as the development of either ascites, variceal bleeding, hepatic encephalopathy or clinical non-obstructive jaundice) and cardiovascular events during the follow-up period, as well as the survival status of each patient were recorded at the end of the data collection (30/06/2016), through the clinical notes, general practitioner enquiries or the national health system-integrated hospital register.

We further evaluated a separate cohort of consecutive contemporary patients with biopsy-proven NAFLD and liver stiffness measurements by transient elastography (Fibroscan), performed within 6 months of the liver biopsy, in order to test the correlation of CPA with liver stiffness. The study was approved by the ethical review board of each participating institution (REC reference number 07/Q0501/50).

Histological assessment

Liver biopsy samples were formalin-fixed, paraffin-embedded and routinely stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS) stain with diastase digestion (DPAS), orcein, Victoria Blue and Perls' Prussian Blue. Another section of tissue was stained with picro-Sirius red for collagen quantification and determination of CPA by digital image analysis.

Liver biopsies samples were centrally reviewed by an expert histopathologist (TVL, Royal Free Hospital) blinded to the clinical data of the patients. NASH was diagnosed based on a compatible morphological pattern of injury and the combination of steatosis, lobular inflammation and hepatocyte ballooning. The NAS score was calculated according to the NASH CRN classification [16]. Liver fibrosis was staged on a 5-point scale, with 0 for absence of fibrosis, stage 1 for zone 3 perisinusoidal/perivenular fibrosis, stage 2 for zone 3 and periportal fibrosis, stage 3 for septal/ bridging fibrosis and stage 4 for cirrhosis [17]. Significant and advanced fibrosis were defined as Brunt stage ≥F2, and ≥F3 respectively.

CPA analysis

CPA was measured as described previously [12]. For the purpose of this study, CPA was measured at different objective magnifications (whole biopsy macro and x4 and x10 objective magnifications).

In summary, whole biopsy images of liver sections stained with picro-Sirius red were captured with a Canon Powershot A640 digital camera attached to a close-up copy-stand with non-flicker backlighting.

High power (x4 and x10) image capture was performed using a microscope and Zeiss Axiocam ICc5 (see supplementary material for more details). For the high-power magnification, the difference between x4 and x10 magnification was evaluated in order to

choose the best feasible technique (a balance between resolution vs. efficiency). Digital image analysis used a visual basic script for Zeiss Axiovision (version 4.8.2.) in which binary segmentation of RGB colour channels was used to distinguish liver tissue from collagen. An editing step was included and confounding artefacts such as major blood vessels and liver capsule were manually edited from analysis. The collagen proportionate area (CPA) was calculated as the area occupied by the collagen as a proportion of the area of the whole parenchyma and expressed as a percentage.

Initially, we compared x4 and x10 CPA measurement on 10 different biopsy slides: since the calculated intra-class correlation coefficient was high (0.98) we used the x4 magnification CPA for simplicity. The whole biopsy macro and x4 objective magnification are hereafter termed as standard (SM) and high magnification (HM) CPA.

The procedure was performed by one of the authors (E.B.) and inter-observer variability was assessed using a separate training group of 20 slides of patients with cirrhosis, with a different observer (A.H.) unaware of E.B.'s assessment: the concordance was excellent (k=0.912) with median CPA difference between observers of 2%.

Statistical analysis

Normal distribution was tested before statistical analysis. Continuous descriptive data are presented as mean and standard deviation when the assumption of normality was met or otherwise as median and interquartile range (IQR). Categorical variables were analyzed using the chi-square test. Between-group comparisons of continuous variables were performed using the Student's *t* test or Anova tests for normally distributed variables and the Mann-Whitney U or Kruskal-Wallis tests when the assumption of normality was not met. The Wilcoxon test was used to compare SM and HM CPA. The Pearson's correlation test was used to determine correlation between CPA measurement and liver stiffness and the Steiger's Z-test for dependent variables was used to compare correlation coefficients for SM and HM

CPA. Cox-regression (univariate and multivariate) analysis was used to determine predictors of clinical outcomes (composite outcome of hepatic decompensation and/or liver-related mortality). Competing risk Cox regression analysis, with non-liver related deaths considered as a competing risk, was also performed. A backward stepwise procedure was used for Cox models. Receiver-operating characteristics (ROC) curve analysis was used to define optimal SM and HM CPA cut-offs for different stages of fibrosis and for prediction of liver-related outcomes. A two-sided p-value <0.05 was considered significant.

All analyses were performed using SPSS (version 22.0, IBM, New York, NY, USA) or MedCalc for Windows (version 14.8.1, MedCalc Software, Ostend, Belgium) except from the competing risk analyses, which were performed using Stata (version 12.1, Statacorp, College Station, Texas, USA).

Results

Baseline characteristics

Table 1 shows the baseline clinical, biochemical and histological data of the 437 patients with NAFLD included in the study, grouped according to the recruiting centre. The mean age was 51±13 years, mean BMI was 30.2 kg/m^2, 40% of patients were females and 74% were of Caucasian ethnicity. A history of type 2 diabetes and hypertension was prevalent in 38.2% and 53% of patients respectively, while 60.2% were dyslipidemic. Furthermore, 46 (10.5%) patients already had a cardiovascular event or were affected by cardiovascular disease (mostly ischaemic heart disease and arrhythmias) at baseline. Twenty percent of patients were already on statins, either for treatment of dyslipidemia or for secondary prevention of cardiovascular disease.

Histology review showed that the median biopsy length was 19 mm (range 4-58 mm) with 0.5%, 7.2%, 54% and 38% of patients having biopsy lengths of <5 mm, 5-10 mm, 11-20 mm and >20 mm respectively. Absence of fibrosis (F0) was found in 233 (53%), mild fibrosis (F1) in 95 (22%), moderate fibrosis (F2) in 37 (8%), severe fibrosis (F3) in 34 (8%) and cirrhosis (F4) in 38 (9%) patients, respectively. NASH was present in 170 (39%) patients.

Clinical outcomes

In total, 32 patients (7.3%) had at least one episode of hepatic decompensation and/or died of liver-related causes (18 in the British cohort, 2 in the Greek cohort, and 12 in the Swedish cohort). Of these, 8 (25%) had a fibrosis stage of <F3 according to the Brunt system at the time of liver biopsy (2 had F0, 3 had F1, 3 had F2).

Twenty-seven patients (6.2%) had at least one episode of hepatic decompensation after a median of 58 (IQR 81, range 1-250) months from their baseline liver biopsy: one patient became jaundiced, sixteen developed ascites, three had variceal bleeding, three were hospitalised for hepatic encephalopathy and three developed liver failure, of which two underwent liver transplantation. One patient decompensated twice, developing ascites and variceal haemorrhage respectively. Five patients had multiple decompensating events on a single admission and subsequently died.

Seventy-one patients (16.2%) developed a cardiovascular complication, with a total of 117 events, mainly ischaemic events (n=34), arrhythmias (n=24), congestive heart failure (n=19) and stroke (n=18).

Fifty-six patients (12.8 %) died after a median follow-up of 103 (IQR 85, range 1-298) months and the larger proportion of events was represented by liver-related mortality (n=16), cardiovascular events (n=15) and non-HCC malignancies (n=14). Of the 16 patients who died

due to liver-related events, 11 had previously developed at least one episode of clinical hepatic decompensation during the follow-up.

CPA values and fibrosis stages

CPA values, both at SM and HM, significantly increased according to incremental stages of liver fibrosis. HM was significantly higher than SM CPA in patients who had F2 fibrosis or lower (Table 2): the median CPA value for patients with F0-F2 was 4.7% if measured at SM and 6.9% if measured at HM ($p<0.0001$), representing a more accurate measurement of the finer peri-cellular component of liver fibrosis. For advanced fibrosis (≥F3) and cirrhosis, the difference between SM and HM values were less pronounced, with a median CPA value of 10.7% and 11.2% for advanced fibrosis ($p = 0.002$) and of 21.3% and 23% for cirrhosis ($p = 0.06$) at SM and HM respectively. An example of the difference in CPA measurements between SM and HM is shown in Figure 1. We further demonstrated that the difference between SM and HM CPA is due to more accurate measurement of peri-sinusoidal fibrosis using HM (Supplementary material).

We further explored optimal CPA cut-offs for individual fibrosis stages (Table 2, Supplementary material): presence of any degree of fibrosis (≥F1), significant fibrosis (≥F2), advanced fibrosis (≥F3) and cirrhosis were predicted by a HM CPA >6.8%, >8.6%, >10.4% and >13.6% respectively. We also determined the SM CPA cut-offs for fibrosis (Table 2, Supplementary material). Importantly, CPA measurements were not influenced by biopsy length (Supplementary Table 3) or steatosis grade except in the absence of fibrosis (Supplementary Tables 4). The accuracy of assigning a specific histological stage using CPA was lower than binary classification (Supplementary Table 5).

Finally, we explored the correlation of CPA with transient elastography values in a consecutive contemporary cohort of patients with available measurements (n=76): CPA, both

at SM and HM, significantly correlated with liver stiffness ($p<0.001$ for both correlations), with HM CPA having a significantly stronger correlation than SM CPA (r=0.73 vs. 0.68, p=0.03, Figures 2a and 2b). Moreover, CPA correlated well with both FIB-4 (r=0.47, P<0.0001) and NAFLD fibrosis score (r=0.26, P<0.0001) in the whole cohort of 437 patients.

CPA and clinical outcomes

In Cox regression analysis, after correction for recruiting center, age at baseline (HR 1.04, 95% CI 1.01-1.08), HM CPA (HR 1.04, 95% CI 1.002-1.08) and presence of advanced fibrosis (HR 15.4, 95% CI 5.02-47.84) were independently associated with the combined outcome (Table 3). In a competing risk Cox regression analysis, where mortality from non-liver related aetiology was considered a competing risk, HM CPA (HR 1.04, 95% CI 1.001-1.08) and advanced fibrosis (HR 9.55, 95% CI 3.15 - 28.9) but not age were still independently associated with liver-related events (Table 3). When the analysis was repeated using SM CPA instead of HM CPA, age at baseline (HR 1.04, 95% CI 1.01-1.09), SM CPA (HR 1.05, 95% CI 1.02-1.09) and presence of advanced fibrosis (HR 13, 95% CI 4.25-39.5) were the independently associated variables (Table 1, Supplementary material). SM CPA and advanced fibrosis remained independent predictors of liver-related events in the competing risk Cox regression analysis (Table 1, Supplementary material). In the subgroup of patients with advanced fibrosis (n=72), SM CPA (HR 1.05, 95% CI 1.01-1.08, P=0.005) was the only independent predictor of liver related decompensation or death.

Figure 3 shows the AUROCs of HM CPA (0.79), advanced fibrosis (0.81), age at biopsy (0.70) and the model obtained by combining the three parameters. The combined model had an AUROC of 0.87, therefore showed a better accuracy at predicting hepatic decompensation and/or liver-related death than independent parameters.

The associated HM CPA cut-off that best predicted the clinical outcome of interest was 7.6% (sensitivity 87%, specificity 62%), while the SM CPA cut-off was 9.0% (sensitivity 70%, specificity 84%). Of the 8 patients with fibrosis stage lower than F3 who decompensated, five had a HM CPA value higher than 7.6 and all of them had a HM CPA value lower than the CPA cut-off for presence of advanced fibrosis (>10.4%).

Discussion

In this study, we describe for the first time an accurate method for the quantitative assessment of fibrosis in patients with NAFLD using CPA with a higher magnification than that conventionally used in chronic viral hepatitis. We further show that CPA is an independent predictor of hepatic decompensation in such patients and could thus be potentially used as a surrogate efficacy endpoint in clinical trials. Therefore, CPA is a useful additional assessment to the standard histopathological evaluation.

CPA was originally developed in patients with post-transplant HCV recurrence and showed good correlation with both HVPG and clinical outcomes[10-12, 18]. We have previously demonstrated that CPA can sub-classify compensated cirrhosis better than the Laennec system, as it is independently associated with clinical decompensation over and above the MELD score [9]. However, CPA has not been sufficiently tested in patients with non-viral disease aetiologies, particularly in the pre-cirrhotic fibrosis stages. This is particularly relevant in patients with NAFLD and alcohol-related liver disease, as these patients develop pericellular and perisinusoidal fibrosis starting from the centrilobular area with subsequent expansion towards the portal tract [19]. In contrast, development of fibrosis in viral hepatitis is initially centered around the portal tract with subsequent rapid expansion toward the centrilobular vein [19]. Existing studies with CPA measurement in NAFLD did not test higher magnifications and importantly did not validate CPA against clinical outcomes [20-22]. We therefore tested different magnifications for digital analysis and CPA measurement in order to accurately quantify the presence of fine pericellular fibrosis that could be missed at a standard magnification. Our results convincingly show that an objective magnification of x4 is far more accurate in measuring fibrosis in pre-cirrhotic patients, with values up to 100% higher than when using the conventional magnification. Increasing the magnification to x10 did not substantially change the accuracy of the method. In addition, our data demonstrate

that x4 magnification measurements have a better correlation with liver stiffness than the conventional magnification. We therefore propose that the x4 magnification should be the standard procedure of quantifying fibrosis with CPA in NAFLD, particularly in stages F0-F2.

Furthermore, this study demonstrates that CPA, along with advanced fibrosis, is an independent risk factor for liver-related decompensation and death. This result was obtained by investigating a cohort of 437 patients with longitudinal follow-up, the majority of whom (n=319) had not been previously described in cohort studies. Such findings were also confirmed in a competing risk analysis, where non-liver related deaths were counted as competing risks. Moreover, in the subset of patients with advanced fibrosis, CPA was the only independent risk factor associated with liver-related outcomes. Interestingly, the NAS was not associated with such events, in accordance with recent results from European [23] and American [24] cohorts. These observations not only confirm that fibrosis is the effective key driver of liver-related complications in NAFLD, but also highlight the need of making the assessment of fibrosis a central element in the design and interpretation of current clinical trials.

NAFLD is currently labeled as a disease of an unmet clinical need and potential treatments are in an accelerated pathway for FDA approval [25]. Therefore, a pharmaceutical treatment will be licensed based on the effect on surrogate outcomes, while the company will have to provide further post-licensing evidence of efficacy on hard clinical endpoints. Current surrogate endpoints include a combination of improvement or resolution of NASH based on the NAS score with no worsening of fibrosis or improvement of fibrosis with no worsening of NASH [25]. The NAS score is a problematic endpoint, given its high inter and intra-observer variability, its heavy reliance on steatosis, the exclusion of portal inflammation and the poor correlation with clinical outcomes [16, 23, 24]. Although it is indisputable that inflammation (loosely termed as steatohepatitis) is one of the key drivers of the fibrogenic process, it is not

easy to quantify and characterize in its multicellular complexity [26]. Therefore, the extent of fibrotic transformation constitutes the hard end-point of this pathophysiological process. Fibrosis is currently measured semi-quantitatively, using a score with a scale of 0 to 4 that takes into account both architectural changes and fibrosis [27]. Assigned scores are overall simple descriptors and do not have a quantitative relation with each other, as also confirmed in the present study. Therefore, progression or regression through stages might not be observed in the relative short duration of trials, while subtle changes in fibrosis might be missed. These shortcomings might provide false assurances or even "false negative" signals for further development of treatments [28]. In that sense, CPA can provide a refinement of fibrosis assessment and is an ideal candidate to explore as a surrogate outcome in trials in combination with "conventional" histopathological staging. Further research is required to understand what magnitude of CPA changes is clinically relevant.

Surrogate outcomes should be able to predict an intervention's effect on a clinically meaningful outcome [29]. CPA is independently associated with liver-related events and is a purely quantitative measure and therefore can offer supplementary information to the semi-quantitative fibrosis stage. It is also biologically plausible that improvement of CPA following therapeutic intervention will translate in a delay in the appearance of clinical events, although this will require further validation. In terms of accuracy, CPA measurements have a high inter and intra-observer agreement (k consistently greater than 0.9), are inexpensive once the necessary equipment and software is purchased and are not time consuming. We have previously shown that sampling variability does not influence this assessment in cirrhosis, as measurements from the left and right liver lobe of explanted livers show similar amounts of fibrosis [30]. Moreover, relatively accurate measurements can be obtained from biopsy samples as small as 5 mm [31, 32].

Lastly, CPA correlated well with liver stiffness as measured by transient elastography. Quantitative non-invasive fibrosis tests are better validated against a pure quantitative measure of fibrosis rather than the semi-quantitative fibrosis stages[33]. This strategy will allow a more targeted approach to liver fibrosis and will also bypass limitations of the traditional staging, such as sampling and inter-observer variability but also the ceiling of cirrhosis with no further ability of sub-classification.

This is a retrospective study with obvious limitations, including reliance on medical records, absence of a standardized protocol for follow-up and most likely a selection bias for patients at greater risk of progression. Nevertheless, we included consecutive patients across three different countries with different characteristics and a long follow-up and have been able to ascertain the final outcomes.

In conclusion, we described an accurate way of quantifying fibrosis using CPA in patients with NAFLD and have demonstrated that this is independently associated with clinical outcomes. Our findings support the routine measurement of CPA in combination with the standard histopathological evaluation in liver biopsies of patients with NAFLD, as it provides additional information to the traditional semi-quantitative staging. More importantly, they suggest the exploration of HM CPA as a surrogate endpoint in clinical trials of patients with NASH.

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Table 1. Baseline characteristics of the 437 patients with biopsy-proven NAFLD included in the study.

Variable	**UK (286)**	**Sweden (118)**	**Greece (33)**	***p****	**Total (437)**
Age (years)	49 ± 13	57 ± 12	57 ± 13	<0.0001	51 ± 13
Caucasian ethnicity[a], n (%)	171 (60)	118 (100)	33 (100)	<0.00001	322 (74)
Females, n (%)	114 (40)	42 (36)	17 (52)	0.25	173 (39.6)
BMI (Kg/m²)	31.2 ± 5.8	29 ± 4.3	27.9 ± 4	0.003	30.2 ± 5.4
Hypertension, n (%)	104 (36)	103 (87)	25 (76)	<0.0001	232 (53)
Dyslipidemia, n (%)	199 (70)	46 (39)	18 (55)	<0.0001	263(60.2)
Diabetes, n (%)	91 (32)	56 (47.5)	20 (61)	<0.0001	167 (38.2)
CVD, n (%)	21 (7.3)	24 (20)	1 (3)	<0.0001	46 (10.5)
NASH, n (%)	100 (35)	50 (42)	20 (61)	0.355	170 (39)
F0, n (%)	161 (56)	55 (47)	17 (52)	0.08	233 (53)
F1, n (%)	53 (18.5)	36 (30)	6 (18)	0.008	95 (22)
F2, n (%)	21 (7.5)	13 (11)	3 (9)	0.23	37 (8)
F3, n (%)	16 (6)	12 (10)	6 (18)	0.1	34 (8)
F4, n (%)	35 (12)	2 (2)	1 (3)	0.001	38 (9)
Platelets (x 10^9/L)	238 (85)	230 (76)	229 (93)	0.29	235 (84)
ALT (U/L)	62 (56)	60 (49)	49 (69)	0.67	60 (55)
AST (U/L)	41 (29)	36 (18)	38 (29)	0.08	39 (25)
Bilirubin (µmol/L)	11 (7)	11 (5)	13 (8)	0.13	11 (7)
Albumin (g/dL)	4.6 (0.4)	4.1 (0.4)	4.5 (0.5)	<0.0001	4.5 (0.6)
INR					

CH (mmol/L)	1 (0.1)	0.9 (0.1)	1 (0.2)	<0.0001	1 (0.1)
HDL (mmol/L)	5.3 (1.7)	5.6 (1.8)	5.4 (2.1)	0.45	5.4 (1.9)
LDL (mmol/L)	1.2 (0.4)	1.2 (0.3)	1.2 (0.4)	0.35	1.2 (0.4)
TG (mmol/L)	2.9 (1.6)	3 (1.5)	3.3 (1.2)	0.46	3 (1.5)
Glucose (mmol/L)	1.8 (1.5)	1.7 (1.1)	1.3 (1)	0.37	1.7 (1.3)
HbA1c (mmol/mol)	5.4 (2)	6.2 (2.3)	5.8 (1.5)	0.001	5.6 (2.6)
Ferritin (pmol/L)	39.9 (12)	27.9 (16)	39 (21)	< 0.001	37.7 (17)
	196 (289)	143 (152)	75 (181)	0.046	164.5 (234)

Values are reported as number (percentages) for qualitative variables, number ± SD or number (IQR) for quantitative variables.

[a.] The British cohort included other ethnicities as follows: Asian/Asian British (n=43), Black/Caribbean/Black British (n=13), Mixed (n=4) or unknown (n=55). * *p* value refers to the comparison between the Swedish and British cohorts.

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; HD: hepatic decompensation; NASH, non-alcoholic steatohepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; INR, international normalized ratio; CH, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; HbA1c, glycosylated hemoglobin.

Table 2. Differences between SM and HM CPA according to fibrosis stage.

	F0	**F1**	**F2**	**F3**	**F4**
HM CPA	5.4 (3.9)	6.6 (4.5)	8.6 (5.2)	11.2 (9.5)	23 (16)
SM CPA	3.4 (2.6)	4.1 (3.2)	6.2 (5.6)	10.7 (9.3)	21.3 (17)
p	< 0.0001	< 0.0001	0.002	0.003	0.06

Values are reported as median (IQR). Abbreviations: HM, high magnification; CPA, collagen proportionate area; SM, standard magnification.

Table 3. Predictors of hepatic decompensation and/or liver-related mortality at the Cox regression univariate, multivariate and multivariate competing risk analysis.

	Univariate Cox regression analysis		**Multivariate Cox regression analysis**		**Multivariate Cox regression analysis Competing risks: non-liver related deaths**	
Variable	**HR (95% CI)**	***p***	**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
Cohort*				NS		
Swedish	0.77 (0.34-1.72)	0.52				
Greek	1.17 (0.27-5.05)	0.83				
Sex	0.96 (0.47-1.97)	0.91				
BMI	1.10 (1.04-1.17)	0.001		NS		
Age at biopsy	1.06 (1.03-1.09)	<0.0001	1.04 (1.01-1.08)	0.03		
Diabetes	3.93 (1.82-8.50)	0.001		NS		
Hypertension	1.8 (0.85-3.81)	0.12				
Dyslipidemia	0.72 (0.35-1.45)	0.38				
HM CPA	1.11 (1.08-1.13)	<0.0001	1.04 (1.01-1.08)	0.04	1.04 (1.001-1.08)	0.04
SM CPA	1.11 (1.08-1.13)	<0.0001				
≥F3	37.9(14.1-101)	<0.0001	15.4(5.02-47.84)	<0.0001	9.55(3.15-28.9)	<0.0001
NASH	6.94 (3.06-15.72)	<0.0001		NS		
Bilirubin	1.02 (1-1.04)	0.06		NS		
ALT	0.99 (0.98-1.01)	0.43				
AST	1.003 (0.99-1.01)	0.29				

*, compared to the British cohort. Abbreviations: HR: Hazards Ration, BMI, body mass index; HM CPA, high magnification collagen proportionate area; SM CPA, standard magnification collagen proportionate area; ≥F3, advanced fibrosis; NASH, non-alcoholic steatohepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Figures legends

Figure 1. Images taken at standard (a) and high (b) magnification of liver sample with fibrosis stage = F2 according to Brunt et al system. The respective SM and HM CPA measurement is 7.7 (a) and 14 (b).

Figure 2.

a. Correlation between HM CPA and liver stiffness (LS) measured by transient elastography (r= 0.73).

b. Correlation between SM CPA and liver stiffness (LS) measured by transient elastography (r= 0.68).

Figure 3. Comparative areas under the ROC (AUROCs) of quantitative and semi-quantitative predictors of mortality for liver related events (death or clinical decompensation) in patients with biopsy-proven NAFLD. AUROCs are: HM CPA = 0.79 (95% CI 0.75-0.83); advanced fibrosis = 0.81 (95% CI 0.76-0.89); age at biopsy = 0.70 (95% CI 0.65-0.74), combination of the three variables (combined variable): 0.87 (95% CI 0.83-0.90).

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	Page 3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Page 3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Page 4
Objectives	3	State specific objectives, including any prespecified hypotheses	Page 5
Methods			
Study design	4	Present key elements of study design early in the paper	Page 6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Page 6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	Page 6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*Pages 6,7,8*
Bias	9	Describe any efforts to address potential sources of bias	Page 6. 8
Study size	10	Explain how the study size was arrived at	Page 6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Page 8, 9
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	Page 8, 9
		(*b*) Describe any methods used to examine subgroups and interactions	Page 8, 9
		(*c*) Explain how missing data were addressed	Page 8, 9

		(*d*) If applicable, explain how loss to follow-up was addressed	NA
		(*e*) Describe any sensitivity analyses	Page 11, 12
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Page 9, 10, 22
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Page 9, 10, 22
		(b) Indicate number of participants with missing data for each variable of interest	NA
		(c) Summarise follow-up time (eg, average and total amount)	Page 10
Outcome data	15*	Report numbers of outcome events or summary measures over time	Page 10
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Page 10
		(*b*) Report category boundaries when continuous variables were categorized	Page 10
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Page 10-12
Discussion			
Key results	18	Summarise key results with reference to study objectives	Page 14
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	Page 16
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Page 14-17

Generalisability	21	Discuss the generalisability (external validity) of the study results	Page 14-17
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Page 2

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.