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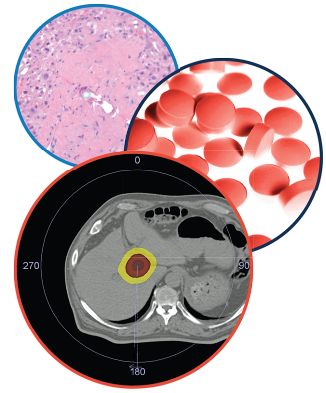

Hepatic Oncology

Endotrophin, a pro-peptide of Type VI collagen, is a biomarker of survival in cirrhotic patients with hepatocellular carcinoma

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Aim: Type VI collagen, is emerging as a signaling collagen originating from different types of fibroblasts. A specific fragment of Type VI collagen, the pro-peptide, is also known as the hormone endotrophin. We hypothesized that this fibroblast hormone would be of particular relevance in cancer types with a high amount of fibrosis activity, namely for outcome in hepatocellular carcinoma (HCC) cirrhotic patients. **Patients & methods:** Plasma C6M, PRO-C6 and alphafeto-protein (AFP) were assessed in 309 patients with mixed etiologies (hepatitis C, hepatitis B, alcohol and nonalcoholic fatty liver) diagnosed as cirrhotics, cirrhotics with HCC, noncirrhotics and healthy controls. Progression-free survival and overall survival (OS) data were collected up to 6120 days after diagnosis. The ability of each marker to predict survival was investigated. **Results & conclusion:** The level of endotrophin assessed by PRO-C6 was able to separate healthy controls, noncirrhotics and cirrhotics from HCC ($p < 0.05$–0.0001). Both endotrophin and C6M provided value in the prediction of OS in cirrhotic patients with HCC. In the multivariate analysis for identifying HCC, in patients with high endotrophin (highest quartile) and that were positive for AFP (≥20 IU/ml), the hazard ratio for predicting OS was increased from 3.7 ($p = 0.0006$) to 14.4 ($p = 0.0001$) when comparing with AFP positive as a stand-alone marker. In conclusion, plasma levels for markers of Type VI collagen remodeling were associated with survival in cirrhotic patients with HCC. A combination of AFP with endotrophin improved the prognostic value compared with AFP alone for predicting OS in cirrhotic patients with HCC.

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Keywords: biomarkers • collagen • endotrophin • extracellular matrix • hepatocellular carcinoma

Hepatocellular carcinoma (HCC) constitutes 70–90% of all liver cancers and was in 2012 the second leading cause of cancer-related deaths worldwide [1]. HCC is often caused by chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) especially in low- and middle-income countries [2]. Risk factors for HCC in developing and developed countries also include obesity, Type 2 diabetes, alcoholic or nonalcoholic fatty liver disease [3], conditions which are increasing to epidemic proportions. Effective treatments are available, but only a third of patients present at a stage where curative therapies are an option. A major problem in the early detection of HCC is the lack of a robust biomarker. Alpha-fetoprotein (AFP), the most widely used, has suboptimal performance in the detection of early stage HCC [4]. While the European Association for the Study of the Liver (EASL) recommends the use of ultrasonography alone [5], the American Association for the Study of Liver Diseases recommends the use of

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ultrasonography with or without AFP [6] and the Asian Pacific Association for the Study of the Liver recommends the use of both ultrasonography and AFP [7]. Though AFP is the gold-standard serological marker for HCC, the utility of AFP for surveillance is limited by its low sensitivity and specificity [8]. A large proportion of HCC patients (42%) do not have elevated levels of AFP (>20 IU/ml) [9], emphasizing the need for novel biomarkers.

An array of alternative (liquid) biomarkers has been proposed, such as PIVKA (des-gamma-carboxyprothrombin), glypican-3, osteopontin and Golgi protein [10]; however, none have been sufficiently validated to be recommended for use and as such, the pursuit of a novel HCC biomarker remains a goal [10,11]. Extracellular matrix (ECM) remodeling plays a pivotal role during HCC development which is skewed toward ECM accumulation as a result of tissue remodeling and the desmoplastic reaction of HCC. Furthermore, the neighboring tumor microenvironment releases ECM-derived signaling molecules to the tumor including endotrophin, a matrikine released from procollagen type [12,13]. There is an evolving understanding of the intricate relationship between HCC and the tumor ECM microenvironment, but while several studies have measured ECM and especially Type III and Type IV procollagen fragments in HCC, using less well-validated tests, these studies largely lacked comparison with other putative HCC markers and often were purely cross-sectional [14–18]. Moreover, since cirrhosis usually goes hand-in-hand with HCC manifestation and progression, a clear separation between cirrhosis as confounder and HCC must be made. The HCC microenvironment comprised tumor cells within a complex milieu of the ECM, stromal cells and the proteins they secrete, with bi-directional signaling between the desmoplastic stroma, the cancer cells and endothelial and immune cells [19].

During the process of excessive ECM remodeling, post-translational modifications are made to the specific proteins [20,21]. Pathology-specific unique protein degradation fragments, so-called neoepitopes are then released into the circulation. These turnover products may theoretically be ideal disease biomarkers as they are thought to be more related to the underlying pathogenesis than unmodified proteins [20]. Furthermore, some have been shown to have endocrine properties by harboring signaling sequences, such as tumstatin, vastatin, restin, endostatin and endotrophin [13]. Several studies have examined the use of various ECM neoepitopes as biochemical serological markers of liver fibrosis, and generally it is seen that Type VI collagen neoepitopes, including endotrophin, have a potential as markers of liver fibrosis as well as general metabolic derangement [22–26].

In this study, we investigated the ability of two ECM markers of Type VI collagen to diagnose HCC and predict patients' overall survival (OS) and progression-free survival (PFS). We included a marker of matrix metalloproteinase (MMP) mediated degradation of the alpha 1 chain of type VI collagen (C6M) [27], and a formation marker of the alpha 3 chain of Type VI collagen (PRO-C6) [28], also known as endotrophin [13]. Endotrophin is found in the C-terminal propeptide of the Type VI collagen a3 chain and has been identified in various tissues including liver and adipose tissue [29]. The pro-peptide of Type VI collagen is released during Type VI collagen formation and further processed by bone morphogenetic protein 1 (BMP-1) and MMP-14 cleavage of the C-terminal pro-peptide which then results in endotrophin [30,31]. C6M, PRO-C6 and AFP were assessed in plasma of patients with cirrhotics with HCC compared with cirrhotics without HCC, noncirrhotics and healthy controls at baseline.

Materials & methods

HCC study population

This case-control study involved four independent groups comprising a total of 309 participants (cirrhotics with HCC [n = 84], cirrhotics without HCC [n = 86], noncirrhotics [HBV infection; n = 84] and healthy controls [n = 55]) recruited from a single tertiary liver clinic in Sydney, Australia, between 2008 and 2018. Data collection were performed prior to performing the index tests in a retrospective manner. The HCC patients had different etiologies and were diagnosed by characteristic radiological appearances on 4-Phase computer tomography (CT) or MRI according to EASL guidelines, or by histology. Clinical staging of HCC was according to the Barcelona Clinic Liver Cancer (BCLC) system. EDTA plasma was taken at the time of diagnosis prior to the initiation of treatment. Plasma samples were stored at -80°C until analysis. None of the patients was eligible for curative resection or liver transplantation according to the BCLC criteria. The cirrhotics with HCC received different therapeutic options: best supportive care (n = 3), radiofrequency ablation (n = 15), selective internal radiation therapy (Sirspheres; n = 5), sorafenib (n = 14), surgical (n = 11) and trans-arterial chemoembolization (n = 31). PFS and OS were estimated from baseline in cirrhotics with HCC at baseline (n = 84). The cirrhotics comprised individuals with different etiologies, diagnosed based on clinical, laboratory and/or imaging evidence or histopathology. The noncirrhotics included only patients with chronic HBV in the absence of cirrhosis. The healthy control group comprised individuals recruited through advertisements in local newspapers at the hospital. All had normal physical examinations and liver tests, negative viral hepatitis serology and no history of liver disease.

Clinical & laboratory data

Demographic and clinical data including age, sex, BMI, etiology (HCV, HBV, alcoholic liver disease, nonalcoholic steatohepatitis), ethnicity (Caucasian, Indian, Asian, Middle Eastern and Polynesian), liver parameters (diabetes status, levels of bilirubin, albumin, alanine transaminase [ALT], aspartate transaminase [AST], platelet count [PLT] and AFP) and tumor related variables (BCLC stage, Child-Pugh score, size of largest lesion, number of lesions, portal vein invasion and existence of metastasis) were collected. Routine biochemical tests including bilirubin, albumin, ALT, AST, PLT and AFP were assessed in fasting blood samples by standard methods at baseline.

Biomarker measurements of Type VI collagen remodeling

Two markers of Type VI collagen remodeling were assessed in plasma samples in a blinded manner. A type VI collagen formation marker of the alpha 3 chain (PRO-C6) [28], also known as endotrophin, and a marker of Type VI collagen degradation of the alpha 1 chain (C6M) [27] were measured by competitive ELISAs developed by Nordic Bioscience (Herlev, Denmark). The ELISAs were performed as previously described [27,28]. Briefly, a 96-well ELISA plate precoated with streptavidin, was coated with the collagen Type VI specific synthetic peptide at 20°C for 30 min by constant shaking at 300 rpm. The plate was then washed five-times in washing buffer. Thereafter, 20 μl of the standard peptide or samples diluted according to the protocol was added, followed by 100 μl peroxidase conjugated mAb in assay buffer. The plate was then incubated at 20°C for 1 h or at 4°C overnight while shaking at 300 rpm. Afterwards, the plate was washed five times. Finally, 100 μl TMB (Kem-En-Tec, Taastrup, Denmark) was added and the plate was incubated for 15 min in the dark, while shaking at 300 rpm. To stop the reaction, 100 μl of stopping solution (1% H_2SO_4), was added and the plate was analyzed on an ELISA reader at 450 with 650 nm as the reference. All samples were measured within the range of the assay.

Statistics

Statistical analysis was carried out using MedCalc (Ostend, Belgium) and GraphPad Prism version 7 (GraphPad Software, Inc., CA, USA). Baseline characteristics are presented as mean ± standard deviation (SD) for continuous variables and as number (frequency) or percentage for categorical variables. Differences between the groups at baseline were assessed using Pearson's chi-square for categorical variables, and ANOVA (parametric) or Kruskal–Wallis test (nonparametric) for continuous variables. Receiver operation characteristics (ROC) analysis was performed for testing the ability of C6M, PRO-C6 and AFP to diagnose HCC in patients with cirrhosis compared with patients with cirrhosis only. The relationship between each marker and PFS (days) or OS (days) was calculated using Kaplan–Meier survival analysis using the median or the upper quartile (Q4) as a cut-off point. The Cox proportional-hazards regression model was used to calculate the hazard ratios (HRs) with 95% CI for prediction of OS and PFS for each biomarker including the following clinical covariates: age, sex, BMI, Child-Pugh score and number of lesions. Multivariate Cox proportional-hazards regression was used to assess the independent predictive value of C6M, PRO-C6 and AFP adjusted for the above noted clinical variables. C6M and PRO-C6 levels above under the 75th percentile cut-off point (high levels, quartile 4) were used as a reference to calculate the HR for patients with levels below the 75th percentile (low levels, quartiles 1–3). AFP levels under 20 IU/ml (negative) were used as a reference to calculate the HR for patients with elevated AFP levels (positive) [32]. For all statistical analysis performed, a p-value below 0.05 was considered significant. Individual p-values are given where appropriate.

Results

Patient characteristics

The clinical characteristics of the cohort summarized in Table 1. Most of the patients were males, the mean age was 52.2–62.4 years, with a BMI of 25.5–29.4 kg/m^2. All HCC patients had underlying cirrhosis. Age, BMI, etiology, ethnicity and diabetes status varied among the disease groups. As expected, bilirubin, ALT, AST were higher in cirrhotics and HCC patients, whereas albumin and platelets were lower in cirrhotics and HCC patients compared with noncirrhotics and healthy controls. Alpha-fetoprotein, PRO-C6 and C6M increase with disease severity. Ethnicity was not matched in the various groups. Between cirrhotics and cirrhotics with HCC a difference in ethnicity, albumin AST, PRO-C6 and C6M and Child-Pugh score was found.

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Table 1. Patient demographics of the studied cohorts.

	Healthy controls	Noncirrhotic HBV	Cirrhosis	Cirrhosis with HCC	p-value four disease groups	p-value cirrhosis vs HCC
n	55	84	86	84		
Age (years), mean ± SD	52.2 (7.7)	58.3 (8.6)	58.8 (10.0)	62.4(11.4)	**< 0.0001**	0.05
Gender (male), n (%)	51 (91.1)	73 (86.9)	75 (87.2)	74 (88.1)	0.89	0.86
BMI, mean ± SD	26.2 (2.9)	25.5 (4.0)	29.4 (5.6)	28.5 (6.5)	**< 0.0001**	0.12
Etiology					**< 0.0001**	0.26
HCV, n (%)	NA	0 (0)	43 (50.0)	40 (47.6)		
HBV, n (%)	NA	84 (100)	23 (26.7)	13 (15.5)		
EtOH, n (%)	NA	0 (0)	7 (8.1)	11 (13.1)		
NASH, n (%)	NA	0 (0)	10 (11.6)	16 (19.0)		
Other, n (%)	NA	0 (0)	3 (3.5)	0 (0)		
Ethnicity					**< 0.0001**	**< 0.0001**
Caucasian, n (%)	44 (78.6)	9 (0.7)	49 (57.0)	54 (65.1)		
Chinese, n (%)	7 (12.5)	55 (65.5)	12 (14.0)	11 (13.3)		
Middle Eastern, n (%)	2 (3.6)	9 (10.7)	20 (23.3)	10 (12.0)		
Indian, n (%)	3 (5.4)	7 (8.3)	3 (3.5)	3 (3.6)		
African, n (%)	0 (0)	2 (2.4)	1 (1.2)	3 (3.6)		
Polynesian, n (%)	0 (0)	2 (2.4)	1 (1.2)	2 (2.4)		
Diabetics, n (y/n)	0/0	74/10	56/29	50/34	**0.0001**	0.40
Bilirubin, mean ± SD	11.4 (5.0)	13.4 (8.2)	21.2 (14.5)	21.8 (23.5)	**< 0.0001**	0.16
Albumin, mean ± SD	43.5 (2.3)	43.7 (2.9)	40.5 (5.3)	36.7 (6.6)	**< 0.0001**	**0.0001**
ALT, mean ± SD	31.2 (15.7)	40.7 (36.45)	65.3 (61.5)	85.7 (87.7)	**< 0.0001**	0.08
AST, mean ± SD	28.6 (7.0)	39.9 (12.8)	75.0 (58.2)	109.8 (98.8)	**< 0.0001**	**0.001**
PLT, mean ± SD	239.2 (56.6)	227.5 (50.9)	131.6 (66.0)	126.2 (64.1)	**< 0.0001**	0.61
PRO-C6 (ng/ml)	4.7 (1.4)	5.3 (2.4)	9.2 (11.4)	10.6 (5.0)	**< 0.0001**	**0.0007**
C6M (ng/ml)	6.6 (1.1)	7.7 (3.3)	8.4 (4.8)	9.6 (5.4)	**0.001**	**0.046**
AFP (IU/ml), mean ± SD	NA	2.6 (1.0)	6.5 (12.3)	2965.7 (13361.4)	**< 0.0001**	**< 0.0001**
Hyperlipidemia (Y/N)	NA	NA	NA	18/66	NA	NA
BCLC staging, 0/A/B/C/D	NA	NA	NA	4/31/32/13/3	NA	NA
Child-Pugh score, A/B/C/n/a	NA	NA	78/6/2/0	54/14/7/9	NA	0.0002
Size of largest lesion, mean ± SD	NA	NA	NA	4.6 (4.0)	NA	NA
Number of lesions, mean ± SD	NA	NA	NA	1.9 (1.5)	NA	NA
Metastasis, Y/N	NA	NA	NA	5/78	NA	NA
Portal vein invasion, Y/N	NA	NA	NA	13/70	NA	NA

Bold p-values indicate statistical significance.
Results are expressed as mean (standard deviation) or frequency (percentage). p-values were calculated using Kruskal–Wallis test with Dunn's multiple comparisons or a chi-square test.
AFP: Alpha-fetoprotein; ALT: Alanine transaminase; AST: Aspartate transaminase; BCLC: Barcelona Clinic Liver Cancer; EtOH: Alcoholic liver disease; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; NA: Not applicable; NASH: Nonalcoholic steatohepatitis; PLT: Platelet count.

Markers of Type VI collagen remodeling & AFP are elevated in HCC

Plasma PRO-C6 increased with disease state ($p < 0.05$–0.0001) and was significantly higher in HCC than in cirrhotics ($p < 0.01$), noncirrhotics ($p < 0.0001$) and healthy controls ($p < 0.0001$) (Figure 1A). Plasma levels of the ECM markers in the HCC and control groups are displayed in Figure 1A–C. MMP-driven degradation of Type VI collagen, plasma C6M, was only elevated in HCC patients compared with healthy controls ($p < 0.01$), with no difference compared with cirrhotics only and HBV patients (Figure 1B). Patients with HCC had high levels of the cancer related marker plasma AFP, though with a high standard deviation, compared with cirrhotics and noncirrhotics ($p < 0.0001$) (Figure 1C).

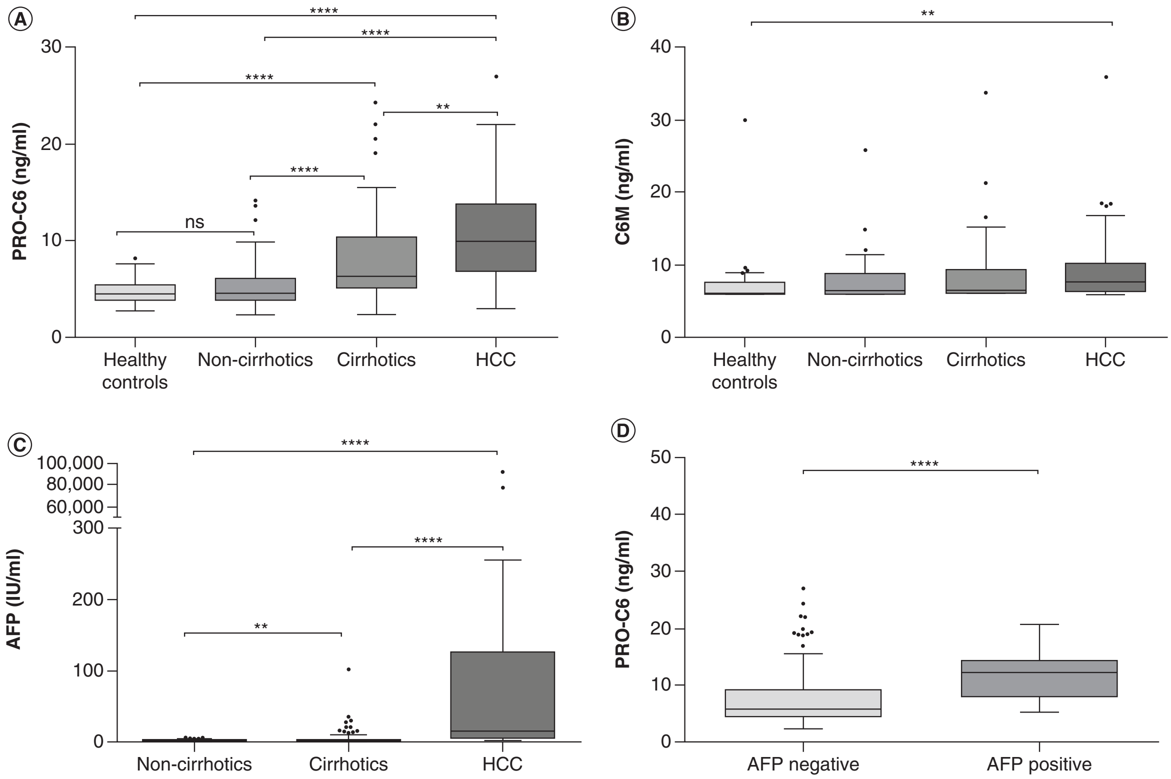

Figure 1. Levels of biomarkers in healthy controls (n = 86), hepatitis B virus noncirrhotics (n = 86), cirrhotics (n = 86) and hepatocellular carcinoma (n = 86) patients. (A) Plasma levels of formation of collagen Type VI (PRO-C6, endotrophin); **(B)** plasma levels of degradation fragments of collagen Type VI (C6M); **(C)** alpha-fetoprotein (AFP) levels; **(D)** PRO-C6 levels in HCC patients divided into AFP negative (AFP<20) or AFP positive (AFP≥20). Statistical difference was calculated using a Mann–Whitney *t*-test. Statistical differences were analyzed using a Kruskal–Wallis test adjusted for Dunn's multiple comparisons test. Data are presented as Tukey boxplots. Significance levels: *p < 0.05; **p = <0.01; ***p < 0.001; ****p < 0.0001.
AFP: Alphafeto-protein; HCC: Hepatocellular carcinoma.

Table 2. Discriminative performance of PRO-C6 and AFP biomarkers for the diagnosis of hepatocellular carcinoma in cirrhotic patients.

HCC vs cirrhotics in all cirrhotic patients	Cut-off value (ng/ml)	Sensitivity	Specificity	AUROC	p-value
PRO-C6 (n = 84 HCC; n = 86 cirrhotics)	6.31	79.8	50.0	0.65	**0.0004**
AFP (n = 84 HCC; n = 86 cirrhotics)	6.00	65.1	80.2	0.78	**< 0.0001**
HCC vs cirrhotics in AFP negative or positive cirrhotic patients (20 IU/ml ≥AFP<20 IU/ml)					
PRO-C6 in AFP negative (n = 48 HCC; n = 80 cirrhotics)	7.49	66.7	73.2	0.69	**< 0.0001**
PRO-C6 in AFP positive (n = 36 HCC; n = 6 cirrhotics)	13.1	44.4	83.3	0.50	0.99

Bold p-values indicate statistical significance.
AFP: Alphafeto-protein; HCC: Hepatocellular carcinoma.

Performance of endotrophin compared with AFP

The performance of the ECM markers versus AFP in discriminating HCC from cirrhosis alone was compared. For the diagnosis of HCC in cirrhotics, ROC analysis for each marker as well as the comparator AFP, were performed. Both PRO-C6 and APF were able to discriminate between HCC patients and non-HCC cirrhotics (Table 2; 0.004 < p <0.0001). AFP had an area under the ROC curve (AUROC) of 0.78 for diagnosis of HCC, while PRO-C6 had an AUROC of 0.65. Sensitivity and specificity were below 80.2% for both markers. The performance of AFP was significantly different from PRO-C6 (p = 0.008 for the difference between AUROC, data not shown). Notably, in AFP negative patients PRO-C6 was able to identify cirrhotic patients with HCC with an AUROC of 0.62–0.69

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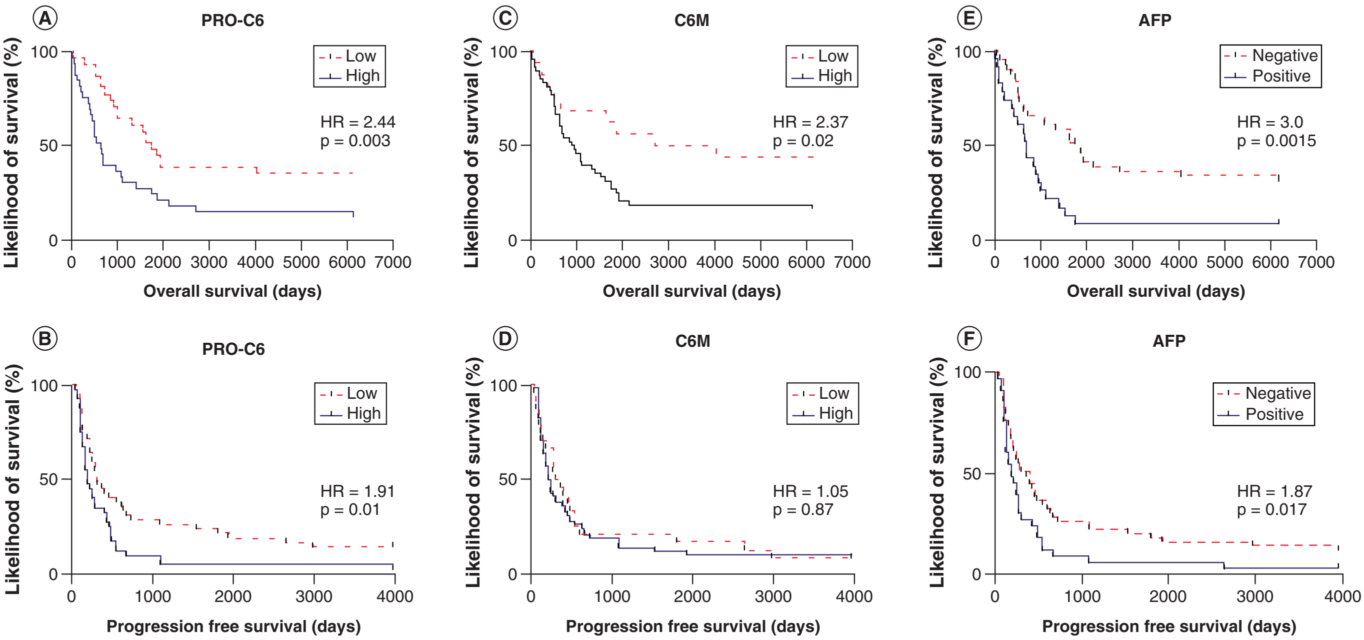

Figure 2. Kaplan–Meier curves for the univariate analysis of each marker in relation to overall survival and progression-free survival. The median is used as cut-off (low vs high) for PRO-C6 (low n = 42; high n = 41) and C6M (low n = 21; high n = 24), and 20 IU/ml cut-off for AFP (negative n = 49/positive n = 37). **(A, C & E)** represents relation to overall survival and **(B, D & F)** represents relation to progression-free survival. Significance levels: ns = nonsignificant, *p < 0.05; ***p < 0.001; ****p < 0.0001.
AFP: Alphafeto-protein.

(p = 0.037–0.0001; Table 2) (n = 48 HCC; n = 80 cirrhotics), however, not in AFP positive patients (n = 36 HCC; n = 6 cirrhotics). Furthermore, PRO-C6 was elevated in patients that were AFP positive compared with negative ($p < 0.0001$; Figure 1). Additionally, AFP as well as C6M were not able to separate patients with Child Pugh Score A, B and C, while PRO-C6 was ($p < 0.001$ and $p < 0.0001$, respectively, Figure 3C & Supplementary Figure 4A–C). PRO-C6 was not related to metastasis, number of lesions, size of largest tumor or BCLC stage, whereas C6M was related to BCLC stage and metastasis (yes/no) (Supplementary Table 4).

Plasma PRO-C6 (endotrophin), C6M & AFP combined improve the prediction of survival in HCC patients

Univariate analysis

In a univariate, Kaplan–Meier analysis, patients with HCC and a level above the median of PRO-C6, C6M or AFP had a lower OS compared with those below the median level when followed up to 6120 days (Figure 2B, D & F). The HR ranged between 2.37 and 2.44 (p = 0.02–0.003) (Table 3). For the univariate prediction of PFS only PRO-C6 was significant with an HR of 1.91 (p = 0.01).

In another univariate analysis, patients in the upper quartile (Q4) of PRO-C6, C6M and AFP had an even higher risk of reduced OS compared with those in the three lower quartiles (Q1–3) when followed up to 6120 days. HRs ranged between 2.89 and 3.4 (p = 0.03–0.003) (Figure 2A, C & E). For the univariate prediction of PFS, PRO-C6 and AFP were significant with an HR of 2.32 and 2.77 (p = 0.01–0.004), respectively. Using the 20 IU/ml cut off for AFP negative versus positive, AFP was significantly related to both OS with an HR of 3.00 (p = 0.0015) and PFS with an HR of 1.87 (p = 0.017).

Multivariate analysis

In COX proportional hazard multivariate analysis, high PRO-C6, C6M and AFP (Q4) remained significant for the prediction of OS when adjusted for age, BMI and sex with HRs ranging from 2.4 to 3.3 (p = 0.01–0.003) (Table 3). Only high AFP (≥20 IU/ml) remained significant for the prediction of PFS when adjusted for age, BMI and sex with an HR of 1.59 (p = 0.07). Patients with high PRO-C6 (Q4) and high AFP (≥20 IU/ml) combined had a significantly lower OS compared with patients with low PRO-C6 (Q1–3) and low AFP (<20 IU/ml) with

Table 3. Association between biomarker levels, clinical covariates and outcome for hepatocellular carcinoma patients.

Variable		Progression-free survival			Overall survival		
Univariate analysis		**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Age	Continuous	1.01	0.99–1.03	0.332	1.01	0.99–1.04	0.280
Gender (male)	Continuous	0.78	0.36–1.71	0.533	1.04	0.41–2.65	0.932
BMI	Continuous	1.01	0.97–1.05	0.672	0.99	0.94–1.04	0.634
Child-Pugh score	B/C vs A	2.39	1.37–4.19	**0.002**	5.06	2.58–9.93	**< 0.0001**
Number of lesions	Continuous	1.18	1.08–1.29	**0.0003**	1.41	1.22–1.63	**< 0.0001**
PRO-C6	High vs low (Median)	1.91	1.16–3.15	**0.01**	2.44	1.35–4.42	**0.003**
C6M	High vs low (Median)	1.05	0.58–1.92	0.87	2.37	1.12–5.03	**0.02**
AFP	High vs low (Median)	1.62	1.00–2.64	0.05	2.4	1.28–4.50	**0.006**
PRO-C6	High vs low (Q4 vs Q1-Q3)	2.32	1.22–4.42	**0.011**	3.18	1.49–6.82	**0.003**
C6M	High vs low (Q4 vs Q1-Q3)	1.51	0.71–3.20	0.28	2.89	1.12–7.46	**0.03**
AFP	High vs low (Q4 vs Q1-Q3)	2.77	1.40–2.51	**0.004**	3.4	1.50–7.75	**0.004**
AFP	High vs low (≥20 vs <20 IU/ml)	1.87	1.12–3.14	**0.017**	3.00	1.52–5.90	**0.0015**
Multivariate analysis		**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Adjusted for age, sex and BMI							
PRO-C6	High vs low (Q4 vs Q1-Q3)	1.63	0.89–3.00	0.12	2.4	1.21–4.7	**0.01**
C6M	High vs low (Q4 vs Q1-Q3)	1.40	0.66–3.00	0.38	3.3	1.34–8.10	**0.01**
AFP	High vs low (≥20 vs <20 IU/ml)	1.58	0.97–2.60	**0.07**	2.51	1.36–4.64	**0.003**
High PRO-C6 and AFP (14.5%)	High PRO-C6 and high AFP vs low/high or high/low PRO-C6/AFP	1.94	0.78–4.83	0.16	6.94	1.19–40.86	**0.03**
High C6M and AFP (12.5%)	High C6M and high AFP vs low/high or high/low C6M/AFP	2.56	0.74–8.93	0.14	4.82	1.42–16.38	**0.01**
Adjusted for Child-Pugh score and number of lesions							
PRO-C6	High vs low (Q4 vs Q1-Q3)	1.38	0.78–2.45	0.27	2.17	1.06–4.44	**0.03**
C6M	High vs low (Q4 vs Q1-Q3)	1.18	0.56–2.51	0.66	2.90	1.24–6.72	**0.01**
AFP	High vs low (≥20 vs <20 IU/ml)	1.81	1.06–3.09	**0.03**	3.68	1.80–7.51	**0.0003**
High PRO-C6 and AFP (14.5%)	High PRO-C6 (Q4) and high AFP vs low/high or high/low PRO-C6/AFP	1.99	0.96–4.11	0.07	5.67	2.30–13.94	**0.0002**
High C6M and AFP (12.5%)	High C6M (Q4) and high AFP vs low/high or high/low C6M/AFP	3.13	1.16–8.21	**0.02**	5.55	1.65–18.61	**0.006**
Adjusted for AFP							
PRO-C6	High vs low (Q4 vs Q1-Q3)	1.78	1.05–3.02	**0.03**	2.40	1.29–4.47	**0.006**
C6M	High vs low (Q4 vs Q1-Q3)	1.48	0.76–2.90	**0.25**	2.22	1.01–4.90	**0.05**
Adjusted for Age, BMI, sex, Child-Pugh score, number of lesions.							
High PRO-C6 (25.3%)	Q4	1.16	0.58–2.31	0.67	1.55	0.67–3.55	0.31
High C6M (25%)	Q4	1.25	0.57–2.73	0.57	3.38	1.32–8.68	**0.01**
High AFP (42.4/100%)	≥20 IU/ml	1.55	0.87–2.77	0.14	3.74	1.76–7.97	**0.0006**
High PRO-C6 and AFP (14.5/100%)	High (Q4) PRO-C6 and high AFP vs low/low, low/high or high/low PRO-C6/AFP	1.51	0.55–4.11	0.42	14.40	2.53–188.14	**0.0001**
High C6M and AFP (12.5/100%)	High (Q4) C6M and high AFP vs low/low, low/high or high/low C6M/AFP	3.90	1.00–15.37	0.0496	9.41	2.42–36.70	**0.0012**

Bold p-values indicate statistically significant HR.
Hazard ratios were calculated by univariate and multivariate Cox proportional-hazards analysis. By univariate analysis, Pro-C6 and C6M were analyzed divided into above or below the median, or quartiles with the lower levels (Q1-Q3) used as a reference to calculate the HR for patients in the upper quartile (Q4). The covariates were analyzed on a continuous scale and Child-Pugh score and AFP were further analyzed on a binominal scale. By multivariable analysis, PRO-C6, C6M and AFP were adjusted as indicated in the text.
AFP: Alpha-fetoprotein; HR: Hazard ratio.

an HR of 6.94 (p = 0.03); also patients with high C6M (Q4) and high AFP (≥20 IU/ml) had a significantly lower OS compared with patients with low C6M (Q1–3) and low AFP (<20 IU/ml) with an HR of 4.82 (p = 0.01) but not of PFS; all adjusted for age, BMI and sex.

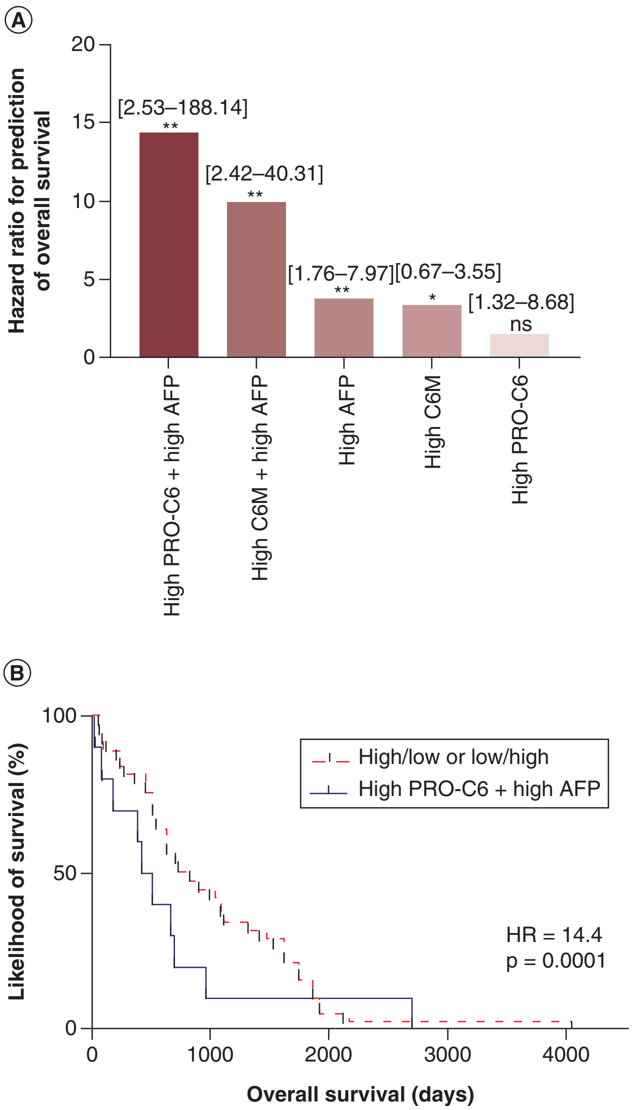

Figure 3. Overall survival analysis. (A) Hazard ratio for the prediction of overall survival by each indicated multivariate model, all corrected for sex, age, BMI, Child-Pugh and number of lesions. High PRO-C6 (Q4) and high C6M (Q4); high AFP (≥20 IU/ml) (HRs from Table 3). **(B)** Kaplan–Meier curves for the multivariate analysis of overall survival for high PRO-C6 (Q4) + high AFP (≥20 IU/ml). The 95% CI is seen for each group in brackets. Significance levels: ns = nonsignificant, *p < 0.05; ***p < 0.001; ****p < 0.0001.
AFP: Alpha-fetoprotein; HR: Hazard ratio.

In a similar manner, in COX proportional hazard multivariate analysis, high PRO-C6, C6M and AFP (Q4) were significant for the prediction of decreased OS with HR ranging between 2.17 and 3.68 (p = 0.01–0.0003) (Table 3) when adjusted for Child-Pugh score and number of lesions, and only AFP remained significant for prediction of PFS with an HR of 1.81 (p = 0.03). Patients with high PRO-C6 (Q4) and high AFP (≥20 IU/ml) had a significantly higher risk of reduced OS with an HR of 5.67 (p = 0.0002). In a similar manner, patient with high C6M (Q4) and high AFP (≥20 IU/ml) had a significantly lower OS with an HR of 5.55 (p = 0.006) and lower PFS with an HR of 3.13 (p = 0.02), all adjusted for Child-Pugh score and number of lesions.

Additionally, in COX proportional hazard multivariate analysis, high PRO-C6 (Q4) was significant for the prediction of both OS and PFS with an HR of 2.40 (p = 0.006) and 1.78 (p = 0.03) (Table 3) when adjusted for AFP, respectively, whereas high C6M (Q4) was not able to predict OS or PFS.

Finally, in COX proportional hazard multivariate analysis adjusted for age, BMI, sex, Child-Pugh score and number of lesions, including only patients with high PRO-C6 (Q4) and high AFP (≥20 IU/ml), the HR for predicting OS was 14.4 (p = 0.0001) (Table 3 & Figure 3A; Kaplan–Meier curve: Figure 3B) versus 9.4 for high C6M (Q4) and high AFP (≥20 IU/ml); p = 0.0012), 3.38 for high C6M (Q4) (p = 0.01) and 3.74 for high AFP (≥20 IU/ml) (p = 0.0006). High PRO-C6 (Q4) was not significantly related to OS. When corrected for the mentioned parameters, only in patients with high C6M (Q4) and high AFP (≥20 IU/ml) the HR was significant (HR = 3.9; p = 0.0496) for the prediction of PFS.

Discussion

In the present study, we investigated whether novel biomarkers of Type VI collagen remodeling and pro-fibrotic signaling by endotrophin may be used to improve diagnosis of and to better predict survival in patients with HCC. The main findings were that – both PRO-C6 and C6M were able to distinguish between non-HCC cirrhotics and cirrhotics with HCC with an AUROC above 0.6. Nevertheless, AFP was superior as a diagnostic marker for HCC in cirrhotics; PRO-C6 was able to stratify cirrhotic patients according to Child Pugh scores, whereas C6M and AFP were not; PRO-C6 was higher in AFP positive versus AFP negative patient; PRO-C6, C6M and AFP were all independent predictors of OS when corrected for confounding factors; AFP was superior in the prediction of PFS when corrected for confounding factors, however; a combination of high AFP and high PRO-C6 or C6M improved the ability to predict OS and PFS in cirrhotic patients with HCC.

AFP as a stand-alone marker for HCC is not recommended by EASL, American Association for the Study of Liver Diseases or Asian Pacific Association for the Study of the Liver since it alone does not provide adequate value for the screening of patients at risk, mainly cirrhotics, for HCC. Therefore, additional noninvasive markers, especially serological markers, that may aid in early detection and in prognostication of the course of HCC are of great interest. In the present work, we set out to test whether two markers of Type VI collagen remodeling may provide additional value to AFP, alone or in combination AFP to identify and predict development of HCC. Endotrophin (PRO-C6) and a serological marker of Type VI collagen degradation (C6M) were investigated for their ability to diagnose HCC in a cross-sectional cohort as well as their capability to predict OS and PFS in cirrhotic patients with HCC that were followed for up to 6120 days.

Interestingly, both formation of Type VI collagen (the alpha 3 chain) quantified as serological PRO-C6/endotrophin and MMP degraded Type VI collagen (the alpha 1 chain) quantified as C6M provided independent value for the diagnosis and prognostication of HCC. This suggest that Type VI collagen independent of chain, and both formation and degradation of these alpha chains, are important for the progression of HCC. There are 5 alpha chains of Type VI collagen, albeit presently only assays for these chains are available. However, there was no relation to tumor characteristics, such as size of largest tumor, metastasis, BCLC and number of lesions. Furthermore, each Type VI collagen marker in combination with AFP increased the HR for predicting OS and PFS. This is plausible, since these collagen markers provided additional information originating from liver fibrosis and the tumor associated ECM, while, as known, AFP is a marker of cancer cell dedifferentiation [33]. AFP was highly elevated in HCC but exhibited a vast range compared with cirrhotics. Likewise, AFP was not related to Child-Pugh score. Only PRO-C6 showed a clear stepwise relationship with the stage of liver fibrosis and the Child-Pugh score. Based on our data and prior mechanistic research, we speculated that PRO-C6, apart from being derived from the profibrotic collagen Type VI measures the serological levels of the 'negative' adipokine endotrophin [28], may provide additional value in combination to AFP for the identification and follow-up of patients with HCC and cirrhosis. In this cohort, PRO-C6, a stand-alone marker did indeed provide additional value, since the diagnostic capability for identifying HCC cross-sectionally was comparably high in AFP negative versus AFP positive patients. The HR for predicting overall survival was dramatically increased from 3.74 for AFP as a stand-alone marker in AFP positive patients to 14.40 in the group that were AFP positive and that also had a plasma level in the highest quartile of PRO-C6 when adjusted for age, BMI, sex, Child-Pugh score and number of lesions. In a similar manner, when adjusted for Child-Pugh score and the number of tumor lesions, the HR for predicting OS increased from 3.7 to 5.7. These data are in alignment with published data showing that the expression of the alpha-3 chain of Type VI collagen which harbors the endotrophin fragment is significantly correlated with HCC presence and growth in HCC tissue and in animal models of liver cancer [12]. Thus, endotrophin in HCC is mainly produced by activated hepatic stellate cells, and increasing levels are related to poor prognosis [12]. Endotrophin activates the c-Jun N-terminal kinase (JNK) pathway that can induce hepatocyte apoptosis and promote further to hepatic inflammation, fibrosis and apoptosis. Furthermore, inhibition of endotrophin by an endotrophin neutralizing antibody seems to ameliorate HCC growth and fibrosis in a mouse liver fibrosis model [12,34]. Outside the liver, endotrophin has been shown to be involved in mammary tumor progression, in part via stimulation of fibrosis and via chemokine upregulation in the tumor microenvironment [13]. Several other studies also indicate that increased levels of endotrophin are related to poor outcome in fibrotic and metabolic diseases, such adipose tissue fibrosis and Type 2 diabetes or can select patients that are more likely to respond to antifibrotic therapy [35]; specifically, it acts as a chemoattractant for macrophages, has effects on endothelial cells and through epithelial-mesenchymal transition enhances fibrosis and tumor progression [29,36].

The assessment of C6M did also provide additional value in HCC detection; however, no signaling function is known for C6M, and the endotrophin marker PRO-C6 was in general superior to C6M. Still, for the prediction of PFS, the HR was increased from 1.8 for AFP as a stand-alone in AFP positive patients to 3.1 in the patient group that were AFP positive and had a plasma level in the highest quartile of C6M when adjusted for Child-Pugh score and the number of lesions. For OS, the HR increased from 3.7 to 5.6 when adjusted for Child-Pugh score and the number of lesions. Notably, also in GWAS analysis a single nucleotide polymorphisms of the alpha 3 chain of Type VI collagen was found to have a negative prognostic value in hepatitis C patients with HCC after hepatectomy [37]. In addition, in the PDGF-C transgenic and Pten null mouse models of HCC, high expression of the alpha-2 and alpha-3 chain of Type VI collagen was associated with HCC severity and growth [38].

Our prior studies already showed that a polyclonal serological assay for triple helical collagen Type VI is highly predictive of advanced liver fibrosis of different etiologies, including children with cystic fibrosis liver disease [39–44]. Of note, the assays used in the present studies are more specific by detecting collagen Type VI fragments derived from defined segments and signaling domains of this complex ECM molecule.

Finally, a promising Type III collagen marker of liver fibrosis stage and progression, PRO-C3, was not evaluated in the present study, although this marker has been highly investigated by the authors. PRO-C3 has been shown to associate to a higher extent than PRO-C6 to liver fibrosis [45,46]. PRO-C3 has previously been investigated as a marker of degree of liver fibrosis as well as survival in cohort presented here [47]. Jensen C *et al.* reported that PRO-C3 was related to the degree of liver fibrosis, however, it was not a predictor of survival in patients with HCC. Nevertheless, a multimeric version of the assay, assessing the cross-linked species of PRO-C3, known as PC3X, was related to survival in HCC patients [47].

Conclusion

In this study, we found that Type VI collagen remodeling is accelerated in patients with HCC. The serological markers PRO-C6 and C6M were able to separate non-HCC cirrhotics from patients with cirrhosis and HCC, and as stand-alone markers already serve as modest diagnostic prognostic markers to diagnose HCC. Most importantly, PRO-C6 and C6M in combination with AFP increased the prognostic value compared with AFP alone for survival in HCC patients. Thus, our study warrants further investigation of these markers for the diagnosis and prognosis of HCC patients and the monitoring of therapeutic response in clinical trials.

Future perspective

The diagnosis and monitoring for minimal residual disease and for recurrence in HCC is a large unmet medical need. The authors speculate that within the next 5–10 years, novel dynamic markers of HCC will emerge to aid in the diagnosis and/or monitoring of HCC progression or regression in combination with ultrasound or more sensitive imaging markers. Concurrent with the use of such markers, the evaluation of novel therapies may be accelerated, hopefully leading to newly approved therapeutics for HCC. These novel dynamic markers may be markers related to liver fibrosis, as suggested in the present manuscript, since liver fibrosis is a known driver of HCC progression.

Summary points

- A specific fragment of Type VI collagen, known as the hormone endotrophin, may be assessed by the serological marker PRO-C6 as well as Type VI collagen degradation by C6M.
- We tested whether endotrophin assessed by PRO-C6 or C6M would be of particular relevance for outcome in hepatocellular carcinoma (HCC) cirrhotic patients.
- Plasma C6M, PRO-C6 and alphafeto-protein (AFP) were assessed in 309 patients with mixed etiologies diagnosed as cirrhotics, cirrhotics with HCC, noncirrhotics and healthy controls.
- Progression-free survival and overall survival (OS) data were collected up to 6120 days after diagnosis. The ability of each marker to predict survival was investigated.
- PRO-C6 was able to separate healthy controls, noncirrhotics and cirrhotics from HCC.
- Both endotrophin and C6M provided value in the prediction of OS in cirrhotic patients with HCC.
- In patients that both had high endotrophin and were positive for AFP the hazard ratio for predicting OS was up to 14.4 ($p = 0.0001$), outperforming AFP as a stand-alone marker.
- Plasma levels for markers of Type VI collagen remodeling were associated with survival in cirrhotic patients with HCC in particular in combination with AFP.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/hep-2020-0030

Author contributions

DJ Leeming and MA Karsdal prepared the manuscript. DJ Leeming and S Holm-Nielsen did the statistical analysis. R Vongsuvanh, P Uchila, Dvd Poorten, M Eslam and J George collected the clinical study; AL Reese-Petersen provided valuable endotrophin supervision. J George, MA Karsdal and DJ Leeming designed the study. All authors read the manuscript.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The study protocol was approved by the Human Ethics Committee of the Sydney West Area Health Service (HREC No.2002/12/4.9 (1564)) in compliance with the Helsinki Declaration. The protocol may be accessed through Westmead Hospital. Written informed consent was obtained from all participants.

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