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# Serum levels of chemokines CCL4 and CCL5 in cirrhotic patients indicate the presence of hepatocellular carcinoma

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**Background:** Most hepatocellular carcinomas (HCCs) are diagnosed at an advanced stage. The prognostic value of serum tumour markers alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) is limited. The aim of our study is to evaluate the diagnostic value of serum growth factors, apoptotic and inflammatory mediators of cirrhotic patients with and without HCC.

**Methods:** Serum samples were collected from cirrhotic potential liver transplant patients (LTx) with ( $n=61$ ) and without HCC ( $n=78$ ) as well as from healthy controls (HCs;  $n=39$ ). Serum concentrations of CRP, neopterin and IL-6 as markers of inflammation and thrombopoietin (TPO), GCSF, FGF basic and VEGF, HMGB1, CK-18 (M65) and CK18 fragment (M30) and a panel of proinflammatory chemokines (CCL2, CCL3, CCL4, CCL5, CXCL5 and IL-8) were measured. Chi square, Fisher exact, Mann–Whitney *U*-tests, ROC curve analysis and forward stepwise logistic regression analyses were applied.

**Results:** Patients with HCC had higher serum TPO and chemokines ( $P<0.001$  for TPO, CCL4, CCL5 and CXCL5) and lower CCL2 ( $P=0.008$ ) levels than cirrhotic patients without HCC. Multivariate forward stepwise regression analysis for significant parameters showed that among the studied parameters CCL4 and CCL5 ( $P=0.001$ ) are diagnostic markers of HCC. Serum levels of TPO and chemokines were lower, whereas M30 was significantly higher in cirrhotic patients than in HCs.

**Conclusions:** High serum levels of inflammatory chemokines such as CCL4 and CCL5 in the serum of cirrhotic patients indicate the presence of HCC.

Hepatocellular carcinoma (HCC) invariably develops within a setting of chronic inflammation caused by hepatotropic viruses, toxins, metabolic liver disease or autoimmunity (Alison *et al*, 2011). HCC is the sixth most prevalent cancer and the third most frequent cause of cancer-related death. Prevention, diagnosis and treatment of HCC are of great concern. Fine-needle biopsy or other investigations such as computed tomography, magnetic resonance imaging or digital subtraction angiography were used as the gold

standard diagnosis in HCC (D'Onofrio *et al*, 2008; Bruix *et al*, 2011; Rahbari *et al*, 2011).

The prognosis at early stages relies on tumour status, liver function and the applied treatment. Tumour status is defined by so-called BCLC Classification (Arii *et al*, 2000; Llovet *et al*, 2003). Early HCC diagnosis is essential, as only in early stages (BCLC A) the tumour is curable by resection, LTx or ablation therapy. Patients with small solitary tumours (single lesion  $<5$  cm) and

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very well preserved liver function are the best candidates for liver resection or transplantation. LTx is most beneficial for individuals who are poor candidates for resection due to cirrhosis as the underlying disease (Forner *et al*, 2012).

Screening of the serum tumour marker alpha-fetoprotein (AFP) and imaging with ultrasound (US) every 6 months in patients with liver cirrhosis have been recommended to detect HCC at earlier stages amenable to effective treatment strategies (Spangenberg *et al*, 2006). Serum AFP is the most widely used marker for HCC. However, its sensitivity and specificity for HCC is poor and varies with the cutoff value, ethnicity of the patient, etiology of liver disease and tumour stage (Spangenberg *et al*, 2006; Toro *et al*, 2014). The sensitivity of AFP decreases from 52% to 25% when tumour diameter is >3 and <3 cm, respectively (Bertino *et al*, 2012). Besides AFP, AFP lectin fraction 3 (AFP-L3) and DCP are widely used clinically as serum tumour markers of HCC. These serum biomarkers (AFP, DCP and AFP-L3) are not accurate enough for the early diagnosis of HCC (Bertino *et al*, 2010, 2012).

Association of immune parameters such as apoptosis markers CK18 (M65) and CK18 fragment (M30), chemokines CCR2, CXCR2, CXCR4, CCL2, CCL4, CCL5, CCL11, CCL15, CCL17, CCL22, CXCL1, CXCL5, CXCL6, CXCL8, CXCL10, CXCL12, CXCL14, Chromogranin A and high-mobility-group-protein B1 (HMGB1) with cirrhosis and HCC have been studied previously in patients with end-stage liver diseases (ESLD; Nahon *et al*, 2008; Dominguez *et al*, 2009; Charni *et al*, 2011; Lavallard *et al*, 2011; Nakamoto *et al*, 2011; Tacke *et al*, 2011; Biondi *et al*, 2012; Bertran *et al*, 2013; Li *et al*, 2013; Waidmann *et al*, 2013; Wang *et al*, 2013; Yan *et al*, 2013; Cao *et al*, 2014; Hefetz-Sela *et al*, 2014; Shi *et al*, 2014).

Development of new reliable serological biomarkers for early stages of HCC is needed to improve clinical diagnosis and outcomes. Furthermore, the marker should be sensitive, easily measurable, reproducible and minimally invasive.

Our aim in the present study is to assess the diagnostic value of cell death markers HMGB1, M65 and M30, growth factors TPO, GCSF, FGF basic and VEGF and inflammatory chemokines CCL2 (monocyte chemoattractant protein-1), CCL3 (macrophage inflammatory protein-1), CCL4 (macrophage inflammatory protein-1beta), CCL5 (RANTES) and CXCL5 (epithelial cell-derived neutrophil-activating peptide-78) and IL-8 (CXCL8) for early detection of HCC in cirrhotic patients (Le *et al*, 2004).

## MATERIALS AND METHODS

**Patients.** The following parameters were measured in 139 cirrhotic patients (aged  $52.0 \pm 11.2$  years, 32 female, 61 cirrhotic HCC + and 78 cirrhotic HCC -) who underwent deceased donor LTx between January 2008 and April 2011. HCC diagnosis was confirmed by pathological reports. Original liver diseases were chronic viral hepatitis C and/or B in 40 patients, alcohol abuse in 41, congenital or autoimmune disease, including cryptogenic cirrhosis, biliary disease, metabolic liver disease, autoimmune hepatitis and amyloidosis, in 58 patients. Preoperative and demographic parameters, including age, gender, severity of liver diseases (determined by MELD score), bilirubin, INR, albumin, viral infection statuses such as CMV, HBV and HCV IgG, retransplantation, serum levels of CRP and neopterin as inflammatory markers and serum levels of cell death markers M65 and M30, TPO, CCL2, CCL3, CCL4, CCL5 CXCL5 and CXCL8 (IL-8), were analysed. We examined the association between HCC with mentioned parameters. Thirty-nine healthy volunteers (HCs) served as controls to establish references for studied parameters such as cytokines and chemokines. Controls were free of infectious and other inflammatory illnesses. The study was approved by the local Ethical Committee.

**Serum separation.** Serum separator tubes were centrifuged at 4000 r.p.m. for 15 min at 4 °C. Serum was collected after the blood clotting process. Serum was snap frozen within 2 h, after the blood was drawn and stored at -30 °C until testing. All serum samples were thawed only once before testing.

**Determination of serum immune parameters.** Cell apoptotic marker (M30; CK18 fragment), growth factors thrombopoietin (TPO) and chemokines C-X-C motif ligand 5 (ENA-78, CXCL5), C-C motif ligand 2 (MCP-1, CCL2), macrophage inflammatory protein-1a (MIP-1a, CCL3), macrophage inflammatory protein-1b (MIP-1b, CCL4) and regulated upon activation normal T cell expressed and secreted (RANTES, CCL5) were measured by ELISA using the Quantikine Kits (R&D Systems, Wiesbaden, Germany).

**Statistical analyses.** Categorical and continuous variables were analysed using chi square, Fisher exact and Mann-Whitney *U*-tests. Continuous variables were modelled stratifying by median. Correlation between variables was analysed using Spearman's rank correlation coefficient. The most sensitive cutoff values were calculated by receiver operating curve (ROC) curve analysis. Univariable and multivariable forward stepwise logistic regression analyses were applied and identified the greatest association of parameters with HCC. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, 18.0; SPSS Inc., Chicago, IL, USA). After Bonferroni correction, *P* values  $\leq 0.05$  were defined as statistically significant.

## RESULTS

**Immune parameters in cirrhotic patients with different original liver diseases.** Serum levels of M30, TPO, GCSF, FGF basic and VEGF, CXCL5, IL-8, CCL2, CCL3, CCL4 and CCL5 were similar among patients with virus-, alcohol- or congenital/autoimmune-induced cirrhosis (data not shown).

**All cirrhotic patients (HCC + and HCC -) vs healthy controls.** Serum levels of HMGB1 ( $3.2 \pm 6.7$  pg ml<sup>-1</sup> vs  $3.7 \pm 2.7$ ; *P* = 0.006), TPO ( $208 \pm 237$  pg ml<sup>-1</sup> vs  $505 \pm 106$ ; *P* < 0.0001), VEGF ( $35 \pm 49$  pg ml<sup>-1</sup> vs  $87 \pm 52$ ; *P* < 0.0001), CCL2 ( $194 \pm 533$  pg ml<sup>-1</sup> vs  $179 \pm 71$ ; *P* = 0.0001), CCL3 ( $33 \pm 280$  pg ml<sup>-1</sup> vs  $70 \pm 199$ ; *P* = 0.002), CCL5 ( $2.7 \pm 5.2$  mg ml<sup>-1</sup> vs  $11 \pm 11$ ; *P* < 0.0001) and CXCL5 ( $167 \pm 238$  pg ml<sup>-1</sup> vs  $766 \pm 540$ ; *P* < 0.0001) were lower, whereas serum CRP ( $20.0 \pm 24.7$  mg l<sup>-1</sup> vs  $3.5 \pm 6.1$ ; *P* < 0.0001), neopterin ( $49.2 \pm 69.1$  nmol l<sup>-1</sup> vs  $6.4 \pm 3.2$ ; *P* < 0.0001), IL-6 ( $74.0 \pm 435$  pg ml<sup>-1</sup> vs  $0.0 \pm 0.0$ ; *P* < 0.0001), IL-8 ( $40.5 \pm 87.3$  pg ml<sup>-1</sup> vs  $11.5 \pm 26.1$ ; *P* = 0.014), M65 ( $2349 \pm 1127$  pg ml<sup>-1</sup> vs  $358 \pm 111$ ; *P* < 0.0001) and M30 ( $1195 \pm 839$  pg ml<sup>-1</sup> vs  $185 \pm 133$ ; *P* < 0.0001) were significantly higher in cirrhotic patients than in 39 healthy controls (16 females, age: mean  $\pm$  s.d.  $39.6 \pm 10.0$  years). The results suggest a decreased chemokine expression and an increased rate of apoptosis and inflammation in cirrhotic patients. Serum levels of GCSF (*P* = 0.08) and FGF basic (*P* = 0.11) were similar in patients and healthy controls.

**Cirrhotic patients with HCC vs cirrhotic patients without HCC.** HCC patients with cirrhosis were older, had more HCV + and lower MELD score ( $11.8 \pm 5.0$  vs  $22.4 \pm 8.6$ ; *P* < 0.001), CRP ( $15.8 \pm 21.8$  mg l<sup>-1</sup> vs  $22.9 \pm 26.1$ ; *P* = 0.009) and neopterin ( $22.2 \pm 21.0$  nmol l<sup>-1</sup> vs  $58.1 \pm 76.8$ ; *P* < 0.001) than cirrhotic patients without HCC - (Table 1). AFP test was carried out in 102 cirrhotic patients; 23 of 49 HCC + and 9 of 53 HCC - patients had positive results (*P* < 0.0001). Serum levels of TPO ( $226 \pm 210$  pg ml<sup>-1</sup> vs  $163 \pm 247$ ; *P* < 0.001) and chemokines CCL4 ( $170 \pm 378$  pg ml<sup>-1</sup> vs  $101 \pm 483$ ; *P* < 0.0001), CCL5 ( $3.6 \pm 5.5$  vs  $2.0 \pm 4.5$  ng ml<sup>-1</sup>; *P* < 0.0001) and CXCL5 ( $230 \pm 301$  vs  $118 \pm 159$  pg ml<sup>-1</sup>; *P* < 0.001) were higher,

**Table 1. Demographic and characteristic data of cirrhotic patient groups**

Parameters	HCC + (n = 61)	HCC – (n = 78)	P
Age (mean ± s.d.; years)	56.5 ± 8.6	50.2 ± 11.6	0.006
Gender, female (n)	8	24	0.01
Child–Pugh category A/B/C (n)	25/19/11 <sup>a</sup>	24/17/35 <sup>a</sup>	0.03
MELD score	11.8 ± 5.0	22.4 ± 8.6	< 0.001
Serum CRP (mg l <sup>-1</sup> )	15.8 ± 21.8	22.9 ± 26.1	<b>0.009</b>
Serum neopterin (nmol l <sup>-1</sup> )	22.2 ± 21.0	58.1 ± 76.8	< 0.001
Previous transplantation (n)	2	15	<b>0.004</b>
Pre-tx encephalopathy (n)	8	33	< 0.001
<b>Viral IgG status<sup>a</sup></b>			
HBV-Ab + (n)	11	6	0.06
HCV-Ab + (n)	20	9	<b>0.002</b>
CMV-Ab + (n)	37	44	0.61
<b>Original liver disease</b>			
Hepatitis/alcoholic/others (n)	25/19/17	15/22/41	0.01

Abbreviations: Ab = antibody; CMV = cytomegalovirus; CRP = C-reactive protein; HBV = hepatitis B virus; HCC = hepatocellular carcinoma; HCV = hepatitis C virus; IgG = immunoglobulin; MELD = model for end-stage liver disease; Pre-Tx = pretransplant. Mann–Whitney U-test, chi square, Fisher exact and Kruskal–Wallis tests were used. P-values < 0.05 after Bonferroni correction were considered significant and are in bold.

<sup>a</sup>Some missed data.

**Table 2. Immune responses and laboratory data of patient groups**

Parameters (mean ± s.d.) (n)	HCs (n = 39)	HCC – (n = 78)	HCC + (n = 61)	P*
M30 (U l <sup>-1</sup> )	185 ± 133	1179 ± 856	1181 ± 870	1.00
TPO (pg ml <sup>-1</sup> )	501 ± 106	163 ± 247	226 ± 210	< 0.001
<b>CCL2 (pg ml<sup>-1</sup>)</b>	179 ± 71	245 ± 689	129 ± 192	0.008
Median	172	111	80	
CCL3 (pg ml <sup>-1</sup> )	70 ± 199	51 ± 370	10 ± 54	0.13
<b>CCL4 (pg ml<sup>-1</sup>)</b>	66 ± 32	101 ± 484	170 ± 378	< 0.0001
Median	63	37	88	
CCL5 (ng ml <sup>-1</sup> )	11 ± 11	2.0 ± 4.8	3.6 ± 5.5	< 0.0001
CXCL5 (pg ml <sup>-1</sup> )	766 ± 540	118 ± 159	230 ± 301	< 0.001

Abbreviations: CCL = C-C chemokine ligand; CXCL = C-X-C chemokine ligand; M30 = Caspase-cleaved cytokeratin 18 fragment; TPO = thrombopoietin. Mann–Whitney U-test was used. P-values < 0.05 after Bonferroni correction were considered significant and are in bold. \*P value of HCC+ vs HCC– patients.

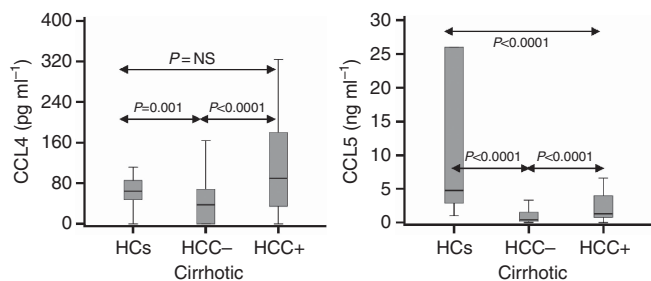


Figure 1. CCL4 and CCL5 serum levels in cirrhotic patients with and without HCC as well as in healthy controls (HCs).

whereas surprisingly CCL2 ( $129 \pm 192$  pg ml<sup>-1</sup> (median = 80) vs  $245 \pm 689$  (median = 111):  $P = 0.008$ ) was lower in HCC+ than in HCC– cirrhotic patients (Table 2 and Figure 1). Interestingly, the apoptosis marker M30 ( $P = 0.60$ ), M65 ( $P = 0.92$ ), GCSF ( $P = 0.66$ ), VEGF ( $P = 0.51$ ) and FGF basic ( $P = 0.28$ ) were similar in the two patient groups. The results indicate high expression of particular chemokines in HCC patients compared with cirrhotic patients without HCC.

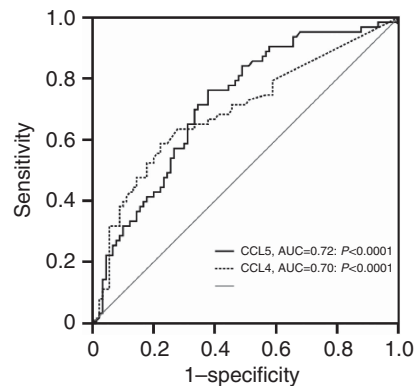


Figure 2. ROC curve analysis for calculation of diagnostic accuracy, cutoff value, sensitivity and specificity of CCL4 and CCL5 plasma levels for HCC screening in cirrhotic patients.

**Sensitivity, specificity, PPV, NPV and cutoff values of parameters.** To calculate sensitivities, specificities and cutoff values, we preformed ROC analysis for significant parameters. The best cutoff values were 170 pg, 66 pg, 0.86 ng and 84 pg ml<sup>-1</sup> for TPO, CCL4,

CCL5 and CXCL5, respectively. The sensitivity, specificity, PPV and NPV were 69, 67, 59 and 70% for TPO, 66, 74, 67 and 73% for CCL4, 71, 68, 62 and 74% for CCL5 and 71, 63, 59 and 72% for CXCL5, respectively (Figure 2). AFP had a sensitivity of 47%, a specificity of 89%, a PPV of 79% and a NPV of 64%.

**Logistic regression analysis for definition of HCC.** Univariate regression analyses showed that AFP  $\geq 20$  IU (Odds ratio (OR) = 5.10, CI 1.71–15.21;  $P = 0.003$ ), TPO  $\geq 170$  pg ml<sup>-1</sup> (OR = 3.74, CI 1.84–7.60;  $P < 0.001$ ), CCL4  $\geq 66$  pg ml<sup>-1</sup> (OR = 4.10, CI 2.00–8.36;  $P = 0.0001$ ), CXCL5  $\geq 84$  pg ml<sup>-1</sup> (OR = 4.00, CI 1.97–8.27;  $P = 0.0001$ ) and CCL5  $\geq 0.86$  ng ml<sup>-1</sup> (OR = 4.42, CI 2.16–9.06;  $P < 0.0001$ ) in cirrhotic patients are diagnostic markers of HCC. Multivariate forward stepwise regression analysis of significant parameters showed that, among the studied parameters, serum CCL4  $\geq 66$  pg ml<sup>-1</sup> (OR = 6.24, CI 2.55–15.23;  $P < 0.0001$ ), CCL5  $\geq 0.86$  ng ml<sup>-1</sup> (OR = 3.63, CI 1.48–8.88;  $P = 0.004$ ) and AFP  $\geq 20$  IU (OR = 5.69, CI 1.58–20.37;  $P = 0.004$ ) have the best association with HCC.

**Correlation between HCC and variables.** Spearman's rank correlation coefficient was used to calculate correlation between significant variables. HCC conversely correlated with MELD score ( $r = -0.382$ ;  $P < 0.0001$ ), serum CRP ( $r = -0.213$ ;  $P = 0.009$ ), neopterin ( $r = -0.353$ ;  $P < 0.001$ ) and IL-6 ( $r = -0.356$ ;  $P < 0.0001$ ) and positively with age ( $r = 0.401$ ;  $P < 0.0001$ ), TPO ( $r = 0.316$ ;  $P < 0.001$ ), CCL4 ( $r = 0.366$ ;  $P < 0.0001$ ), CCL5 ( $r = 0.363$ ;  $P < 0.0001$ ), CXCL5 ( $r = 0.319$ ;  $P < 0.001$ ) and AFP ( $r = 0.421$ ;  $P < 0.0001$ ).

**TACE procedure, tumour size, AFP and immune parameters.** TACE procedure was performed in 40 HCC patients. MELD score, liver function tests, chemokine levels, neopterin, CRP and albumin levels were similar in patients with and without TACE and in patients with and without detectable AFP ( $P =$  not significant for all investigations). Tumour size was only correlated with TPO ( $r = 0.41$ ;  $P = 0.002$ ).

## DISCUSSION

**Association of cytokines, chemokines and apoptotic markers with ESLD.** In the present study, we investigated the serum levels of biomarkers, including cytokeratin 18 (M65), cytokeratin 18 (CK18; M30) fragment, TPO, CC and CXC motif chemokines and AFP, to find a diagnostic marker for HCC. A detailed comparison of serum levels and/or production of CXCL5 and CC chemokines between healthy controls and patients with ESLD has not been performed so far. The results of our study indicate that patients with advanced fibrosis and ESLD are unable to produce sufficient chemokines, in accordance with previous studies showing low expression and production of chemokines in cirrhotic patients (Nischalke *et al*, 2004; Tacke *et al*, 2011; Zhang *et al*, 2014). CK18 is an apoptosis marker and a major intermediate filament protein in liver cells and a popular marker for detecting liver fibrosis in recent years (Feldstein *et al*, 2009; Chitturi *et al*, 2011; Jazwinski *et al*, 2012; Sumer *et al*, 2013). This apoptosis marker increased in sera of patients with cirrhotic liver diseases compared with healthy controls, reflecting the higher rate of apoptosis (Chitturi *et al*, 2011; Jazwinski *et al*, 2012). Chemokines – biomolecules that induce chemotaxis – have a fundamental role not only in inflammation and immune surveillance but also in cancer progression (Aldinucci *et al*, 2012). Chemokines are important mediators of host defense functioning in the recruitment and activation of leukocytes and other cells at the sites of injury, infection and neoplasia (Driscoll, 1994).

C-C receptor 5 is a receptor for CCL3, CCL4 and CCL5 and is expressed in effector and effector memory subsets of Th1 CD4+ T cells and CD8+ T cells (Kang and Shin, 2011). Previous

studies showed high expression of CCL4 and CCL5 chemokines in viral, alcoholic and autoimmune inflammatory diseases in the liver (Bautista, 2001; Bautista and Wang, 2001; Maghazachi, 2010; Kang and Shin, 2011). Intrahepatic and peripheral blood levels of chemokines CCL4 and CCL5 are increased during chronic hepatitis C (Larrubia *et al*, 2008). In patients with HCV infection intrahepatic expression of CCL4 and CCL5, especially in periportal and lobular areas is increased, and their levels of expression were significantly associated with the degree of liver inflammation (Larrubia *et al*, 2008). CCL4 and CCL5 expression have also been associated with liver histological activity index in chronic hepatitis C (Larrubia *et al*, 2008; Moura *et al*, 2009). CCL4 and CCL5 are potent chemoattractants for memory T lymphocytes, monocytes, NK cells and eosinophils, and they attract cells through an interaction with their receptor CCR5 (Navratilova, 2006; Maghazachi, 2010; Ingelsten *et al*, 2011). CCL5 was found to be highly expressed in activated T lymphocytes, macrophages, fibroblasts, platelets, mesangial cells, epithelial cells, megakaryocytes and some tumours (Navratilova, 2006; Charni *et al*, 2009; Takai *et al*, 2009; Maghazachi, 2010).

**Association of soluble factors and cancer.** Interaction between host cells and cancer cells happens via a large variety of soluble factors. The association of chemokines with cancer is not surprising, as they both promote and restrict tumour onset and/or progression (Gonzalez-Martin *et al*, 2012). CCL4 and CCL5 are expressed in human breast cancer and CCL5 is associated with the progression of particularly triple-negative (oestrogen, progesterone and tyrosine-protein kinase erbB-2 negative) breast cancer (Niwa *et al*, 2001; Wolf *et al*, 2003; Lv *et al*, 2013). This chemokine can regulate the function of effector cells and chemotaxis relies on its concentration. Therefore, the increased level of CCL5 can enhance its role in chemotaxis (Yoshie *et al*, 2001). CCL5 is minimally expressed by normal breast epithelial duct cells but is highly expressed by breast tumour cells, suggesting that this chemokine has a role in breast cancer development and/or progression (Soria and Ben-Baruch, 2008; Lapteva and Huang, 2010). Upregulation of CCL4 in human colon and gastric cancer was reported previously (Saito *et al*, 2003; Erreni *et al*, 2009). Following prostatectomy, the expression of CCL4 is associated with recurrence of prostate cancer and the absence of CCL4 expression supported recurrence-free survival after prostatectomy (Blum *et al*, 2008).

**Correlation of C-C chemokines to HCC.** HCC is the commonest primary malignant cancer of the liver in the world (Bosch *et al*, 2004). Some cancer staging systems, grading severity of dysfunction and tumour burden can estimate the survival of HCC patients (Hsu *et al*, 2010).

CCR1 binds a particular set of C-C chemokines, including CCL3, CCL4 and CCL5, and is expressed by a variety of cells, including lymphocytes, monocytes, basophils, neutrophils and bone marrow progenitor cells (Lu *et al*, 2003). CCR5, another specific receptor for CCL3, CCL4 and CCL5, was also detected in tumour-infiltrating lymphocytes but not in hepatoma cells (Yoong *et al*, 1999). Lu *et al* (2003) provided that hepatoma cells express CCR1 *in vitro* and *in vivo*. The correlation between CCL5 and HCC is a controversial issue (Yoshie *et al*, 2001), whereas an association between HCC susceptibility and genetic polymorphism of CCL5-28 and CCL5-403 was described (Yoshie *et al*, 2001). A study by Liu *et al* (1999) provides evidence of CCL5-28G mutation with increased transcription of the RANTES gene.

To our knowledge, the association of HCC with CCL4 gene expression and/or serum levels was not studied so far. Thus our report is the first study that evaluates this association. CCL4 and

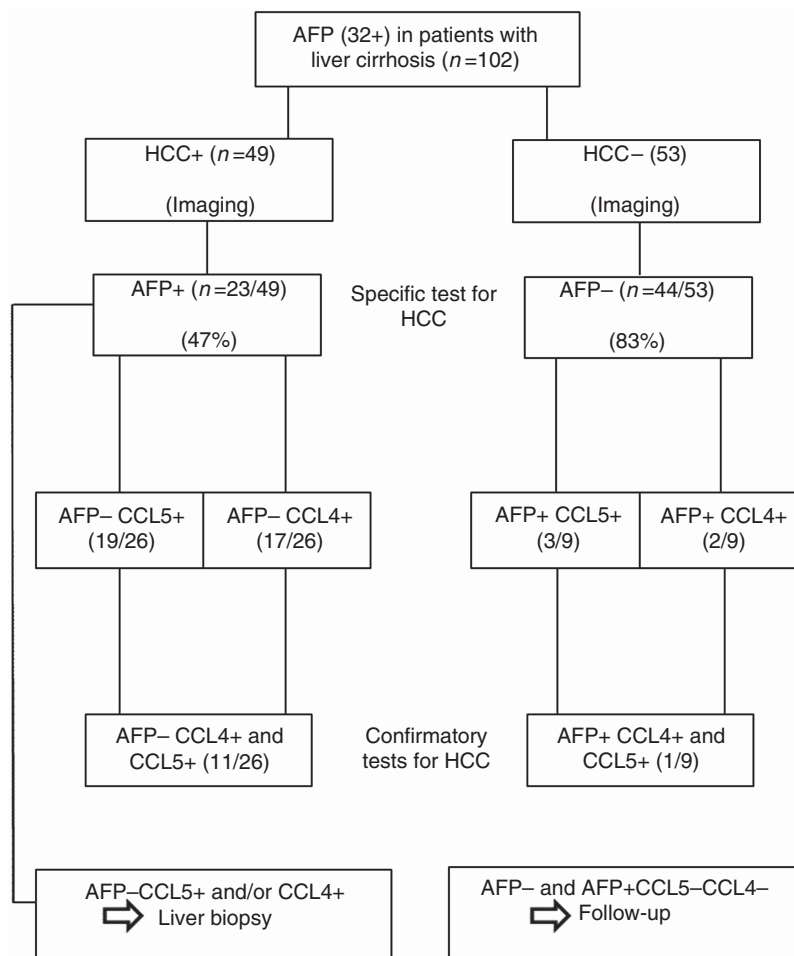


Figure 3. Algorithmic approach to AFP, CCL4 and CCL5 tests in cirrhotic patients.

CCL5 can bind to their receptors in the carcinomatous tissues and Kupffer cells in the liver and induce the infiltration of various inflammatory cells. We speculate that cirrhotic high producers of CCL4 and CCL5 are at higher risk of HCC. It can be postulated that high serum levels of CCL4 and CCL5 indicate the presence of HCC in patients with liver cirrhosis and along with other indicators can help in early detection of HCC. Algorithmic approach of our results (Figure 3) indicates that AFP is a specific test in patients with suspected HCC (imaging). When imaging is positive but AFP is negative, surrogate markers such as CCL5 and CCL4 are confirmatory tests. We recommend a liver biopsy in patients with positive imaging and negative AFP, when CCL5 and/or CCL4 are positive.

Furthermore, future large prospective studies may be needed to investigate the association between these chemokines and hepatocellular carcinoma. Until now, AFP together with imaging and pathology detection are commonly used in the clinical early diagnosis of liver cancer. However, the specificity and sensitivity of AFP used in screening for liver cancer are not satisfactory (Zhao *et al.*, 2013). The results of the present study indicate that CCL4 and CCL5 are more sensitive and AFP more specific markers of HCC and that monitoring the combination of these markers might be an innovative approach in the early diagnosis of HCC.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### AUTHOR CONTRIBUTIONS

Mahmoud Sadeghi designed, analysed and wrote the manuscript; Imad Lahdou assisted to design and performed the chemokines and cytokine tests. Hani Oweira operated patients and collected the serum samples. Jan Schmidt and Peter Schemmer treated the patients. Volker Daniel, Peter Terness, Karl Heinz Weiss, Thomas Longerich and Gerhard Opelz assisted in writing the manuscript. Ariane Mehrabi treated the patients and assisted in preparing and writing the manuscript.

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