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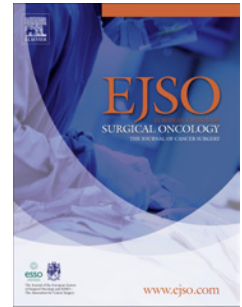
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# **Title: Haptocorrin as marker of disease progression in fibrolamellar hepatocellular carcinoma**

DL Lildballe,<sup>a\*</sup> KQT Nguyen,<sup>b1</sup> SS Poulsen,<sup>c</sup> HO Nielsen,<sup>d</sup> E Nexø<sup>e</sup>

a: Department of Clinical Biochemistry; Aarhus University Hospital; Norrebrogade 44, DK-8000 Aarhus; E-mail: dolild@rm.dk

b: Department of Clinical Biochemistry and Pharmacology; Odense University Hospital; Sdr. Boulevard 29, DK-5000 Odense; E-mail: kirstennguyen@gmail.com

c: Institute of Biomedical Sciences; Panum Institute; University of Copenhagen Blegdamsvej 3, DK-2200 Copenhagen N; spoulsen@sund.ku.dk

d: Department of Surgical Gastroenterology, Odense University Hospital; Sdr Boulevard 29, DK-5000 Odense; E-mail: henning.nielsen@ouh.regionsyddanmark.dk

e: Department of Clinical Biochemistry; Aarhus University Hospital; Norrebrogade 44, DK-8000 Aarhus; E-mail: ebbanexo@dadlnet.dk

1: Current address: Department of Clinical Pathology; Lillebaelt Hospital, 7000 Vejle, Denmark

\*Corresponding author: Dorte Launholt Lildballe; Department of Clinical Biochemistry; Aarhus University Hospital (AS), Norrebrogade 44, DK-8000 Aarhus; e-mail: dolild@rm.dk; fax: +45 8949 3060; phone: +45 8949 9743

## Abstract

**Aims:** No valid markers are routinely available to follow disease progression in patients with fibrolamellar hepatocellular carcinoma (FLHCC). We report data suggesting that the vitamin B12 binding protein haptocorrin (HC) may prove a suitable marker.

**Methods:** We monitored a 15-year-old boy diagnosed to have FLHCC by measuring the common markers alanin aminotransaminase, alkaline phosphatase, lactatdehydrogenase, and bilirubin, as well as vitamin B12 (B12), and the forms of the B12 binding proteins. Tumour biopsies were examined immunohistologically. DNA and RNA were extracted from tumour and normal tissue and examined for content of HC DNA and mRNA.

**Results:** The only markers indicative of disease progression were HC and (B12), levels of which were markedly elevated to 84 (11) nmol/L at the time of diagnosis and returned to values within the reference interval (0.43 (0.33) nmol/L) after an apparently radical removal of the tumour. The disappearance rate of HC followed a biphasic curve, the unsaturated protein displaying a half-life of 2.8 days and B12 and saturated HC one of 13 days. Before each diagnosed relapse, an increased concentration of HC was observed. We found a strong immunoreaction against HC in tumour tissue and a high mRNA expression of HC supporting the notion that HC was tumour derived.

**Conclusions:** Plasma HC proved to be a useful tumour marker in a patient with FLHCC, and we suggest the use of this protein as a marker of disease progression in these patients.

## Keywords

Fibrolamellar hepatocellular carcinoma; Vitamin B12; haptocorrin

## Introduction

Fibrolamellar hepatocellular carcinoma (FLHCC) is a subtype of primary liver cancer usually diagnosed in young patients with no underlying liver diseases. Liver enzymes are typically normal or only mildly elevated (1) and elevation of the tumour marker  $\alpha$ -foetoprotein (AFP) is rarely seen (1;2). Currently, a routine biomarker to monitor disease progression in patients with FLHCC is not available.

The vitamin B12 (B12) binding protein haptocorrin (HC, previously known as R-binder, TCI, and TCIII) is normally present in the circulation where it carries most of the circulating B12. Its function remains unknown but its concentration has been reported to be greatly elevated in patients with FLHCC (3). Therefore, HC has previously been suggested as tumour marker (2;4-7) but few studies have used the concentration of HC to follow the disease for a prolonged period of time.

In this study, we demonstrate the usefulness of measuring total HC in order to monitor the progression of the disease in a young patient diagnosed with FLHCC.

## Patients and Methods

### Patient

In April 2007, a 15-year-old boy presented with hepatomegaly, and fatigue, and weight and growth retardation compared with his monozygotic twin. Laboratory tests showed a mildly elevated alanin aminotransaminase (ALAT) and increased lactate dehydrogenase (LDH). Further data are summarised in Table 1.

A CT-scan of the abdomen revealed a large tumour-suspicious lesion occupying nearly the entire right hepatic lobe; no enlarged lymph nodes were observed.

Microscopic examination of a liver biopsy revealed lamellar bands of fibrosis and large, polygonal cells with abundant granular eosinophilic cytoplasm, and prominent nucleolus. A diagnosis of FLHCC was made (Fig. 1A).

A hemihepatectomy (OP1) was performed successfully and follow-up magnetic resonance imaging (MRI) revealed satisfactory removal of the tumour. However, 7 months later a new MRI disclosed a 1 cm lesion, this time in the left liver lobe. At operation, this and a second tumour found during the operation were removed (OP2). Histological examination confirmed recurrence of FLHCC. Two months after OP2, a new tumour was found. The patient underwent surgery again (OP3) and subsequently received alternating thalidomide and cyclophosphamide for 12 months. Throughout the study, HC was employed to monitor the disease progression. Three months before completing chemotherapy, HC levels increased again suggesting a new relapse. The suspicion of recurrence remained strong although ultrasound and MRI of the liver as well as a CT-scan of the thorax were normal, and the patient was referred for a liver transplantation (OP4). Two tumours were observed in the explanted liver and microscopic examinations revealed tumour cells in an adjacent

lymph node (Figure 1D). Metastatic lesions in the mediastinum were identified by an MR scanning 5 weeks after the liver transplantation. HC declined following the transplantation to values around 1 nmol/L but never reached levels as low as after OP1. In accord with the occurrence of metastatic lesions the HC levels increased during the following months to reach values around 10 nmol/L (data not shown). This study was carried in accordance with the Helsinki Declaration and the patient and his parents gave informed consent allowing for publication of the study.

## Methods

### Biochemical methods

Routine laboratory methods were used for measurements of total **B12** based on immunoassay (*Cobas 6000 E*; Roche, Mannheim, Germany), imprecision <5%. Samples with >6nM unsaturated B12 binding capacity were diluted with B12-free 0.9% NaCl (VWR, Denmark) to ensure accurate measurement of B12 (8). **ALAT**, **LDH**, **alkaline phosphatase (AP)**, and  **$\gamma$ -glutamyltransferase** were measured using enzymatic activity assay (Modular P; Roche), imprecision <5%, <4%, <3% and <7%, respectively. **C-reactive protein**, **orosomucod**; **immunoglobulin G**, and **immunoglobulin A** were measured using immuno-turbidimetric analysis (Modular P; Roche), imprecision <3%, <6%, <3%, and <3%, respectively.  **$\alpha$ -foetoprotein (AFP)** and **choriongonadotropin** were measured using fluoroimmunoassay (1235 AutoDELFIA™; Wallac Oy, Turku, Finland), imprecision <10%. **Albumin** and **bilirubin** were measured using a dye-binding photometric method (Modular P), imprecision 1.6% and 5%., respectively **Haemoglobin** was measured by spectrophotometry (Beckman Coulter LH750), imprecision <2%. **Carcinoembryonic antigen** was measured with an enzyme enhanced chemiluminescence assay

(IMMULITE), imprecision <6%. **Hepatitis** vira were measured by enzyme immunoassay (Architect, Abbott).

Unsaturated B12 binding capacity (**UBBC**) was analysed according to (9). The concentration of total **TC** and total and B12-saturated (holo-)**HC** were measured with ELISA-based assays, imprecision 4-7%, as described previously (10;11).

Unsaturated (apo-)**HC** was calculated as the difference between **HC** and holo**HC**.

### **Immunohistochemistry**

Specimens from the carcinoma (OP1) and a lymph node with infiltration of tumour cells (OP2) were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Histological sections were stained with haematoxylin–eosin and periodic acid Schiff (PAS). Immunohistochemistry was performed by means of an in-house rabbit polyclonal antibody as described previously (12). A specimen of normal trachea was used as positive control for the immunoreactions.

### **Molecular biology**

Genomic DNA was extracted from tissue samples taken at OP4 as described previously (13). For standard curves, DNA extracted from MCF-7 cells (ATCC-LGC Standards) in a dilution serie of 0.10 to 100ng/μL was used. RNA extraction from tissue samples obtained at OP4 and the cDNA synthesis thereof were done as described previously (14;15). For standard curves, total RNA extracted from MDA-MB-231 cells (ATCC-LGC Standards, Sweden) was used in dilution series of 0.001 to 1 μg/μL RNA.

Real time PCR was performed as described previously (14; 15), the only modification being that 96 well plates were used instead of glass capillaries. Further information is given in Supplementary Data (Table A).



**Literature search**

To review previous information concerning FLHCC and HC, we conducted the following searches in Pubmed: “vitamin B12 binding protein AND hepatocellular carcinoma”, “vitamin B12 AND hepatocellular carcinoma” and “Fibrolamellar hepatocellular carcinoma AND marker”. The search gave a total of 29, 60, and 64 publications, respectively. After duplicates were excluded, the titles and abstracts of the remaining 122 publications were reviewed. A total of eight articles proved to be of relevance based on the presence of FLHCC or APF-negative HCC and measurements of B12, UBBC, and/or HC.

**Data analysis**

The disappearance-rate ( $t_{1/2}$ ) for HC following removal of the tumour was analysed by linear regression of a semi-logarithmic plot with time against  $\ln(\text{Conc}(\text{HC}))$  employing GraphPad Prism 4.03 (GraphPad Software Inc).

## Results

### A boy with FLHCC and increased concentrations of HC

We report data on a 15-year-old boy diagnosed as having fibrolamellar hepatocellular carcinoma (FLHCC) (Fig. 1A). At the time of diagnosis, only minor biochemical changes were observed, and the only significantly abnormal parameters were plasma B12 and HC (Table 1).

HC, B12 and the commonly used liver markers ALAT, AP, bilirubin, and LDH were monitored during two periods: 1) The parameters were measured at least weekly from the initial operation (OP1) and until the patient was believed to be cured (3 months after OP1) and 2), from the time point at which a relapse was suspected (7 months after OP1) and until the patient was referred for a liver transplantation 18 months later during which time the parameters were measured monthly (Fig. 2).

Both before and after OP1, AP and bilirubin remained within the reference intervals; LDH and ALAT were initially above the reference interval but normalised within 2 and 9 days after OP1, respectively. During months 7 to 25 after OP1, only minor fluctuations were observed. After the third operation (OP3), these four parameters remained within the reference intervals.

The most dramatic alterations were observed for HC and B12 (Figs. 2A & B). At diagnosis, the plasma concentrations were elevated for both HC (~100 fold) and B12 (~20 fold). The other B12 binding protein transcobalamin (TC) remained unchanged and within normal ranges throughout the study (data not shown).

Both apoHC and holoHC were measured before and after OP1. Interestingly, we observed a rapid decline in apoHC ( $t_{1/2} = 2.8$  days) whereas holoHC showed a  $t_{1/2}$  of 13 days (Fig. 3). Within 2.5 months after OP1, totalHC, apoHC, holoHC, and B12

were within the central part of the reference intervals and at this point, the patient was considered cured.

When a relapse was suspected 7 months after OP1, slight increases in HC and B12 were apparent. The concentrations of HC and B12 increased prior to tumour removal and decreased again after both OP2 and OP3. HC and B12 increased steadily from 9 months after OP3 and until the end of the study (patient referred for liver transplant). Immunohistochemical examination of liver tissues revealed a high level of HC in tumour cells especially in the strands of tumour cells seen at the peripheral part of the tumour tissue (Fig. 1B-C). and in an adjacent lymph node (Fig. 1D-E), there were several cells immunoreactive to HC.

The, mRNA transcript level of HC was 16-fold higher in tumour tissue as compared with normal liver tissue ( $p < 0.001$ ), while no difference was observed in DNA level ( $p = 0.79$ ).

### **A review of the literature of patients with FLHCC and increased levels of HC**

Review of the literature revealed 20 published cases of FLHCC with measurement of B12, HC, and/or unsaturated B12 binding capacity (UBBC) (Table 2). As is apparent from the table, UBBC was increased at the time of diagnosis, ranging from slightly increased to 200-fold above the normal range. In nine cases, the increased UBBC was accompanied by elevated HC levels.

## Discussion

### HC as a marker for FLHCC

We report a patient with FLHCC and a marked increase in HC and B12 but only a slight elevation of other markers of liver disease. FLHCC most often occurs in young individuals without other liver diseases, and most often biochemical changes are moderate. A few cases with increased concentrations of des-gamma-carboxy prothrombin and neurotensin have been reported (16;17) but the clinical applicability of these markers is still unclear.

A significant increase in the concentration of HC has been reported in a number of malignant conditions, notably in most patients with chronic myeloid leukaemia (18;19) and in sporadic cases of other types of cancer including breast cancer (20). As apparent from Table 2, an increased concentration of HC also occurs in patients with FLHCC. 13 cases published before 1980, but never sub-typed as FLHCC, showed an increased plasma B12, HC, and/or UBBC (3;7;21). They were young, had no underlying liver disease and showed no increase in AFP and they, all probably had FLHCC. Small-scale studies analysing the frequency of elevated UBBC or HC in FLHCC have shown that elevated concentrations can be found in 80% to 87% of the patients with this subtype (2;22). In contrast, only 3% of patients with other types of HCC show a marked increase in the plasma levels of these parameters (2).

Although HC is not observed from hepatocytes of healthy individuals (23), it is commonly seen in the noncancerous hepatocytes of FLHCC patients (23;24).

Immunohistochemistry showed a similar pattern in this patient, in whom HC was very abundant in tumour tissue (Fig. 1B+C) and only sporadically present in normal liver tissue (data not shown). Real time rtPCR showed that HC transcript was significantly increased in the tumour tissue compared with the patient's normal liver tissue. This

supports the supposition that the cancer caused the increased level of HC measured in the circulation of this patient with FLHCC and not, as previously suggested, because of a diminished clearance of HC in the liver (25) . We found no duplication of the gene coding for HC, and at present we do not know the reason for the increased expression of HC.

### **HC level is linked to disease progression**

Our patient showed fluctuations in HC that closely followed the progression of the disease (Fig. 2), and this pattern of HC following disease progression is in accord with previously published cases (Table 2): Five reported patients who experienced remission after chemotherapy or resection all had significantly reduced or normalised UBBC and/or HC. UBBC/HC levels increased in two out of three cases prior to radiological evidence of recurrence. UBBC continued to rise in five other patients, in whom no response to treatment was seen. Taken together, the results therefore suggest that HC can be used to monitor disease progression in patients with FLHCC.

### **HC disappears in a biphasic manner after tumour removal**

After an apparent radical removal of the liver tumour our patient showed a biphasic disappearance curve for HC. Interestingly, apoHC showed a fast disappearance rate, whereas holoHC and B12 showed a disappearance rate in accord with the previously described half-life for HC (24).

Burger and coworkers have purified HC from two patients likely to have had FLHCC (24) and Nexo has purified HC from plasma of a third patient likely to have had FLHCC (26). In all three cases, the proteins were characterised by a high content of sialic acid compared with HC from other sources (27). HC is cleared in the liver by the asialo receptor (28), and high contents of sialic acid are known to protect the protein from degradation yielding a half-life of 8 to 11 days (29) . In contrast, after

removing the sialic acid enzymatically, the half-life of HC is significantly reduced (28).

Previously, no differences in metabolism have been seen between apoHC and holoHC (30). Our study is the first to analyse the disappearance of HC in the circulation after an apparently complete removal of the HC-producing tumour. We expected to observe a half-life comparable with the previously reported data for sialated HC, and we did so for holoHC and B12. To our surprise we observed a much shorter half-life for apoHC. The results imply that the tumour release isoforms of HC with various half-lives. The differences in half-lives for apoHC and holoHC may be explained if all HC is released in its apo form and meets B12 in the circulation in agreement with the recent suggestion that B12 meets its binding proteins in the circulation (31). Assuming a steady influx of B12 into the circulation, proteins with short half-lives have less chance of meeting a B12 molecule than do proteins with a longer half-life. The net result will be that HC with a fast clearance occurs mainly as apoHC, whereas HC with a slow clearance occurs mainly as holoHC. Independent of the explanation for the biphasic disappearance of HC, the observed heterogeneity in the turnover is obviously of importance for the interpretation of measurements of total HC as a marker for successful surgical removal of FLHCC tumours.

### **High HC levels may induce megaloblastic anaemia but only in extreme cases**

HC competes with TC for binding to B12 released into the circulation. Since TC is the protein responsible for delivering B12 to the cells in the body, there has been concern that patients with high apoHC are at risk of developing B12 deficiency (29;32). We did not find signs of B12 deficiency in the patient described here, and to our knowledge, only one patient with a HC-induced megaloblastic anemia has been described. The patient had HC levels 10 000-fold above the reference interval (7),

levels which are considerably higher than the values of 1.5-220 times above the reference interval reported in other patients with FLHCC (Table 2).

### **Laboratory methods for HC as a marker of progression of FLHCC**

The concentration of HC can be measured using ELISA methods, although such assays are not widely available. However, routine assays for B12 may be a suitable proxy for the concentration of HC provided the absorption of B12 is normal and provided that the concentration of the other circulating B12 binding protein, TC, is normal. A further caution for the use of B12 as a proxy for HC relates to the recent observation that a high concentration of apoHC may interfere with current routine assays used for measurements of B12, giving rise to spuriously too high or too low results depending on the methods employed (8).

### **Conclusion**

In conclusion, our study, together with previously reported data, strongly supports the use of HC as a marker of disease progression in patients with FLHCC.

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## Abbreviations

AFP, alpha-foetoprotein; ALAT, alanin aminotransaminase; AP, alkaline phosphatase; apo, unsaturated; B12, vitamin B12; FDG-PET, fluorodeoxyglucose-positron emission tomography; (FL)HCC, (fibrolamellar) hepatocellular carcinoma; holo, saturated; HC, haptocorrin; LDH, lactatdehydrogenase; MRI, magnetic resonance imaging; OP, operation; PAS, Periodic Acid Schiff PAS; TC, transcobalamin.

## Conflict of interests statement

None declared.

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**Table 1** Laboratory data of a 15-year-old boy at the time of diagnosis of a fibrolamellar hepatocellular carcinoma.

<b>Haematology</b>	
B-Haemoglobin (Fe); mmol/L	8.2 (8.0-11.0)
B-Leucocytes; 10 <sup>9</sup> /L	8.3 (3.0-10.0)
B-Neutrophils; 10 <sup>9</sup> /L	4.65 (1.50-7.50)
B-Thrombocytes; 10 <sup>9</sup> /L	<b>480</b> (120-400)
<b>Liver function</b>	
P-Albumin; g/L	40 (40-51)
P-Lactate dehydrogenase (LDH); U/L	<b>400</b> (105-205)
P-Alanine aminotransaminase (ALAT); U/L	<b>80</b> (10-70)
P-Alkaline phosphatase (AP); U/L	200 (100-400)
P-Gamma-glutamyltransferase; U/L	76 (10-80)
P-Bilirubins (total); µmol/L	4 (<20)
<b>Immunology/Inflammation</b>	
P-C-reactive protein; mg/L	<b>13</b> (<10)
P-Orosomucoid; g/L	<b>1.8</b> (0.54-1.17)
P-Immunoglobulin G; g/L	11 (6.1-15.1)
P-Immunoglobulin M; g/L	0.96 (0.33-2.02)
<b>Infection</b>	
HBsAg	Negative
Anti-HBc	Negative
<b>Tumour markers</b>	
S-Alpha-1-foetoprotein (AFP); 10 <sup>3</sup> IU/L	2 (<14)
S-Carcinoembryonal antigen; µg/L	0.9 (<5.0)
S- Human choriongonadotropin; IU/L	< 2 (<10)
<b>Haptocorrin and related parameters</b>	
P-Total haptocorrin (totalHC); nmol/L	<b>84</b> (0.25-0.84)
P-Holo-haptocorrin (holoHC); nmol/L	<b>16</b> (0.24-0.68)
P-Apo-haptocorrin (apoHC); nmol/L	<b>68</b> (0.07-0.35)
P-Cobalamin (B12); nmol/L	<b>11</b> (0-.2-0.6)

**Table 2** A review of patients with fibrolamellar hepatocellular carcinoma (FLHCC) in whom plasma vitamin B12 (B12), unsaturated vitamin B12 binding capacity (UBBC), and/or haptocorrin (HC) had been examined.

Case <sup>(Ref)</sup>	Age/sex	AFP <sup>a</sup>	FLHCC	B12, nmol/L (ref. interval: ~0.16-0.6)	UBBC, nmol/L (ref. interval: ~0.40-1.4)	HC <sup>b</sup>	Key findings
1 <sup>(2;6)</sup>	15/F	N	Yes	11	310	n.d.	UBBC ↑ with disease progression
2 <sup>(2;6)</sup>	16/M	N	Yes	2.6	32	n.d.	UBBC ↓ during remission but rose with relapse
3 <sup>(2;6)</sup>	30/F	N	Yes	9.6	58	n.d.	UBBC normalized after resection
4 <sup>(2)</sup>	26/M	N	Yes	n.d.	130	n.d.	UBBC ↑ in 3 patients who did not respond to chemotherapy. In 1 patient UBBC ↓ after operation
5 <sup>(2)</sup>	20/M	N	Yes	n.d.	110		
6 <sup>(2)</sup>	16/F	N	Yes	n.d.	140		
7 <sup>(2)</sup>	21/F	N	Yes	> 0.7	27		
8 <sup>(2)</sup>	n.a.	N	Yes	n.d.	N		
9 <sup>(4)</sup>	12/F	N	Yes	n.d.	2	↑	UBBC and HC normalized after operation and chemotherapy, but started increasing again 18 mo before recurrence could be detected
10 <sup>(31)</sup>	17/F	N	Yes	n.d.	↑ 4-6 fold	n.d.	↑ UBBC at recurrence which normalized after resection. UBBC rose again when peritoneal dissemination was found
11-20 <sup>(22)</sup>	16-50 /M&F	N/↑ /n.d. <sup>c</sup>	Yes	n.d.	↑ in 8 (range: 0.38- 72) n.d. in 2	↑ in 8 n.d. in 2	In 1 patient, UBBC and HC rose post-operatively. After resection of a lymph node detectable by FDG-PET <sup>d</sup> but not CT, UBBC and HC fell
21 <sup>this work</sup>	15/M	N	Yes	12	83	↑	↑ HC at every relapse but normalized after each operation

n.d.: not determined.

<sup>a</sup>: AFP:  $\alpha$ -foetoprotein; N: normal level within the reference interval.

<sup>b</sup>: HC levels were measured employing various methods. Arrow indicates an increase relative to the appropriate reference interval.

<sup>c</sup>: AFP was analysed in 6/10 patients in whom four had normal and two had slightly increased levels.

<sup>d</sup>: FDG-PET: fluorodeoxyglucose-positron emission tomography.

