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# Diagnostic and mechanistic implications of serum free light chains, albumin and alpha-fetoprotein in hepatocellular carcinoma

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**Background:** Mass spectroscopy analysis suggested low serum albumin and high immunoglobulin free light chain (sFLC) levels may have diagnostic value in hepatocellular carcinoma (HCC). Our aims were to apply quantitative assays to confirm these observations, determine their diagnostic utility, and investigate the mechanisms involved.

**Methods:** Albumin, sFLC, routine liver and renal function tests were measured in patients with chronic liver disease with ($n=102$) and without ($n=113$) HCC. The discriminant performance was compared with the current standard serological test alpha-fetoprotein (AFP) using receiver operating characteristic (ROC) and area under the curve (AUC) analyses.

**Results:** sFLC and serum albumin were each confirmed to have discriminatory utility in HCC with AUC values of 0.7 and 0.8, respectively. sFLC were strongly correlated with gammaglobulin levels and both these were inversely related to serum albumin levels. The discriminatory utility of sFLC was retained after adjusting for renal and liver function.

**Conclusions:** Serum levels of sFLC and albumin were strongly associated with HCC as predicted by mass spectroscopy. Discrimination of HCC by AFP was improved by the addition of either albumin or sFLC. Larger prospective studies are required to determine how AFP, sFLC and albumin might be combined in a useful diagnostic approach for HCC.

Early diagnosis of hepatocellular carcinoma (HCC) is widely considered to be the key to effective therapy. Radical, potentially curative treatments, such as surgical resection, radiofrequency ablation and orthotopic liver transplantation are only applicable to that minority of patients who present with, or who are detected by screening to have, smaller tumours, typically $<5$ cm in diameter (Bruix and Sherman, 2005). This observation has led to routine screening/surveillance of patients at high risk of HCC, namely those with advanced chronic liver disease and certain carriers of the hepatitis B or C viruses (HBV, HCV). Both in the screening situation and the clinical situation where it is required to determine if HCC has developed in a patient with symptoms, an imaging procedure, usually ultrasound, followed by a CT or MRI are the primary diagnostic modalities supplemented where necessary with histological confirmation by biopsy (Bruix and Sherman, 2005). Characteristic imaging features such as arterial enhancement and portal phase 'washout' have been recognised to be highly sensitive and specific features for the diagnosis of HCC (Torzilli *et al*, 1999; Sangiovanni *et al*, 2010). Nonetheless, such an approach is expensive and ultrasound examination is time-consuming and highly operator-dependent so, a simple serological diagnostic test would be invaluable. Estimation of serum alpha-fetoprotein (AFP) has been widely used in this respect and indeed it is well recognised to be highly specific and useful in the clinical setting when levels are elevated above a particular threshold typically set between 200 and 500 ng ml$^{-1}$ (Johnson, 2001). However, the role of AFP in the screening setting remains controversial, some supporting its utility (Marrero and El-Serag, 2011) and others, because of its low

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sensitivity in the early stages of the disease, have suggested that AFP has no role in the screening setting (Bruix and Sherman, 2005; Bruix and Sherman, 2011).

In a search for an alternative or additional serum markers for HCC, we and others (Poon *et al*, 2003; Schwegler *et al*, 2005; Ward *et al*, 2006b) have used surface-enhanced laser desorption/ionisation (SELDI) mass spectroscopy to profile the serum proteomes of patients with HCC and chronic liver disease and those with chronic liver disease alone with a view to identify a specific HCC signature. Although our studies showed that, in principle, there was a characteristic 'signature', the early studies did not identify the proteins responsible for the discrimination and as such were regarded as 'black box' models (Poon *et al*, 2003). Subsequently, in a study of hepatitis C-related HCC, we developed a model that appeared to have high sensitivity and specificity and that performed well on an independent test set (Ward *et al*, 2006a). Furthermore, we identified the most discriminatory of the identified peaks as representing serum immunoglobulin free light chains (sFLC) and albumin. Recognising that the spectroscopic profiling techniques for measuring these proteins were only semi-quantitative, and that is difficult to envisage a plausible explanation for the apparent diagnostic value of sFLC or albumin, the model was not pursued further.

More recently, a sensitive assay for sFLC has become available (Bradwell *et al*, 2001) and it has been shown that concentrations of sFLC are elevated in chronic liver disease (Assi *et al*, 2010). We have, therefore, revisited this area to examine the extent to which the mass spectroscopy-based prediction of diagnostic utility could be confirmed when specific assays were used.

## MATERIALS AND METHODS

Hundred and two subjects with HCC were recruited from patients referred to the Queen Elizabeth Hospital, Birmingham, UK. For all the patients, the diagnosis was established by histology or the combination of characteristic radiology together with an elevated AFP level according to international guidelines (Bruix and Sherman, 2005). Control samples were recruited from 113 patients who were attending out-patient clinics for chronic liver disease in the same institution. The various aetiologies were classified as HBV-related, HCV-related, alcoholic-related and other. The latter group comprised patients with haemochromatosis, primary biliary cirrhosis, non-alcoholic steatohepatitis, or cryptogenic cirrhosis. The diagnosis of chronic liver disease was made on the basis of liver biopsy and/or typical clinical and imaging features. None of the control groups had evidence of HCC at the time when the sample was taken or with a follow-up period of 9 months.

Samples were collected with full informed consent and after approval by the local ethics committee, prospectively and specifically for the purpose of biomarker discovery and validation. Routine liver and renal function tests (including cystatin C) were measured and the severity of the liver disease was defined according to the Child's classification (Child and Turcotte, 1964; Pugh *et al*, 1973) and the model for end-stage liver disease (MELD) score (Kamath and Kim, 2007). Standard liver and renal function tests were measured using the Roche Modular Analytics system (Roche Diagnostics Ltd, Burgess Hill, UK). Hepatitis B surface antigen and anti HCV were measured using the Roche Hepatitis system.

Samples were assessed for total IgG, total IgA and total IgM (Dade Behring, Siemens, Germany), serum free kappa (FLC$\kappa$) and serum free lambda (FLC$\lambda$) light chains (Freelite, The Binding Site Group Ltd, Birmingham, UK) and cystatin C (The Binding Site Group Ltd). The reference range used for the sFLC analysis were: FLC$\kappa$ 3.3–19.4 mg l$^{-1}$, FLC$\lambda$ 5.71–26.3 mg l$^{-1}$ and FLC ratio 0.26–1.65 (Katzmann *et al*, 2002). The reference range for cystatin C was 0.56–0.99 mg l$^{-1}$ and the reference range for IgG, IgA and IgM were 6–16 g l$^{-1}$, 0.8–4 g l$^{-1}$ and 0.5–2 g l$^{-1}$, respectively (Ward *et al*, 2004).

**Statistical analysis.** Statistical analysis was conducted using Stata11 (StataCorp, College Station, TX, USA). The distribution of FLC$\kappa$ and FLC$\lambda$ was plotted in box and whisker format. One extreme value of FLC$\kappa$ (592 mg l$^{-1}$) with a highly abnormal FLC ratio of 619 suggesting undiagnosed multiple myeloma/B-cell dyscrasia was excluded. In descriptive statistics, Mann–Whitney *U* test was used to assess the difference in distribution of sFLC and albumin between the HCC and the control group. Logarithmic transformation was applied to transform skewed data to correct for overdispersion. Univariate analysis was performed for each potential biomarker and serum parameter. Receiver operating characteristic (ROC) analysis was used to assess the discriminant performance of various biomarkers alone and in combination by comparing the area under the curve (AUC). Serum AFP was used as the current standard serological test for comparison and as a baseline to determine whether sFLC or albumin could add discriminatory power. A *P*-value of $<0.05$ was considered statistically significant.

## RESULTS

There was a highly significant increase in the concentration of both FLC$\kappa$ and FLC$\lambda$ in the HCC patients (median of 34.81 mg l$^{-1}$ and 32.50 mg l$^{-1}$ for the HCC patients *vs* 18.20 mg l$^{-1}$ and 18.20 mg l$^{-1}$ for the control group, respectively, $P<0.0001$, Mann–Whitney *U* test) (Figure 1A and B). Serum albumin levels were signficantly lower in the HCC patients (medians of 37 g l$^{-1}$ in the HCC group and 45 g l$^{-1}$ in the control group, $P<0.0001$, Mann–Whitney *U* test) (Figure 1C). Levels of sFLC varied with disease aetiology. In particular, the elevation of both FLC$\kappa$ and FLC$\lambda$ was most marked in HCV-positive HCC patients (Figure 1A and B).

Both albumin and either of the light chains had discriminatory power with AUC ROC values between 0.73 and 0.77, respectively (Figure 2). We then used multivariant logistic regression analysis to assess the impact of sFLC when adjusted for individuals' renal function (as measured by creatinine or cystatin C) and/or liver function (as measured by bilirubin, INR, or MELD). Both cystatin C and MELD were significant in univariate analyses but both dropped out of the model at 95% significance when either of the sFLC were included into the model. Being aware that the standard serological marker for HCC was serum AFP, we then examined the extent to which either of the light chains or serum albumin increases the diagnostic accuracy of AFP. The AUCs of FLC$\kappa$ and FLC$\lambda$ were 0.91 and 0.90, respectively, when each was individually combined with AFP (Figure 3). However, their impact was no longer significant when serum albumin was included into the model. The simple combination of albumin (AUC = 0.77) and AFP (AUC = 0.87) was marginally more effective, than sFLC, with an AUC of 0.92 (Figure 3).

As noted in Figure 1, most values within the control group fell within the reference range, but in the HCC group, levels were signifcantly higher across all aetiology groups. Gamma globulin was moderately directly related to both FLCs ($\kappa$and $\lambda$) with an $R^2$ figure of 0.37 and 0.33, respectively, while it was moderately inversely related to serum albumin with an $R^2$ value of 0.34 (Figure 4A–C).

Finally, we explored the relationship between serum albumin and gamma globulin as assessed by the difference between total protein and albumin, or by individual immunoglobulin subgroups, that is, IgG, IgA and IgM. There was a moderate inverse

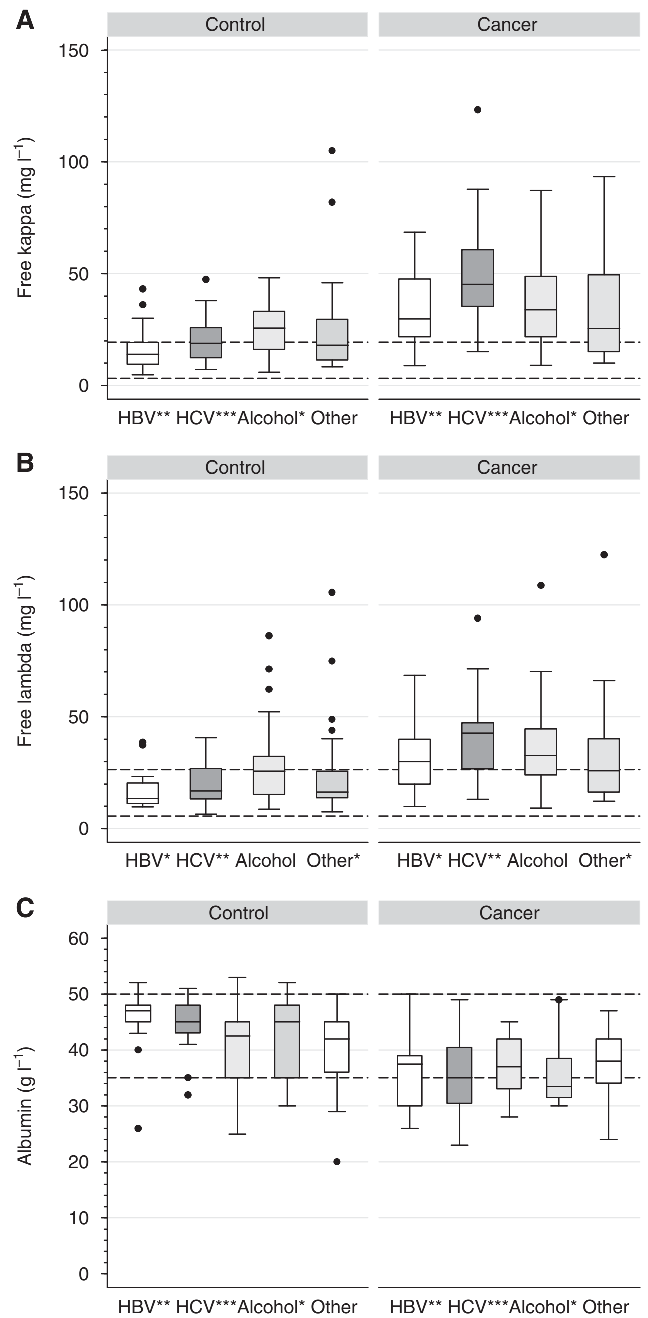


**Figure 1. Serum levels of (A) free kappa, (B) free lambda light chains and (C) albumin in relation to disease aetiology.** Mann–Whitney *U* test was used to assess the differences in distribution between HCC (Cancer, $n = 102$) and the control group ($n = 113$) within the same aetiology group. (*$P<0.05$, **$P<0.005$ and ***$P<0.0005$). Dashed lines indicate the upper and the lower reference values.

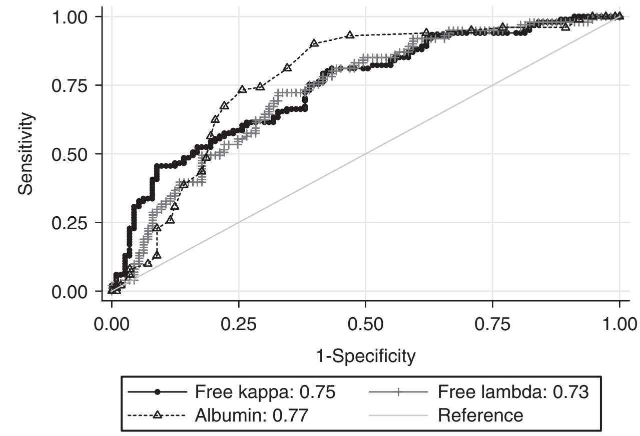


**Figure 2.** ROC curves for serum free kappa, serum free lambda and albumin.

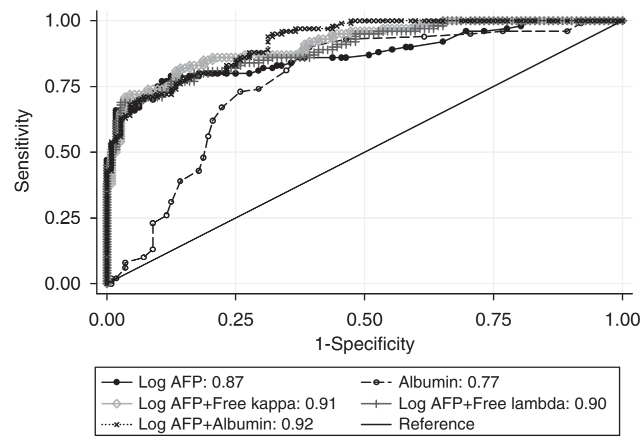


**Figure 3.** ROC curve for serum albumin and AFP and their combinations.

relationship between serum albumin and the difference between total protein and albumin (i.e., the gamma globulin fraction) with an $R^2$ value of 0.42 (Figure 5A). Among all the immunoglobulins, the relationship of IgG and albumin was most akin to the overall gamma globulin in relation to albumin as indicated by equal slopes (Figure 5B).

## DISCUSSION

The data presented here validate the conclusions of our original SELDI study using quantitative assays in a large independent patient cohort (Ward *et al*, 2006a). We confirm that the most significant changes identified by SELDI (albumin and sFLC) (Ward *et al*, 2006a) indeed do have a considerable discriminatory potential.

AFP is the standard serological test for HCC and returned an AUC value of 0.87. By simply combining either albumin or sFLC with AFP, an impressive AUC ROC value of $>0.90$ was obtained. However, sFLC concentrations correlated closely with albumin, and combining sFLC and albumin with AFP did not further improve the AUC. Serum AFP and albumin are simple, cheap and are routinely used in hospital laboratories for the assessment of liver disease (Carr *et al*, 2007; Leerapun *et al*, 2007; Sterling *et al*, 2007). Therefore, these observations indicate that albumin used in combination with AFP, might be a useful diagnostic screening test for HCC, although this would require further validation in larger prospective studies.

A low serum albumin level has been consistently associated with a markedly increased risk of HCC. Thus, Wong *et al*, (Wong *et al*, 2010) using a large prospective cohort of patients with HBV-related chronic liver disease, showed that low serum albumin, at the time of entry into the study, was a key determinant of subsequent HCC development. Nagao and Sata (Nagao and Sata, 2010) reported that, in a population-based study, in an area of high HCV endemicity, hypoalbuminaemia was associated with significantly increased mortality, particularly from HCC. Bonis *et al* (1999) similarly found albumin to be a predictive factor for HCC development among a large group of patients with chronic

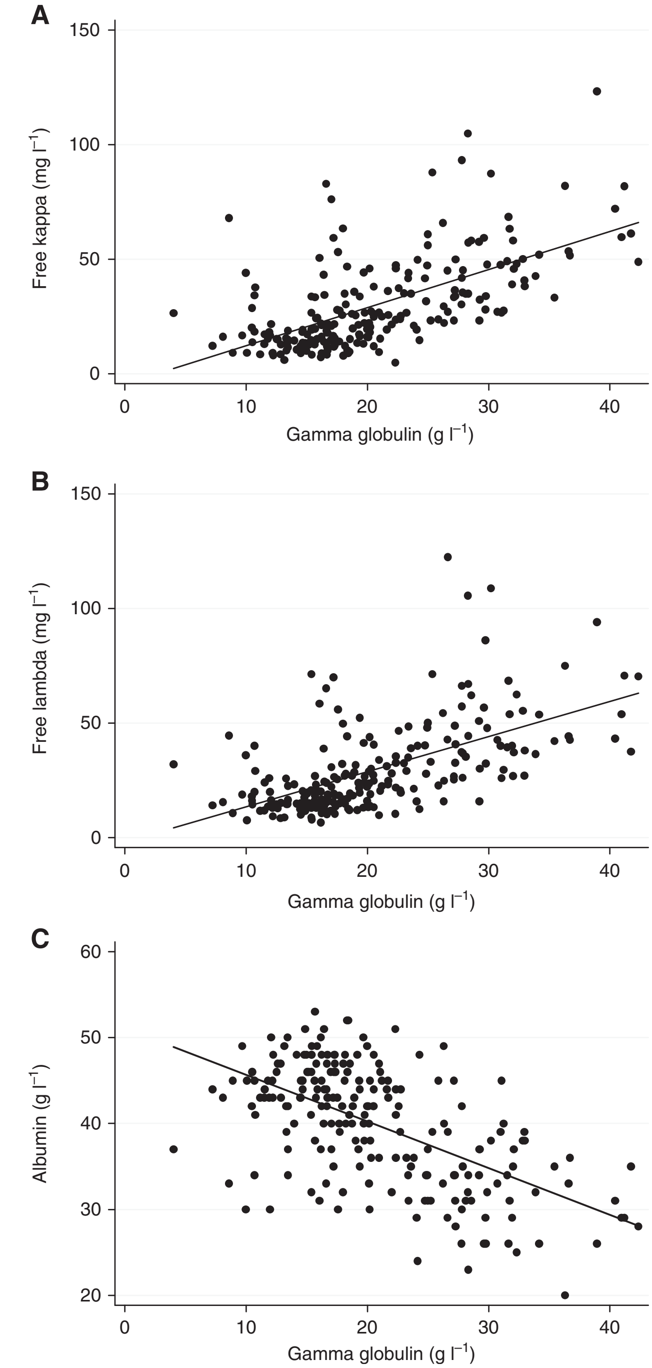


Figure 4. Correlation between gammaglobumin and (**A**) serum free kappa, (**B**) serum free lambda and (**C**) albumin.

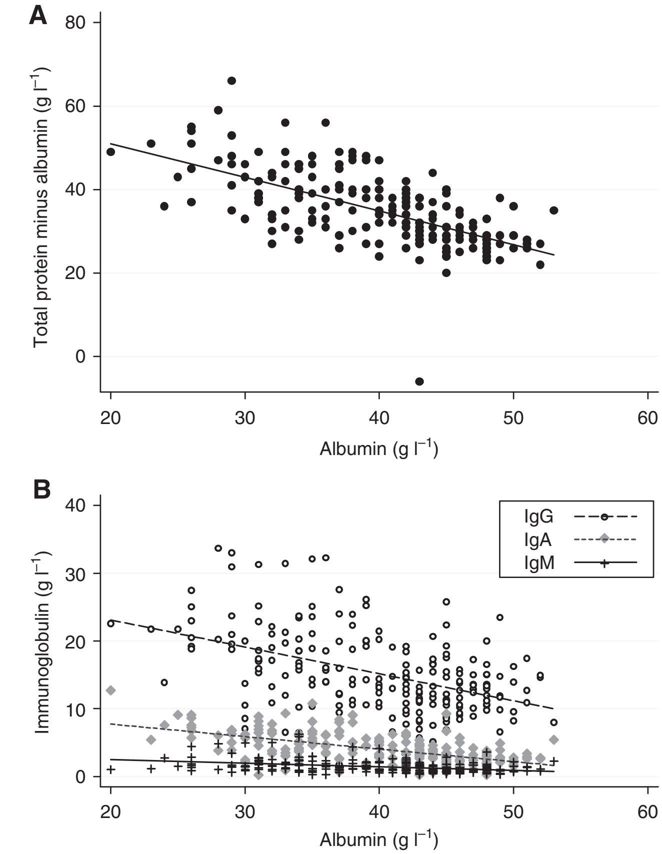


Figure 5. Correlation between albumin and (**A**) total gamma globulin and (**B**) individual immunoglobulins (IgA,IgM andIgG).

hepatitis C infection. Above an albumin level of 41 $g l^{-1}$, the risk of HCC was only 3% compared with 40% for an albumin level below 41 $g l^{-1}$. These authors considered a standard hepatological interpretation of hypoalbuminaemia, that is, a reflection of poor synthetic liver function (Schuppan and Afdhal, 2008). Others, however, have questioned this and suggested that the hypoalbuminaemia may be related to the level of hypergammaglobulinaemia as depression of albumin is consistent across a wide range of diseases and inversely proportional to the gamma globulin levels (Keshgegian, 1984). This was the case in the present study, both in the HCC patients, the control subjects and in the group as a whole. The reason for an elevation of gamma globulin in chronic liver disease is again unclear, but seems likely to reflect an increased exposure of gastrointestinal antigens to the systemic circulation as a result of portal hypertension.

Elevated sFLCs occur in several chronic conditions particularly those with generalised B-cell activation including systemic lupus erythematosus (Aggarwal *et al*, 2011), rheumatoid arthritis and Sjörgrens syndrome (Gottenberg *et al*, 2007), and have been shown to correlate significantly with disease activity (Aggarwal *et al*, 2011). Elevated sFLC have also been shown to be associated with an increased risk of non-Hodgkin's lymphoma in patients with HCV (Terrier *et al*, 2009) or HIV (Landgren *et al*, 2010), and are associated with poorer overall survival in chronic lymphocytic leukaemia patients (Maurer *et al*, 2011). The association between disease activity and sFLC was stronger than for immunoglobulin levels, suggesting that sFLC may act as more sensitive surrogate markers of B cell function (Landgren *et al*, 2010; Aggarwal *et al*, 2011).

In the current study, sFLC concentrations were significantly elevated in the HCC patients, who were HBV or HCV-positive. Antibodies to HBV and HCV viral epitopes are more frequently detected in patients with HCC than in patients with cirrhotic liver disease and are often evident before the development of HCC and prior to elevations in AFP (Hann *et al*, 2004). Elevated sFLC may reflect the developing B-cell response to viral infection and could provide an early marker of HCC onset in virally infected patients. In addition, as sFLCs have been implicated in the induction of chronic inflammatory disease, and have been shown to bind to carcinoma cells (van der Heijden *et al*, 2006) and other intracellular and extracellular proteins (van der Heijden *et al*, 2006), sFLC could potentially influence the onset of HCC. Further work is required to elucidate the role of sFLC in HCC.

Overall, there are two possible interpretations of our data. Firstly, low serum albumin, as a result of poor 'synthetic liver function' is compensated for by a rise in gamma globulin as a homoeostatic response to maintain plasma oncotic pressure and that the elevation of sFLC is simply a consequence of this hypergammaglobulinaemia. Alternatively, as suggested by Keshgegian (1984), the primary event may be the hypergammaglobulinaemia. In any event, this interpretation would suggest that any of these three parameters (low albumin, and raised gamma globulins or sFLC) somehow reflect an aspect of liver function that is permissive to HCC development.

The observation that a combination of albumin and AFP both appear to have some diagnostic utility individually, but more so in combination, one rising and one falling in the presence of HCC, may still be noteworthy as the two proteins have a high degree of sequence homology (Law and Dugaiczyk, 1981; Belanger *et al*, 1994). AFP is often described as 'fetal albumin'. Thus, levels of albumin are low in the fetus but rise rapidly, immediately after birth, whereas our current results appear to suggest the reverse happens during malignant change. It is conceivable that the rise in AFP and fall in albumin represents a recapitulation of the fetal state or a block in differentiation during malignant change, at the fetal stage.

In conclusion, we have identified two easily quantifiable serum markers that can be combined individually with AFP to improve the discrimination of HCC patients from patients with chronic liver disease. However, an important question to address is, to what extent albumin and sFLC changes may address the limited sensitivity of AFP for early HCC detection. This would require suitable diagnostic thresholds to be established for each marker. Additional prospective studies are required to determine how a combination of albumin, sFLC and AFP may be used to predict risk of HCC onset or to identify cirrhotic patients at a higher risk of developing HCC, who would benefit from more frequent surveillance.

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## CONFLICT OF INTEREST

LKA and RGH are employees of The Binding Site Group Ltd. The remaining authors declare no conflict of interest.

## AUTHORS CONTRIBUTIONS

PJJ, RGH conceived the original idea and designed the study with LKA. MT, DGW and LKA collected the data for the study, which was analysed by MT, DS and SP. The manuscript was written by PJJ. All authors reviewed the manuscript and gave final the approval for submission.

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