THE APPLICATION OF MOLECULAR PROFILING AND PROTEOMICS IN THE STUDY OF LIVER CANCERS

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ABSTRACT

Background: Hepatocellular cancer [1] is increasing worldwide. The majority are detected too late for curative surgery as effective identification of the at risk population and their subsequent surveillance is inadequate. My **specific aims** were to study and characterise our own patients, evaluating the known and predicted biomarkers for HCC in patients with non-alcoholic fatty liver disease and exploring novel biomarker methodologies for the detection of either cirrhosis or HCC complicating it.

Methods: Details of all HCC patients presenting to the Newcastle hepatopancreatobiliary (HPB) multidisciplinary team were recorded in an NHS intranet database, and a subset were consented for tissue collection. Established and candidate biomarkers were assessed in serum by western blotting and/or ELISA assay and the role of microarray analysis of HepG2 cells, and 2D-gel electrophoresis of immunodepleted serum in novel candidate biomarker identification were explored.

Results:

The number of HCC cases referred has increased dramatically over the last decade, as has the numbers arising in a background of non-alcoholic fatty liver disease (NAFLD). In our cohort, patients with earlier stage cancers detected by either - surveillance or incidentally had a better prognosis, but only 10% were eligible for definitive treatment.. While HCC patients treated with liver transplant had an overall 5 year survival was 62.1%, the median survival of the transplant cohort was 137 months. Exploration of serum levels of Glypican 3, Squamous Cell Carcinoma Antigen-1 and follistatin were poor surveillance biomarkers, but the combination of Alpha-fetoprotein and Protrhombin induced by vitamin-K absence was encouraging. Serum proteomic analysis in a small subgroup identified four isoforms of apolipoproteins as well as CD5L as differentially expressed between patients with no cirrhosis, cirrhosis and HCC, although subsequent CD5L ELISA failed to confirm its ability to specifically detect HCC.

Discussion:

While the incidence of HCC is increasing, the prognosis for those affected remains poor. Biomarkers identifying both the at risk individuals and those with cancer are urgently required.

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'This work is dedicated to my father late Mr G.S.Chatterjee'

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CHAPTER 1.   INTRODUCTION

1.1   Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma is a major health problem worldwide. It is the fifth commonest neoplasm in the world, and the third most common cause of cancer related death[2]. More than 500,000 new cases are currently diagnosed yearly, with an age adjusted worldwide incidence of 5.5 – 14.9 per 100,000 population. The incidence of HCC has been reported to be rising during the last 5-8 years   [2-6]. Commonly but not exclusively, HCC (60-80%) develops in a setting of liver cirrhosis[7]. Liver cirrhosis has a very diverse aetiology which includes chronic hepatitis B and hepatitis C infection, alcohol excess, non-alcoholic fatty liver disease (NAFLD), diabetes, certain medications (e.g. imipramine, phenothiazine, tolbutamide, phenytoin) genetic metabolic diseases as well as toxic exposures including dietary aflatoxins. The annual incidence of HCC in cirrhotic patients ranges from 2-8% with a cumulative 5 year incidence rate ranging from 15% to 20%[8, 9].

Cirrhosis development is characterized by inflammatory cell infiltration, hepatocyte regeneration, necrosis and parenchymal remodelling. Residual hepatocytes are stimulated to proliferate and this is eventually associated with remodelling of the hepatic sinusoids and subdivision of liver parenchyma by fibrous tissue. Hepatocarcinogenesis is a multifactorial, multistep process in which external stimuli induce genetic changes in key growth regulatory genes in hepatocytes. This can lead to changes in cellular proliferation, cell death and the emergence of clonal populations with a genetic damage induced survival advantage. These clonal cells may become dysplastic hepatocytes, evolving into dysplastic nodules[10]. These grow more rapidly and have a tendency to acquire additional genetic damage, progressing into higher grade dysplastic nodules. High grade dysplastic nodules are considered premalignant in nature. No consistent pattern of genetic damage at different evolutionary stages of HCC has been described. This may be because the molecular pathways leading to HCC differ according to the underlying aetiology.

As cirrhosis is clearly a major factor pre-disposing to HCC development, one would hope that monitoring of individuals with known chronic liver disease would increase the likelihood of early HCC detection, facilitating effective treatments, and improving prognosis. While surveillance programmes are thought to aid the diagnosis of early HCC (single tumour less than 5cm or not more than 3 tumours and none more than

3cm) in 30 – 60%, the benefit is restricted to those patients with known cirrhosis, already in surveillance programmes, largely in developed countries [11]. The reality is that in up to 80% of patients, an HCC is detected at an advanced stage and prognosis for those presenting after the onset of symptoms, is dismal. The overall survival for HCC patients is 0-10% at 5 years [12], with many surviving just a few weeks or months after diagnosis. This failure is attributed in part to the lack of effective surveillance tools in patients with cirrhosis, but also because of our inability to satisfactorily identify the at risk population with chronic liver disease who would benefit from regular follow-up.

1.2   The surveillance population – individuals with cirrhosis

Progressive hepatic fibrosis with the development of cirrhosis is a feature of all chronic liver diseases. Development of fibrosis is a step by step process starting from minimal fibrosis limited to the portal tracts, followed by more extensive fibrosis with septa extending into the liver parenchyma, that can form bridges between portal tracts. On this background, nodules of regenerating hepatocytes further distort the liver architechture. Studies on the natural history of the disease suggest that advanced fibrosis and cirrhosis develop in about 20 – 40% of patients with chronic hepatitis B or C and in a similar proportion to those with alcoholic and non-alcoholic steatohepatitis[13-15]. The progression of chronic liver disease through liver fibrosis to cirrhosis may take years to decades to develop and needs the perpetuation of the underlying injury. Up to 80% of patients with HCC have an underlying cirrhosis. One factor important in a successful HCC surveillance programme is the identification of the at risk population. This population includes individuals with chronic hepatitis B, hepatitic C virus hepatitis as well as those with cirrhosis of any cause. This suggests that staging of hepatic fibrosis is of paramount importance to assess the risk of disease progression as patients with necro-inflammation and fibrosis are at highest risk for end stage liver disease and hepatocellular carcinoma. To diagnose the aetiology of liver disease and stage the severity of fibrosis or confirm the presence of cirrhosis, a liver biopsy with subsequent histopathological assessment is the recommended procedure[16]. However, for a number of reasons there is a lot of interest in developing non-invasive methods as alternatives.

Liver biopsy has been used as the ''gold standard'' for almost all studies evaluating noninvasive markers of liver fibrosis[17]. Liver biopsy has the advantage of allowing

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the acquisition of diagnostic information in making a diagnosis in chronic liver diseases, but also plays a key role in the staging of the disease and in their follow up. For both the physicians and the patient, the decision to proceed with a liver biopsy is not a trivial one due to the risk of complications. Following a liver biopsy 1-5% of patients experience severe adverse events needing hospital after care prolonged by more than one day with a reported mortality rate between 1:1000 and 1:10,000[18-22]. Furthermore, many patients are reluctant to have repeated biopsies, which limits our ability to monitor disease progression and the effects of treatment.

Moreover, a needle liver biopsy only removes 1/50,000th of the liver and hence carries the risk of substantial sampling error and cirrhosis can be missed on a single blind liver biopsy in 10-30% of cases[23-25]. Review of the available data on the accuracy of needle liver biopsy to define the stage of fibrosis is affected by significant interpretative error[17]. Interpretation of the sample can also be affected by sample size as Scheuer concluded that bigger specimens are more conclusive [26]. Colloredo et al also concluded that an adequate specimen should be at least 20 mm in length with at least 11 complete portal tracts[27]. The current British guideline considers that most hepatologists are satisfied with a biopsy containing more than 6 – 8 portal triads[28]. The need for obtaining an adequate liver sample contrasts with the patient needs of limited pain and reduced haemorrhagic risks and in fact this procedure could be particularly risky for advanced liver disease[29, 30]. The different adverse factors associated with biopsy, along with intra/ interobserver variability [31] and considerable sampling variability[32], limits the role of liver biopsy in regular surveillance. It is also a practice that should not be widely employed as a surveillance or screening tool for wider populations. The development of potential non-invasive markers, with high sensitivity and specificity, that can be repeated at regular intervals, without adding any risk to the patient, are being actively pursued.

Presently, non-invasive markers for fibrosis can be broadly divided into those that are serum based, and those that are imaging based. Assessment of liver fibrosis by a combinations of serum markers have shown encouraging results, and the ideal characteristic of a serum biomarker as defined by Sebastiani G et al [33] will: (1) be specific for fibrosis in the liver; (2) provide measurements to stage the fibrosis and fibrogenesis activity; (3) not be influenced by co-morbidities; (4) have a known half-life; (5) have a known excretion route; (6) be sensitive and reproducible. They can be divided into 'direct' and 'indirect' markers.

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Direct markers of fibrogenesis are defined as measurable biochemical markers in the peripheral blood which reflect the turnover of extracellular matrix of the liver. Examples include several glycoproteins (laminin, hyaluronan), the collagen family (procollagen III), collagenases and their tissue inhibitors (metalloproteinases, tissue inhibitors of matrix metalloproteinase) and cytokines associated with fibrogenesis (TNF-β, TGF- β1). These markers have the potential to clinically assess the stage and progression of fibrosis, but also to monitor response to treatment. As yet, this has not been achieved, but progress has been made.

Indirect marker of fibrosis are defined as single or combined haematological or biochemical parameters that reflect the stage of liver disease, rather than the process. Of the direct markers, hyaluronic acid has been studied in all the common forms of chronic liver disease and shows an overall good accuracy to discriminate early from significant fibrosis. However, the studies had very small cohorts and needed validation in larger studies. In 2004, Rosenthal et al in a multicenter cohort study with 1021 patient samples developed an algorithm combining age with direct markers of fibrogenesis i.e. hyaluronic acid, amino-terminal propeptide of type III collagen, and tissue inhibitor of matrix metalloproteinase along with markers for inflammation and extracellular matrix degradation. The results were compared with tissue samples and it detected severe fibrosis with sensitivity of 90% and also accurately detected the absence of fibrosis in patients with alcoholic and non-alcoholic fatty liver diseases[34]. This algorithm is now described as the 'Enhanced Liver Fibrosis Panel'(ELF). Two studies so far has validated the ELF panel of markers in detecting fibrosis in adult and paediatric patients with non-alcoholic fatty liver disease. It is restricted by the limited capacity to diagnose the intermediate stages of fibrosis i.e. can diagnose cirrhosis. Regarding the ability to predict clinical outcomes in patients with chronic liver disease, a prospective study with a 7 year follow up, reported its efficacy to be similar to liver biopsy. However, more data is required from longitudinal studies to evaluate the performance of these non-invasive markers in evaluating disease progression or regression.

Indirect markers can predict fibrosis either as independent markers like ALT/AST ratio (aspartateaminotransferase ratio)[35] or as multicomponent indirect fibrosis test. Of the latter, the 'Fibrotest' is the most extensively studied. This test which uses a combination of five blood tests including γ glutamyl transferase, haptoglobin, bilirubin, apolipoproteinA1, α2 macroglobulin, was initially proposed by Imbert-

Bismuth and colleagues in 1999[36].The data from different studies suggest that the Fibrotest performs well in detecting the two extremes of stages of liver fibrosis but is again less accurate in detecting the intermediate stages of fibrosis[17, 37]. Radiological or imaging methods considered as liver fibrosis detection methods include CT, ultrasound and MRI. Subjective measures such as 'an irregular liver edge' are poorly reproducible. More quantitative attempts have identified that the right lobe exhibits relatively greater shrinkage, while the caudate lobe undergoes relative enlargement, and that the ratio of the transverse caudate lobe width to the transverse right lobe width can separate cirrhotic livers from the non-cirrhotics ones [38]. Using these technique, cirrhosis could be correctly diagnosed with a sensitivity of 84%, specificity of 100% and a diagnostic accuracy of 94%. The lower sensitivity is attributed to these morphological changes only being present in those with more advanced disease[17]. Aube et al studied 11 ultrasonographic variables (in 243 patients with chronic liver disease), including measurements of liver morphology, assessment of portal venous flow, spleen size and liver nodularity and detected a diagnostic accuracy of 82-88%. Here, the limitation was significant interobserver variability and inability to gain all the required measurements in all patients due to anatomic constraints [39]. Comparing ultrasonography to clinical and biochemical parameters, Oberti et al found that USS had a diagnostic accuracy of only 70% as compared to biochemical parameters which correctly diagnosed cirrhosis in $91 - 94\%$ of patients with chronic liver disease, with serum hyaluronidate appeared the most sensitive marker for screening[40].

Another non-invasive imaging modality that is gaining popularity in the assessment and staging of liver fibrosis is Hepatic ultrasonic transient elastography/Fibroscan, first reported by Sandrin L et al in 2003 [41]. The Fibroscan includes a probe, an electronic analysis system, and a control unit installed on a computer. The technique uses both ultrasound (5mHz) and low frequency (50Hz) elastic waves whose propagation velocity in the liver tissue is directly related to the elasticity of the tissue. A metanalysis in 2009 showed that Transient Elastography can be performed with excellent accuracy in the diagnosis of cirrhosis irrespective of the aetiology [42] However, it is again less accurate in differentiating the different stages of liver fibrosis [43]. The results of the test can also be limited by obesity, especially increasing waist line/central obesity and also by the experience of the operator [44].

In summary, liver biopsy cannot be generally applied for fibrosis detection, and although some of the non-invasive methods being explored show promise, none has as yet been widely adopted as an alternative.

1.2.1 Poor sensitivity and specificity of surveillance for HCC

During the last 30 years, there have been enormous advances in the understanding of the mechanisms of carcinogenesis. However, these advances have not seen a parallel improvement in the treatment of cancers[45]. A surveillance programme in cirrhotic patients has the potential advantage of detection of HCC in its early stages. Early detection of HCC is the only way to avoid mortality as the disease is always fatal when diagnosed too late for surgical and / or ablative treatments. However, to diagnose HCC early and appropriately has proved to be very difficult. The complexity of cancer disease can be attributed to be one of the important causes of impediment to this process. It is also known now that the response of individual tumours to various forms of treatment is often not possible to predict by histological evaluation of tumour tissue alone. An important mission in cancer research is the identification of accurate biological markers that can be used to diagnose a disease early, as well as predict prognosis and response to treatment options.

A biological marker is defined as a ''characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes or pharmacological responses to therapeautic intervention''. An ideal marker would be a protein or protein fragment that can be easily detected in the patient's blood or urine, but not detected in a healthy person. Today the most common use of biomarkers is for detection of early and recurrent disease [46]. Despite the large number of investigations devoted to their identification, reliable biomarkers are still elusive in many malignant diseases, including HCC.

Biomarkers can be grouped into three different classes[47]:

(1)Diagnostic: These are used to aid histopathological tumour classification. Accurate diagnostic markers can aid in screening helping early diagnosis of tumours and optimal treatment choices. Diagnostic markers include Calcitonin – elevated in the serum of patients with thyroid medullary carcinoma; and PSA(Prostate Specific Antigen) – elevated in the serum of patients with prostate cancer [48]. Diagnostic markers can also aid the detection of tumour recurrence, especially if the treated

primary tumour secreted a particular marker, such as CA-125 (ovarian carcinomas), or CEA (colorectal carcinomas) [46].

(2)Prognostic: These provide information about the malignant potential of the tumours, and can be instrumental in optimum treatment choice. An adequate evaluation of the metastatic spread is of particular importance. Examples include hormone receptors, proliferation markers, markers of angiogenesis (VEGF), growth factor receptors (HER-2/neu) and p53. Her-2 oncogene expression in tissues and serum is the most commonly used predictive biomarker for breast cancer. Expression of HER-2 is significantly related to positive lymph nodes, poor nuclear grade, lack of steroid receptors and high proliferative activity and has been shown to identify a subgroup of patients node-specific breast cancer patients with poor prognosis [49-51]. Absence of oestrogen and progesterone receptors, upregulation of Ki-67 (MiB1) antigen are additional indicators of poor prognosis [52-54]. For ovarian cancer cyclin D1 overexpression is related to an aggressive phenotype and poor prognosis

(3) Predictive: These can also guide alternative treatment modalities. Breast cancer patients that exhibit oestrogen receptor positive tumours are usually treated with anti-oestrogen compounds, as the presence of the marker predicts their response to that therapy. Similarly, patients with tumours positive for the Her-2 poor prognostic marker, respond to treatment with Herceptin, a monoclonal antibody that binds the Her-2 epidermal growth factor receptor isoform, blocking its mitogenic signalling to tumour cells[46, 47]

An assay that is used to determine the presence or absence of disease must be validated using a series of analyses that determines how well the test performs in diagnosing the disease (since no test is 100% accurate). The simplest measures are sensitivity (true positive rate) and specificity (true negative rate), which are inversely related. The diagnostic accuracy of a test further relies on the prevalence of the disease in the population and can be ascertained by the positive and negative predictive values, i.e. the rates at which positive and negative results are correct. The Youden index (sensitivity + specificity − 1) can be used to give an estimate of the efficiency of the test, free of the influence of the disease incidence.

Current surveillance tests for HCC are divided into two categories: serological and radiological tests

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1.2.2 Surveillance - Serum biomarkers

1.2.2.1 Alpha-foetoprotein

Alpha foetoprotein is a glycoprotein produced primarily by the foetal liver. Its concentration falls after birth and its production is repressed in adult life. Since its discovery in 1963 [55], and its recognised association with the presence of HCC [56], serum alpha –fetoprotein has become the most commonly performed serological test for hepatoma screening and surveillance in cirrhotics patients.

Trevisani et al reported, using Receiver Operating Characteristics (ROC) for AFP, that with a normal serum level below 10ng/ml, 20ng/ml reportedly showed the optimal balance between sensitivity and specificity. 20ng/ml is currently considered the limit above which investigations for HCC are needed[57]. However, at this level the specificity is not particularly good and the sensitivity is only 60%, which is inadequate for general use. At higher cut-offs, the specificity is increased, but progressively fewer HCC are detected. If the AFP cut-off is raised to, *e.g.*, 200 ng/mL (This is the level set by the European Association for the Study of Liver Disease (EASL) for HCC diagnosis in a cirrhotic patient with a typical hypervascular mass on imaging) the sensitivity drops to 22%[58]. Some investigators have suggested that the usefulness of AFP for HCC detection in viral hepatitis is even worse, and that it should be reserved for detecting HCC of non-viral etiology. [59] Certainly transient increases and fluctuations in serum AFP may occur in chronic liver disease and cirrhosis, especially during exacerbations of hepatitis. Moderately raised levels in some patients with uncomplicated chronic liver disease contributes to a relatively low specificity of AFP [60]. Therefore some increases can be misleading and all patients need retesting at a fortnight after a raised result, especially if obtained in the absence of positive imaging. It is a persistently elevated serum AFP, or one steadily rising, that alerts to the presence of a tumour. In specialist liver clinics, where the prevalence of HCC among cirrhotic patients is high at approximately 5%, serial testing still has a role, even though its inadequacy as a screening test is widely acknowledged [57].Serum AFP estimation may be useful in monitoring response to therapy despite its poor performance as a surveillance diagnostic marker. Indeed, there is preliminary evidence that changes in serum AFP may be a more accurate and sensitive way of determining the degree of response to treatment than conventional imaging procedures that rely on physical determination of tumour size[61].

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Total AFP can be divided into three different glycoforms, namely AFP-L1, AFP-L2 and AFP-L3, according to their binding capabilities to lectin lens culinaris agglutin. AFP-L1, as the non-LCA bound fraction, is the major glycoform of AFP in the serum of non-malignant hepatopathy patients. On the contrary, AFP-L3, is the LCA bound fraction, is the major glycoform of AFP in the serum of HCC. Some researchers suggest that the sensitivity of AFP can be improved by measuring the glycoforms of AFP. Measurement of the lens culinaris agglutin-reactive fragment of AFP (AFP-L3) has been developed and reflects HCC related changes in the AFP carbohydrate side chain[62]. Early studies suggested that the AFP-L3 assessment could be used to detect early HCC in patients with cirrhosis [63, 64] and that AFP-L3 could be used as an adjuvant to imaging and AFP in the surveillance for HCC[65]. These studies had the advantage of being prospective in nature but they were under powered (patient numbers ≤ 51) and performed only in single centre studies. However, a large phase 2 biomarker case control study which included 417 cirrhosis controls and 419 HCC cases showed that AFP-L3 was not sensitive in detecting early HCC. The study concluded that for the diagnosis of early stage HCC, AFP-L3% was not useful, partly because of the need for an elevated total AFP, which limits its effectiveness [66] AFP-L3 could possibly be used as a prognostic marker. According to a study by Kumada et al, the tumour doubling time is shorter in patients with high levels of AFP-L3 and failure of AFP-L3 to fall after treatment could signify residual disease. Recurrence of HCC is likely if AFP-L3 starts to rise after returning to baseline after treatment.[67]. It has also been found that relapse with multifocal HCC and portal vein invasion is more frequent in patients with elevated AFP-L3 as compared to the patients with re-elevation of AFP-L3 after treatment[67]. Unfortunately, as the sensitivity of AFP-L3 is suboptimal it is unlikely to improve surveillance strategies if widely accepted. The assay of AFP-L3 is not routinely available, [68] which is also a limiting factor in its routine use.

1.2.3   Surveillance - Imaging modalities

a) Abdominal Ultrasound (USS) of the liver is the most commonly used test for surveillance of HCC because it is simple and non-invasive [69, 70]. Lesions can take on a number of appearances. The lesion can be echogenic because of the fat content, or give a hypoechogenic or target lesion appearance. None of these appearances are specific. The sensitivity of ultrasound has been reported to be

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between 65 and 80% with a specificity of 90% when used as a screening test for HCC in patients with cirrhosis[71]. In patients with cirrhosis a positive predictive value to detect HCC has been reported to be 78%[72]. However, a study by Sherman et al showed a positive predictive value of only 14% when ultrasound was used for screening in chronic HBsAg carriers [73]. Thus, the value of USS is highly variable. The major drawback is that its performance depends on the experience of the examiner and the physical size of the patient. Although these performance characteristics are not ideal, they are superior to any available and validated serological test.

Further development in ultrasound technology can improve the efficacy of the technique in screening. Colour Doppler imaging provides real time imaging of the haemodynamics of liver tumours, and power Doppler imaging has added to a better detectability of blood flow. However, limitations in the detection of slow flow and vessels located deeply from the skin surface have prevented the use of these modes regularly in the evaluation of tumour haemodynamics. Against this background the development and use of micro bubble contrast agents was expected to provide a stable enhancement effect that are useful for the detection and characterisation of liver tumours. Recent literature suggests that contrast enhanced ultrasound has a reported sensitivity of 98 – 100% and specificity of 63 – 93% in discriminating benign from malignant liver lesions. The use of contrast enhanced ultrasound to characterise nodular lesions in cirrhosis has been recommended by the clinical practice guidelines issued by the European Federation of Societies for Ultrasound in Medicine and Biology and the American Association for Study if Liver Diseases. However, contrast-enhanced US has not resulted in any significant improvement in the ability of US to detect small tumour foci, since a comprehensive assessment of the whole liver parenchyma cannot be accomplished during the short duration of the arterial phase [74]. Secondly, contrast enhanced ultrasound is not cheap and is not routinely available.

    b) **Computerised Tomography (CT)** is suggested when there is suspicion of HCC by ultrasound in a patient with cirrhosis of the liver[75]. Evaluating spiral CT alone Kim et al showed that the sensitivity of HCC detection was up to 100% from results of imaging with the four phase CT in tumours greater than 2cm in size, 93% in tumours 1-2cm in size and 60% in tumours less than 1cm in size[76]. Comparing ultrasound with CT scan in a screening programme Chalasani et al reported a

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sensitivity of 59% with ultrasound and 91% with CT scan[77]. In another study comparing the two modalities for evaluation before liver transplantation sensitivity to detect HCC was reported to be 79.4% for ultrasound and 81.6% for CT scan[78]. Teefey et al, however, mentioned that the sensitivity of ultrasound (89%) was much higher than CT (67%) and magnetic resonance imaging (56%). Given the poor performance of alpha foetoprotein and the observer dependant variation with ultrasound some groups have suggested the use of CT for surveillance. This however poses a number of logistic problems. First, a screening test is usually not the diagnostic test of choice. Second, the performance characteristics of CT scanning have been developed in diagnostic/imaging studies and the performance characteristics of CT scanning in HCC surveillance are unknown. Furthermore, if CT`

` scan is to be used as a screening test, i.e. every 6-12 months over many years, there is a significant radiation exposure [58], as well as significant increase in costs to be considered. Current practice is to use CT for surveillance only in poor sonar subjects e.g. markedly obese individuals, or in those where a suspicious nodule on USS or mid to moderately raised AFP, suggests a higher individual risk of developing HCC.

c) Magnetic Resonanace Imaging (MRI) of the liver has a sensitivity of 40-70% for detection of early HCC [79, 80]. The arterial phase is critical for detection of HCC[81]. Lesions more than 1cm which are hyperintense in the T2 phase of imaging are likely to be malignant which helps to differentiate from dysplastic nodules and regenerative nodules which are T2 hypointense [82]. These characteristics make it a useful tool for diagnosis of HCC but this modality is expensive with a poor sensitivity which does not make it an ideal imaging for surveillance.

d) 2-[fluorine-18]fluoro-2-deoxy-D: -glucose Positron Emission Tomography (FDG-PET) detects increased glucose metabolism associated with neoplastic lesions, provides high accuracy in most cancer imaging. Only 30-50% of HCC show 18F FDG uptake above background levels [83] which limits its application in the evaluation of HCC. To improve visualisation of these lesions an alternative tracer (11-acetate) has been used in conjuction with FDG [84]. The degree of differentiation determines its relative avidity for one of the two probe molecules. This can be used as a diagnostic tool but the limited availability and and high cost do not make it an effective surveillance tool.

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1.2.4 Current practice of surveillance

Our current practice for HCC surveillance is to use six monthly USS with serum AFP measurements. HCC screening is recommended in the high risk patients as mentioned below[85]

- Hepatitis B carriers (HBsAg positive)
- Asian males >40 years
- Asian females >50 years
- All cirrhotic hepatitis B carriers
- Family history of HCC
- Africans over age 20 years
- Nonhepatitis B cirrhosis
- Hepatitis C
- Alcoholic cirrhosis
- Genetic haemochromatosis
- Primary biliary cirrhosis
- Possible α1-antitrypsin deficiency, non-alcoholic steatohepatitis, autoimmune hepatitis

The cost effectiveness of this programme is still debatable. The detection rates with this practice are increased, but so also the costs and false positive rates. AFP alone had false positive rate of 5% and ultrasound has a false positive rate of 2.9% but when used together the false positive rate rises to 7.5%[86]. The study by Izzo et al supported the six monthly surveillance by AFP and USS for patients with severe chronic active hepatitis and liver cirrhosis[87]. Fasani et al reported that patients with multiple risk factors the six month surveillance might be inadequate and a tailor made surveillance programme might be required [88], complicating matters further.

Two randomised controlled trials from China have reported contradictory findings. Investigating 5581 patients with chronic hepatitis infection Chen et al reported that biannual screening with AFP and USS resulted in earlier diagnosis of HCC but the gain in lead time did not result in overall reduction in mortality[89]. Zhang et al, however, in a study of 18816 patients with HBV or chronic hepatitis, reported that in the screening group patients the HCC related mortality was reduced by 37%[90]. Examining their surveillance programme based on six monthly AFP and USS in a European cohort Bolondi et al reported that the cumulative survival for the 61

patients with HCC detected through the surveillance programme was significantly better than that of controls not participating in any surveillance programme[71]. Other non-randomised trials and observational studies have also shown survival benefit in patients diagnosed with HCC in 6-12 monthly surveillance programme using AFP and USS[91, 92]. Studies have also shown a survival benefit in those diagnosed with small and early tumours[93].

Surveillance programmes benefit the population if the increased survival with early diagnosis is associated with gains in lead time when compared to those diagnosed with the disease in late stages. If there is no gain in survival time beyond the time when death would occur if diagnosed late then the apparent gain is called a ''lead time bias''. The above mentioned, not so well controlled studies, suggesting improved survival by surveillance are plagued by lead time bias, differential tumour growth and poor compliance with surveillance. However, a recently reported study by Tong et al reported that surveillance for HCC identifies patients with a smaller tumour burden after correction for lead time bias these patients show improved survival as compared to patients presenting with symptomatic HCC [94]. Another study reported a overall reduced survival with delayed diagnosis of HCC irrespective of lead time bias[95]. The above mentioned studies are heterogenous but there is some evidence that surveillance for HCC may have a positive impact on survival. Due to the availability of curative therapy for early HCC it would be difficult to plan further randomised trials to accurately evaluate the effect of lead time bias on the survival of patients with HCC diagnosed through surveillance versus symptomatic patients. On the basis of available data surveillance is performed as per EASL guidelines on a 6 monthly basis in the liver units. There is little doubt, however, that the performance needs to be improved. It is hoped that more sensitive biomarkers and improved imaging technologies will facilitate this.

1.2.5   Diagnosis of HCC

HCC is commonly diagnosed with non-invasive methods. For lesions more than 2cm or more on a background of cirrhosis the diagnosis of HCC is made using the EASL imaging criteria of the presence of typical imaging features showing areas of early arterial enhancement and delayed washout in the venous phase of multidetector CT or dynamic enhanced MRI. For lesions measuring 1-2cm the diagnosis is made by characteristic imaging findings in one or two modalities. For lesions less than 1cm the

lesions should be imaged in 4 months later in order to note a change in size or the presence of new imaging characteristics[96]. In difficult cases PET-CT is another modality which is now being used in specialised centres. In patients where imaging is inconclusive for lesions more than 1cm biopsy is suggested in the EASL guidelines.

1.2.6 Staging of HCC

An effective staging classification of HCC should help to decide the choice of therapy and also predict survival. In addition it is an important research tool which permits comparison between differenrt research groups. Well defined staging systems are available for almost all cancers. In HCC however, standard tumour staging systems, such as the widely adopted tumour, node metastases (TMN), are not helpful as they do not take into account the co-existent liver disease. There are a number of HCC specific prognostic scoring systems, which include Cancer of the Liver Programme (CLIP), Barcelona Clinic Liver Cancer (BCLC), Chinese University Prognostic Index (CUPI) and Japanese Integrated System (JIS) and Okuda Scoring systems. All these staging systems use permutations of different variables including number and size of the liver lesions, spread of the disease to the portal veins or systemically, as well as having a measure of the severity of background liver disease. Largely because of the variability in the latter, HCC has proved somewhat of an exception in that here is no consensus as to which one to be used universally. Some groups have adopted the BCLC staging system (*figure 1.1*) as this system aids management by stratifying patients into treatment groups, according to the extent of the disease, underlying liver function and performance status (PST).

Figure 1.1(below) showing the BCLC staging of HCC

Stage	Tumour status			
	PST	Tumour stage	Okuda	Liver function
O very early HCC	0	Single <2cm	I	No portal hypertension, normal bilirubin
A early HCC				
A1	0	Single	I	No portal hypertension and normal bilirubin
A2	0	Single	I	Portal hypertension and

				normal bilirubin
A3	0	Single	I	Portal hypertension and abnormal bilirubin
A4	0	3tumours <3cm	I-II	Child-Pugh A - B
B Intermediate HCC	0	Large multinodular	I-11	Child-Pugh A-B
C Advanced HCC	1-2	Vascular invasion or extrahepatic spread	I-11	Child-Pugh A-B
D End stage HCC	3-4	Any	III	Child-Pugh C

Another commonly used staging system is the CLIP score, which uses a mathematical score based on the Child Pugh criteria for assessing liver function, tumour morphology, AFP and the presence of vascular invasions. This scoring system has been analysed in a prospective manner and has been suggested as the best documented and analysed staging system for prognostic purposes[97]. Using a combination of two staging systems is an acceptable alternative and many centres prefer this.

1.2.7   Current treatment modalities

Therapies for HCC can be broadly classified into 4 categories:

Surgical

Resection

Transplantation

Percutaneous ablative techniques

Radiofrequency Ablation (RFA)

Percutaneous ethanol injection (PEI)

Microwave ablation

Experimental strategies

High intensity focussed ultrasound

Electroporation

Radiofrequency based treatment with nano particles

Local embolic therapies

Transarterial chemoembolisation

Transarterial embolisation

Radioembolisation

Systemic

Chemotherapy

Molecular targeted therapy

a. Surgical

Surgical intervention is the treatment of choice for HCC, as resection and transplantation provide the curative options in well selected patients.

Resection is the preferred modality of treatment for patients with HCC without concomitant liver cirrhosis and in selected patients with Child-Pugh A cirrhosis. Because of the prevalence of cirrhosis, however, surgical resection is suitable for less than 5% of western patients with HCC. In contrast, it is offered in up to 40% in Asian countries, where HBV is endemic and HCC in the absence of cirrhosis is common[98].

Historically, resection was associated with significant mortality, most commonly due to post-operative haemorrhage, liver failure and sepsis but the reduced operative mortality to <5% reported by major centres in recent times has made resection a first line therapeutic option [99, 100]. Pre-operative assessment of the liver remnant after resection is an important predictor. The Makuuchi algorithm uses the Indigo-Cyanine Green retention rate (ICG). An ICG 15, value of less than 20% confers better prognosis [101]. Patients with oesophageal varices, splenomegly, high bilirubin (> 2 mg/dl) and a low platelet count are poor candidates for liver resection. On the contrary, hepatic venous wedge pressure of <10mm Hg and single tumours afford the lowest mortality [102]

Refinements of surgical techniques, understanding of the Couinaud's segmental anatomy of the liver, development of ultrasonic dissectors and vascular staplers have aided in reducing blood loss and thereby have played a significant role in reducing post resection morbidity [103].

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The selection of patients for resection and the margin of resection vary on a case to case basis but in general the tumour is considered unresectable if the tumour is large with insufficient hepatic remnant after resection, the tumour is multifocal and bilobar, there is extra hepatic metastases, portal vein thrombosis, or hepatic vein or inferior vene cava involvement [104]. Tumour size also predicts survival and outcome. A tumour size of 5cm also appears to be a prognostic factor: 5-year survival is 32% for tumours more than 5cm compared to 43% when less than 5cm [105, 106].

Other studies have also reported favourable results for solitary tumours with the five year survival reported to be 35 -50% for all resections [107] but more than 50% for small, solitary HCC with preserved liver function [99, 108]. Another major clinical problem after resection is tumour recurrence in 50% to 80% of patients after 5 years. Accurate identification of disease free survivors based on clinico-pathological characteristics continue to be a challenge. It has been suggested that neo-adjuvant or adjuvant therapy which can decrease or delay the incidence of intrahepatic recurrence may improve the result of liver resection[109], and the international STORM (Sorafenib as Adjuvant Treatment in the Prevention of Recurrence of Hepatocellular Carcinoma) study aims to investigate the role of the VEGF inhibitor in reducing tumour recurrence after radical surgery. If carefully selected liver resection and liver transplantation may act in a complimentary way to achieve the best outcome.

Liver transplantation for HCC has evolved over the last two decades and is now considered the optimal therapeautic option for the disease arising in a cirrhotic liver as confirmed by the Organ Procurement Transplant Network data [110]. The potential advantage of this modality over resection is that it not only removes the tumour but also the liver with the background disease and thereby reducing the chances of recurrence. With a limited supply of life saving organs and with post-transplantation outcomes dependant on the complex relationship between tumour and immunosuppression, choosing the patient who will receive maximum benefit is of paramount importance[109].

The commonly used criteria for liver transplantation in HCC was initially proposed by Mazzafero et al in 1996 [111] and is now routinely referred to as the Milan criteria. (Table1.2) The reported 4 year survival rates are more than 70% with recurrence rates of less than 15%.

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Table 1.2 Milan criteria for consideration of OLTx in HCC

One HCC smaller than 5cm in diameter
or
Up to three HCC nodules less than 3 cm in diameter
In the absence of extrahepatic disease or macroscopic vascular invasion.

As the number of patients with HCC falling outside the Milan Criteria is increasing these criteria have since been challenged with suggestions that these criteria are too restrictive and expansion of these could achieve comparable survival rates. In 2001,Yao et al proposed to expand the criteria by including solitary tumours up to 6.5cm or three or less nodules with the largest not more than 4.5cm and the combined diameter is not more than 8 cms. They reported a five year mortality of 75.2% [112], thus proposing the UCSF criteria. The dilemma this criteria presented was that the results were based on explant tumour characteristics rather than on the preoperative characteristics based on imaging.This study was corroborated at UCLA by Duffy et al who even demonstrated the use of pre-operative imaging as opposed to explant histology to expand the criteria for transplant. They noted in a retrospective study that tumours which were beyond the Milan criteria showed a 5 year survival rate near or above 50%with a mean follow up of 7 years [113]. Other critical studies using different expanded criteria include the studies from Tokyo [114], Kyoto [115] and Hangzhou[116]which report five year survival rates up to 87%. Though the data is limited but it presents an opportunity for consideration of extension of the criteria for transplantation. Following the success of the expanded criteria indications for LT in the UK have been revised allowing patients with a solitary lesion between 5–7 cm to be considered provided this has not increased bymore than 20% over 6 months. The new criteria also allow for up to 5 tumours – all < 3 cm in size.

The discrepencies between radiological and pathological data for HCC prompted a search for more reliable morphological data and Toso et al evaluated total tumour volume (TTV) for transplant assessment[117]. A TTV of $> 115cm^3$ was used as cut-of and the radiological accuracy increased to 91% (69% and 75% for Milan and UCSF criteria respectively). A score combining TTV $>115cm^3$ and an alpha-fetoprotein level $>400ng/ml$ provided the best predictive outcome in a group of 6478 adult recipients of liver transplantation.

Liver transplantation is the best treatment option for HCC and with the expanded criteria an increasing number of cases of HCC are being considered for transplantation. This generates a constant struggle to strike a balance between the HCC and non-HCC recipients of liver transplantation. There is also the discrepancy between demand and supply of livers, which results in a drop out rate from the waiting list between 25 – 37.8% in twelve months owing to disease progression[118]. It is because of these constraints that hepatologists and hepatobiliary surgeons are obliged to follow a strict selection criteria. As a consequence patients with HCC who fall outside selection criteria are considered for alternative therapeutic options. To reduce these drop out rates during the waiting time, bridging therapies like transarterial chemoembolisation (TACE) and percutaneous ablation techniques are used, but there is no consensus at present as to the optimal bridging therapy before transplantation.

Live Donor Liver Transplantation (LDLT) for HCC has developed as an acceptable alternative to cadaveric transplantation over the last decade because of organ shortages, increasing waiting time and the expectation that many patients listed for liver transplantation will die while they are waiting for suitable organ. LDLT, however, is a complex procedure that is associated with a morbidity of 20 – 40% and a donor mortality of 0.3 – 0.5% [119, 120]. Donor safety is of paramount importance but the mortality data in donors raises some ethical and legal issues. Currently there is no consensus about an acceptable donor risk and an acceptable recurrence risk. Three studies have reported higher rates of tumour recurrence after live related liver transplantation though overall survival rates are not significantly different[121-123]. The difference in recurrence could be a result of selection bias as the longer waiting time for cadaveric transplants results in drop out of patients with aggressive HCC but when live related transplant is offered on a fast track basis it is conceivable that many patients with aggressive HCC did not wait long enough for the biological behaviour of the tumour to be assessed.

As for the criteria for selection, the data is not clear as to whether the centres should use the same criteria for DDLT and LDLT or use extended criteria for LDLT.

In many regions deceased donation rates are poor. In these areas live related liver transplant may offer the only chance of cure and should be considered a private gift. But there are conceptual reasons for concern about coercion and potential harm to the donor when only LDLT is offered for HCC.This is a difficult balace to achieve and

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centers preferentially offering LDLT for HCC should prospectively monitor and share data for both donor and recipient outcomes and balance their responsibility to protect the living donor with their respect for autonomy[124].

b. Local ablative techniques

Image guided tumour ablation is now a conventional treatment for tumours less than 3cms. Ablation induces rumour necrosis by injection of chemicals e.g ethanol or by temperature modification by radiofrequency, microwave or cryoablation and most of these procedures can be performed percutaneously.

Percutaneous ethanol injection, introduced in the 1980's was the most commonly studied. For single tumour upto 5cm or $\leq$ 3 tumours measuring <3cm the five year survival is 50% but for single tumours less than 3cm a 80% response is seen. This procedure is limited by a high recurrence noted to be up to 43%. Radiofrequency ablation has rapidly evolved, and is considered potentially curative for small HCCs. PEI involves injecting absolute alcohol into the liver lesion under ultrasound guidance, while RFA is a thermo ablative technique, inducing temperature changes by applying high frequency alternating current through electrodes placed in the tumour. RFA can be applied laparoscopically or percutaneously under ultrasound guidance. Randomised trials suggest that RFA is superior to PEI in the treatment of HCC of $\leq$ 3cm, both in terms of tumour response and long term survival [125-127] and a recent meta-analysis confirms the same [128]. This meta-analysis also concluded that RFA was superior to ethanol injection in ablating all viable tumour tissue and creating an adequate tumour free margin.

For tumors less than 2cm, RFA can achieve a 5-year survival of 68% with the preservation of liver parenchyma and thereby present a challenge to liver resection for this cohort of patients. Cho et al concluded that RFA and hepatic resection are to be considered equally effective for the treatment of HCC less than 2cm. Therefore, in patients with HCC measuring less than 2cm RFA can be offered a first line treatment, with resection reserved for individual variables, including tumour location, would make RFA not feasible or unsafe. For tumours larger than 2cm the the published data comparing RFA with resection is controversial. A randomized trial comparing resection with RFA including patients with tumours less than 5cm and Childs A disease of the liver failed to show any statistical difference in overall and disease free survival [129]. However another randomized trial comparing the two modalities in

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patients of HCC whose disease conformed to the Milan criteria suggested that resection may provide better survival and lower recurrence rates than RFA[130]. The quality improvement guidelines for radiofrequency ablation of liver tumours suggest that the target tumour should not be more than 3cm in its longest axis to achieve the best rates of complete ablation [131]

Microwave ablation (MW) is emerging as an effective alternative to RFA for thermal ablation for HCC. This modality heats matter by agitating water molecules in the tissue producing friction and thereby inducing cell death by coagulation necrosis[132]. Microwave ablation when compared with RFA show consistently higher intratumoral temperatures, larger tumour ablation volumes and faster ablation times.This modality is less affected by tumour proximity to vessels as compared to RFA [133]. However, a RCT comparing MW with RFA found no statistically difference in effectiveness of the two procedures [134].

A new, non-chemical, non-thermal image guided technique currently undergoing clinical investigation for management of HCC less than 3cm is irreversible electroporation (IRE). This modality induces disruption of cell membrane by changing the transmembrane potential, resulting in cell death without the need for additional pharmacologic injury [135]. This modality has the ability to to sharply delineate the treated from the non-treated tissue. There appears to be complete ablation to the margin of blood vessels with no compromise to the functionality of blood vessels [135]. With all the suggested benefits of this technique clinical trials required to determine its clinical effectiveness

c. Local embolic therapies

Transarterial embolisation(TAE) is a loco-regional modality for palliation of HCC not suitable for surgical resection or curative therapy but have relatively preserved liver function, absence of cancer related symptoms and no evidence of vascular invasion or extrahepatic spread [58]. Once the HCC is vascularised the blood supply is from the hepatic artery and not from the portal vein. TAE induces tumour necrosis by hepatic obstruction and when chemical agents mixed in lipiodol as the embolising agent then it is termed as Trans Arterial Chemoembolisation (TACE). The most commonly used agents are adriamycin and cisplatin [136]. Response rates of 16 – 60% are noted with TACE [12, 137, 138]. A meta-analysis of RCTs comparing TAE/TACE to a control group showed only a modest effect in terms of survival due to the advanced nature of the disease [138]. Tumour factors specifically related to good prognosis include

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tumour less than 5 cm, replacement of liver by tumour tissue of less than 50% and unilobar tumour [109]. Unfortunately even for this palliative treatment, a study reported that only 12% of patients were eligible.

An ideal TACE regime should achieve sustained concentration of the chemotherapeutic agent in the tumour with minimum systemic exposure.This has led to the introduction of embolic microspheres that have the ability to actively sequester doxorubicin from the solution and release it in a controlled and sustained fashion locally to the tumour with minimal amount of the agent reaching the systemic circulation as compared to the standard TACE[139].Results of the PRECISION trial indicate that TACE with drug eluting beads are better tolerated with significant reduction in toxicity. Significantly improved objective response rates were also noted in this trial [140]. A trial comparing TACE with drug eluting beads with TAE showed significantly lower tumour progression with the drug eluting beads [141]. Radioembolization is emerging as another form of tumour embolization. The most popular radioembolization technique uses microspheres coated with yttrium 90, a β-emitting isotope. Given intra-arterially the injected microspheres are preferentially delivered to the tumour bearing area selectively emitting high energy radiation to the tumour with minimal collateral damage [142]. Salem et al reported in a comprehensive analysis of 291 patients with HCC that yttrium microspheres could be an effective loco-regional treatment option especially in patients with portal vein thrombosis when TACE is not an option [143]. The results suggest that a well controlled trial is required to compare radioembolization with conventional TACE to establish its role in clinical practice.

d.   Systemic chemotherapy

Systemic chemotherapy is useful in a select few as a palliative option. The response is limited and is dictated by the underlying state of cirrhosis. Until recently, no phase 3 trial had demonstrated a survival benefit for HCC patients. This changed in 2007. The SHARP trial reported a modest but significant survival benefit for patients with advanced HCC treated with the RAF-kinase / VEGF-2 antagonist, sorafenib [144]. This drug has limited availability for NHS patients in the UK, although it has increasingly been adopted by regional cancer drug advisory groups under the 'Intermediate Cancer Drug Fund' introduced by the coalition government elected in 2010. Numerous other trials of targeted biological agents, either alone or in combination with surgical or ablative therapies, are ongoing and it is likely that more

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medical treatments will become available. As this happens, it is even more important that

we diagnose these cancers early enough to treat patients effectively,

We learn how to employ relevant staging systems and biomarkers to help to choose the best therapy for each patient.

1.2.8   The need for biomarkers

In the absence of markers for early detection of this disease, HCC continues to be diagnosed largely at a late stage. While advanced HCC has a dismal prognosis, there are therapeutic and even curative options for those detected earlier. Thus we urgently need to improve our means of performing surveillance in known at risk populations.

While improvements in radiological imaging advance in parallel, our own focus is on the identification of new serum biomarkers. The ideal serum surveillance marker for HCC would be specific for HCC and not detected in the individuals with predisposing liver disease (i.e. cirrhosis, regardless of the cause). The ideal marker should also be sensitive, enabling the detection of HCC at an early stage, when curative treatment is possible. It should be easily measurable, and the test highly reproducible, minimally invasive, and acceptable to both patients and physician[145].

Recent developments of gene-expression microarrays, proteomics and tumour immunology have raised the possibility of screening thousands of genes or proteins simultaneously. Whilst we struggle to handle the huge amount of data generated by these techniques, it is anticipated that they will play a role in the identification of new biomarkers for HCC within the in the next decade.

1.3   Experimental biomarkers

1.3.1   Proposed biomarkers

Serological tests that have been suggested for surveillance are PIVKAII (Prothrombin Induced by Vitamin K Absence), glycipan3 and the 8900Da fragment of Vitronectin. However none of these have been validated as surveillance tests for HCC. These and other novel proposed serum markers will be discussed in turn.

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a.Des-gamma carboxy prothrombin/Prothrombin induced by vitamin K absence (DCP/PIVKA-II)

PIVKA-II is an inactive prothrombin deficient in γ-carboxyglutamic acid and is produced by malignant hepatocytes. Recent evidence suggests that the elevated levels of PIVKA-II in patients with HCC is influenced by the increased production of PIVKA-II / DCP not only by the cancerous tissue but also by the surrounding non-cancerous tissue [146]. PIVKA-II results from an acquired post-translational defect in the vitamin K dependant carboxylase system. PIVKA-II production is independent of vitamin K deficiency, although pharmacological doses of vitamin K can suppress production of PIVKA-II in some tumours[147]. PIVKA-II has been reported to be a growth factor which increases the expression of angiogenic growth factors like VEGF, TGF-alpha and β-FGF in HCC cells which suggests its role in the progression of HCC [148].

Liebman et al in 1984 first reported the use of des-gamma carboxy (abnormal) prothrombin / PIVKA in the detection of hepatocellular carcinoma[149]. They detected des-gamma-carboxy prothrombin in the serum of 69 of 76 patients (91 per cent) with biopsy-confirmed hepatocellular carcinoma (the mean level of the abnormal prothrombin was 900 ng per millilitre). In contrast, levels of the abnormal prothrombin were low in patients with chronic active hepatitis (mean, 10 ng per millilitre) or metastatic carcinoma involving the liver (mean, 42 ng per millilitre), and undetectable in normal subjects. Using a cut-off value of 40mAU /ml, Cui et al reported a sensitivity and specificity of 51.7% and 86.7% in discriminating HCC from cirrhosis and also found that 36.84% of patients with small HCC also had a raised serum PIVKA-II values [150]. Marrero et al used a cut off value of 125mAU/ml in American patients and found PIVKA-II to have a sensitivity and specificity of 89% and 86.7% in discriminating HCC from, non-malignant hepatopathy [151]. However, regarding the role in detecting early tumours, Koike et al in 2001 in a study on 227 patients found that the serum DCP level is the most useful predisposing clinical parameter for the development of portal venous invasion.[152]. Other studies also confirm that HCC with raised PIVKA-II values are associated with higher frequency of HCC with indistinct margin, large nodule more than 3cm and moderate to poor differentiation [153, 154]. This would suggest that it detects late disease and not early disease, which doesn't make it a good candidate for surveillance. Similar to AFP,

PIVKA-II is an excellent marker for monitoring treatment efficacy, with changes correlating with clearance of HCC after curative treatment, and its subsequent recurrence.[68] Since PIVKA-II and total AFP are independent of each other in the setting of HCC and neither one is an ideal marker of HCC, Grazi et al find that the combination of the two is better because in their study, the sensitivity, specificity, and diagnostic accuracy for PIVKA-11 alone and total AFP alone are 53.3, 88.1 and 71.1% and 54.9, 97.4 and 76.6%, respectively but on combining the two markers these figures become 74.2, 87.2 and 80.95 respectively [155]. Other studies also have concluded that combination of the two markers increases the sensitivity as well the specificity[156-158]

b.Glypican 3

The name glypican has been assigned to a family of heparin sulfate (HS) proteoglycans that are linked to the cell membrane by a glycosyl-phosphatidylinositol anchor. To date, six family members of this family have been identified in mammals (GPC1 to GPC6). Glypicans play an important role in cell growth, differentiation and migration [159, 160]. Although down regulation of these proteins are noted in several tumours and cell lines, a study in 2001 showed increased glypican 3 (GPC3) expression at the mRNA levels in HCC as compared to healthy livers and benign hepatic lesions [161, 162].

Glypican 3 may enhance the growth of HCC by stimulating the canonical wnt pathway[163]. In 2003, studies showed the increased expression of GPC3 at the protein level in HCC. In one such study Capurro et al reported that GPC3 is elevated in more than 71% of HCCs but not detected in healthy livers and benign liver diseases [164, 165]. In addition GPC3 is detected in the serum of subset of patients with HCC. Nakatsura T et al in 2003 detected GPC3 in the sera of 40% of HCC patients. It was reported negative in 13 patients of liver cirrhosis, 34 patients of with chronic hepatitis and 60 healthy individuals, giving it a specificity of 100% [165]. Capurro et al also noted elevated serum levels of GPC3 in 50% of patients with HCC but in only 1/20 patient of chronic viral hepatitis [164]. In most cases of HCC no correlation was noted in the serum levels of GPC. These two markers in combination could improve the sensitivity to detect HCC[164] [166].

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GPC-3 and AFP has also been reported to be elevated in patients with testicular germ cell tumours [167]. Overall, however, the sensitivity of GCP-3 in isolation as a biomarker of HCC has been a little disappointing. The elevated levels in other malignancies also necessitates further studies needed to validate its usefulness as a surveillance tool

c. 8900Da fragment of Vitronectin

Paradis V et al identified 8900 Da fragment of vitronectin as a potential marker for HCC by serum proteomic profiling of HCC patients. Because they also demonstrated that vitronectin gene is down regulated in HCC they postulated that the occurrence of the 8,900-Da carboxy terminal fragment in sera of patients with HCC may derive from an increase in vitronectin catabolism associated with liver carcinoma[168]. No further studies have been published validating the data.

d. Heat shock protein 27 (HSP27)

Heat shock protein 27 belongs to a family of heat shock proteins that are ubiquitous and highly conserved proteins whose expression is induced in response to a wide variety of physiological and environmental insults. As molecular chaperones they perform an important role in protein folding, translocation, and refolding of intermediates, proteases, such as the ubiquitin-dependent proteasome, which ensure that damaged and short-lived proteins are degraded efficiently.

Heat shock protein 27 (HSP27) is ubiquitously present in many normal tissues[169]. Studies have shown that HSP 27 may play a role on thermo-tolerance, cellular proliferation and apoptosis, oestrogen response and molecular chaperoning[170-173]. Hsp27 can prevent apoptosis downstream of caspase-3(casp.3) activation by interacting with caspase-3.[174] The molecular mechanisms responsible for overexpression of heat-shock proteins in cancer cells are not known but a plausible scenario is that oncogenic mutations create an increased demand for chaperone activity within cells expressing protein variants that possess less than optimal folding characteristics. This cancer-specific stress response represents an adaptive process that allows cells to configure signal transduction pathways in such a way as to permit unrestrained proliferation. These oncogenic proliferative signals are tied to apoptotic processes that must be circumvented. This requirement may be met by the antiapoptotic function of heat-shock proteins[174].

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HSP 27 was elevated in the HCC tissues in 28 of 45 tissues and was correlated with a poor survival compared to HCC (17 of 45) with low expression of HSP27 [175]. Feng et al reported that HSP 27 was elevated in 90% of patients with HCC and in 2 of 20 patients with HBV but none in healthy controls[176]. However the cohort in the reported study was small and the results need to be validated in a larger group of patients. Furthermore, Heat Shock Proteins induced by cell stress are expressed at high levels in a wide variety of tumours and are not specific to the liver tissue, which does not make it the first choice for a surveillance marker for HCC.

e. Alpha-L-fucosidase(AFU)

Alpha-l-fucosidase is an enzyme which hydrolyses fucose glycosidic linkages of glycoproteins and glycolipids. Giardina et al in 1992 reported that serum alpha-l-fucosidase is a useful marker in detecting HCC, especially in conjunction with AFP and ultrasonography. They reported a sensitivity of 76% and a specificity of 90.9% for alpha L-Fucosidase. Its activity in patients with HCC was significantly higher than that found in cirrhosis, chronic hepatitis, other tumours and controls. They concluded that patients with cirrhosis who have a marked increase in serum AFU should be closely monitored for HCC development. El-Hosseini ME et al in 2005 also reported that adding AFU to measurement of AFP increased the detection of HCC from 68% to 88.6% [177]. These encouraging findings also, have yet to be validated in an independant and larger group of patients before the value of AFU as a surveillance tool can be affirmed.

f. Thioredoxin

Thioredoxin (TRX), a reductase enzyme, is known to contain an active site with a redox-active disulfide and has various biological activities. Tumour cell proliferation, de-differentiation and progression depend on a complex pathway of altered intracellular processes including cell cycle regulation, excessive growth factor pathway activation and decreased apoptosis. Metabolites from these processes result in significant oxidative stress that must be buffered to prevent cell death. It has been hypothesized that redox-sensitive signalling factors such as thioredoxin and thioredoxin reductase may represent central pro-survival factors that allow the tumour cell to evade the damaging and potentially cytotoxic effects of endogenous and exogenous agents that induce oxidative stress [178].

Hepatocellular carcinoma tissue shows increased expression of thioredoxin and thioredoxin reductase as compared to controls [179]. It has also been detected in sera

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of patients with HCC and has been shown to be significantly higher in patients with advanced HCC as compared to normal volunteers and patients with chronic hepatitis and liver cirrhosis without HCC [180]. Its utility in HCC diagnosis and surveillance is unknown.

Squamous Cell Carcinoma Antigen-1 (SCCA-1)

Squamous cell carcinoma antigen, a member of the high molecular weight family of serine protease inhibitor(serpins), is physiologically found in the spinous and granular layer of normal squamous epithelium and typically over expressed by the neoplastic cells of epithelial origin and so used as a clinical marker. Reports indicate that SCCA expression makes cancer cells resistant to several killing mechanisms by inhibiting apoptosis involving the caspase 3 pathway and/or upstream proteases.
Pontisso et al first reported the over expression of SCCA in liver tumours(14/18) and not in normal livers[181]. However, the first report of increased serum levels of SCCA1 in HCC patients as compared to cirrhotics was published in 2005 by Gianneli et al. They suggested that level of SCCA1 was not related to the size or spread of the tumours and that it could potentially be an early tumour marker. In a study of 120 patients with HCC and 90 patients with cirrhosis SCCA1 was found to have sensitivity of 84.2% but a specificity of only 48.9% but when combined with AFP a correct serologic diagnosis could be made in 90% of patients[182]. This performance was detected in patients with viral hepatitis and its value in patients with HCC developing against a background of other aetiologies such as alcoholic and non-alcoholic liver disease is unknown.

1.3.1 Dissemination of tumour cells in the peripheral blood

Solid malignancies are characterised by spreading of tumour cells from the tissue of origin to distant parts of the body at some point in the course of the disease. This spreading or dissemination of cancer cells from the primary tumour is the most important factor affecting prognosis in carcinoma patients. Once metastatic spread has occurred, the cancer disease is often no longer regarded as curable and medical treatment is generally considered palliative. Haematogenous spread of solid tumours represent a major challenge in oncology and has a fundamental influence on the outcome of the disease.[183]

The early seeding of metastatic cells in distant organs can contribute to metastatic relapse and is missed by traditional tumor staging. It has, therefore, been hypothesized that very sensitive monitoring of single or small clusters of tumor cells that disseminate into the blood would allow improvement of individual outcome prediction because it addresses metastasis formation more directly. Metastases currently are diagnosed by clinical manifestations, imaging studies like computerized tomography, and serum biomarker assay such as CEA for colorectal tumours. However, these methods are insensitive to the cancer cells at the small cell level or small cell clusters. The amount of tumour markers are related to the tumour mass and does not reflect viable disseminated tumour cells. Therefore, detection of cancer cell dissemination at an early stage could have great impact on cancer mortality, making eradication of cancer cells more probable by applying treatment modalities before clinically overt metastases appear. Moreover, detection of tumour cells in peripheral blood after resection could help early prediction of relapse in comparison to traditional tumour markers.

HCC is a solid tumour and liver being a very vascular organ there is likelihood that tumour cells may be shed from the HCC during treatment like Radiofrequency ablation, transplantation or resection. However, the number of tumour cells in circulation could be extremely small to be detected morphologically. In order to detect disseminated tumour cells in blood or bone marrow at an expected frequency of 10^{-6} or 10^{-7} nucleated haemopoietic cells requires highly sensitive techniques. During the last decade molecular techniques such as polymerase chain reaction (PCR) or reverse transcriptase polymerase chain reaction(RT-PCR) has been suggested as one of the most sensitive techniques.

Molecular detection methods such as PCR or RT-PCR target nucleic acids as discrimination markers between carcinoma and nucleated hematopoietic cells. A major drawback in the use of PCR with genomic DNA as starting material is that only a few tumor types show characteristic genomic alterations, and high sensitivity detection of mutations can only be obtained in cases where these occur in a few specific codons of a gene or when the mutation is already known.

The other main strategy for the detection of occult tumor cells involves detection of tissue-specific mRNA. The procedure is known as RT-PCR. This approach is based on the fact that malignant cells often continue to express markers that are characteristic of or specific for the normal tissue from which the tumor originates or

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with which the tumor shares the histotype. The appearance of these tissue-specific mRNAs at body sites where these transcripts are not normally present implies tumor spread[184] Because of the instability of mRNA in the extracellular environment, the detection of mRNA in peripheral blood requires the presence of viable tumor cells. Matsumara et al (1995) and Komeda et al (1995) using the polymerase chain reaction (PCR) to identify tumour-specific gene transcripts showed that the detection of isolated tumour cells in a small blood sample is possible and that the method is sensitive[185, 186]. Using PCR to detect target DNA or RNA of tumour cells it is possible to detect isolated tumour cells with a sensitivity of one cell in $10^5 - 10^6$ normal cells[187]. The clinical relevance of detecting these peripheral cells by molecular techniques has been the subject of ongoing debate. Recent data, however, suggest that detection of tumour mRNA in the peripheral blood is associated with a increased recurrence and poor prognosis in patients with colorectal or gastric carcinoma [188, 189] . In HCC, detection of peripheral tumour cells predict tumour recurrence[190].

 The above suggests that if this method is reproducible and correlates with the clinical condition then it could possibly be used as a molecular marker to detect HCC, either for diagnostics during surveillance, or providing prognostic information post treatment.

1.4   *Application of current techniques to identify novel biomarkers*

1.4.1   Genomics

Genomics is the study of genes and their function. Recent advances in genomics are bringing about a revolution in our understanding of the molecular mechanisms of disease, including the complex interplay of genetic and environmental factors

At present, histopathologic evaluation of a tumour and tumour staging are the mainstays for guiding therapeutic interventions and predicting outcomes. However, the limitations of the conventional methods are obvious. Tumours with identical histopathology may progress differently, responding differently to therapy and may be associated with different clinical prognosis, suggesting that additional parameters should be identified to predict disease outcomes. Gene expression profiles of cancers may be able to serve as a complimentary tool providing useful information.

Like most solid tumours, the development and the progression of HCC are believed to be caused by the accumulation of genetic changes resulting in altered expression of cancer related genes, such as oncogenes and tumour suppressor genes, as well as genes involved in different regulatory pathways, such as cell cycle control, apoptosis, adhesion and angiogenesis[192, 193] Comprehensive analysis of gene expression patterns of thousands genes in certain tumour cells and comparison to the expression profile obtained from healthy cells and/or other cancer cells of different phenotype can provide insight into the consistent changes in gene expression that are associated with tumour cellular dysfunction and concomitant regulatory pathways. Current cDNA microarray technology enables investigators to measure the expression of thousands mRNAs simultaneously in a biological specimen, providing comprehensive information which may ultimately aid diagnostic and prognostic methods, as well as identify novel therapeutic targets.

1.4.1.1 Microarray

Microarray technology which was developed to study differential expression of thousands of genes simultaneously in complex populations of RNA, allowing scientists and clinicians to identify qualitative and quantitative changes at RNA level in the development and progression of cancer[194-196]. Spotted arrays are manufactured using robots that deposit cDNA (PCR products) or short oligonucleotides onto specially designed glass microscope slides[197]. Spot sizes range between 80 and 150μm in diameter, and arrays that contain up to 80,000 spots can be obtained. Gene expression sequences to be arrayed are selected from public databases and the clones chosen are amplified from appropriate cDNA libraries by PCR and purified before spotting on the slide[195]

In general, the use of microarrays in HCC can be categorised into three purposes:

1. to define the molecular profile of HCC as distinct from non-cancerous liver and other tumour types identifying the tumour and liver specific genes.

2. to describe gene expression profiles that correlate with clinical subsets, which mechanisms of HCC development and progression.

3. to identify tumour specific molecular markers, which will be helpful for cancer diagnosis, prediction of prognosis and response to treatment.[198-200]

We are aware that progression of HCC is a stepwise process that proceeds from preneoplastic lesions , including low grade and high grade dysplastic nodules to early and then advanced cancer. Although the stepwise molecular changes associated with each stage are not clear, genome based research in this field is growing. Suk Woo Nam et al, 2005, used DNA microarray to show a clear molecular demarcation between dysplastic lesions and overt HCC lesions. They noted a heterogeneity in G1(low grade Edmondson grading) HCC suggesting that G1 HCC might border between preneoplastic lesion and outright carcinoma representing a transition state from dysplasia to carcinoma. They also identified 3,084 grade associated genes whose transcript levels were either positively or negatively correlated with tumour progression. The majority of oncogenes and tumour suppressors identified in the study demonstrated expression patterns that systemically change from dysplasia to carcinoma, and in some cases, with alteration in expression already detectable in the pre-neoplastic state. The latter suggests that these genes, acting together or separately, could be directly involved in common pathways of HCC pathogenesis [201]. This study showed that systemic approaches such as genome wide transcripts and regulatory pathways in precancerous lesions and HCCs may help us to gain the much needed molecular insight into hepatocarcinogenesis.

Microarray technology has also been used in an attempt to predict clinical outcome and survival in HCC. Ju-Seog Lee et al characterised gene expression profiles in 91 human primary HCC and 60 matched non-tumour surrounding liver tissue using cDNA microarray. Hierarchical clustering of data revealed two subclasses of HCC strongly associated with the length of survival. The group also noted that enhanced activation of ubiquitin-dependant protein degradation may account for deregulation of cell cycle control and faster cell proliferation in the poor survival group, perhaps indicating that deregulated components in ubiquitin-mediated protein degradation may provide attractive targets for novel HCC treatment modalities[202]. The data available from the microarray analysis can also be used to predict serum biomarkers. Using gene ontology analysis of the microarray data, upregulated genes encoding for the secretory or transmembrane proteins could be identified. Using this method in the data generated from HBx transgenic mice Quang Sun et al in 2007 identified four genes encoding TFF3, IGF2, LPL and SPL1 as promising diagnostic biomarkers for the detection of HCC in the tissue[203]. This appears to be a very

promising new method to mine for biomarkers for diseases both for diagnosis and for targeting treatment.

1.4.2 Proteomics

The term 'proteomics' was first coined in 1995 by Wilkins and co-workers[204, 205]. Proteomics may be defined as the direct qualitative and quantitative analysis of the full complement or subset of proteins present in an organism, tissue, cell under a given set of physiological and or environmental conditions[206]. The proteome is the time and cell-specific protein complement of the genome, encompassing all proteins expressed in a cell at any given time, including protein isoforms as well as co- and post- translational modified forms. The study of the proteome is more daunting than the study of the genome for several reasons. While the genome of the cell is constant, nearly identical for all cells of an organ and organism, and consistent across a species, the proteome is extremely complex and dynamic as it continuously responds, at both transcriptional and post transcriptional levels to multiple external factors as other cells, nutritional status, temperature, drug treatment, to name only a few. Therefore, there is no fixed proteome and the analysis of the proteome at any one time is a ''snapshot''. Serum and tissue are the most commonly studied for proteomics.

There has been significant interest in the study of global protein expression, an approach known as expression proteomics. It is well established that for only a subset of mRNA does expression significantly correlate with protein abundance. Moreover, post translational modifications, including phosphorylation, glycolsylation, and degradation, which are aberrantly regulated in many types of cancer, can be predicted neither by measuring the amount of RNA, nor by studying nucleoside sequences. Therefore, proteomic studies may overcome some of the limitations of global mRNA studies. As the functions of proteins are more directly linked to the aberrant phenotypes and malignant behaviour of cancer cells, proteomic studies may represent a better strategy to investigate cancer.

The technology of proteomics has generated great interest to apply the technique to investigate the proteome of diseased samples, with the very goal of mining biomarkers for different disease conditions because it is a non-invasive and can be repeated easily.

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1.4.2.1 Serum Proteomics

Of all accessible physiological reservoirs of human proteome, human plasma is considered one of the richest and most representative, with more than 10,000 distinct proteins[207]. Since blood has direct contact with most of the tissues of the human body, pathological changes are likely to be reflected with changes in the serum. The cancer proteome is an exceptionally complex biological sample containing information on perhaps every biological process that takes place in cancer cells, cancer tissue microenvironment, and cancer cell-host information. Cancer cells release protein biomarkers into the extra-cellular fluid through the secretion of intact or cleaved peptides. In addition, cancer-associated circulating biomarkers can be contributed by the tumour microenvironment e.g. surrounding host cells such as fibroblasts and macrophages. Some of these products can end up in the serum and can serve as potential biomarkers. This is one of the important reasons for exploiting the human serum in the search for biomarker discovery. However, it is difficult to identify these low abundance proteins in the serum. The study of the human serum proteome is complicated by the dynamic range of protein expression which may vary by as much as 7-12 orders of magnitude as compared to only five orders of magnitude for DNA[208, 209].

One of the major obstacles in mining these low abundance biomarkers from serum or plasma is caused by the fact a small number of proteins, including albumin, α2-macroglobulin, transferrin and immunoglobulins constitute about 80% of total proteins. At present, no single technique in a one-stop operation can provide the identification and quantification of all proteins in a complex sample like body fluids, cell lystaes or tissue extracts. Therefore, to achieve this objective of analysing the complex proteome concerted approach is needed which includes sample preparation, protein/peptide separation, mass spectrometric analysis and the use of bioinformatics tools for database search and quantification. Currently the tools for resolution and analysis of plasma proteins can be broadly categorised into the two-dimensional gel electrophoresis (2D-GE) approach and mass spectrometry (MS) based approach.

The high abundance proteins in the serum and tissues provide a significant background and make it difficult to identify the low abundance proteins. Therefore, a highly promising first step for most analysis strategies of serum or plasma is to deplete as many of the major proteins as possible, followed by pooling and

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concentration of the low abundant protein fraction to bring its concentration to a level consistent with the sensitivity range of current analytical instrumentation.

1.5   *Overview of current proteomic technology*

1.5.1   Elimination of the high abundant proteins

A range of methods to deplete high-abundance proteins have been evaluated which include Molecular Weight Cut Off Filters (MWCO), Cibacron blue – a chlorotriazine dye which has a high affinity for albumin, protein A or G to deplete immunoglobulins and the recently developed multiple affinity removal system.

A.   Low molecular weight cut off filters

Georgiou et al in 2001 using 30 kDa cut off membranes attempted to remove the high abundant proteins from the plasma following the manufacturer's protocol. They concluded that centrifugal ultra filtration of whole plasma prior to 2-DE does not adequately remove proteins and suggested that they are unlikely to be useful[210]. However, Tirumalai RS et al in 2003 separated low molecular weight protein using centrifugal ultrafiltration with 30kDa cut off molecular weight filters and get consistent results. Harper et al in 2004 using 50kDa cut off filters and modifying the manufacturer's protocol were able to successfully separate the high abundant proteins from the low abundant proteins efficiently[211]. The numbers of studies using low molecular weight cut off filters, however, are limited and this technique has not been validated or become routinely applied for removing high abundant protein.

B.   Cibacron Blue

Cibacron blue is a chlorotriazine dye which has a high affinity for albumin[212, 213]. While these dye based kits bind the majority of albumin they often also bind a large number of non-specific proteins, resulting in potential losses. This non-specific binding probably includes both proteins that bind to the dye as well as minor proteins that bind albumin[214]. Cibacron blue and other dye based methods are known to bind proteins with nucleated binding domains as well as via ionic and hydrophobic interactions[212]. This multifuntionality may result in the removal of proteins of interest through a rather broad and non-specific interaction, which can only be partially mitigated by judicious selection of mobile phase conditions

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C.   Multiple affinity removal system (MARS)

Another approach is the use of antibodies targeted to the common abundant proteins. Individual antibody methods have shown to be more specific in depleting targeted proteins like albumin and this strategy could be extended to the removal of any protein for which specific antibodies or affinity reagents are available[215]. Monoclonal antibodies are a promising target choice for their high specificity, but since they target a specific epitope of such proteins, they may not recognise all forms of the targeted proteins, e.g. proteolytic fragments, the post translational modified forms of the antigen, or proteins where the epitope is occluded due to protein-protein interactions[215, 216]. Polyclonal antibodies are more likely to remove multiple structural forms of the protein.

Ideally for a biomarker discovery it is desirable to deplete as many abundant proteins as possible and at the same time minimising incidental losses of the non-targeted proteins. Removal of high abundant proteins by multi-component immunoaffinity based protein subtraction chromatography introduced by Anderson and co-workers specifically for analysis of plasma was in important step towards this process[217]. The ability of a commercially available MARS HPLC column to efficiently remove the six most abundant serum proteins and to aid in the identification of more low abundant proteins efficiently, have been recently published[214].

1.5.2   Fractionation of serum low abundance proteins and protein identification

The next step following sample preparation is the separation of proteins. The most common method for separating complex mixture of proteins is called 'two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis' (2D-PAGE). In this approach the complex mixture is subjected to two dimensional chromatographic separation, using iso-electric focussing as the first dimension which separates proteins according to their isoelectric points and the second dimension is SDS-PAGE electrophoresis which separates proteins according to their molecular weights. The differential proteins of interest can be identified by two approaches:

A)'Top down' approach

B)'Bottom up' approach

A)   'Top down' approach

A conventional mass spectrometry approach to proteomics is generally initiated by one- or two- dimensional(2D) electrophoretic separation of a protein mixture, after

which protein bands are excised from the gel and subjected to reduction, alkylation and several washing steps, and finally enzymatic digestion followed by peptide extraction[218]. The masses of these peptides are characteristic of the protein, and provide a peptide 'mass fingerprint' which can be used in database searches to identify the protein of interest[219, 220]. The 2-dimensional gel technique has been used since 1975 when Klose, O'Farrel and Scheele almost simultaneously published the methods of isoelectric focussing in the first dimension and gel electrophoresis in the second. This technique has been very successful and will continue to be improved and refined. This technique is capable of producing highly reproducible results and there is good coverage of proteins with isoelectric point 3-11 and molecular weight 10 – 100kDa. Since it separates intact proteins, different isoforms of the same protein can also be separated and detected. In conjunction with other techniques of fractionation coverage of the proteome can be high. When combined with mass spectrometry, proteins can be identified and fully characterised [221].

2-Dimensional gel electrophoresis is currently the principal technique for the separation of complex protein mixtures[222]. 2-Dimensional electrophoresis not only generates information regarding protein information regarding protein modification and/or expression level changes but also allows for the isolation of proteins in significant amounts for further structural analysis using MALDI-TOF MS or Erdman microsequencing techniques[223]

 However, no technique is without its limitations and there are several practical and fundamental limitations associated with the 2-dimesional electrophoresis approach. Despite the potential and resolution of 2-dimensional gel approaches it remains a labour intensive technique to produce reproducible results. To overcome gel to gel or intrinsic biological simple variations, it is considered that, for this type of expression proteomics study, at least three different gels of three different samples of the same biological state are required. In any case, the total proteome coverage by 2-dimensional electrophoresis is experimentally limited to proteins with molecular weight in the 10 – 120 kDa range, with neutral – acidic end points. Basic proteins are very difficult to focus[224] and this technique rarely displays hydrophobic proteins weight[224]. Despite these drawbacks, Kim M and Kim C in 2007 concluded that despite a variety a techniques being attempted so far, no generally accepted technique has yet been developed for the identification of biomarkers that can replace 2-

dimensional electrophoresis with regard to its ability to separate and display several thousand plasma proteins simultaneously[225] [226]

B)   'Bottom up' approach

A widely used strategy in the bottom up approach is based on the use of isotope coded affinity tag (ICAT) reagents by the Aebersold group[227]. This technique consists of chemical labelling of protein sulph-hydryl groups with the 'light' and 'heavy' versions of the ICAT reagent followed by mixing of both samples and digestion by an endoprotease. The peptides are then fractionated usually using Mud-LC – strong cation exchange chromatography followed by reversed phase liquid chromatography. Relative abundance of the peptides and their identification is then established by MS/MS analysis by tandem mass spectrometry.

This approach has some inherent disadvantages with it. An important disadvantage is that proteins lacking cysteine residue are not detected.[224]. Also, digestion of an unfractionated protein mixture greatly increases the number of components to be analysed and condenses the peptide mixture into a narrow mass range, thereby complicating the task of isolating individual components for subsequent analyses. Furthermore, while the identification of only one peptide from a protein digest is required to identify that protein unambiguously, provided that peptide is unique to single protein, a significant amount of time during the analysis of complex peptide mixtures is spent analysing the same peptide ion or different peptides of the same ion. The peptide digest often yields significant number of peptide tandem mass spectra which are unassignable. Finally, it is inevitable that information is lost when upon transformation of an intact protein or protein complex into a family of polypeptides. Additionally, the mass of an intact protein or protein complex, which can provide important protein characterisation information such as the identity of post-translational modification, is not directly accessible via the 'bottom up' approach[226] This technique is also limited to the study of >30 kDa proteins only and there is obviously less coverage of the proteome it studies limited to proteins of MW <30kDa. [221].

1.5.3   Chromatographic techniques

1.   Ion exchange chromatography

Ion Exchange Chromatography relies on charge-charge interactions between the proteins in the sample and the charges immobilized on the resin of choice. Cation exchange chromatography is a form of ion exchange, in which positively charged ions bind to a negatively charged resin

Cation exchange chromatography retains biomolecules by the interaction of the sulfonic acid groups on the surface of the ion-exchange resin with histidine, lysine and arginine. Commonly used cation exchange resins are S-resin, sulfate derivatives; and CM resins, carboxylate derived ions. The bound solutes are then eluted differentially by adding increasing gradient of salt in the mobile phase.

2.   Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Reverse phase chromatography was originally developed in the 1960s for the separation of small organic molecules. The separation mechanism in reversed phase liquid chromatography depends on the hydrophobic interaction between the solute molecules in the mobile phase and the immobilised hydrophobic ligand i.e. the stationary phase. Reverse phase chromatography is an adsorptive process by experimental design which relies on a partitioning mechanism to effect separation. The solute molecules partition between the mobile and the stationary phases. The distribution of the solute between the two phases depends on the binding properties of the medium, the hydrophobicity of the solute and the composition of the mobile phase. Bound solutes are then desorbed from the stationary phase by adjusting the polarity of the mobile phase so that bound solutes will sequentially desorb and elute from the column. The gradual decrease in polarity (increasing mobile phase hydrophobicity) is achieved by increasing linear gradient from 100% initial mobile phase A containing no organic modifier to 100% (or less) mobile phase B containing a higher concentration of organic modifier.

Modern RP-HPLC utilises a wide selection of chromatographic materials to separate proteins and peptides. The choice of packing material has the greatest impact on the separation and resolution of proteins or peptides of interest. The separation efficiency of the packing material is determined by the particle size, pore size, surface area, stationary phase, as well as the chemistry of the substrate surface. The most

popular materials for RP-HPLC column packing are based on spherical silica. Typically particle size of 3-5μm are used for analytical separation of proteins, peptides or other small molecules. Separation efficiency increases by 30-40% when particle size is reduced from 5 to 3 μm.[228].

For this procedure it to be effective for human serum samples the most abundant proteins need depleting before subjecting to reverse phase chromatography. The RP-HPLC when coupled with high resolution mass spectrometry can lead to enhanced identification of low abundance proteins[229] .

1.5.4   Protein Identification

A.   Matrix Assisted Laser Desorption Ionisation (MALDI)

The proteins in the different fraction from different forms of Liquid Chromatography, described above, can be identified with help of Mass Spectrometers. The Mass Spectrometers require charged molecules for analysis. Large biomolecules are not easily transferred to the gaseous phase and ionised. However, the development of Matrix Assisted Laser Desorption of Ionisation (MALDI) and the Electrospray in the late 1980s should be credited with most of the success of Mass Spectrometry (MS) in life sciences.

MALDI was developed by Karas and Hillenkamp in the late 1980s. To generate gas phase, protonated molecules, a large excess of matrix material is coprecipitated with analyte molecules by pipetting a submicrolitre volume of the mixture onto a metal substrate and allowing it to dry. The resulting solid is then irradiated with nanosecond laser pulses, usually from small nitrogen lasers with a wavelength of 337nm. The matrix is usually a organic molecule with absorbance at the wavelength of the laser employed. Work with biomolecules almost exclusively uses α-cyano-4-hydroxycinnamic acid or dihydrobenzoic acid(DHB).

For MALDI time of flight (TOF) Mass Spectrometry the short burst of ions, generated by the laser pulses, are accelerated to a fixed kinetic energy and then they travel down a flight tube. The small ions have higher velocity and are recorded on the detector [182] before the larger ones, producing the time of flight spectrum. Several dozen to hundred of laser shots are averaged to produce the final MALDI spectrum. Performance in modern reflector MALDI Mass Spectrometers is typically in the range

of a few parts per million in mass accuracy, and only about a femtomole of peptide material needs to be deposited on the MALDI target to produce a signal

The mass range below 500 daltons is often obscured by matrix related ions in MALDI. The proteins generally undergo fragmentation to some extent during MALDI, resulting in broad peaks and loss of sensitivity, therefore MALDI is mostly applied to the analysis of peptides.

B.   Electrospray and nanospray

Electrospray mass spectrometry has been developed for use in biological mass spectrometry by Fenn et al [230]. Liquid containing the analyte is pumped at low microlitre-per-minute flow rates through a hypodermic needle at high voltage to electrostatically disperse, or electrospray, small, micrometer-sized droplets, which rapidly evaporate and which impart their charge onto the analyte molecules. This ionisation process takes place in atmosphere and is therefore very gentle (without fragmentation of analyte ions in the gaseous phase). The molecules are transferred into the mass spectrometer with high efficiency for analysis. To stabilise the spray, a nebuliser gas or some other device is often employed.

Nanospray is a miniaturised version of electrospray that operates without pumps and at very low flow rates in the range of a few micro litres per minute[231, 232]. It is performed in pulled glass capillaries with an inner diameter at the tip of about one micrometer. A microliter volume of sample can be analysed for more than one hour at full signal strength, which allows complete sequencing experiments to be performed[218].
The ions can be trapped for analysis in three-dimensional electric fields. These ion traps capture the continuous beam of ions up to the limit of their space charge. This is the maximum number of ions that can be introduced without distorting the applied field. The ions are then subjected to additional electric fields, which eject one ion species after another from the trap, and are detected, to produce a mass spectrum.
A large number of ions can be analysed by the electrospray mass spectrometry; the only requirement is that the molecule is sufficiently polar to allow attachment of a charge. The large ions are typically multiply charged which bring them into the range

of mass-to-charge ratios of typical mass spectrometers. The distribution of charges give rise to the typical multiple charged envelope[218].

C.   Fourier ion transform ion cyclotron resonance

Another version of ion trapping is embodied in the Fourier Transform Ion Cyclotron Mass Spectrometry. This technique was first published in the mid 1950s where it was demonstrated for measurement of very small mass differences at very high precision. It was applied to proteins only in the late 1980s, when electrospray made it possible to ionise the large protein molecules without disintegrating them.

In the basic FT-MS instrument the ions generated from the source are passed through a series of pumping stages at increasingly high vacuum. When the ions enter the cell pressures are in the range of 10^{-10} to 10^{-11} mBar with temperatures close to absolute zero. The cell is located inside a spatial uniform static superconducting high field magnet (typically 4.7 to 13 tesla) cooled by liquid helium and liquid nitrogen. When the ions enter the magnetic field they are bent into a circular motion in a plane perpendicular to the field. They are prevented from falling out of the cell by trapping plates at each end of the cell. The frequency of the motion is dependant on their mass/charge (m/z) ratio. At this stage no signal is observed, because the radius of the motion is small. Excitation of each individual m/z is achieved by a swept radiofrequency(RF) pulse across the excitation plates of the cell. Each individual excitation frequency will couple with the ions natural motion and excite them to higher orbit where they induce an alternating current between the detector plates. The frequency of this current is the same as the cyclotron frequency of the ions and the intensity is proportional to the number of ions. This results in the measurement of all the ions simultaneously producing a complex frequency *vs* time spectrum containing all the signals. The signal is then deconvoluted into a deconvoluted frequency vs intensity spectrum which is then converted to the mass vs intensity spectrum. Due to the ion trap nature of this instrument it is possible to measure the ions without destroying them.

1.6   Statistical Analysis

Statistical methods are required for analysis of data achieved during research. Variability in data is inevitable and to gain any relevant conclusion from this data for clinical application an appropriate statistical analysis of the same is essential.

Statistical analysis is hence needed to

- Explore and summarise data
- Analyse the data and derive conclusions
- Make relevant decisions

1.6.1 Types of data

Data can be classified into

- Qualitative data
- Quantitative data

Qualitative data refers to measured attributes e.g gender, blood groups, social groups, racial origin etc

Quantitative data refers to numerically measured data which can be continuous e.g blood pressure, temperature, or discrete e.g. number of admissions to the surgical ward.

1.6.2 Analytical methods

Analysis of differences

For analysis of data it is important to understand whether the data follows a normal or not normal distribution. The statistical tests can be either or non-parametric.

Parametric tests e.g. t-tests, ANOVA (Analysis of Variance) makes a number of assumptions about the data

- The data is continuous
- The data has a normal distribution
- When differences or measures of statistical association are being analysed between two or more samples the variance between these samples is not significant.

If the assumptions of a para-metric test are not fulfilled a non-parametric equivalent e.g. Mann-Whitney test, Wilcoxon, Kruskall-Wallis test, and for categorical data Fisher or Chi-square test is applied.

The significance of the difference calculated is reported as a probability. The probability (p-value), from zero to one, that the results observed in a study could have occurred by chance. By convention, the p-value of less than 0.05 is considered significant. That means the chance is less than 1 in 20 which is not very likely.

Multivariate analysis

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Multivariate analysis consists of a set of dedicated statistical methods used to analyse data sets with more than one variable to investigate relationship with the variables.

Survival analysis

Survival analysis is concerned with studying the time between entry to a study and a subsequent event. The event may death, disease free survival, recovery from illness. Two survival curves with the same condition but different treatment options could be compared with the log rank test.

1.6.3 Software package

The data is statistically analysed with suitable software. The software packages commonly used are

- SPSS (Statistical Product and Service Solutions)
- SAS/STAT
- Prism
- Minitab

1.6.4 Statistics used in this study

Linear regression was used to explore associations between two characteristics. The diffference between two groups of continuous variables obtained in serum ELISA assays were assessed by t-test (paramateric data) and Mann-Whitney test for two variables and Kruskall-Wallis test for more than two variables (non-parametric data). Differences between categorical variables were assessed by Pearson Chi Square, or Fishers Exact tests approximated using a Monte Carlo approach where cells within a contingency table of greater than 2x2 contained low numbers (<5). A p-value of <0.05 was considered significant.

Survival statistics in the prospective epidemiological study and the study of patients receiving liver transplant for HCC was performed using Kaplan-Meier analysis and to compare two groups of survival data log-rank test was used. To analyse any association of a patient or tumour characteristic with survival univariate analysis was performed. In the epidemiological study the factors included in this analysis included age, sex, Child-Pugh status, Performance status, mode of presentation, portal vein thrombosis,tumour number and AFP values.A cut-off of <0.05 was considered for factors to be included in the multivariate analysis. In the analysis for the subset of patients receiving liver transplant for HCC tumour characteristics affecting survival

which were considered for univariate analysis included aetiology, number of tumour nodules, degree of differentiation, AFP values, pre-operative treatment, lobar distribution, microvascular invasion and multifocal or unifocal tumour. A p-value of less than 0.05 was considered for the factor to be included in the multivariate analysis. All statistical analalysis was performed using using SPSS for windows, version 14 (SPSS Inc. Chicago Illinois, USA), licensed to the Newcastle University.

1.7   Aims

The **Primary Aim of this thesis** is to identify the disrupted genes or proteins in primary liver cancer development with a view to identify novel biomarkers for the disease. We want to correlate these with the clinical stage of the disease and identify those that are diagnostic, or which predict prognosis. We believe that this may improve our ability to accurately both diagnose and treat the disease.
Specific Aims included:

- The creation of a user friendly database incorporating all relevant patient information for exploring staging systems and their correlation with outcome, as well as identifying the characteristics and trends within our own patient population.

- To explore known and predicted serum surveillance biomarkers for HCC in patients with non-alcoholic fatty liver disease (NAFLD)

- To explore proteomic strategies for identifying novel serum biomarkers identifying advanced fibrosis in patients with NAFLD, as well as HCC in those with NAFLD cirrhosis.

CHAPTER 2.   PATIENTS AND METHODS

2.1.   Ethical Approval and Patient Recruitment

Recruitment of patients to this project was commenced in December, 2004 after receiving formal ethical approval from the regional ethical committee (Appendix D). All patients with suspected or confirmed HCC referred to the hepatology clinic were considered for the study. The diagnosis of HCC was made according to the EASL guidelines (European Association for Study of Liver diseases) as summarised in Table 2.1 and the patients were then staged accurately according to the Okuda and CLIP score and the performance status assessed. These scoring systems are as summarised in Table 2.2

Table 2.1 EASL criteria for diagnosis of HCC

Investigation	Criteria for diagnosis
Radiological & serum AFP	Non-invasive criteria is based on imaging techniques obtained by 4-phase multidetector CT or dynamic contrast enhanced MRI. Diagnosis should be based on the identification of the typical hallmark of HCC (Hypervascular in the arterial phase with washout in the portal and delayed phases and serum AFP levels >400ng/ml. While one imaging modality is required for nodules beyond 1cm in diameter, a more conservative approach with 2 techniques is recommended in suboptimal settings.
Histopathological	For nodules of 1-2cm, HCC diagnosis should be based on non-invasive criteria of imaging or biopsy proven pathological confirmation. However, there is a 30-40% false negative rate and a negative biopsy does not rule out malignancy[233]

Table 2.2 Scoring systems for HCC

Okuda Score	0	1	
Tumour size	<50% of liver	>50% of liver	
Ascites	No	Yes	
Albumin(µmol/l)	>30	<30	
Bilirubin (g/l)	<35	>35	

Okuda Stage	Stage 1: score 0; Stage 2: score 1 or 2; Stage 3: score 3 or 4		
CLIP scoring	0	1	2
Childs Pugh stage	A	B	C
Tumour morphology	Uninodular and extension <50%	Multinodular and extension <50%	Massive or extension >50%
AFP(ng/ml)	<400	>400	
Portal vein thrombosis	No	Yes	

Barcelona Clinic Liver Cancer scoring is described in Figure 1.1 (Chapter 1)

The patients were recruited after a fully informed written consent was obtained.

2.2 *Central Database*

A central database was the key to the study. A Microsoft access database was set up in conjunction with the Freeman Hospital information technology (IT) department, linked to the hepatopancreatobiliary (HPB) patient database recording all new referrals to the Newcastle upon Tyne NHS Trust. Unfortunately, this database lacked full functionality and ongoing IT support. Thus, the HPB patient database was used to identify all patients with HCC. Subsequently the main clinical, epidemiological, pathological and radiological variables were entered into a patient proforma (see appendix) and subsequently recorded in an excel spreadsheet database on a secured hospital intranet server. All therapeutic options were recorded, as was any invasive diagnostic procedure such as a biopsy. Patient progress was monitored until death.

2.3 *PATIENT DATA AND SAMPLES FOR ANALYSIS*

For the analysis of the data from the central database 130 consecutive patients with HCC referred to the Freeman Hospital between January 2004 and June 2006 were considered for analysis. The follow up data was cut off in June 2011. The data included demographics, Child Pugh status, CLIP, BCLS and Okuda staging and the treatment provided.

To explore and suggested biomarkers for HCC pre-treatment samples from 50 patients with HCC, all of whom had an underlying cirrhosis, were selected for study. Of these, 31 patients had alcoholic liver disease (ALD) and 19 patients had NAFLD. The serum

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was immediately separated by centrifugation and frozen at - 80°C. These serum samples were compared to an independent group of 41 patients with biopsy proven ALD or NAFLD cirrhosis. The diagnosis of NAFLD cirrhosis was made in patients who had clinical features and liver biopsies compatible with NAFLD. Females and males consuming greater than 14 or 21 units of alcohol per week respectively were excluded from this category, as were any individuals with viral or autoimmune liver diseases.

In the proteomics study for HCC serum samples from 5 patients with biopsy proven NAFLD without cirrhosis, 5 patients with NAFLD cirrhosis and 5 patients with AFP negative HCC were used for immunodepletion and 2 dimensional gel electrophoresis. A novel biomarker identified from the above experiments was assessed in the serum of 45 individuals with steatohepatitis-related HCC and compared to levels in 49 individuals with biopsy proven steatohepatitis-related cirrhosis and 64 patients with biopsy proven NAFLD steatohepatitis without cirrhosis or HCC.

2.4 EDTA blood and serum collection and storage

At each visit a blood sample was taken from those individuals who had consented to ethically approved (by the Newcastle and North Tyneside Ethics Committee) tissue collection. Serum from 5ml of blood was harvested after centrifugation at 3500g for 10 min and then stored at -80°C for serum proteomic studies and for later validation of markers. 5ml of EDTA blood was also taken and stored at -80C for preparation of leukocyte DNA.

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2.5 Serum Analyses

Successful serum proteomics, identifying and comparing quantities of proteins in similar serum samples, needs careful preparation. Collected samples were centrifuged and serum stored at -20degrees c within 2h wherever possible. Within 1-2 days, linked-anonymised samples were transferred to -80^{0}C storage at the Northern Institute for Cancer Research Central Tissue Resource. Protein quantification was as described in the Pierce BCA protein assay (section 2.5.1). Polyacrylamide gel electrophoresis for separation of proteins in a single phase is described (section), as are relevant standard laboratory techniques such as western blotting and ELISA assay for specific protein detection. In addition, for novel

protein detection, a methodology to remove the abundant proteins enabling the subsequent analysis of the depleted serum for change in any protein(s) to characterise a disease condition (which in our study was cirrhosis and hepatocellular carcinoma) was required. So we initially explored low molecular weight filter as a method for removing the abundant heavy proteins. We subsequently explored the use of immunodepletion to remove the top six abundant proteins, prior to separating samples by two dimensional gel electrophoresis. Each of these techniques are summarised.

2.5.1 Protein Quantification

Protein quantification with the Pierce protein assays was estimated relative to a standard curve created with Bovine serum albumin (BSA), as summarised below.

1. The BSA standards provided as vials in the kit were diluted in 'RIPA' buffer.

Vial	Vol of diluent (RIPA)	Vol and Source of BSA standard	Final (BSA) mg/ml
A	0	300(stock)	2
B	125	375(stock)	1.5
C	325	325(stock)	1
D	175	175(vialB)	0.75
E	325	325(vialC)	0.5
F	325	325(vialE)	0.25
G	325	325(vialF)	0.125

2. 10 μl of each standard or blank was placed in quadruplicate wells according to the following layout in a 96 well plate, with diluted unknowns (uk) in remaining wells.

Blank	2	1	0.5	0.125	Uk1	Uk3						
Blank	2	1	0.5	0.125	Uk1	Uk3						
Blank	2	1	0.5	0.125	Uk1	Uk3						
Blank	2	1	0.5	0.125	Uk1	Uk3						
Blank	1.5	0.75	0.25		Uk2	Uk4						
Blank	1.5	0.75	0.25		Uk2	Uk4						

| Blank | 1.5 | 0.75 | 0.25 | | Uk2 | Uk4 | | | | | | |
| Blank | 1.5 | 0.75 | 0.25 | | Uk2 | Uk4 | | | | | | |

3. The working Pierce reagents were made up according to manufacturer's instructions (50 parts Reagent A and 1 part Reagent B: 19.6 ml of Reagent A to 400 μl Reagent B in a universal container, followed by gentle inversion).

4. 190 μl of working reagent was added to each well using a multichannel pipette, followed by brief mixing on a plate shaker.

5. The plate was covered with cling film and placed in a 37°C incubator for 30 mins.

6. The plate was read on a spectra max plate reader at 562 nm, with software creating a standards curve and calculating the concentration of protein in each unknown.

2.5.2   Western blotting

i)   *Casting the gels using Biorad*

1. Clean glass plate and spacer plate were slid into the casting frame. After ensuring a tight seal at the bottom, the pressure clamps were engaged. The gel cassette assembly was then placed in a casting stand. A sample loading comb was placed on top of the assembled gel cassette to mark the level up to which the resolving gel is poured.

2. The resolving gel was prepared from the recipe in appendix 1. The chemical 'TEMED' is added last, just prior to pouring, as in the presence of APS, it helps to initiate the solidifying process. A 10% resolving gel was used as it is effective for the proteins in the range of 20 – 300 kDa. The solution was then poured up to the mark in the casting apparatus taking care to prevent mixing with air. It was then quickly overlaid with isopropanol, to keep the top of the gel smooth while setting

3. The gel was allowed to polymerise for 1h and then the alcohol overlay was removed by blotting and replaced with the stacking gel, prepared as per recipe in appendix 2. Again, the APS and Temed were added last and the solution was poured between the glass plates till the top of the short plate was reached.

4. The sample loading comb (creates the spaces in which the samples will be placed after the loading/stacking gel has set) was then inserted between the plates and the stacking gel allowed to polymerise for 45 minutes.

5. The comb was gently removed and the wells are rinsed thoroughly with running buffer. The gel cassette assembly was then removed from the casting stand and then the gel cassette sandwich released from the casting frame and was ready for electrophoresis after sample loading
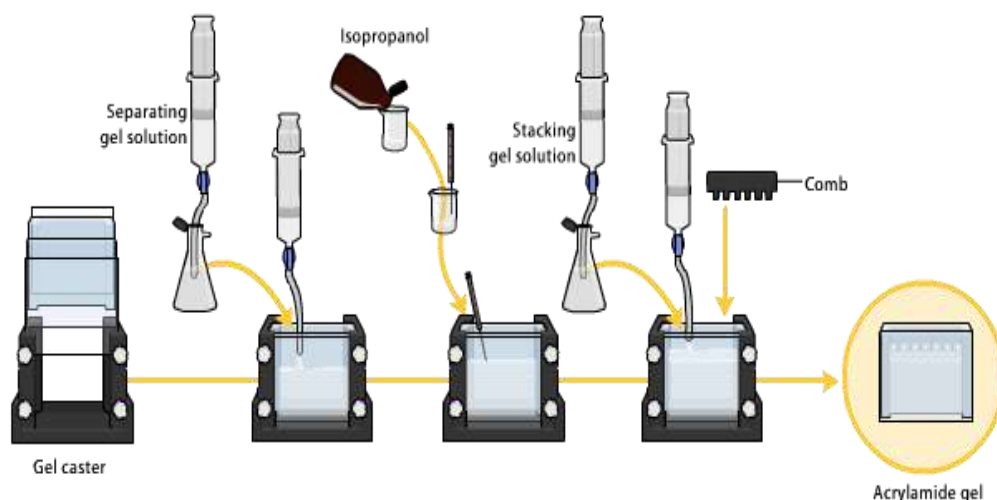
Figure 2.1 below shows the preparation of SDS gel for electrophoresis

ii) SDS Polyacrylamide gel electrophoresis

1. .Running buffer was prepared while the stacking gel was setting. The stock recipe is used (appendix 3), diluted 1:10 (900ml distilled water: 100ml of running buffer) prior to use.

2. The prepared serum samples were thawed on ice and 50 μg protein equivalent is aliquotted into each eppendorf and RIPA buffer added to make total volume of 15 μl. To each of these aliquots 5 μl of SDS loading buffer was added. The aliquots are then placed in a preheated hot block at 100ºC for 2 minutes.

3. Before loading the samples the wells were flushed gently to ensure they were free of 'unset' acrylamide. The samples were then loaded into the wells, with a record kept of their order, relative to the marker loaded into the end well.

4. The lid was then placed on the tank ensuring that the colour coded plugs (determining the direction of current passed) were in the correct orientation.

5. Ice was packed around the apparatus. The leads were then inserted into the power supply, with volts set as follows:

      a) 90v for the running in the stacking solution

      b) 150v for running in the resolving gel.

6. The Hybond P (Polyvinylidene difluoride, PVDF) membrane to which separated proteins would be transferred was prepared by cutting a piece slightly bigger than the size of the gel along with two similar sized filter paper while the gel was running. Transfer buffer was also prepared using protocol in appendix 4. Two pieces of sponge more than the size of the gel was also assembled.

iii)   *Protein transfer onto the membrane and antibody fixing*

1. After the electrophoresis was complete, the power supply was turned off, the electrical leads disconnected and the gel running assembly removed from the tank. The gel cassette was freed, and the gel plates were separated. The gel removed by gently floating it in a container of transfer solution.

2   . For assembling the transfer of proteins from the gel to the hybond P membrane, a sandwich of plastic cassette (black side), sponge, filter paper, gel, hybond P, filter paper, sponge cassette (red side) was made, as described in the figure below

--------------------------------red (+)
--------------------------------pad/sponge
--------------------------------filter paper
------------------------------membrane
-----------------------------gel
--------------------------------filter paper
--------------------------------pad/sponge
--------------------------------black (-)
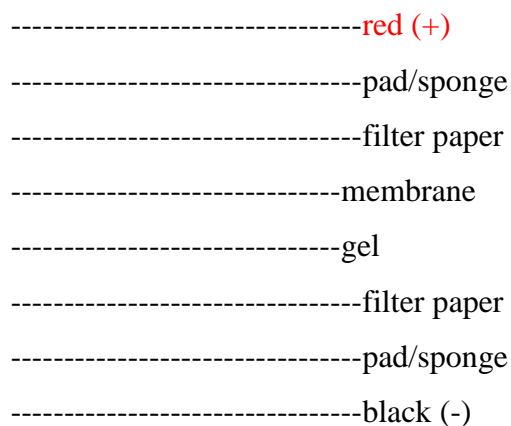
3. The closed sandwich cassette was then placed in the correct orientation in the transfer tank (black side of the cassette facing the black side of the tank)

4. The gel transfer was done at 40mA overnight on the bench using cooling packs around it and stirrer within the tank to ensure circulation of the buffer. The transfer of the ladder proteins was used to check for transfer.

5. After removal, antigenic proteins on the transfer membrane were blocked with 100ml of 5% milk in TBS, shaking gently for 1h. The membrane was subsequently washed in TBST 5-10 minutes, repeated 3 times.

6. Incubating transfer membrane with antibodies was as follows:

Description	Antibody raised in	Dilution factor	Diluted in	Incubation time	Incubation temperature
Anti Granulin	Rabbit	1:100	5% milk in TBST	Overnight	Room
Anti FST	Mouse	1:250	5% milk in TBST	Overnight	Room

7. After the incubation with primary antibody the membrane was washed three times with TBST, as it was after secondary antibody incubation.

Description	Dilution factor	Diluted in	Incubation time (hours)	Incubation temperature
Anti rabbit	1:1000	5% milk in TBST	1	Room
Anti mouse	1:1000	5% milk in TBST	1	Room

iv) *Developing the 'Western Blot'*

1. The inside of the radiographic cassette was lined with cling film. The Pierce West Dura Substrate kit was used. The TBST was removed, leaving the membrane in its empty wash container.

2. The membrane was covered with 1ml of 'Dura' solution (500μl of stable peroxide buffer plus 500μl of luminal enhancer) for five minutes.

3. The membrane is then transferred to the clingfilm within the radiographic cassette and carefully wrapped and smoothed.

4. In the dark room, radiographic film was placed over the membrane and the cassette is closed for 1min, prior to the film being removed and placed into the automatic film developer. On film removal, additional time points for incubation with film were chosen pending the initial result.

2.5.3   ELISA (Enzyme Linked Immunosorbent Assay)

Western blotting was used to confirm the presence of discriminatory identified in our projects. However, an ELISA assay is a more quantitative technique and a background to the technique and summary of our methods are summarised below. 'ELISA' evolved in the 1960s from a radio-immuno assay, with the observation that either the antibody or the analyte / antigen could be adsorbed to a solid surface and still participate in specific high affinity binding. Engvall and Perlman published their first paper on ELISA in 1971 and demonstrated quantitative measurement of IgG in rabbit serum using alkaline phosphatase as the reporter label.[234]. The basic purpose of an ELISA is to determine whether a particular protein is present in the sample, and is so, quantify it.

In an ELISA, the antigen of interested must be immobilised to a solid surface, which is also called the 'solid' phase. The antigen is then complexed with an antibody that is linked to an enzyme. Detection is accomplished by incubating this enzyme-complex with a substrate that produces a detectable product. The most crucial element of the detection strategy is a highly specific antigen-antibody interaction. The ability to wash away the non-specifically bound materials makes the ELISA a powerful tool for measuring specific analytes within a crude sample preparation.

I)   Types of assays

i)   Direct and Indirect ELISA

A detector enzyme may be linked directly to the primary antibody (***Direct ELISA***) or introduced through a secondary antibody that recognises the primary antibody (***Indirect ELISA***). The most common enzymes used are Horse radish peroxidise (HRP) and Alkaline phosphates (AP).Other options have been used but they have not gained widespread acceptance because of limited substrate options. The choice of

substrate is also determined by the necessary sensitivity level of the detection and instrumentation available for detection (eg spectrophotometer, fluorometer or luminometer).
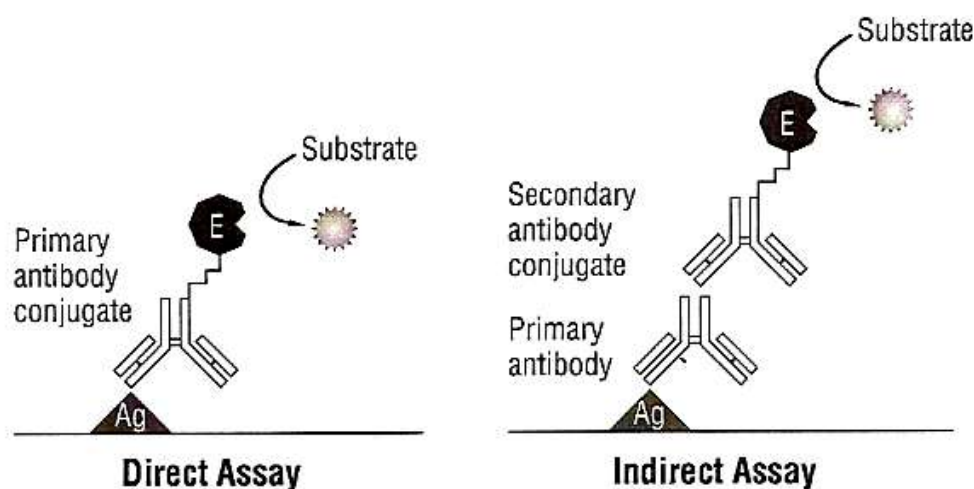
The advantage of direct assay is that it is quick and cross reactivity of secondary antibody is eliminated. It has the disadvantage that immunoreactivity may be reduced by labelling of the primary antibody. In addition, labelling of the primary antibody is time consuming and expensive, with no flexibility in choice of primary antibody label from one experiment to another.

Cross reactivity of the secondary antibody can be a disadvantage with the indirect ELISA, but this assay has some significant advantages. Firstly, a wide variety of labelled secondary antibodies are available; secondly, the immunoreactivity of the primary antibody is not affected by labelling of the primary antibody. Thirdly, the sensitivity is increased because each primary antibody has several epitopes that can be bound by the labelled secondary antibody allowing for signal amplification.. Lastly, different visualisation markers can be used with the same primary antibody.

ii)   Capture ELISA / Sandwich ELISA

Another commonly used format for ELISA is the sandwich assay. The name is derived from the fact that the analyte to be measured is captured between two antibodies – the capture antibody and the detection antibody i.e. two antibodies directed to two different epitopes of the same protein to prevent interference in binding. It is more sensitive and robust. Either monoclonal or polyclonal antibodies can be used. Monoclonal antibodies have an inherent monospecificity towards a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as a capture antibody to pull down as much

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antigen as possible, and then a monoclonal antibody is used as the detection antibody to provide improved specificity.

Capture Assay
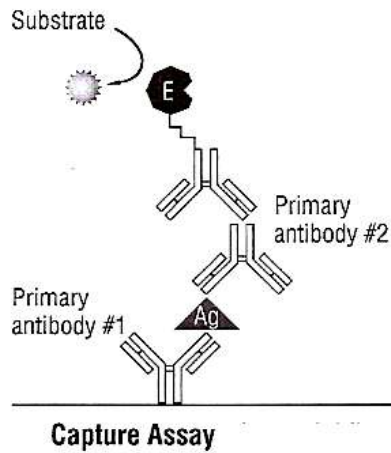
II) Sensitivity of an ELISA assay

The antibody is the key to the sensitivity and specificity of an ELISA assay. It is the three dimensional configuration of the antigen binding site found in the F(ab) portion of the antibody that controls the strength and specificity of the interaction with the antigen. The stronger the interaction, the lower the concentration of the antigen that can be detected. A competing factor is the specificity of the binding or the cross reactivity of the antibody to serum proteins other than the target proteins.

The sensitivity of the assay can also be compromised by the presence of detergents in the coating solution, which can prevent binding of the protein to the plate. Excessive concentration of coating protein occasionally lead to less binding, a phenomenon known as the 'hook effect'. This can therefore cause poor detection of the protein in the sample.

III) Detection molecules

Common detection molecules used are Horse radish peroxidase and alkaline phosphatase.

Horseradish Peroxidase (HRP) Substrates

Horse radish peroxidase is a 40 kDa protein that catalyses the oxidation of substrates (e.g.TMB; tetramethylbenzidine) by hydrogen peroxide, resulting in a coloured or fluorescent product or the release of light as a by-product. High turnover rate, low cost, wide availability of substrates and superiority of antibody-HRP conjugates in comparison to antibody-AP (Alkanine phosphates) makes it the enzyme of choice for most applications.

Alkaline phosphatise (AP) Substrates

Alkaline Phosphatase is a 140 kDa protein that catalyses the hydrolysis of phosphate groups from a substrate molecule, resulting in a coloured or fluorescent product or the release of a fluorescent product as by-product.

A.   Indirect ELISA for FST and CD5L

Due to the availability of antibody to only one epitope of our target proteins studied (FST and CD5-L), we developed the indirect ELISA. FST and CD5L recombinant protein was used in serial dilution (*figure 2.2*) to obtain a 'standard' graph. After measuring serial dilutions of FST and CD5L, the optimum dilution for FST was noted to be 1:50, while for CD5L it was 1:2. Reproducibility was confirmed by duplicate analyses.
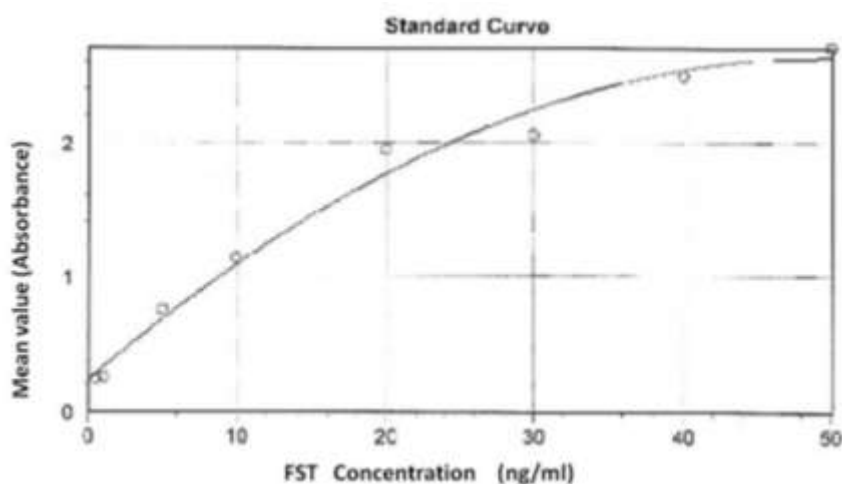
Figure 2.2: The standards curve generated with known concentration of the target protein Follistatin (FST)

The materials are summarised below:

Coating buffer (1 × TBS) (Appendix 6)

Wash buffer (TTBS; TBS + 0.05% Tween 20)

Tween 20 = 0.5gm per litre of TBS

Blocking buffer

3% BSA (Bovine serum albumin) in TBS

Dilution buffer

% BSA in TTBS

Primary antibody

Mouse antibody for FST (Sigma Aldrich SAB14025150

Goat antibody for CD5L (Abgent AT1443A)

63

HRP(Horse raddish peroxidise) secondary antibody

Anti-mouse antibody for FST

Anti-goat antibody for CD5L

Substrate solution (appendix 7) was prepared fresh

The method developed was as follows:

Serial dilutions (in coating buffer) of the recombinant protein were placed in triplicate in the wells of the ELISA plate.

Appropriate dilution of the serum (1:10) was made in the coating buffer and 100µl was then placed in triplicate wells. The plate was covered for overnight incubation at $4^0\,C$

The following day the plate was emptied. The plate was then inverted and tapped on a paper towel.

Each plate was rinsed 4 times with wash buffer.

The primary antibody was diluted (1:250 for FST and 1:500 for CD5L). 50µl of primary antibody was then added to each well and the plate incubated for 1h at 37^0C.

Step 4 was repeated to wash the plate.

50µl of HRP antibody (anti-mouse for FST 1:2000 and anti-goat for CD5L 1:5000) in dilution buffer was added to each well. The plate was then incubated at room temperature for 1h.

The plate was washed as in step 4.

100µl of substrate solution was added to each well and then left at room temperature for 30 minutes. A blue colour should develop.

The reaction was stopped by adding 50µl of 1m sulphuric acid, turning the colour yellow.

The plate was then read in the Spectra Max plate reader at optical density (OD) 650 nm and 450 nm. The reading at at 650nm was then subtracted from the OD reading at 450 nm. The plates were read within 30 min of stopping the reaction.

2.5.4   ELISA for PIVKA-II (Prothrombin induced by vitamin-k absence)

As a part of our project we also analysed known serum biomarkers PIVKA-II and glypican 3 in our serum samples, exploring whether they would add any value in our

surveillance for HCC in our patients. Custom made ELISA kits were used for these analyses, used according to manufacturers instructions, described in appendix B.

2.5.5   ELISA for Glypican-3 (GPC-3)

The glypican 3 assay was performed with an ELISA kit manufactured by Biomosaics limited (Catalog no B1500) available for research purposes. It is ''sandwich'' ELISA employing two GPC-3 specific antibodies. The assay was performed as per manufacturer's protocol described in appendix C.

2.5.6   Optimising serum samples for proteomic studies

From the available literature it was obvious that to mine for biomarkers we needed to analyse the less abundant proteins. To achieve that objective it was important that we optimised a method with reproducible results to remove the abundant proteins from the serum samples before subjecting it to two dimensional fractionation. The first step was to assess the effectiveness of low molecular weight cut off filters in removing the abundant high molecular weight fractions

Low Molecular weight filters

To 100µl serum aliquots 300µl of TBS buffer was added and centrifuged at 10,000g for 10 minutes. The supernatant was then applied to the Low Molecular Cut Off Filter (Microcon/Millipore) and centrifuged at 10,000g for 30 minutes at 4° C. Multiple filtered fractions of the same sera were prepared at the same time and are pooled together. The pooled fractions were then concentrated by lyophilisation and the protein concentration is checked BCA assay. The retentate from the filters was also treated in the same way.

To check for the efficiency of the cut off filters we subjected the depleted serum and also the retentate through one dimensional SDS-polyacrylamide gel electrophoresis which was performed as per protocol mentioned in section 2.4.2.(ii). The gel was then stained with Coumassie.

The 30kDa filter showed no low molecular weight protein bands in the filtrate and the 50kDa revealed low molecular weight bands but could not effectively remove the high molecular weight proteins in the filtrate. Two 50kDa filters with the same serum sample show different filtration fractions suggesting significant

inconsistency. The failure of the low molecular weight cut off filters led us to revisit our strategies.
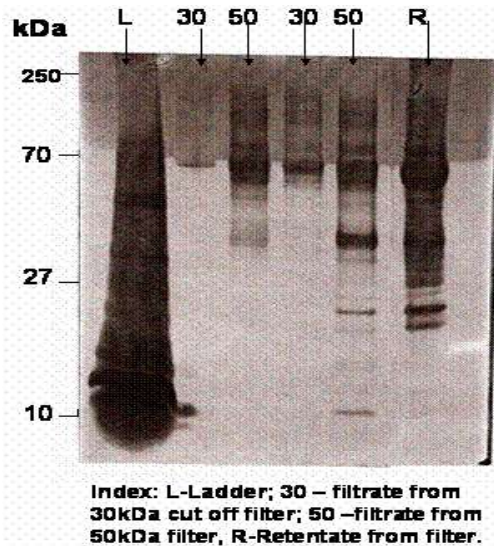
Figure 2.3. Picture of gel comparing retentate with the filtered fractions from a 50 kD and from a 30kDa filter. The ladder has stained as a control and the raw serum shows some protein bands to confirm the performance of the PAGE. The filtered sera from the 30 kDa filters fail to reveal any bands in the gel and the filtrate from the 50 kDa filter shows bands from more than 50 kDa proteins

2.5.7 Immunodepletion of abundant proteins and 2-dimmensional gel electrophoresis

Following the encouraging reports about the MARS (Multiple Affinity Removal System) immunoaffinity column in effectively depleting the top 6 abundant proteins with reproducible results we used this as our first step. The serum fraction depleted of the top 6 abundant proteins was then subjected to 2-dimensional gel electrophoresis for the second dimension.

Immunoaffinity Column (Agilent Technologies)

An 1100 HPLC (Agilent Technologies) apparatus was used for the immunoaffinity column. The HPLC apparatus is first purged with Buffer A and B (Proprietary buffers for the column). Buffer A is a salt containing neutral buffer, pH7.4, used for loading, washing and requilibrating the column. Buffer B is a low-pH urea buffer used for eluting the bound high abundance proteins from the serum A time table is set up in the apparatus for the running of the serum samples as mentioned in the manual.

LC method for 4.6×100mm column

Time	%B	Flow Rate ml/min	Max Press
0.00	0	0.500	120
10.00	0	0.500	120
10.10	100	1.00	120
17.00	100	1.00	120
17.01	0	1.00	120
28.00	0	1.00	120

The MARS column is attached after two runs with buffer A the HPLC apparatus, For each run, 80µl of serum is prepared by diluting with 300µl of buffer A and then centrifuged through a 0.22µm filter at 16,000 for one minute to remove particulates. The fraction eluting from between 2.5 min and 6 minutes corresponding to the less abundant proteins was collected. The protein quantities were assessed by the BCA protein assay kit and then the depleted samples are then stored at -20° C before two dimensional electrophoresis.

Two dimensional gel electrophoresis

This involves isoelectric focussing as the first dimension and gel electrophoresis as the second dimension

For isoelectric focussing the sample was prepared with a rehydration buffer to completely solubilise the proteins, prevent protein modification, remove interfering components and maintain protein in solution during the isoelectric focussing. Isoelectric focussing was performed using 18cm, 3-10 non-linear strips (GE Healthcare Immobiline^{TM} Dry Strip pH 3-10 NL, 18cm). For each strip depleted sera with 500µg of protein was prepared by adding rehydration buffer (appendix) (total volume 450µl). After incubating in room temperature for 30 minutes the sample was pippeted into the strip holder. The non-Linear strip is then placed in the holder with the gel side down taking care to avoid any air bubbles

After incubating at room temperature for 12 hours, isoelectric focussing was performed using high voltage and low current using the following protocol

Step	Voltage	Duration (h:min)	Kilo volt hours (KVh)	Gradient Type
1	Rehydration	12:00	00	
2	500	6:00	3	Step-n-hold
3	3500	01:30	3	Step-n-hold
4	3500	06:00	30	Step-n-hold

After focussing, the strips were stored at -20° C before proceeding to the second dimension gel electrophoresis. The second dimension gel electrophoresis required the preparation of 25×25cm gel and then equilibrating and saturating the strips before gel electrophoresis.   To prepare the gel for electrophoresis the components in appendix 10 were mixed and degassed. Then Temed (337.5µl) and 10% APS (3.375µL) were added to the above solution. 10ml of the solution was then poured into each gel stack (Amersham Biosciences) and the gel allowed to settle.

The isoelectric focussed strips were first treated with 15ml of equilibration buffer with added DTT (100 µg/10ml) on a rocker for 15 minutes and then with 15ml of equilibration buffer with IAA (250µG/10ml) for a further 15 minutes on a rocker.

The equilibrated focussed strips were then placed over the top of the 1mm gel and secured with agarose. The gel in the plates was then placed in the DALT six ([TM]GE Healthcare Life Sciences) and electrophoresis was performed using the following conditions

Step	Current (mA/gel)	Duration (h:min)
1	10	00:15
2	20	05:00

The gel run was stopped within 1cm of the lower edge of the gel and the gels were washed with double distilled water. The gels were then stained with Coumassie for one hour and then destained with double distilled water overnight.

Next day the gels were imaged under the scanner at 16 bits and stored for further analysis. The gels were then analysed using the software.

2.5.8   Microarray data for serum biomarkers

The complete unravelling of the human genome and the increasing amount of data from a DNA microarray of different disease conditions has opened up a new window to mine for biomarkers. Microarray data provides us with a vast amount of information about genes expressed in a given tissue. The mining of this data can identify genes or clusters of genes which transcribe cell surface or secreted proteins, but needs careful and complex statistical analysis.

RNA was previously extracted from HepG2 cells (hepatoblastoma cell line) stably expressing either empty vector (pBABEpuro) or the KLF6 tumour suppressor gene. Proliferation was suppressed in the cells over-expressing KLF6. The RNA obtained from both the cell lines was converted to cDNA and biotin labelled, prior to hybridisation to Codelink Bioarrays (Unpublished data from Dr Reeves Group). The Bioarrays were previously scanned using an Axon Laser Scanner and Genepix Software, prior to importing the data into Gene Spring version 6 (Silicon Genetics) for analysis.

My own analysis of this data identified 20 genes with a particularly high basal level of expression in HepG2 cells, whose expression was suppressed by at least 2 fold in the slower growing KLF6 overexpressing cells. Of these, I identified genes which were secretory proteins/transmembrane proteins, which were targets of MMPs, or involved in regulation of the cell cycle/survival. I also looked for those to which commercial antibodies were available. Consistency with other data sources was also explored, including the Stanford university website of HCC microarray data. Finally, I chose two genes Follistatin (FST) and Granulin) suspected of having a role in hepatocarcinogenesis, which were predicted to be present in serum, for characterisation at the protein level.

Western blotting was then performed with immunodepleted serum obtained from the previous part of the study. The samples were then blotted separately against antibodies for Follistatin (FST) and granulin. The results with FST appeared discriminatory for HCC and cirrhosis. But granulin was non-discriminatory and was discarded from further study.

Chapter 3 Results 1. Hepatocellular cancer in the North-East of England

3.1 Introduction - Approach and management of HCC patients in Newcastle

With 200,000 cancers diagnosed annually in the UK and 120,000 people dying from cancer, cancer treatment has become one of the central priorities for the NHS. It was recognised in the 1990s that there were many regional variations in the quality of cancer patient care and treatment. In the year 2000, the department of health introduced a comprehensive 'Cancer Plan' aiming to deliver the fastest improving cancer care services in Europe. The plan had four objectives:

To save more lives

To ensure people with cancer get the right professional support and care as well as the best treatments.

To tackle the inequalities in health that meant unskilled workers were twice as likely to die from cancer as professionals

To build for the future through investment in the cancer workforce, through strong research and through preparation for the genetics revolution, so that the NHS did not fall behind in cancer care.

The success of the plan would rely on reducing the risk of cancer, detecting cancers earlier, improving cancer services in the community and ensuring faster access to treatment. The goal for faster access to treatment was that no patient should wait longer than one month from an urgent referral by their GP with suspected cancer to the start of treatment. The progress of the cancer plan has been closely followed (www.nhs.uk/nhsengland/NISF/pages/Cancer.aspx) and some of the important achievements are outlined below

Better prevention – A ban on smoking in private places has resulted in a reduction in smoking rates

More early stage cancers are detected through screening – National screening programmes for bowel, breast and cervical cancer are considered successful and further screening programmes will be introduced.

Faster diagnosis and treatment – waiting times for cancer have reduced dramatically Improved access to better treatment – there has been a major increase in the use of drugs approved by the National Institute of Clinical Excellence to treat cancer with less variation between cancer networks.

Free prescription charges for patients with cancer – since April 1, 2009, patients undergoing treatment for cancer, including the effects of past cancer treatment, have been able to apply for medical exemption.

Building on the success of the Cancer plan 2000, the Cancer Reform Strategy was published in December, 2007. It detailed how by 2012 cancer services in England could be among the best in the world. The continuing achievement in the early diagnosis and treatment of cancer could be facilitated by having dedicated databases in trust hospitals with prospectively entered data, with regular monitoring of progress. A step in the right direction was taken at Freeman Hospital at the time of the inception of the NHS cancer plan 2000, when a Microsoft Excel database was initiated in the same year, with the primary aim of auditing the success of radiofrequency ablation(RFA) as a recently introduced therapeutic intervention for colorectal liver metastases and HCC. In this database a record of interventions and outcomes for patients, treated with RFA, was maintained by clinical surgical staff. The majority of patients receiving RFA at this time received the treatment for colorectal liver metastases rather than primary HCC.

In parallel a separate NHS IT staff supported database was set up – the hepato-pancreatobiliary (HPB) database. This database was central to the newly created HPB multidisciplinary team; supporting improved care for cancer patients following the government initiated Cancer Plan 2000. All the HPB cancer patients in the Northern region are now discussed and managed with agreement of this multidisciplinary team – including physicians, surgeons, radiologists, oncologists, pathologists and specialist nurses. Details for all patients with primary or secondary liver tumours and pancreatic tumours, irrespective of the treatment they received, have been regularly recorded in the HPB database.

Whilst the HPB database had the potential to be used as a powerful research tool, its major purpose was for patient tracking. In addition, the large number of patients being discussed and managed in this forum hampered focus on HCC. Restructuring of the multidisciplinary meetings, with grouping together of subclasses of diseases has improved the efficiency of this service. In parallel an intranet based HCC excel patient database has been created. The data entered includes patient demographics, baseline blood tests and radiology and facilitates patient staging according to Childs-Pugh, Okuda and CLIP score. This database has become a

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powerful audit and research tool. The maintenance of a central database is key to the management of cancer patients as explained in figure 3.1

NHS cancer plan 2000
&
Guidelines for management of HCC
|
Referral to appropriate centres with necessary skill and services
&
Management decided in MDT
|
Central database to acquire all patient information
|
Patient management plans and regular update
Different clinical score of the disease and regular updates
Different treatment and its effects
Survival /Mortality

Figure3.1 (left) showing the central database in relation to cancer management

In 2004 we successfully obtained permission to collect and store patient tissues as well as data for research purposes, initiating a project entitled 'The application of molecular biology and proteomics to the management of patients with liver cancer'. We collected serial blood samples from all patients, as well as any biopsy tissue available, hoping to improve the value of the HCC database as a research tool, by accompanying it with genomic and proteomic studies.

3.2   *Staging of HCC*

As for any malignancy, clinical staging for HCC is an essential pre requisite as it provides the tools for assessment and a guide for therapeutic strategies. We not only need it to predict prognosis at the time of diagnosis but a standard validated staging system is required for comparing results from trials before we can incorporate them in daily clinical practice. However, the assessment for HCC is more complex than other tumours as the prognosis and treatment modalities are not only guided by the tumour characteristics and extent of spread (local and distal,) but also by the background function of the liver, as about 80% of HCC develop in the background of chronic liver disease. This complexity of the disease has resulted in the failure to develop a uniform standard staging system for HCC management.

A staging classification for solid tumours was first proposed by French surgeon, Pierre Denoix more than 50 years ago which then formed the basis of the first edition of TNM (Tumour, Node, Metastasis) classification in 1968. This has since then undergone modifications and the sixth edition were published in 2003. The assessment of the tumours in this system is based on the size of the tumour and the extent of local and distant invasion. In HCC, the presence of chronic liver disease/cirrhosis prevents accurate assessment. This limitation has resulted in the development of different staging systems for HCC with each of them trying incorporating different parameters of chronic liver disease.

The staging systems commonly used in contemporary clinical practice are the Okuda staging, the CLIP (Cancer of the Liver Italian Program) score, and the BCLC (Barcelona Cancer Liver Clinic) staging. There are other staging systems which are used more locally and they include CUPI (Chinese University Prognostic Index), JIS (Japanese Integrated Score) and the GRETCH (Groupe d'Etude et de Traitement du Carcinome Heaptocellulaire) systems. The Okuda Staging (Table 2.2) was proposed in 1985 and included the significant variables of liver function and tumour extension [235]. This staging stratified the disease more successfully when patients presented with advanced disease (Okuda Stage 3 or more), but in present day practice, with the disease being diagnosed early, the Okuda system fails to stage the disease accurately, failing to separate individuals into distinct prognostic categories, therefore failing as a tool to guide management [236].

While the failure of the Okuda staging system to differentiate the disease in the early stages suggested the need for more complex staging systems, the Okuda system represents an important step forward for HCC staging, suggesting the importance of combining liver function with the tumour characteristics. In 1998, the CLIP (**Cancer of the Liver Italian Program**) (please refer to table 2.2) was proposed from a retrospective evaluation of 435 Italian patients diagnosed with HCC between 1990 and 1992 [237] and includes the Child-Pugh stage, tumour morphology and extension, serum AFP levels and portal vein invasion. The CLIP score was then prospectively validated internally by the same group in 2000 [237, 238] and also in separate series of Italian and Canadian patients[236, 239]. However, in a series of 662 Japanese patients the predicted mortality rates were significantly different from the actual survival which compromised the external validation [240]. A major problem encountered with this staging system is the CLIP score of '0' for uninodular tumours

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with a tumour extent of less than 50%, which is a fairly large tumour in present day practice, where more tumours are being diagnosed when small in size, thereby compromising the predictability. It has also been reported that CLIP scores of 4, 5 and 6 have no statistical difference and that it is also not able to stratify treatment option s for different CLIP scores [241]. Despite these limitations it has fared better than Okuda scoring system and is now commonly used for prognostic assessment but not for planning management.

A further step was taken when the EASL (European Study of Liver Diseases) panel of experts recommended inclusion of general condition of the patient and treatment efficacy along with tumour stage and liver function status in the prognostic modelling of HCC patients which formed the basis of the Barcelona-Clinic Cancer Liver staging system. The Barcelona group constructed the BCLC (**Barcelona-Clinic Liver Cancer**) staging (Figure 1.1) system after reviewing survival data of early tumours selected for radical therapy and the natural outcome of non-surgical HCC patients. This staging includes the variables related to physical status, cancer related symptoms along with the tumour stage and functional status of the liver categorising HCC into four stages (Stages A – D) with a treatment algorithm. The BCLC has been prospectively validated and has been suggested to more accurate than the other systems available [242, 243]. However, a recent study, in 2009, concluded that BCLC was the ablest in predicting survival in early treated patients but none of the scoring systems (CLIP, BCLC and GRETCH) used in the study was able to predict confidently survival in individual patients[244]. The authors suggested that a more accurate staging system was still needed.

In our study, we prospectively scored the patients into OKUDA, CLIP and BCLC stages, as they are the most commonly used scoring systems, and regularly accrued treatment and survival data to assess the prognostic ability of these staging systems and comparing them with the available data in the literature. This is an ongoing study to recruit a large cohort of patients to continuously monitor patients, analyse response to treatment and regularly check the mortality from the disease.

3.3 *Increasing incidence and changing aetiology of HCC in the North East of England*

The number of patients included in the study from its inception in January 2004 until mid 2006 was 130, and this cohort has been the focus of my research. Comparison of

the trends in disease characteristics were facilitated by the inclusion of HPB data from 2000 until 2004. In addition, a cohort of 69 consecutive HCC patients identified and characterised by Dr Mark Hudson between 1993 and 2000, was also available for comparison. The most obvious difference is one of numbers, namely 69 over a 7 year period pre 2000, versus 130 in a 2.5 year period just 4 years later. The referrals to the HPB team documented in figure 3.1 confirm a steady increase from 10 – 12 patients per year, to over 50 in 2006. This dramatic increase most likely reflects, at least in part, the success of the Cancer Plan 2000, ensuring that all patients in the region were referred to specialist team. The increase may also be due to a true increase in the numbers of patients with HCC. Certainly a comparison between the 1993 – 2000 and the 2004 – 2006 cohorts suggest a dramatic change in the aetiology of underlying liver disease in more recent years. Table 3.1 demonstrates a proportional increase in the numbers of patients with underlying NAFLD – an entity hardly recognised in the previous decade.

The irony of the Cancer Plan 2000 success for HCC patients was that all too often this was a 'paper exercise' only, with no treatment to offer the referred patients other than best palliative supportive care. With careful characterisation of our 2004 -2006 cohort, we aimed to audit care and outcomes, hoping to facilitate improvements in both.

Table 3.1 (below) comparing the background liver disease in patients with HCC between the 1993 – 2000 and 2004 – 2006 cohort

Aetiology	1993 – 2000 N = 69	2004 – 2006 N = 130
Alcoholic liver Disease	16 (23%)	50 (38.5%)
Non-alcoholic liver disease	02 (3.0%)	21 (16.2%)
Hepatitis B	06 (9.0%)	06 (4.6%)
Hepatitis C	10 (14%)	08 (6.2%)
Haemachromatosis	05 (7.0%)	11 (8.5%)
Autoimmune hepatitis	03 (4.0%)	04 (3.1%)
Primary Biliary Cirrhosis	06 (9.0%)	04 (3.1%)
Alpha-antitrypsin deficiency	04 (6.0%)	01 (0.7%)
Cryptogenic	04 (6.0%)	07 (5.3%)
Primary HCC without cirrhosis	13 (19%)	18 (13.8%)
Other	4 (6.0%)	0 (00%)
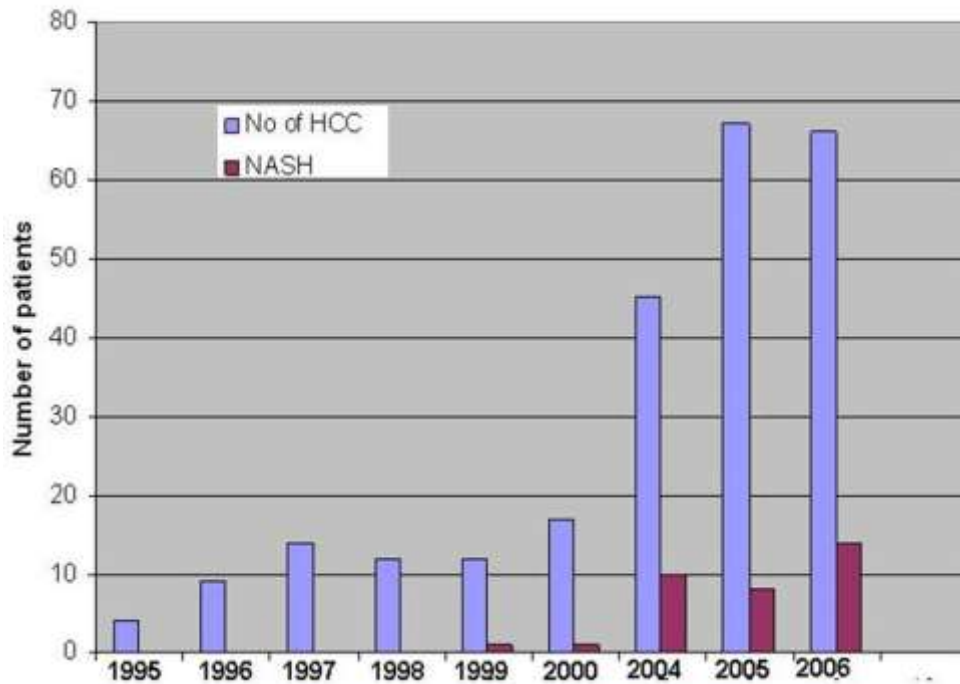
Figure 3.1 showing the trend in HCC from 1995-2006

3.4 Patient demographics, Childs Pugh status and tumour staging

Our cohort of 130 patients was followed up until June 2011, completing a minimum of five years follow up. There were 22 females and 108 males with a male to female ratio of 5:1. The most common aetiology was chronic alcoholic liver disease, comprising 38.2% of patients. The demographic, clinical and tumour staging characteristics of our set of patients are described in table 3.2

Table 3.2 (below) showing the demographic, clinical and tumour staging information of our cohort of patients with HCC.

Demographic, clinical and tumour characteristics	Number of patients	%
Sex Male Female	 108 22	 83.1 16.9

Age group		
<40	2	1.5
40 - 50	4	3.0
50 – 60	24	18.5
– 70	52	40
>70	48	37
Mode of presentation		
Incidental	55	42.3
Surveillance	34	26.2
Symptomatic	41	34.5
Child – Pugh score		
A	82	63.1
B	25	19.2
C	23	17.7
AFP		
<400	55	42.3
400 – 1000	30	23.1
>1000	45	34.6
Portal vein invasion		
None	92	70.8
Branch invasion	18	13.8
Main trunk invasion	20	15.4
Okuda stage		
1	66	50.8
2	47	36.2
3	17	13.1
CLIP score		
0	23	17.7
1	32	24.6
2	24	18.5
3	26	20
4	16	12.3
5 / 6	09	6.9

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BCLC stage		
A	20	15.4
B	19	14.6
C	64	49.2
D	27	20.8

The primary outcome of our observational study was survival. Follow-up times were measured as the number of months from diagnosis of HCC until death or the last available information of the patient. The patients were periodically assessed in the clinic at Freeman Hospital using clinical and biochemical parameters, and interval imaging.

The cumulative survival was ascertained using the Kaplan-Meier method and the differences in survival between different stages in any staging system were determined using the Log-Rank test. The Cox-regression model was used to identify prognostic value of baseline parameters in predicting survival. A P-value of <0.05 was considered significant. All analysis was performed using the SPSS 10.0 for Windows (SPSS Inc. Chicago Ill, USA).

3.4   Results - Treatment strategies and its effect on survival

The available treatment options in this unit are the following:

Surgical resection for patients with preserved liver function. Peri-operative adriamycin was administered and selective post operative adriamycin was considered for tumours with adverse prognostic factors

Cadaveric liver transplantation with per-operative chemotherapy for patients with HCC which met the Milan criteria. Selective post operative adriamycin was recommended for tumours with adverse prognostic factors (tumour >2cm, evidence of lymphovascular invasion) in patients who were fit for adjuvant chemotherapy four weeks post liver transplant.

Percutaneous ablation using radiofrequency. This technique was considered for patients with small volume disease unfit for resection. It could also be seen as a bridge to transplantation. This modality was also used as an adjunct to transarterial chemoembolization

Transarterial chemoembolization (TACE) was and still is recommended as first line palliative therapy for non-surgical patients with intermediate/multifocal HCC who do not have vascular invasion or extrahepatic spread.

The ***main contraindication to TACE*** is the lack of portal blood flow (because of portal vein thrombosis, porto-systemic anastomoses or hepatofugal flow) and poor hepatic functional reserve. Particularly patients with lobar or segmental portal vein thrombosis are poor candidates for TACE, as this will cause necrosis of the tumour and of the non-tumour bearing liver deprived of its blood supply. This increases the risk of treatment-related death due to liver failure. Patients with advanced liver disease (Child-Pugh class B or C) and/or clinical symptoms of end-stage cancer should not be considered for transarterial embolization and chemoembolization as they have an increased risk of liver failure and death

Hepatic artery adriamycin infusion therapy was considered for patients with advanced tumours with portal vein invasion but preserved synthetic function Options for patients with more advanced tumours included consideration for entry into medical trials or for palliation. Sorafenib is now approved as the first line medical therapy for patients with advanced liver cancer and preserved liver function following the success of the SHARP trial (LLovet et al 2007/2009), in which 2 of our 2004 – 2006 took part. Sorafenib was not available as an NHS treatment during that time and will not be discussed further. All the patients receiving treatment other than best palliation were followed up at regular intervals by AFP assessment and ultrasound and / or CT. Table 3.3 shows the distribution of our cohort of patients with HCC receiving any modality of treatment. Curative therapy in the form of resection or liver transplantation was available to only 8.5% (n = 11) of the patients. This small cohort had a median survival of 71 months. Of the palliative treatments, a combination of TACE and RFA appeared to be more effective than TACE alone. Treatment with RFA only was undertaken in 2 patients only and it would be improper to make any inference.

Treatment option	Number of patients	%	Median survival
Transplant	09	6.9	55mo
Resection	02	1.5	36mo
3. TACE	50	38.5	13 mo
4. RFA	02	1.5	7 mo
5. RFA + TACE	13	10	30 mo
6. None	54	41.5	3 mo

Table 3.3(left) showing the different treatment groups
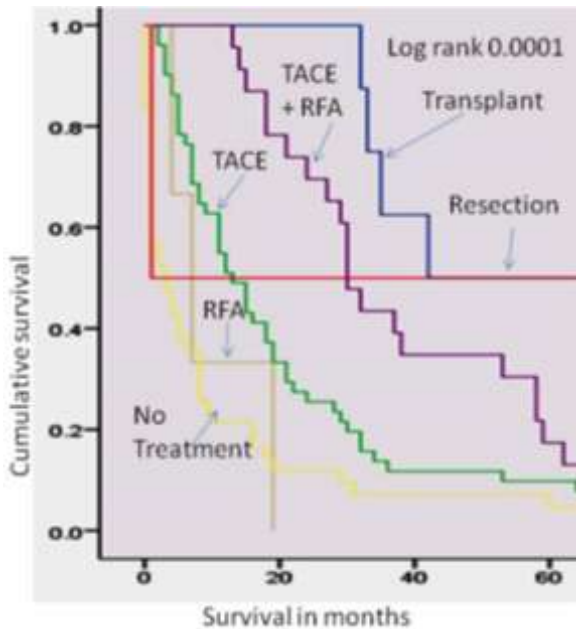
Figure 3.2 (left) showing the survival curves with the different treatment modalities.

3.5   *Results - Survival and baseline characteristics*

In the initial univariate analysis performed on the whole set of 130 patients it wasconfirmed that the mode of presentation (symptomatic, incidental or surveillance), tumour extension in to a branch or main trunk of the portal vein, increasing tumour number and rising AFP values were independent predictors for survival. Cox regression analysis also confirmed the same parameters in predicting survival. Age, sex did not show any correlation with survival. The results of the multivariate analysis using different demographic and clinical parameters are shown in table 3.4

Table 3.4 (below) showing the clinical characteristics of our patients with HCC in predicting survival

Parameter	Median survival (months)	Univariate	Cox regression model (Multivariate)
Sex Male Female	 10 12	0.575	0.59
Age <40 40-50 50-60 60-70 70+	 10 13 08 13 11	0.045	0.058
Mode of presentation Incidental Surveillance Symptomatic	 19 14 03	0.001	0.002
Childs Pugh stage A B C	 18 04 04	0.001	0.002
Performance status 0 1 2 3 4	 24 16 08 01 0	0.002	0.002
Portal vein thrombosis		0.001	0.001

No thrombus	18		
Thrombus in a branch	05		
Main trunk thrombus	03		
AFP		0.001	0.002
<400	19		
400-1000	12		
$\geq$1000	04		
Tumour number		0.001	0.002
1			
2-3	19		
4 or more	18		
	08		

Table 3.4 shows that patients in whom tumour was diagnosed incidentally or by surveillance shows better median survival as compared to the symptomatic patients suggesting that intense surveillance with more sensitive biomarkers would diagnose tumours early which could improve the median survival of the disease. Regarding the patient and basic tumour characteristics, the table shows that deteriorating liver function, worsening performance status, increasing AFP levels, portal vein thrombosis and multiple tumours are independent poor prognostic markers.

3.6   RESULTS – Survival and the staging systems

The long term survival of these patients with or without treatment is calculated using the Kaplan-Meir survival analysis. The cumulative five year survival of patients of HCC in our series was 8.5% as seen in **figure 3.3** and the median survival was 12 months.
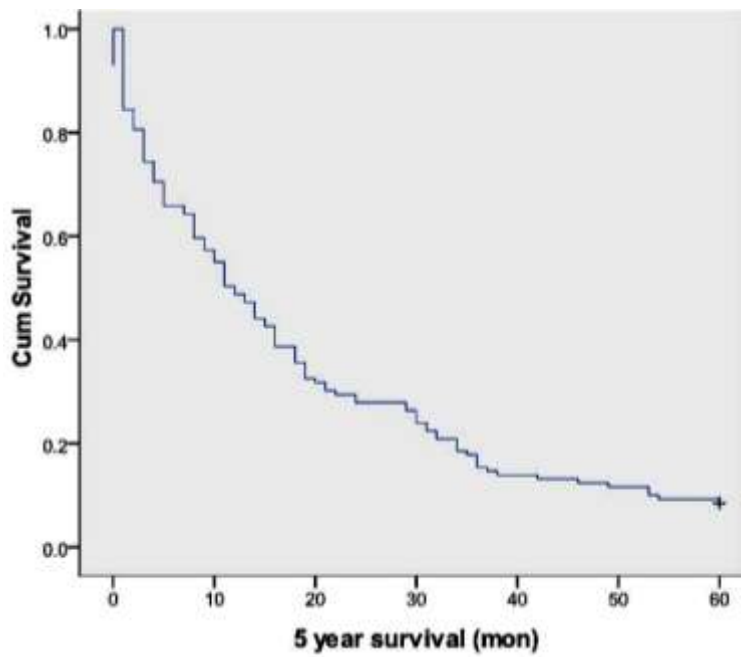
Figure 3.3(left) showing cumulative survival of HCC patients for five years in our study.

The survival pattern of the patients was then calculated for the different tumour stages and any association of survival with any tumour or patient characteristics were also evaluated.
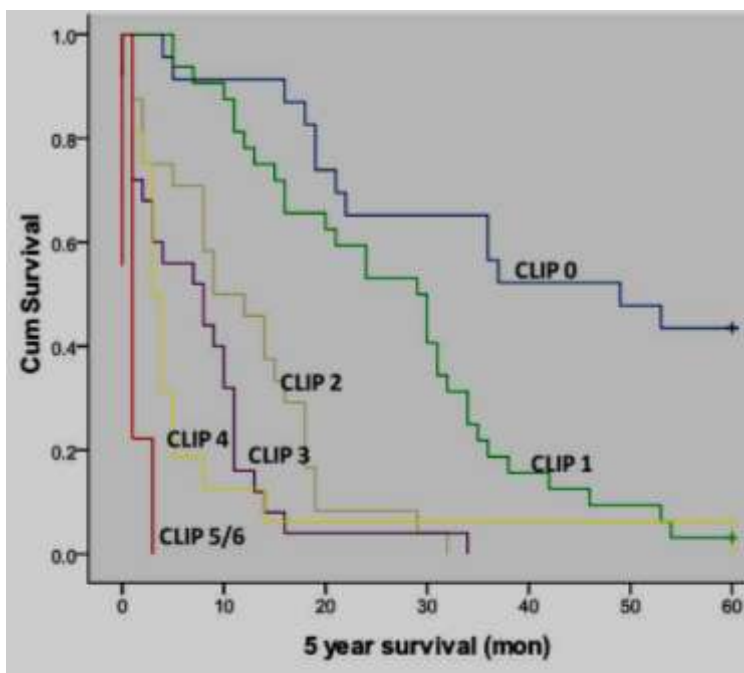
Figure3.4 (left) showing the difference in survival with different CLIP scores.

CLIP score	Predicted Median survival	P-value
		0.001
0	49	
1	29	0.001
2	09	
3	08	0.001
4	03	
5/6	01	0.082
		0.497
		0.001

Table 3.5 (left) showing the median survival in each CLIP stage and the significance in the inter-group difference in survival.

The median survival over 5 years, decreases with increasing CLIP scores (figure 3.4). The difference in median survival is statistically significant for stages 0 and 1 and also for the advanced stages 5/6. However, the difference in survival between the intermediate stages 2, 3 and 4 was not statistically significant. The early CLIP scores of 0 and 1 were heterogeneous as they did not identify the patients for curative treatment separately from the patients who could only be considered for palliative treatment i.e. TACE and RFA.

The survival pattern of the patients with HCC in different Okuda stages is noted in Figure 3.5. The median survival reduces with increasing stage of the tumour but Stage 1 fails to identify the patients with early tumour who would benefit from the curative surgical options. No statistical difference in survival is noted in the median survival between stages 2 and 3.
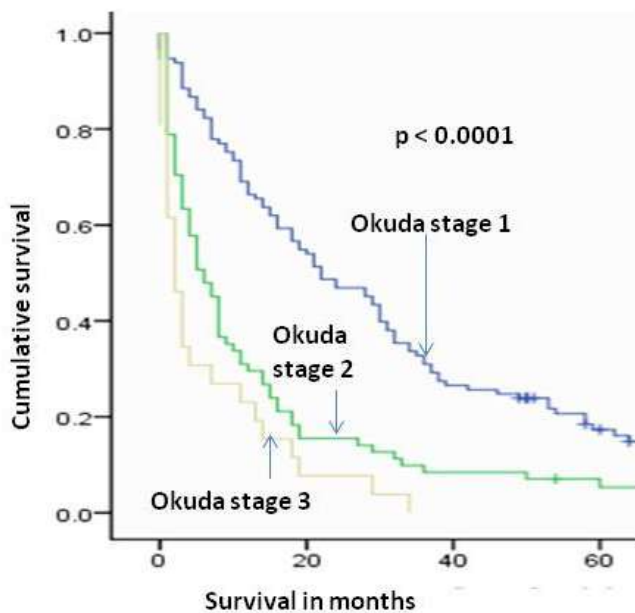
Figure 3.5 (left) showing probability of survival in different Okuda stages in the 130 patients.

Okuda stage	Predicted Median survival (in months)	Difference P-value
1	22	0.001
2	8.2 ⎤	0.001
3	1.6 ⎦	0.168

Table 3.6 (left) showing the median survival in the different Okuda stages and the difference in survival between the stages
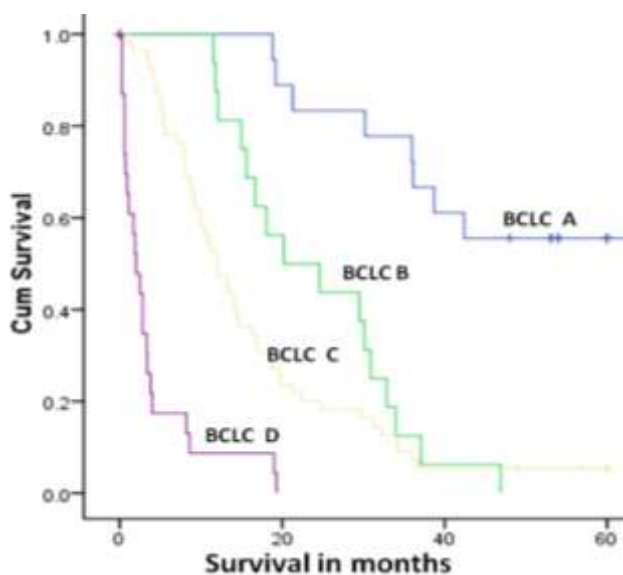
Figure 3.6 (left) showing survival probability in different BCLC stages of HCC.

The survival pattern in the BCLC stages are shown in figure 3.6 and the median survival with the difference in between stages are shown in table 3.7. Stage A representing the early tumour shows the best survival among all the three stages due to the fact that it contains all the patients who underwent definitive treatment which significantly affects survival. It also predicts the poor prognosis of the most advanced group in stage C. The staging, however, fails to differentially predict survival in the intermediate stages B and C.

BCLC Stage	Predicted Median survival (in months)	Statistical difference (p-value)
A	48.0	0.001
B	20.2	0.001
C	12.1	0.112
D	2.1	0.001

Table 3.7 (left) shows the medial survival in the different BCLC Stages and the statistical significance

Finally, the predicted survivals for each of the stages other than early (CLIP 0, BCLC A) was poorer in our cohort of predominantly ALD and NAFLD patients, than that predicted by the schemes developed in patients with predominantly HCV.

3.7   Discussion

The setting up of the central database for HCC in Newcastle was a key step in devising management strategies for patients with HCC and the initial data has shown some interesting results. Hepatitis B and C virus infections are the major causes of HCC worldwide[245, 246]. However, in our series we note that the major cause of HCC was alcoholic liver disease and non-alcoholic liver disease, which accounted for 71 of 130 patients, comprising 54.6% in total. This difference in demographic profile is likely explained by a relatively low immigrant population in the North East UK, in combination with a high prevalence of alcohol abuse and obesity. The emerging epidemic of the obesity associated metabolic syndrome in North America and Western Europe is believed to have contributed to the increase in non-alcoholic steatohepatitis, it being the liver component/sequelae of this syndrome [247]. In a

review in 2005, Bugianesi E et al observed that 7 – 30% of HCC develops against a background metabolic syndrome, which includes obesity, Type-2 diabetes and dyslipidaemia[248]. Obesity now appears to be increasing worldwide, especially in the developing countries undergoing economic transition to an open market economy[246]. The after effects of this growing problem in the east could be felt in the years to come. In the west the compulsory vaccination against hepatitis B and the scrupulous screening of blood before transfusion could contribute to the low incidence of viral hepatitis B and C (less than 10%) related HCC in our series. In the west drug abuse is the principal cause of hepatitis C as compared to the developing world where blood transfusions from unscreened donors and unsafe therapeutic practices account for the majority of cases [249]

The prognosis of HCC without specific treatment is poor but the patients diagnosed with the disease in early stages can be offered definitive therapeutic intervention which has a definitive impact on survival. So early diagnosis of the disease remains the key goal in improving the management which also remains the goal of the NHS Cancer Plan. Progress has also been made in multimodality therapy which improves survival in patients with unresectable HCC with preserved synthetic function with no involvement of the portal vein. In our series of patients, 54% received TACE with or without radiofrequency ablation with a median survival of 18.8 months and three year survival of 42%, as compared to the group which did not receive any treatment showed a median survival of 3.4 months. This compares with the studies in the literature with the 2 year survival varying from 25% to 65% and all these studies also documented a definite improvement from the group treated expectantly [250].

We have used Okuda, CLIP and BCLC systems of scoring. Since the Okuda system, based on findings from advanced tumours, is more than 20 years old and did not take into account important prognostic factors like tumour size, portal vein thrombosis and number of neoplastic lesions, we evaluated the CLIP scoring in addition. The latter is widely used now, as it incorporates additional HCC tumour characteristics, and we wanted to evaluate its ability to predict survival.

In our series of 130 patients the overall median survival with CLIP scores of 0 – 5/6 were 49, 29, 9, 8, 3 and 1 months (Table 3.5 and figure 3.4), which was similar to the large series reported by Lin CY et al in 2009[251]. This suggests its ability to discriminate patients with early tumours from patients with advanced tumours

Studies have reported accurate predictive values of CLIP score for early and advanced

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tumours.[1, 236, 237, 240, 244, 252]. The CLIP scores of 2-4 have a total of 65 patients showing worsening survival with increasing scores, which was not statistically significant Table 2.5). This group with three intermediate CLIP scores has poor discriminating ability. Even for patients with scores of 0 or 1 the groups are very heterogenous because not all patients in these groups receive definite treatment. There could be more than one reason to explain this lack of statistical significance in our cohort compared to other series. The small number of patients in our study could be one of the factors responsible for this finding. The validation of the CLIP score has been principally done in centres with hepatitis B and C being the most important cause of HCC, in contrast to our series which has NASH and alcoholic liver disease as the principal group. This suggests that thegenetic heterogeneity of a tumour make up due to its different aetiologies could be responsible for the difference noted. Alternatively, other characteristics of the patient such as co-morbid conditions, may account for these differences. Often with human data there is some degree of random variability and a model score always works best in the given set of patients within which it was created. This necessitates the need to validate any scoring in different centres with varying aetiology for HCCs. We also note that the CLIP score depends a lot on the human assessment of the nodularity and size of the tumour which can be subject to observer variability as has been suggested by Ray Kim in a recent review[252]. Besides, the score of 0 for <50% of liver involvement also encompasses lesion from 2cms to large lesions which could still be less than 50% of liver volume which could make this group very heterogeneous. In a study of 3868 patients, Lin CY et al concluded that though the CLIP scoring system is a reasonable ordinal scale the clinician must be aware of this heterogeneity of mortality within a given score[251]. Our centre is continuing to collect data of all newly diagnosed HCC patients in to the database which should be able to provide more information about the discriminatory ability of the CLIP scoring system.

The BCLC scoring also has limitations in our series. The overall median survival with scores of A,B,C and D are 53, 24, 11 and 1 months respectively (Table 3.7 & figure 3.7). Only 20 patients (15.4%) were diagnosed in the early curable stage. The intermediate stages of B and C are less discriminatory, and account for the majority (61.5%) of the patients.

The independent prognostic value of AFP is not very clear and various cut-off levels have been used in different studies. Analysis of our prospective series of 130 patients

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showed a correlation of increasing AFP values with poor overall survival with a median survival of 4 months for patients with AFP levels more than 1000 ng/ml compared to a median survival of 19 months for patients with AFP values less than 400 ng/ml (Table 3.4). CLIP score takes that into account and assigns more points for AFP values more than 400 ng/ml, which suggests the increased risks associated with elevated levels of AFP. A Study by Farinati F et al separated patients into three groups of <20 ng/ml, 21 – 400 ng/ml and 400 ng/ml and reported that the high value of AFP correlated with the overall survival in untreated patients or those treated with transplant or loco-regional therapies, but not in those surgically treated [253]. Levels more than 400 ng/ml was associated with vascular invasion and HCC progression predicting poor outcome for these subset of patients after resection[254]. A recent review analysing multiple prognostic factors, reported that appropriate cut-off values are yet to be determined but raised levels of AFP are associated with advanced tumours and a poor prognosis [255].   Our series also shows an inverse relationship between rising AFP values and survival, but it is obvious that a uniform cut-off value is essential to determine its prognostic efficacy in different centres. With more patients being added to our dataset on a regular basis, this needs to be re-evaluated in future years to add more data to our initial findings of the prognostic significance of AFP but that is beyond the scope of this work

The most disturbing finding from this series was that only 10% of patients diagnosed in this two year period could undergo definitive treatment which suggests that the disease continues to be detected in late stages. Comparing the previous data from Dr Hudson in our Hospital it appears that more cases are being diagnosed, but as suggested that could be a result of increased surveillance, increased incidence and / or increased referral from the District hospitals. However, to improve the number of patients to be considered for definitive treatment the following measures might need to be considered:

The primary care trusts (PCTs) need to be updated with the increased burden of HCC and need to identify the at risk population from their demographic profile and habits

A robust surveillance system not only in the apex hospitals but also in the district hospitals, where most cases are diagnosed.

Any suspicious lesion in the district hospital screening programme needs to be referred to the apex hospital for thorough evaluation and follow up.

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From the effectiveness of district nurses in the community it might be worthwhile to have specialist community nurses to identify high risk patients in the community, or who regularly fail to make their appointment.

To continue to use the central database for data collection and also making the database available to the specialists at the district hospitals to enter any patient of HCC enabling them to be seen at the earliest by making referrals quicker.

To continue to strive towards an ideal biomarker or a panel of biomarkers to suit our cohort of patients to identify effectively the surveillance population and also to identify the tumour at an early treatable stage.

Chapter 4   Results 2.   AFP, PIVKAII, GP3, SCCA-1 and follistatin as surveillance biomarkers for hepatocellular cancer in non-alcoholic and alcoholic fatty liver disease

4.1   Introduction

Hepatocellular carcinoma (HCC) is a major health problem worldwide, with more than 500,000 cases diagnosed annually [1]. While the incidence of HCC has reportedly risen over the last 5–8 years, the survival of those affected has not changed significantly in the last two decades [2, 4, 6] . This is related to both its late detection and the lack of effective therapies for advanced stage disease [9]. Up to 80% of HCCs develop against a background of cirrhosis of the liver and while we believe that surveillance of the at risk cirrhotic population could aid earlier detection of the disease and decrease the cancer related mortality rate, our present success is limited by the lack of sensitive biomarkers. Currently, standard surveillance includes a combination of a 6 monthly abdominal ultrasound scan (USS) and a serum alpha-fetoprotein (AFP) measurement, but this strategy does not reliably detect early disease. The diagnostic performance of AFP is inadequate [256] as it is only elevated in 40–60% of cases, while abdominal USS is difficult in cirrhotic nodular livers and notoriously user dependent [8]. Alternative serum biomarkers are being actively sought and proposed candidates include Prothrombin Induced by Vitamin K Absence (PIVKA-II), glypican-3 (GP3), and more recently, Squamous Cell Carcinoma Antigen -1 (SCCA-1). PIVKA-II is an abnormal prothrombin, identified as an HCC biomarker in 1984 [7] and since reported, is elevated most notably in advanced cases with portal vein invasion [152, 257]. It is proposed that PIVKA-II may be useful primarily as a prognostic biomarker, predicting rapid tumour progression and a poorer prognosis [258]. The oncofetal antigen glypican3 (GP3) is a heparan sulfate proteoglycan that is expressed in more than 70% of HCC [164]. When combined with AFP it has a sensitivity of up to 82% for HCC detection on a background of viral hepatitis [163]. SCCA-1 is a member of the high molecular weight serine protease family called serpins [259] initially reported to be elevated in epithelial tumours such as the cervical cancer [260] and more recently in the serum of individuals with HCC and cirrhosis [261]. On a global scale, viral causes of chronic liver disease are the commonest predecessors of HCC and these proposed biomarkers [262] have largely been studied in this disease group. Our own HCC patients have tumours arising predominantly on a

background of alcoholic (ALD) and non-alcoholic fatty liver diseases (NAFLD). In this chapter we present the data on a cross-sectional study comparing the efficacy of these markers, as well as a novel candidate biomarker, Follistatin, for the diagnosis of HCC arising on a background of steatohepatitis related cirrhosis. Follistatin is a monomeric protein overexpressed in rat and human liver tumours and reportedly contributing to hepatocarcinogenesis by the inhibition of activins [263]. Follistatin mRNA was markedly overexpressed in a HCC cell line microarray study performed in our own (Dr Reeves' group) laboratory (unpublished data). Our data indicate differences in biomarker performance in NAFLD and ALD patients compared to performances reported in viral hepatitis. Neither PIVKAII, GP3, SCCA-1, nor the novel candidate Follistatin, has a role independent of AFP in HCC surveillance in steatohepatitis related cirrhosis. We show that the combination of AFP and PIVKAII is more valuable than AFP alone and suggest this approach should be adopted as standard surveillance in this disease group.

4.3   Methods

Patient serum samples

All patient serum and clinical information were collected with patient consent after approval by The Newcastle and North Tyneside Ethics Committee for our study. Patients were diagnosed as having HCC as per guidelines proposed by the European Association for the Study of the Liver [8]. Pre-treatment samples from 50 patients with HCC, all of whom had underlying cirrhosis, were selected for study. Of these, 31 patients had alcoholic liver disease (ALD) and 19 patients had NAFLD. The serum was immediately separated by centrifugation and frozen at - 80°C as per protocol in 2.3. As 80% of HCC develops against the background of liver cirrhosis, these serum samples were compared to an independent group of 41 patients with biopsy proven ALD or NAFLD cirrhosis. The diagnosis of NAFLD cirrhosis was made in patients who had clinical features and liver biopsies compatible with NAFLD. Females and males consuming greater than 14 or 21 units of alcohol per week respectively were excluded from this category, as were any individuals with viral or autoimmune liver diseases. The presence of steatosis was necessary for the diagnosis to be made, as was stage 4 fibrosis defined by the modified Brunt criteria [264].

The biochemical serum tests, including serum AFP, were measured using routine automated methods in the Biochemistry Laboratory at the Freeman Hospital,

Newcastle upon Tyne. No patient positive for either HBsAg or HCV were included in this study.

Western blotting and serum ELISA assay

PIVKA-II was measured using a commercially available ELISA kit (Asserachrom PIVKAII kit, Stago, France), according to the manufacturer's instructions (protocol 2.4.5) . The detection limit is 1.0 ng/ml. The cut-off value was set as 20 ng/ml for differentiation between HCC and cirrhosis based on the findings in this study.

Glypican-3 was measured using commercially available ELISA kit (Biomosaics limited) following the manufacturer's protocol (protocol 2.4.6). Serum samples for this study was also sent to Rome where SCCA-1 was measured in an ELISA kit purchased from Xeptagen (Xeptagen, Naples, Italy) and following the manufacturer's instructions (This study was performed in collaboration with Prof Gianelli's group in Italy as they were the first group to report the importance of SCCA-1 antigen in HCC)[261].

Follistatin was selected for study based on its marked expression in HCC cell lines on microarray analysis and a literature review identifying it as a secretory protein with a previously suspected role in hepatocarcinogenesis. Ten samples each from patients with NAFLD, NAFLD and cirrhosis, or NAFLD with cirrhosis and HCC were immunedepleted by multiple affinity removal (MARS HPLC column; Agilent technologies) and desalted using 5K molecular weight cut off spin filters (Agilent technologies). Subsequently, 50 μg of protein was separated by SDS-PAGE and transferred to PVDF membrane (250 mA for 90 min). The membrane was then probed with mouse anti-follistatin antibody (R&D Systems) at 1:500 dilution at room temperature overnight. After washing in Tris Buffered Saline (0.1% Tween), the membranes were incubated with secondary peroxidase conjugated rabbit anit-mouse immunoglobulin and developed using ECL (Amersham) (protocol 2.4.3 for Western Blot)).

Subsequently, a direct ELISA assay for quantitative analysis, was developed using different concentrations of serum (raw; 1:10; 1:50; 1:100: 1:1000) with serial dilution of the primary antibody. Optimal conditions were using a raw serum dilution of 1:10 and an antibody dilution of 1:250 (protocol 2.4.4).

Statistical analysis

Quantitative variables were expressed as median, mean and standard deviation. Comparison between groups was by Mann-Whitney U, Pearson Chi-square, Wilcoxon

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or Student's t-test, as appropriate. Qualitative variables were expressed as count and percentage and comparisons between independent groups was by Pearson Chi-squared test. The diagnostic accuracy of each of the candidate biomarkers was evaluated using receiver operating characteristic (ROC) curve analysis, reporting the area under the curve (AUC) and its 95% confidence interval (CI). The diagnostic cut-off and the related sensitivity and specificity were determined. Statistical analysis was performed with SAS V8.2 software for PC, MedCalc version 7.4.3.0, as well as SPPS version 14.

4.3 Results

Serum AFP, PIVKA-II, GP3 and SCCA-1 levels were determined in 50 patients with HCC arising in a background of ALD or NAFLD cirrhosis. A control group of 41 patients with cirrhosis from ALD/NAFLD was used for comparison. The clinical characteristics of the patients in these groups are shown in Table 4.1

Variable	Cirrhosis	Cirrhosis with HCC	P-value
Number	41	50	
Age (years)	54.3 $\pm$ 9.62	62.67 $\pm$ 12.02	0.001
Male: Female	28 : 13	40 : 10	0.205
ALD:NAFLD	33 : 08	30 : 20	0.074
Childs-Pugh A:B:C	22 : 14 : 05	27 : 18 : 05	0.853
Portal vein invasion	NA	08	NA
Single nodule	NA	22	NA
Two nodules	NA	10	NA
$\geq$ 3 Nodules	NA	18	NA

Table 4.1: Clinical characteristics of the patients with cirrhosis and cirrhosis plus HCC

Serum AFP and PIVKAII as biomarkers of HCC in steatohepatitis related cirrhosis The median AFP value determined in our patients with HCC and those with cirrhosis were 92.4 ng/ml and 5.9 ng/ml respectively. These data are represented using a log scale in Figure 4.1A (see page 102) and also summarised in Table 4.2. This difference

was statistically significant (p value 0.0004). The ROC curve analysis (Figure 4.2A) confirmed an area under curve of 0.71 (CI 95% 0.61 – 0.8), with a cut-off value of 15 giving a sensitivity of 58% (CI95% 43.2% 71.8%) and a specificity of 100% (CI 95% 91.3 – 100).

Mean PIVKA-II levels were also significantly different between patients with HCC and liver cirrhosis, as shown in 4.1B and Table 4.2.

Table 4.2: Levels of candidate biomarkers (ng/ml) as detected by specific ELISA assays

	Cirrhosis		Cirrhosis + HCC	
	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median
AFP	5.96 $\pm$ 2.65	5.00	5934.66 $\pm$ 13025.02	92.50
PIVKA II	23.47 $\pm$ 59.69	7.83	135.17 $\pm$ 168.96	42.75
GPC 3	125.41 $\pm$ 281.05	29.62	161.41 $\pm$ 422.33	56.57
Follistatin	72.41 $\pm$ 76.16	50.20	87.33 $\pm$ 131.31	61.35

The median value in the former was 42.74 ng/ml and in the latter 7.8 ng/ml (interquartile range: 2.8 – 17.8). The area under the ROC curve was 0.81 (CI95% 0.715 – 0.886) with a cut-off 20.24 ng/ml. This predicted a sensitivity of 79.6%. (CI95% 65.7% – 89.7%) and a specificity of 80.5% (CI95% 65.1 – 91.2%). As shown in Figure 4.1F, the level of PIVKAII is significantly raised in patients with tumours >5 cm in size (n = 24) relative to those 3–5 cm in size (n = 23) (ANOVA p = 0.001). In fact, the level from tumours 3–5 cm in size was not significantly different from the level in patients with cirrhosis alone. While there was similarly a difference between these two size groups using AFP (7369 +/- 14361 ng/ml; n= 36 versus 2417 +/- 8889 ng/ml; n = 13), the difference was not statistically significant. Serum AFP therefore is better at specifically detecting early malignant disease than PIVKAII. However, the combination of serum AFP and PIVKA-II in these patients is better than either alone,

with a combined sensitivity of 94% and a specificity of just over 80%, as shown in Table 4.3.

Serum GP3 and SCCA-1 have no role in HCC surveillance in steatohepatitis-related cirrhosis

The data for GP3 and SCCA-1 in this group of ALD/NAFLD patients with and without HCC is also presented in Figures 4.1C, 4.1D, 4.2C and 4.2D and tables 4.2 and 4.3. These data demonstrate that neither had any value in HCC detection in this group of patients. While their expression was elevated in the serum of patients with chronically diseased livers and HCC, there was no significant difference between the levels detected in cirrhotic patients with and without a cancer.

Follistatin is raised in the serum of individuals with cirrhosis and HCC, but its specificity for HCC is poor.

Levels of follistatin were studied in immune depleted serum from individuals with either NAFLD (n = 10), NAFLD with cirrhosis (n = 10), or NAFLD with cirrhosis and HCC (n = 10) by western blot analysis. Representative data from 24 of these individuals is presented in Figure 4.3. While there was little evidence of follistatin in the serum of individuals with NAFLD without significant fibrosis or HCC, it was detectable in all individuals with HCC as well as some individuals with cirrhosis and no HCC. We went on to develop an ELISA assay for more quantitative raw serum analysis between the latter two groups. Unfortunately, while follistatin is clearly increased in individuals with cirrhotic NAFLD, it fails to distinguish between those with and without HCC, as shown in Figures 4.1E and 4.2E.

Table 4.3 Performance of combinations of candidate biomarkers

Combination	TP	FP	TN	FN	Sensitivity (HCC=50)	Specificity (LC=41)	PPV	TPN	LR
AFP	28	0	41	22	56.00%	100.00%	100%	65.10%	--
AFP+PIV	47	8	33	3	94.00%	80.50%	85.50%	91.70%	4.82
PIV	39	8	33	11	78.00%	80.50%	83.30%	75.00%	4
AFP+SCCA	36	11	30	14	72.00%	73.2%	76.60%	68.20%	2.68
AFP+PIV+SCCA	45	16	25	5	90.00%	61.00%	73.80%	83.30%	2.31
PIV+SCCA	42	16	25	8	84.00%	61.00%	72.40%	75.80%	2.15
GCP+SCCA	36	16	25	14	72.00%	61.00%	69.20%	64.10%	1.85
AFP+GCP	41	22	19	9	82.00%	46.30%	65.10%	67.90%	1.53
AFP+PIV+GCP	48	27	14	2	96.00%	34.10%	64.00%	87.50%	1.46
GCP+PIV	46	27	14	4	92.00%	34.10%	63.00%	77.80%	1.4
All	48	31	10	2	96.00%	24.40%	60.80%	83.30%	1.27
GCP	34	22	19	16	68.00%	46.30%	60.70%	54.30%	1.27
GCP+PIV+SCCA	47	31	10	3	94.00%	24.40%	60.30%	76.90%	1.24
AFP+GCP+SCCA	36	28	13	14	72.00%	31.70%	56.30%	48.10%	1.05
SCCA	9	11	30	41	18.00%	73.20%	45.00%	42.30%	0.67

The true and false positive (TP and FP), as well as true and false negative (TN and FN) for each candidate either alone or in combination is presented, along with sensitivity, specificity, Positive Predictive Value (PPV) and true percentage negative (TPN) values and the likelihood ratio

(LR). The LR is the ratio of true and false positives (sensitivity and 1-specificity respectively), where higher values reflect the probability of a better performance.

(AFP- alpha-fetoprotein, PIV – Prothrombin induced by vitamin K absence II, GCP3 – Glypican 3, SCCA – Squamous cell carcinoma antigen
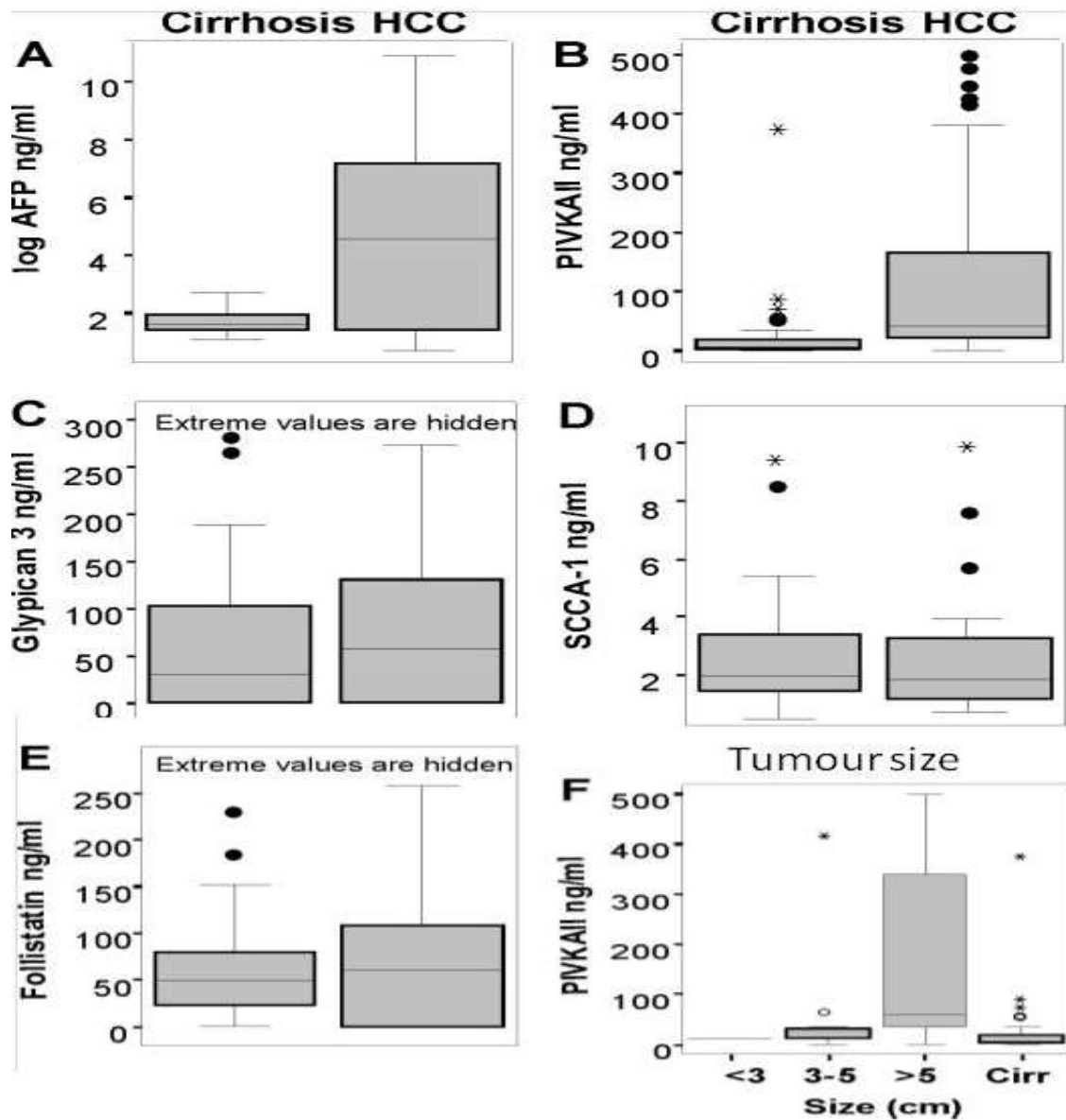
Figure 4.1 Levels of Candidate Biomarkers in cirrhotic patients with and without HCC. Box plots comparing levels of AFP, PIVKAII, GP3, SCCA-1 and Follistatin are shown. Levels are presented as ng/ml, except for AFP where the log data are presented in order to accommodate the wide range. The mean between the two groups is significantly different for both AFP and PIVKAII. For the latter, this is predominantly a result of a marked increase in levels in individuals with tumours greater than 5 cm in size.
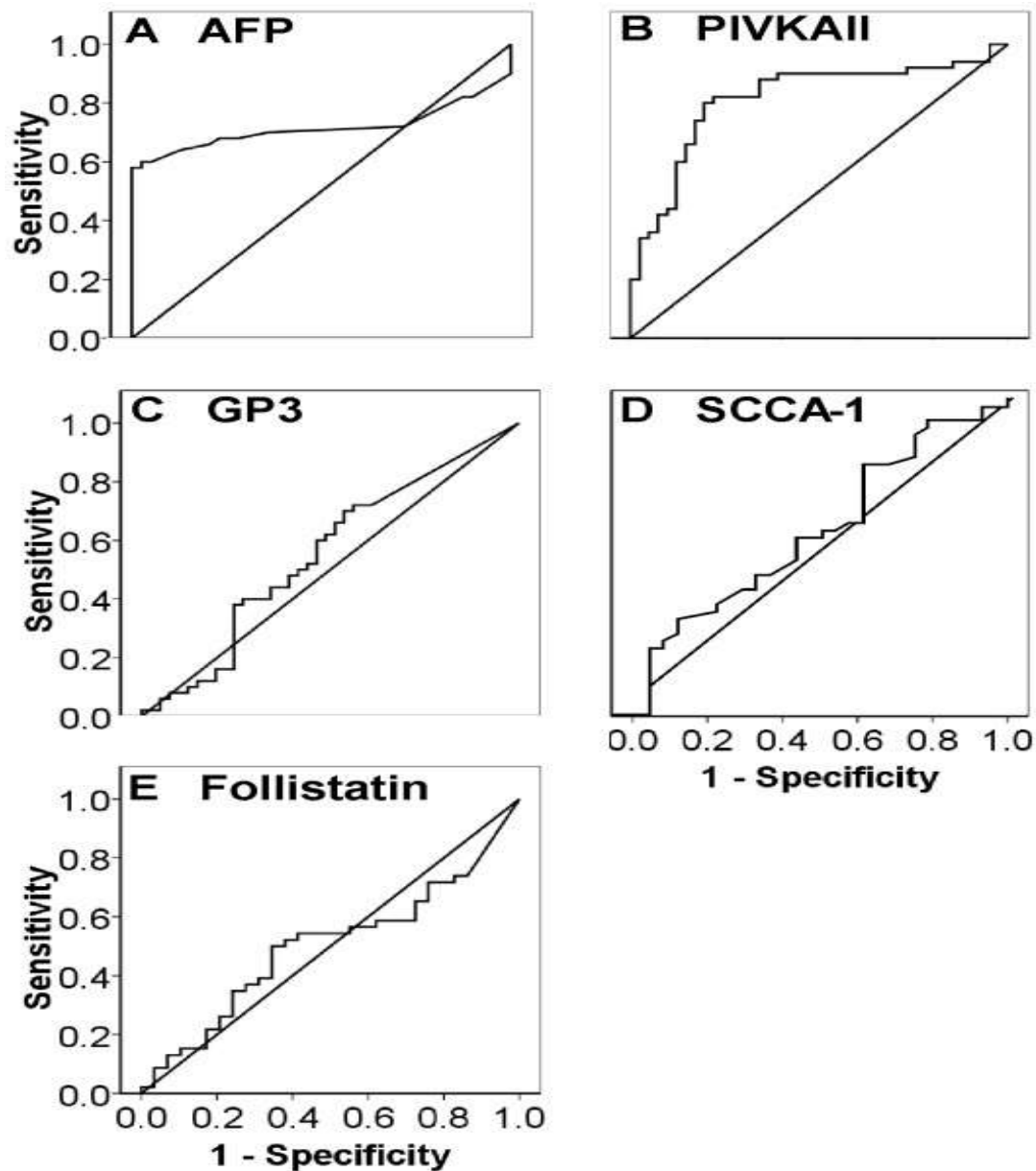
Figure 4.2

ROC Curve analyses of the candidate biomarkers. The diagnostic accuracy of each candidate biomarker, in terms of sensitivity and specificity, are presented after receiver operating characteristic (ROC) curve analysis. In figures 4.2A and 4.2B, corresponding to AFP and PIVKAII, the area under the curve is markedly better than for the other markers.

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Figure 4.3

Follistatin is detectable in serum in patients with NAFLD related HCC. Immune depleted serum samples from individuals with non-fibrotic NAFLD (N), NAFLD cirrhosis(Ci), and NAFLD cirrhosis with cancer (C) have been separated by SDS-PAGE and analysed by western blot. The majority of HCC patients had detectable levels of follistatin in their serum, as did one or two individuals with NAFLD cirrhosis and no cancer
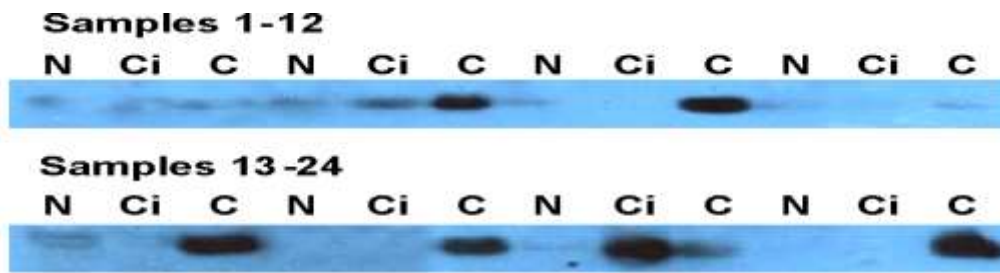
4.4   Discussion

The increasing incidence of HCC[3], compounded by the fact that the majority of these tumours are diagnosed at a late stage when curative treatments are not possible[19], has prompted the international community into performing regular surveillance of high risk individuals. Unfortunately, surveillance programmes are hindered by the poor performance of the commonly used serum marker, namely AFP [6], even in combination with abdominal USS. A tremendous amount of effort has been and continues to be applied to the search for improved HCC biomarkers.

As yet, none has proved superior to AFP in performance, but in combination some may have complimentary roles in HCC arising on a background of viral hepatitis [20]. Our own particular concern relates to the marked increase in the prevalence of ALD and NAFLD related HCC on our own unit.

In our study, serum AFP performs moderately well as a biomarker of HCC in ALD/NAFLD patients, with a sensitivity of 58% (15 ng/ml) in combination with a specificity of 100%. The AASLD recommended cut off level for diagnosis of HCC is 200 ng/ml[21], although lower levels, particularly if rising, should be deemed suspicious and followed very carefully. In ALD/NAFLD patients, where a mild to moderately elevated but stable AFP level similar to that occasionally observed in individuals with viral hepatitis is rare, it may be possible to attach a more sinister

connotation to much lower levels of expression. While this data is encouraging, the sensitivity of AFP is not good enough for it to be used in isolation, as over a third of cancers will be missed.

Both the sensitivity and specificity for PIVKAII as an HCC biomarker were in the order of 80% at a level of 20 ng/ml. The addition of PIVKAII serum analysis to that of AFP increases the combined sensitivity to 94%. While this is at the modest expense of the specificity (reduced to 80.5%), the combination of both AFP and PIVKAII analyses may well be justified in our patients. It should be noted, however, that the added benefit is only in the detection of more advanced disease – as indicated in previous viral hepatitis studies and confirmed in our own NAFLD/ALD patients -the encouraging performance of PIVKA-II is predominantly a result of detection of larger, more advanced cancers.

Assessment of the other candidate biomarkers was disappointing. Both the sensitivity and specificity of GP3 were poor in our patient set, indicating that it has no role at all in the surveillance of HCC in individuals with steatohepatitis related cirrhosis. Follistatin is an expressed transcript in the foetal liver and has previously been identified by microarray as an up-regulated gene in HCC relative to dysplastic nodules [265]. Although we had high hopes for this activin antagonising protein, [266] based on both our preliminary microarray data and a pilot study in immune depleted sera, the ELISA data assessing its discriminatory function between cirrhotic individuals with and without HCC was poor. The discrepancy between the western and ELISA data is most likely a result simply of assessing a greater number of patients using the latter method, but it is also that follistatin, or an isoform of it, was enriched during the column preparation phase of the serum of HCC patients assessed by western blotting. Perhaps the most surprising of results, however, in this homogenous group of patients with steatohepatitis related HCC, was the disappointing performance of SCCA-1. SCCA-1 has previously shown promise, particularly as an AFP complementary biomarker, in viral hepatitis related HCC [182, 267]. In our study in NAFLD/ALD, however, there was no significant difference between levels in patients with and without HCC and while the combination with AFP does modestly improve its sensitivity (78% from 56%); this is at an unacceptable cost to specificity (73% from 100%). Why this serum protein should be significantly elevated in the serum of HCV related HCC patients relative to HCV cirrhosis alone, and not similarly elevated in steatohepatitis related HCC patients relative to steatohepatitis cirrhosis

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alone is unclear. It is possible that the study of these novel candidate biomarkers complexed to immunoglobulins, rather than the study of their free forms, may yet improve their performance, as has been shown for other biomarkers [268]. Whether or not there is room for improvement, however, our SCCA-1 data clearly indicate that as we come to consider further candidate biomarkers it is important to assess HCC arising in different disease backgrounds independently when performing validation studies.

Chapter 5.   Results 3.   A Proteomic Strategy to Identify Novel Serum Biomarkers for Liver Cirrhosis and Hepatocellular Cancer in Fatty Liver Disease.

5.1   Introduction

Globally, viral infections such as Hepatitis B (HBV) and Hepatitis C (HCV) are the principal causes of chronic liver injury, while in western nations steatohepatitis secondary to alcoholic liver disease (ALD) or non-alcoholic fatty liver disease (NAFLD) contribute significantly. NAFLD is the liver manifestation of the metabolic syndrome, characterised by central obesity, insulin resistance, hypertension and atherogenic dyslipidaemia. It is now the commonest cause of chronic liver disease in western countries[15]. Whatever the insult, chronic injury generates a persistent wound healing response associated with a changing extra cellular matrix (ECM) and the accumulation of fibrous, type I collagen rich, scar tissue. Cirrhosis describes the end stages of this process and is characterised by the disruption of normal liver architecture by both fibrotic bands and disorganised nodules of regenerating hepatocytes. There is presently no medical treatment to reverse the changes of cirrhosis, although it is hoped that improved therapies (e.g. antiviral therapy or those targeting the metabolic syndrome and/or NAFLD) introduced at lesser stages of disease may have an impact on their rate of progression to cirrhosis.

As well as deteriorating liver function and significant clinical morbidity, cirrhosis is associated with a markedly increased risk of developing hepatocellular carcinoma (HCC). With more than 500,000 cases diagnosed annually, HCC itself is a major health problem[2]. It is frequently detected at an advanced, incurable stage [9] and the survival of those affected has not altered significantly in the last two decades [2, 4, 6]. It was hoped that surveillance of cirrhotic individuals would facilitate early diagnosis of HCC and improve survival. Surveillance using liver imaging with abdominal ultrasound (USS) in combination with serum alpha fetoprotein (AFP) measurement is performed 6 monthly in many centres. This strategy, however, has limited value. There is no safe and cost-effective population-based means to identify the at risk cirrhotic population requiring surveillance. Liver biopsy is presently the best means of diagnosing cirrhosis, but it carries a significant risk and has well recognised

limitations such as sampling error. It is only performed if there is a clinical indication. There are panels of serum-based tests,[269] some in conjunction with clinical parameters, [270, 271] now proposed as useful in diagnosing cirrhosis. These too are largely aimed at individuals in the clinical setting, rather than being advocated as population-based screening tools. Despite the prevalence of NAFLD being 20-25% of the population [272], the reality is that many of those who progress and develop cirrhosis remain unaware of their disease until a complication, such as an HCC, develops. Secondly, and even more disheartening for those with known cirrhosis, the diagnostic performance of AFP for HCC detection is inadequate [256] as it is only elevated in 40-60% of positive cases. Abdominal USS is a little better as it is difficult in cirrhotic nodular livers and is notoriously user dependent [8]. USS in NAFLD patients is particularly difficult owing to the frequent association with central obesity. Improved non-invasive means of detecting both cirrhosis and HCC are urgently required if we are to have an impact on the survival of the increasing numbers of individuals affected by these diseases. Although a number of alternative biomarkers for HCC have been proposed, largely in individuals with HCV and some in combination with AFP, none has yet had an impact on clinical practice. Here we report our own pilot study in individuals with either ALD or NAFLD. We have used immune depleted and filtered serum from individuals with liver disease, with and without cirrhosis, and from cirrhotic individuals with and without cancer. This serum has been studied using gel-based proteomic techniques in conjunction with mass spectroscopy to identify novel biomarkers distinguishing the three conditions. From the analysis of the gels we have identified 5 candidate biomarkers capable of discriminating at least one or other of the patient groups. One novel serum protein, CD5L, was selected for further characterisation in a larger patient group. We have confirmed that it is an independent predictor of cirrhosis in NAFLD, and may also identify individuals at greater risk of HCC development.

5.2   Methods

Patient serum samples

All patient serum and clinical information was collected with patient consent after approval by The Newcastle and North Tyneside Ethics Committee. The liver histology of patients with NAFLD (steatosis on biopsy and compatible clinical

features in the absence of an alcohol intake greater than 14 units weekly for women and 21 weekly for men) was staged according to the Brunt scoring system [264]. A fibrosis score of 4 describes cirrhosis. Serum samples from patients with chronic liver disease (including both ALD and NAFLD patients) were taken at the time of liver biopsy. Individuals with HCC were diagnosed as per guidelines proposed by the European Association for the Study of the Liver (mentioned in Table 2.1, Chapter2) [8]. The majority of these were not biopsied, but each of them had underlying clinical cirrhosis in association with a hypervascular lesion visible on two imaging modalities and compatible with the diagnosis of HCC. Serum samples from the individuals with HCC were pre-treatment samples taken at the time of diagnosis. Details of the HCC patients and controls with biopsy proven cirrhosis are included in Table 1. All serum samples were separated by centrifugation within 4 h and subsequently stored at -80°C. The standard biochemical serum tests, including serum AFP, were measured using routine automated methods in the Biochemistry Laboratory at the Freeman Hospital, Newcastle upon Tyne. No patient positive for either HBsAg or HCV were included in this study.

Table 5.1

Clinical characteristics of cirrhotic patients with and without HCC.

	Cirrhosis	**HCC**
Number	49	45
Age (years)	56.73 ± 8.9	67.8 ± 7.4
Male: Female	35:14	37:8
ALD:NAFLD	28:21	28:17
Childs-Pugh A:B:C	30:14:05	24:16:05
AFP (ng/ml)	6.04 ± 2.68	6551 ± 13602
Bilirubin (μmol/L)	33.42 ± 30.81	22.87 ± 22.29
Albumin (g/l)	36.36 ± 7.76	35.71 ± 5.02
Portal vein	NA	8

	Cirrhosis	HCC
invasion		
Single nodule	NA	19
Two nodules	NA	8
≥ 3 nodules	NA	18

The difference in age between patients with cirrhosis and HCC versus those with cirrhosis alone was statistically significant ($p < 0.001$). There was no significant difference between sex or Child-Pugh stage between the two groups

Proteomic studies

Serum samples from 5 patients with NAFLD but no significant fibrosis, 5 patients with NAFLD and biopsy proven cirrhosis, and 5 patients with NAFLD cirrhosis and advanced AFP negative HCC were prepared for study. AFP negative samples were used hypothesising that these patients secrete other proteins. The samples were immunodepleted and de-salted using protocol 2.4.2 A. 500 μg of total protein was separated per 2-dimensional gel electrophoresis run. The prepared protein was then subjected to 2 dimensional electrophoresis as per protocol in 2.4.2 C.

All samples were analysed using duplicate gels. Gel images after scanning were stored as TIFF files and analysed using Progenesis Software (Nonlinear Dynamics). Protein spots of interest were excised, destained, and digested with trypsin. Peptide mass fingerprinting was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems Inc.) operated in positive ion reflectron mode with α-cyano-4-hydroxycinnamic acid as the matrix. Protein identifications were performed using the Mascot Peptide Mass Fingerprint search program (Matrix Science Ltd).

Serum ELISA assay

A direct ELISA for novel candidate CD5L was optimised using different concentrations of serum (raw, 1:10, 1:100, 1:500 and 1:1000), primary anti-CD5L antibody (R&D systems) and recombinant CD5L protein (R&D systems). Serum dilutions of either 1:10 or 1:100 generated a linear absorbance response over a protein range of 0.01 - 1.0 ng/ml using a primary antibody dilution of 1:500. This antibody

dilution was then used to analyse all subsequent patient serum samples in triplicate at a dilution of 1:10 (protocol 2.4.4).

Liver Tissues CD5L mRNA analysis (performed by Dr HL Reeves' group)

Liver biopsy tissues surplus to diagnostic requirements were available from 21 patients with histologically staged pre-cirrhotic NAFLD, collected with appropriate ethical approvals in either Newcastle Hospitals or the Policlinico Gemelli Hospital, Rome. Histological staging was as defined by Brunt [264], although no RNA yield sufficient for analysis was obtained from a stage 4/cirrhotic biopsy. In addition, liver tissues obtained at the time of liver resection or radiofrequency ablation for benign or malignant primary or secondary tumours was obtained in the Newcastle Hospitals, with ethical approval, from an additional 14 individuals. One of these had histologically confirmed HCC and steatohepatitis related cirrhosis. The other 13 had 'normal livers', although mild steatosis or a mild focal mononuclear cell infiltrate were occasionally noted on histological assessment post resection. Semi-quantitative real time PCR analysis was performed as previously described [273] using GAPDH as a control gene and the following CD5L primers: Forward: 5' CAA CAA GCA TGC CTA TGG CCG AAA, Reverse: 5' TCA CAT TCG ACC CAC GTG TCT TCA. CD5L expression was expressed relative to the control gene GAPDH and a normal liver sample from an individual patient undergoing resection of a benign focal nodular hyperplasia.

Statistical analysis

Quantitative variables are expressed as mean and standard deviation. Comparison between groups were performed by Pearson Chi-square, Wilcoxon or Student's t-test, as appropriate. The diagnostic accuracy of CD5L was evaluated using receiver operating characteristic (ROC) curve analysis, reporting the area under the curve (AUC) and its 95% confidence interval (CI). Statistical analyses were performed with SPSS version 14.

5.3   Results

Novel candidate biomarkers identified using proteomic techniques

Figure 5.1 shows representative 2D gels of each of the three groups and identifies a number of spots, labelled 1-5. These spots, particularly in combination, enable the differentiation between the three patient groups, namely NAFLD without fibrosis, NAFLD with cirrhosis, and NAFLD with cirrhosis and HCC.

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Table 5.2 (below) lists the the 5 proteins with differential expression ($\geq$ 2 fold change) in between the conditions of steatohepatitis, cirrhosis and HCC

| Spot number	Accession code (Entrez protein)	Identification method	Mascot Score	Sequence Coverage	Protein description	Mass	Function
1	CAA00975	Matrix MS	247	80%	ApoA1 Protein (fragment)	28061	Cholesterol transport to the liver
2	CAA00975	Matrix MS	236	75%	ApoA1 Protein (fragment)	28061	Cholesterol transport to the liver
3	CA00975	MatrixMS, then MS MS	195	62%	Pro-ApoA1 Protein (fragment)	28061	
4	AAA51748	Matrix MS	338	64%	Apo A iv Protein	43358	Required for efficient activation of lipoprotein lipase
5	AAD01446	Matrix MS	218	68%	AFO11429 (CD5L)	38063	Apoptosis inhibitor secreted by macrophage
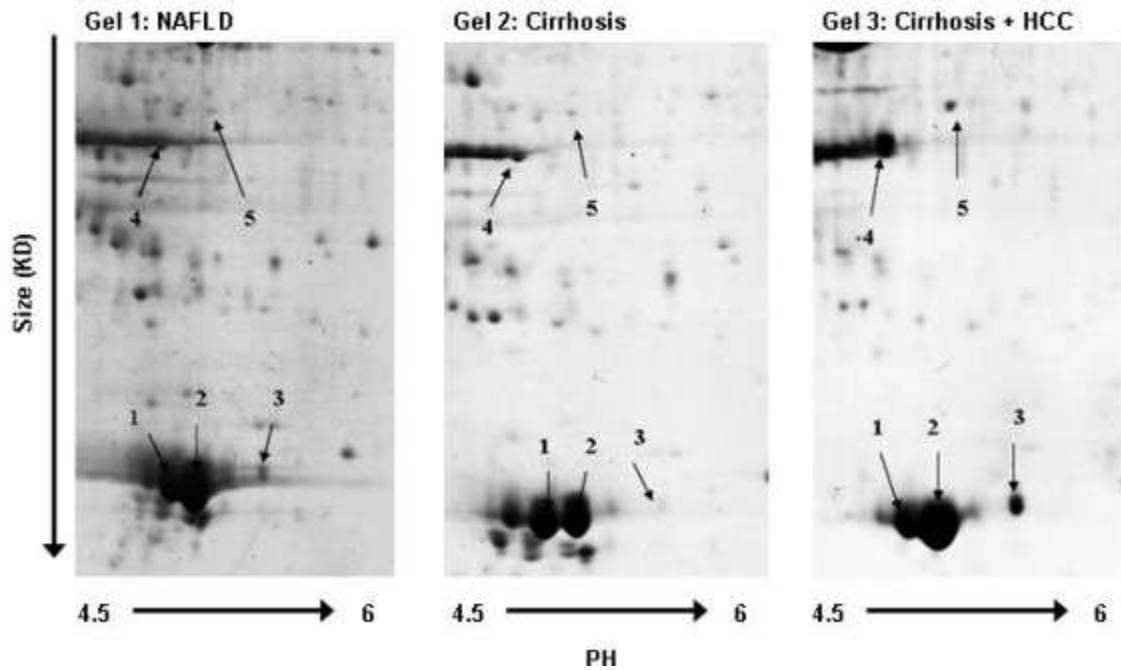
Figure 5.1

Serum proteins separated by two dimensional gel electrophoresis in early and advanced NAFLD, with and without HCC. These are representative blots comparing immune depleted and desalted serum from a patient with NAFLD and no fibrosis, a patient with NAFLD cirrhosis, and a patient with NAFLD cirrhosis and cancer. Serum profiles separated by 2D gel electrophoresis from 5 patients in each group, run in duplicate, were compared using Progenesis software. 5 spots were characterised by MS after excision. Spots 1,2 and 3 were isoforms of apolipoprotein A1, spot 4 was apolipoprotein A4, and spot 5 was identified as CD5 antigen like, or CD5L

Protein identification of spots 1-5 was by Mascot search after mass spectroscopy. The identities and Mascot scores are summarised in Table 5.2. Spots 1-3 were all identified after mass spectroscopy as apolipoprotein A1 (ApoA1). Spot 2 in particular was reduced in cirrhotic individuals relative to those with pre-cirrhotic NAFLD. In addition Pro-ApoA1, spot 3, was markedly present in all individuals with cirrhosis and HCC relative to only trace amounts in simple steatosis patients and cirrhotic patients without HCC. Protein spot 4 appeared lower in pre-cirrhotic NAFLD patients, compared to those with cirrhosis and in individuals with cirrhosis and HCC. This spot was identified as Apolipoprotein A4 (ApoA4). Protein spot 5 was identified as CD5 antigen like (CD5L). Relative to pre-cirrhotic NAFLD, this protein was identified in

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the serum of individuals with both cirrhosis and also those with cirrhosis and cancer, markedly so in the latter group.

CD5L determined by ELISA assay was very highly expressed in the serum of individuals with cirrhosis and HCC

Serum CD5L was assessed in the serum of 45 individuals with steatohepatitis-related HCC and compared to levels in 49 individuals with biopsy proven steatohepatitis-related cirrhosis. Patient characteristics are shown in Table 5.1. The CD5L serum level was not significantly associated with age or sex. The box plots depicting the mean levels of the two groups of patients are shown in Figure 5.2 and are not significantly different (194 ± 167 without HCC versus 218 ± 221 with HCC). The AUC determined by ROC analysis (shown in Figure5.2B) was 0.495 and indicated a poor serum CD5L discriminatory capacity between cirrhotic individuals with and without cancer. Some individuals with HCC did have, however, particularly high CD5L levels. A level greater than 400 ng/ml has a specificity for HCC of 88%. The sensitivity at this level was unacceptably poor (20%).
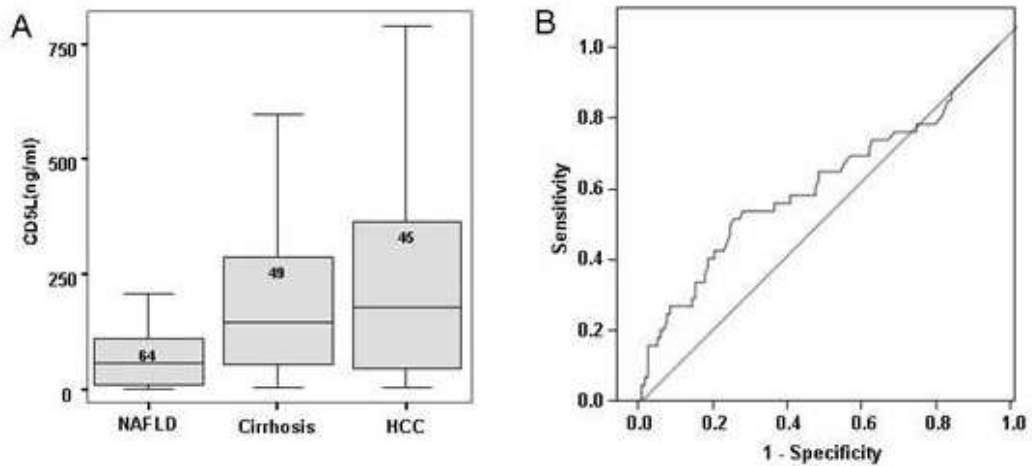
Figure 5.2

CD5L discriminates poorly between cirrhotic patients with and without HCC.
Serum levels of CD5L were measured by ELISA assay in cirrhotic patients with (218
± 221; n = 45) and without (194 ± 166; n = 49) HCC, as shown in 2A. Characteristics
of the individuals are shown in Table 5.1. There was no significant difference between
the two groups. ROC analyses is shown in 2B. The area under the curve is 0.495 (95%
confidence intervals 0.376 and 0.614). A level > 400 ng/ml has a sensitivity of only
20%, but a specificity of 88%. Levels > 500 ng/ml have a specificity of 96%, > 600
ng/ml of 98% and > 700 ng/ml of 100%. For comparison, the mean CD5L serum level
from patients without cirrhosis (detailed in figure 5.2A) is also shown.

CD5L ELISA in individuals with different stages of NAFLD was a good predictor of those with cirrhosis

Serum from 77 patients, all of whom had biopsy proven NAFLD and had an alcohol intake of < 21 units per week for men and < 14 units for women, was studied. In this group of individuals the serum level of CD5L ng/ml in cirrhotic individuals (236 ± 223) was similar to those cirrhotic individuals with and without HCC described above. This level for stage 4 fibrosis/cirrhosis was significantly elevated compared to individuals with all other pre-cirrhotic stages (ANOVA p = 0.001, Bonferroni correction applied). Mean levels of CD5L were not significantly different between any other histologically defined group (steatosis or the presence or absence of necroinflammation). The level of CD5L was significantly associated with fibrosis independently of age, sex, steatosis or necroinflammation, as assessed by General Linear Model analysis (GLM, SPSS). The ROC analyses of CD5L as a predictor of stage 4 fibrosis in this independent group of patients with NAFLD is shown in Figure5.3 B. The area under the curve was 0.712 (95% CI 0.534-0.889).
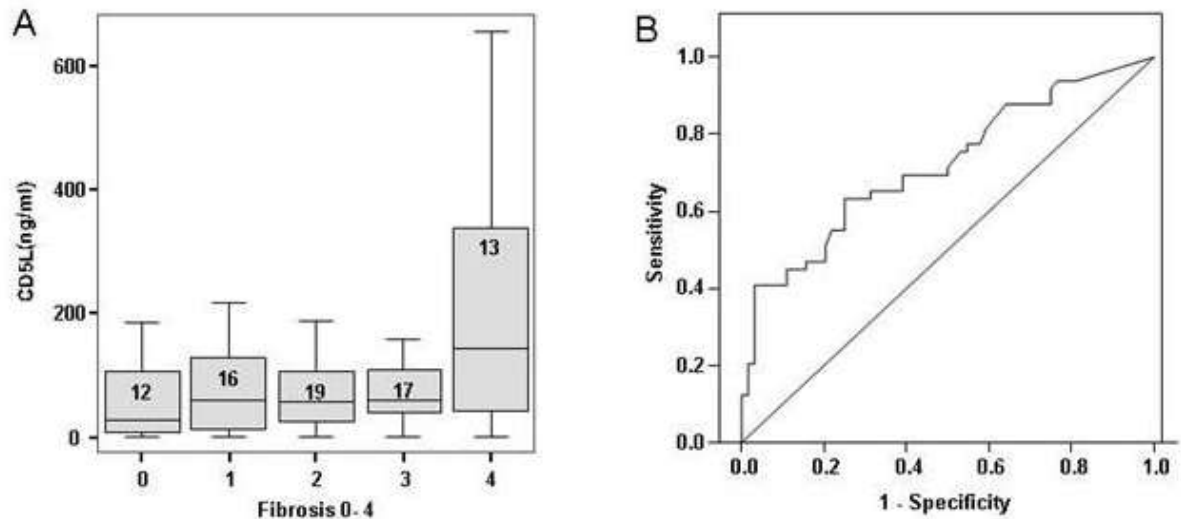
Figure 5.3

CD5L discriminates between steatohepatitis patients with and without cirrhosis.
Serum levels of CD5L were measured in a total of 113 patients with either ALD or
NAFLD. Fibrosis was scored histologically on liver biopsy as per the Brunt scoring
system. The numbers of individuals in each group are shown within the boxes of
figure 5. 3A. The difference in CD5L levels between the different stages of fibrosis is
statistically significant by univariate analysis (p = 0.004) controlled for both age and
sex. This difference The ROC analyses for the identification of those individuals with
stage 4 fibrosis (cirrhosis) is depicted in 5.3B. The area under the curve is 0.719 (95%
confidence intervals 0.623 and 0.816), $p < 0.0001$. A level of CD5L 50 ng/ml has a
sensitivity of 78% and a specificity of 46%, while a level of 100 ng/ml has a
sensitivity of 63% and a specificity of 72%. A level greater than 200 ng/ml has a
specificity of 95% (sensitivity 41%), and greater than 300 ng/ml of 97% (sensitivity
27%).

CD5L mRNA expression was elevated in individuals with NAFLD versus those with
no underlying liver disease
CD5L mRNA expression was quantified in one set of liver biopsy tissues from
patients with histologically staged NAFLD, and a set of normal liver tissues collected
at the time of resection for benign or secondary cancers (See methods). Data are
expressed relative to the GAPDH control gene and a single normal liver sample from
an individual undergoing resection for focal nodular hyperplasia. In the pre-cirrhotic

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NAFLD biopsy tissues, there was no significant difference in association with either fat, inflammatory or fibrosis score, as shown in Figure 5.4. What was most notable, was that the liver mRNA expression of CD5L was significantly increased in the diseased NAFLD group as a whole (n = 21), relative to the normal liver group (n = 13) (6.945 ± 0.722 versus1.68 ± 0.269; p = 0.000). Although some of the 'normal' liver samples from patients undergoing resection for secondary malignancies had a 'mild steatosis' or a 'minimal focal mononuclear cell infiltrate' noted at the time of histological examination, there was no significant difference in CD5L expression in association with either of these minor changes (data not shown). The marked CD5L mRNA expression shown in the single cirrhotic tissue and HCC pair (Figure5.4C) were in keeping with the serum ELISA assays from the much larger patient group.
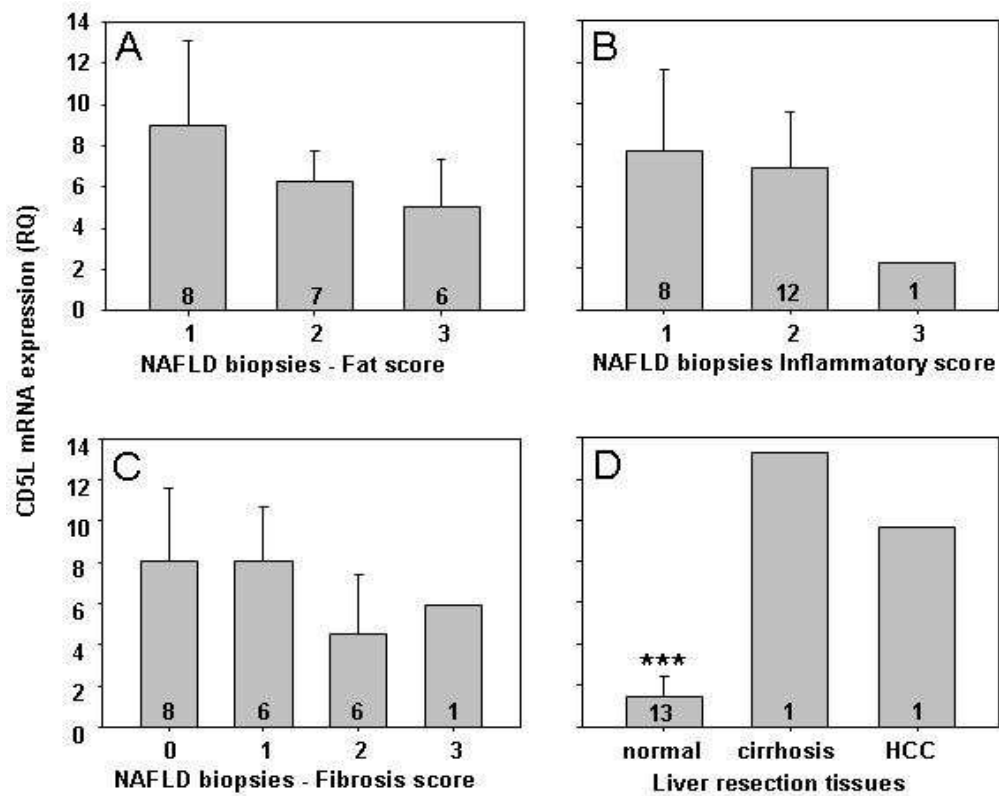
Figure 5.4

CD5L mRNA expression is not altered in association with either fat, inflammation or fibrosis scores in pre-cirrhotic NAFLD liver tissues. mRNA CD5L expression was quantified by real-time PCR, relative to GAPDH and a normal liver sample, in 21 pre-cirrhotic NAFLD biopsy tissues, 13 normal liver samples taken at the time of liver resection, one cirrhotic liver and one HCC. As shown in 4A-C, there was no difference in any pre-cirrhotic NAFLD biopsy tissues in association with the degree of fat, inflammation or fibrosis. There was a significant increase in the NAFLD tissues as a group (n = 21), compared with normal liver tissues (n = 13) as represented in 4D (6.945 ± 0.722 versus1.68 ± 0.269; p = 0.000, ***). The elevated CD5L mRNA expression in one cirrhotic and HCC tissue pair, obtained at the time of laparoscopic radiofrequency ablation, is also presented in 4C and is in keeping with the elevated serum CD5L levels identified in the larger cohort of patients studied.

5.4   Discussion

The prevalence of chronic liver disease is increasing steadily in the UK, as is the population at risk of developing and dying from HCC. An increasing incidence of HCC has already been documented [6] and this is unfortunately compounded by the majority of tumours being diagnosed at a late stage when curative treatments are not

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possible [274]. Regular surveillance of high risk individuals is recommended but is presently hindered by the poor performance of the commonly used serum marker, AFP [8], even in combination with abdominal USS. A significant effort has been and continues to be applied to the search for improved HCC biomarkers. As yet, none has proved superior to AFP in performance, but in combination some may have complimentary roles [275, 276]. Our particular concern relates to the marked increase in the prevalence of ALD and NAFLD related HCC on our own unit. These individuals are often over 65 years of age and have cardiovascular co-morbidities precluding curative resection or transplantation. Much attention has been focused on validating quantitative fibrosis or cirrhosis markers in these individuals, with combinations of serum markers [271] showing encouraging if still suboptimal improvements in performance. Imaging methods such as the fibroscan may improve the efficiency of cirrhosis detection [277-279], but presently this technique needs to be interpreted with caution in obese individuals [279-282]. The need for further improvements in serum biomarkers for early detection of both advanced fibrosis and HCC grows ever more pressing.

We have applied a proteomic strategy using an optimised method of patient serum preparation in order to identify candidate biomarkers of either cirrhosis or HCC. This includes immune depletion prior to separation of serum by 2D gel electrophoresis. While this might remove some key biomarkers, we believe that this strategy improves the sensitivity of the technique applied to the remaining serum proteins. Having identified a number of spots able to discriminate between patients in at least one of our pre-defined disease groups, namely pre-cirrhotic NAFLD, cirrhotic NAFLD, and cirrhotic NAFLD with cancer, we selected candidates for identification by peptide mass spectroscopy.

The novel serum protein CD5L was identified in the serum of cirrhotic individuals with and without HCC by our proteomic strategy. CD5L, also known as Sp alpha, is a soluble protein belonging to group B of the scavenger receptor cysteine-rich (SRCR) superfamily for which little functional information is available [283]. It is expressed by macrophages present in lymphoid tissues (spleen, lymph node, thymus, and bone marrow), and it binds to myelomonocytic and lymphoid cells, suggesting that it may play an important role in the regulation of the innate and adaptive immune systems. It was recently identified in the sera of individuals with liver fibrosis related to HCV infection and, based on its proposed role in immune system regulation, was thought

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most likely associated with viral infection rather than cirrhosis [284]. While this may yet be true, as a mRNA transcript, it has been previously reported AS upregulated in HCC relative to dysplastic nodules [265]. We have investigated the potential of CD5L as a candidate biomarker for either advanced liver disease, or advanced liver disease and cancer. Our ELISA assay indicated a poor performance for CD5L as a surveillance tool for HCC, but again suggested value for cirrhosis detection. Our data clearly indicate, however, an association with cirrhosis in individuals without viral infection, which is independent of the presence or absence of histologically assessed inflammation. Our CD5L mRNA expression data from pre-cirrhotic NAFLD liver biopsies indicate an increase in association with fatty liver disease which, again, is not altered by the grade of either fat or inflammation. As CD5L does not increase incrementally with the level or stage of fibrosis, we propose that its dramatic increase in the serum of individuals with a background cirrhosis reflects hepatocyte regeneration, rather than the advanced fibrosis *per se*. The recent identification of CD5L by microarray as one of 30 mRNA transcripts expressed in patients with cirrhosis with a 'high risk' of HCC development [285] was one reason for our focus on this serum protein. Certainly some of our HCC patients had markedly high CD5L levels, while those with cirrhosis who had particularly high levels may be at high risk and may yet develop HCC. As a cirrhosis biomarker, the ROC analysis for CD5L indicates an accuracy of 72%. While this falls short of the accuracy of the European Liver Fibrosis (ELF) panel, recently validated in NAFLD patients and having an AUC of 0.9 for advanced fibrosis, CD5L is a single biomarker whose performance in conjunction with others may yet improve. In addition, it may have a value complementing that of AFP, which is predominantly elevated in patients with advanced HCC, in highlighting individuals with cirrhosis who are at greater risk of HCC development. At a level > 200 ng/ml, CD5L had a specificity for cirrhosis of 96% and a specificity for cancer of 60%.

Particularly prominent on our gel images were three spots identified as apolipoprotein A1 (ApoA1). ApoA1 is the major protein component of high density lipoprotein in plasma. It promotes cholesterol efflux from tissues to the liver for excretion and it is a cofactor for lecithin cholesterolacyltransferase which is responsible for the formation of most plasma cholesterol esters. It is not that surprising that this liver function associated protein alters in the serum of individuals with chronic liver disease and this has been previously reported in serum proteomic studies [286]. The mass of spots 1

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and 2 (Figure 5.1) varied by approximately 30 daltons using MALDI analysis and are most likely attributable to post translational modification by oxidation. Notably, these two spots were reduced in cirrhotic individuals relative to those with pre-cirrhotic NAFLD, which is in keeping with previous reports and validates the clinical relevance of our methodology. In fact, a reduction of ApoA1 in the serum of patients with cirrhosis has remained a constitutive part of combined peptide panels used to predict fibrosis for a number of years [269, 287]. In addition, however, we identified a novel isoform - spot 3. The difference in mass between spots 1 and 2 and spot 3 was much greater and in the order of 900 daltons (Additional files 1, 3 and 5). MALDI MS analysis of this spot has identified it as pro-Apolipoprotein A1 (Additional files 6 and 11), similarly detected and reported by an independent group of researchers studying patients with lung cancer [288]. Pro-Apolipoprotein A1 is proposed as a novel serum marker of brain metastases in lung cancer patients [288] and there may well be a rationale for its upregulation also in HCC patients. ApoA1 is secreted as the proprotein (pro-Apolipoprotein A1/spot 3) and is then cleaved, regulating its activation for lipid binding, by a metalloproteinase. One candidate metalloproteinase responsible is the c-terminal procollagen endoproteinase, Bone morphogenic protein 1 (BMP-1) [289]. BMP-1 is secreted by the liver, but protease inhibitors, such as alpha-2-macroglobulin (A2M), are also secreted by the liver, often at elevated levels in inflammation or chronic disease [290]. Either a reduction in BMP-1, or an increase in inhibitors such as A2M - as reportedly occurs in HCC [291], could block the maturation of pro-Apolipoprotein A1, hence contributing to relative increase in this isoform (spot 3). In fact, the relative decrease in mature apoA1 in cirrhotic patients (spot 2) may also reflect increases in protease inhibition and A2M has similarly contributed significantly to serum diagnostic tests for fibrosis [290-292]. Further investigation has yet to determine the sensitivity and specificity of pro-Apolipoprotein A1 as a novel candidate biomarker in patients with chronic liver disease and HCC, but our pilot study suggests that its up-regulation is specifically a feature in the serum of patients with HCC.

5.5   *Conclusion*

While non-hypothesis driven methodologies may be criticised by some as 'fishing expeditions', encouraging agreement is beginning to emerge when comparing data generated by proteomic techniques in the field of chronic liver disease, particularly

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when it is interpreted in the context of the ever growing academic literature generated by gene expression profiling. The data presented in this pilot study have identified a pattern of serum apolipoproteins which, in combination CD5L, can discriminate between precirrhotic NAFLD, cirrhotic NAFLD, and cirrhotic NAFLD with HCC. These methodologies, even in small studies requiring additional validation, contribute significantly to this field and provide the hope that improved serum biomarkers may yet become available as surveillance, diagnostic and prognostic tools in patients with chronic liver disease.

Chapter 6   Results 4.   Liver transplant for HCC – Newcastle experience

6.1   Introduction

The definitive treatment for HCC is surgical, removing the tumour either by resection or liver transplantation. Resection is usually only considered for patients with early malignant disease and with preserved liver function and no portal hypertension. For those in whom the liver function is poor, or in whom there is significant portal hypertension, liver transplantation to replace both the tumour and the diseased liver offers the only chance of cure. Selection of patients for liver transplantation in the UK is carefully governed by use of the Milan criteria, as detailed in Chapter 1. Other critical limiting factors include the age of the patient and the presence of other significant co-morbidities.

Unfortunately, despite careful selection of our patients by these means, the outcome of liver transplantation for non-malignant conditions remains superior. Recurrence of HCC in the transplanted liver occurs in approximately 10 - 60% of recipients, adversely affecting their long term survival. [111, 293-296]. The role of immunosuppressive drugs in HCC recurrence post liver transplant remains unclear. As yet there are not sufficient data to implement evidence based changes to standard triple therapy regimes, but many transplant centres do modify immunosuppressive regimes in the HCC setting. The role of adjuvant therapy, for which there is similarly no proven benefit in these individuals, remains an area of keen interest. Ideally, we would like to be able to extend rather than further restrict the numbers of patients transplanted for HCC, but this is not appropriate when there are growing waiting lists with restricted donor organs. To achieve long term survival in HCC patients, we need not only to more accurately predict those individuals at higher risk of recurrent disease, but also to offer them an adjuvant therapy which will reduce tumour recurrence.

Liver transplantation has been ongoing at the Freeman hospital (Regional centre for Liver diseases) in Newcastle-Upon-Tyne since 1993. We have retrospectively audited our patients transplanted for HCC. A number of them have received adjuvant chemotherapy with Doxorubicin. Our Newcastle survival and recurrence data are presented.

6.2   *Patient and methods*

Between 02/10/1993 and 18/07/2007, a total of 553 patients were transplanted on our unit at the Freeman Hospital, Newcastle Upon Tyne. HCC co-existent with chronic liver disease was present in 51 of these patients. The pre-transplant diagnosis of HCC was made using a combination of contrast enhanced cross-sectional imaging by CT and MRI scanning, as per recommended EASL guidelines, in 38 of these individuals (HCC being an incidental finding in the explanted liver in 11 patients). Post 1996, only those individuals radiologically within the Milan criteria (a single tumour $\leq$ 5cm; or 3 tumours none >3cm and no macrovascular invasion) were accepted onto the transplant list. Additional imaging included a thoracic CT scan, a bone scan, and bone densitometry. Routine serum tests included the tumour markers, CA19-9, CEA and AFP. Patient details are summarised in Table 6.1. The diagnosis of HCC was confirmed histologically in the explanted liver. Some patients received transarterial chemoembolisation (50mg doxorubicin mixed with 6-8ml lipiodol, followed by polyvinylalcohol particles) whilst on the list.

From the time of transplantation, patients were managed according to our own protocols modified for the HCC setting. Postoperative immunosupression was modified relative to non-malignant liver diseases. Our protocol included patients with a preoperative diagnosis of HCC being given a perioperative dose of intravenous doxorubicin 20 mg/m^2 during the anhepatic phase of the liver transplant.

A careful pathological study of the explanted liver was performed in all cases. The tumour characteristics based on the pathological findings were compared to preoperative imaging. The recorded tumour characteristics included the number of tumours, unilobar/bilobar involvement, the size of the tumour(s), grade of differentiation and presence/absence of vascular invasion. The total tumour diameter was calculated as the sum of the greatest diameter of each lesion [297].

28 days post transplant, patients (HCC diagnosed pre transplant and incidental tumours) who had unfavourable histology (moderately to poorly differentiated tumour, microvascular invasion) and multifocal tumours were considered for adjuvant doxorubicin chemotherapy (weekly doxorubicin of 25mg/m^2 for a total of 200mg/m^2). Peri-operative mortality was defined as patient death, irrespective of the cause, within 28 days of the liver transplant. Follow up of LT recipients included a CT scan and AFP measurement at every 4 months for the first year, six monthly for second year

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and annually thereafter. The recurrence rate was defined as percentage of HCC recurrence including all HCC cases during the follow up period. All patients were followed up at the Freeman Hospital and no patients were lost to follow up. Follow up data was collected in February, 2009.

Statistical analyses were using SPSS software version 16.0 and Graph pad prism version 4.02. Actuarial survival was calculated using the Kaplan-Meier method. Differences in survival were examined using the log rank test. Cox-proportional hazards regression analysis used for multivariate analysis of variables to test the significance of variables removing the confounding variables. Students t test, Mann-Whitney test, Fisher exact test, and the Chi squared test were used to compare groups. $P<0.05$ was considered significant.

6.3   Results

6.3.1   Demographic profile of patients transplanted for HCC

The majority of patients transplanted with HCC were male, with an underlying ALD or HCV related cirrhosis

Table 6.1 Demographic data of patients who underwent OLT and the explant confirmed HCC	
Characteristics	Number of patients (%)
Male/Female	43/8
Age (M/F)	59/56.5
Aetiology	
ALD	20 (39.2)
NAFLD	3 (5.8)
HCV	15 (29.4)
HBV	3 (5.8)
PBC	2 (3.9)
AIH	2 (3.9)
Haemochromatosis	3 (5.8)
Cryptogenic	3 (5.8)
Follow-up (months)	
Median	
Mean	55
	67.6 ± 50.32

The demographic data for the 51 patients with HCC transplanted between 1993 and 2007 are summarised in *Table 6.1*. Eight were female and 43 were male, their median ages being 56.5 (42 – 67) and 59 years (28 - 69) respectively. Alcoholic liver disease (ALD) and Hepatitis C (HCV) were the commonest underlying aetiologies. The follow-up times until 1st of July, 2011 are a median of 55 months (range 03 – 202) and a mean of 67.6 ± 50.52 months.

6.3.2 Cross sectional imaging underestimated the stage of malignant disease

The pathological and histological characteristics of the tumours identified are summarised in Table 6.2 (In one patient histopathological data was not available). Of these 51 patients, 11 were diagnosed of HCC in the excised liver and the preoperative imagings did not reveal any tumour i.e. incidental tumours. While it is not possible to determine the histological grade of an HCC pre-transplant without biopsy, it is proposed that some pre-operative factors such as radiological (eg size or number of

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Table 6.2. Tumour characteristics		
Distribution	Unilobar	36
	Bilobar	14
Differentiation	Well	17
	Moderate	19
	Poor	14
Number	1	27
	2-3	10
	4-5	05
	≥ 6	08
Total size	≤ 30 mm	17
	$30 - 50$ mm	17
	≥ 50 mm	16
Microvascular Invasion	Present	22
	Absent	28
Explant fitting Milan criteria	Yes	32
	No	18

tumours, the presence of large vessel invasion) can be useful to predict outcome. One third of our patients transplanted with HCC had poorly differentiated cancers on explant histology, with a similar number having evidence of microvascular invasion. In fact, this histological grading does reflect tumour size and number, as a similar proportion of patients had larger or more than ideal numbers of tumours in their explanted liver.

In summary, 18 (36%) of these individuals had disease at explant that would have been deemed beyond 'Milan criteria', if radiological imaging could be more sensitive.

6.3.3   The survival of our cohort of patients who underwent liver transplant for HCC (including incidental tumours) was 61.3% at 5 years.

In the first 18 years of liver transplant for HCC in this centre, 25 deaths were observed in 51 patients. Of these, two deaths were in the post operative period (death within post-operative 4 weeks), one as a result of sepsis and the other as a result of reperfusion syndrome. The causes of the additional 23 mortalities are shown in Table 6.3.

| Table 6.3 Cause of death	Number of patients	Median survival (months)
Recurrence of HCC	5	10
Primary lung carcinoma	1	06
Primary gastric carcinoma	1	20
Intracranial Haemorrhage	1	06
Myocardial Infarction	2	120
Status epilepticus with aspiration	1	01
Sepsis	2	01
Reperfusion syndrome	1	0
Coagulopathy	1	0
Graft failure	7	32
Recurrent hepatitis with cirrhosis Post-Transplant	1	137
Lymphoproliferative disorder	1	152
Hodgkin's disease	1	149
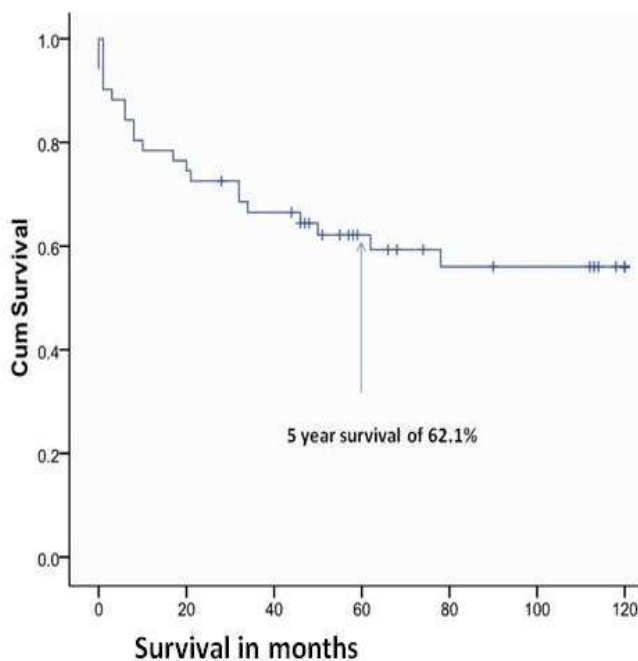
Figure 6.1 The overall 5 year survival for patients transplanted for HCC was 62.1%.

The causes for early death are reperfusion syndrome, sepsis and coagulopathy whilst recurrence of HCC and graft failure accounted for deaths within 2 years of transplant. Lymphoproliferative disorders and recurrent cirrhosis were responsible for those patients who died in the long term.

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Recurrence of HCC was observed in 5 patients, occurring within two years in all cases. The overall survival was at 62.1% at 5 years, as shown in figure 6.1, with a recurrence rate in the explants or distant site of 10% (n=5). The median survival noted for this period of the study was 137 months.

The survival in the incidental cohort was longer than the preoperatively diagnosed cohort but the difference was not statistically different as shown in Figure 6.2.
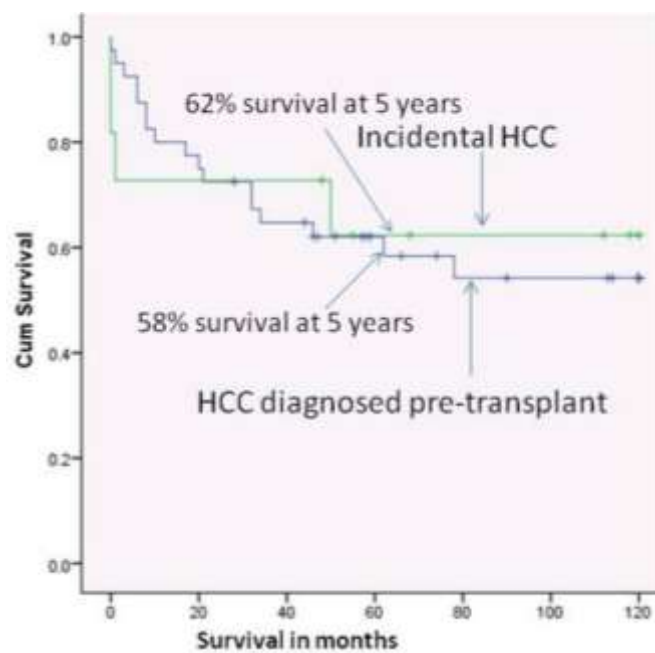
Figure 6.2 comparing the survival after liver transplant with a known preoperative diagnosis of HCC and incidental HCC in the explants (log rank 0.8)

6.3.4   Tumour and epidemiological characteristics in 5 year survival

A Cox regression model was used to analyse the effect of different tumour and epidemiological characteristics which include aetiology of cirrhosis, number of tumour nodules, tumour differentiation and microvascular invasion. 19 of 39 patients had preoperative TACE and was also included in the analysis to assess whether it affects survival after liver transplantation. The results are shown in Table 6.4 and Figures 6.3 and 6.4.

Characteristic	Number	Log rank	Cox regression
Aetiology			
ALD			
HCV	15		
HBV	03		
NASH	03		
PBC	02	1.000	
Haemachromatosis	03		
AIH	02		
Cryptogenic	03		
Tumour Nodules			
One nodule	26		
2-3 Nodules	11	0.136	
>3 nodules	13		
AFP			
<400	40	0.031	0.039
>400	11		
Pre op TACE			
Yes	20	0.687	
No	31		
Lobar distribution			
Unilobar			
Bilobar	36	0.058	
	14		
Differentiation			
Mild	17		
Modearte	20	0.043	0.021
Poor	13		
Microvascular invasion			
Yes	28	0.003	0.004
No	22		
Multifocal			
Yes	26	0.08	

Table 6.4 Univariate and multivariate analysis of patient survival with tumour and epidemiological characteristics using log rank test and Cox regression model

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No	24		
Tumour diameter			
<3cm	24		
3-5 cms	13	0.005	0.008
>5cm	13		

Table 6.4 (above) shows the correlation of survival with different tumour parameters and the aetiology of background liver disease

Long term survival was adversely affected by raised AFP (>400), poor differentiation and tumours more than 5cms. Pre-transplant TACE showed no improvement in survival.
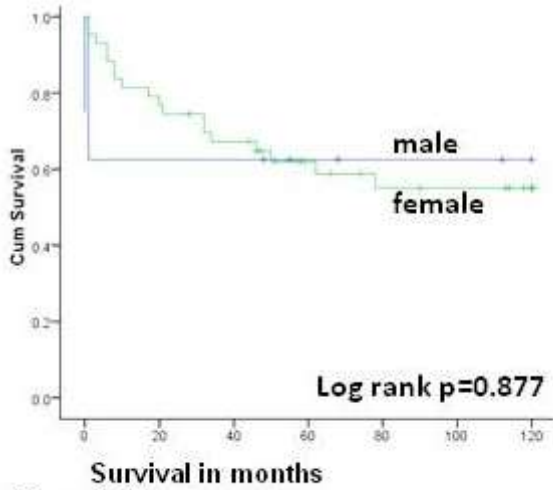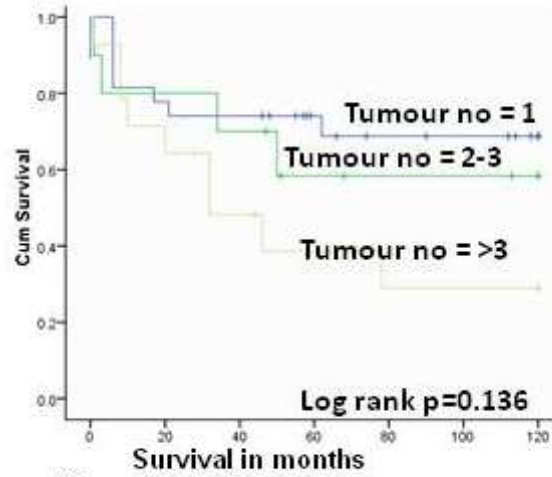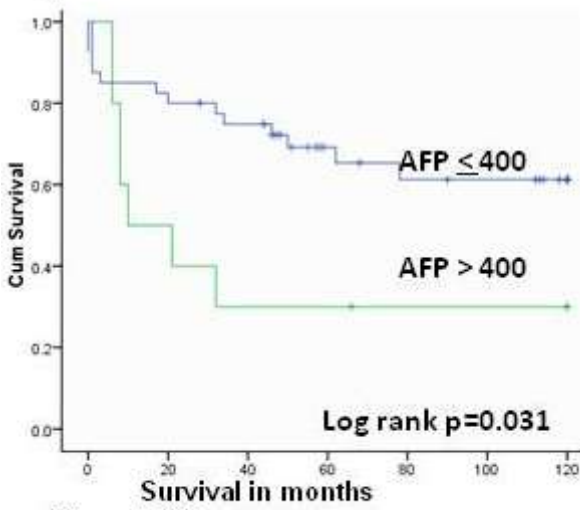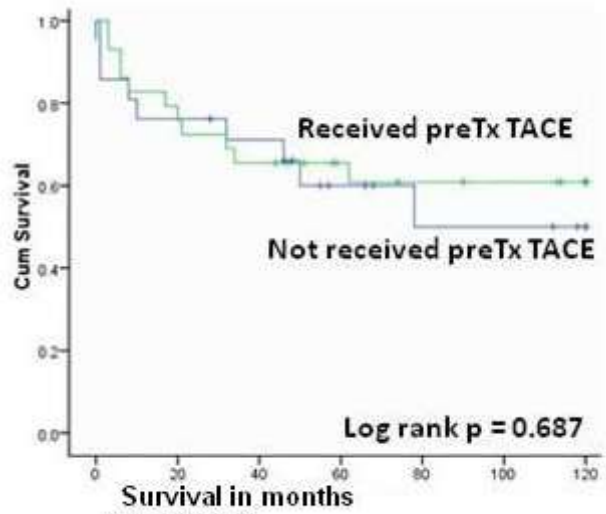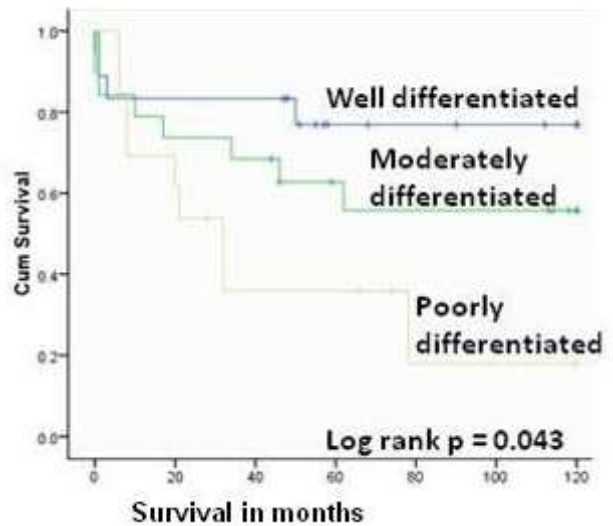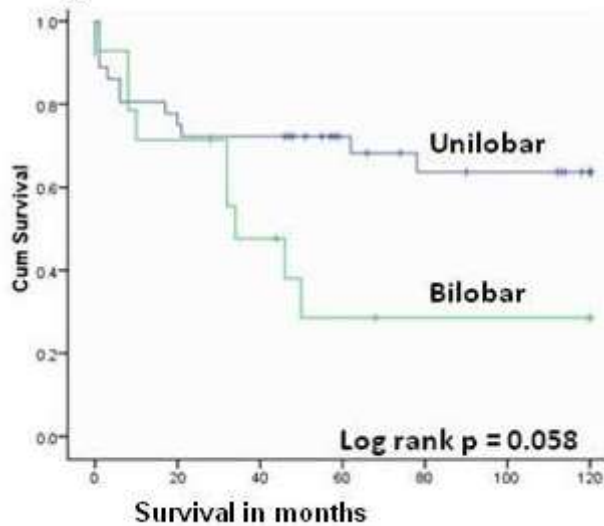
Figure 6.2 shows comparisons of survival in relation to sex (A), tumour number (B), AFP levels (C), pre-operative TACE (D), lobar distribution (F) and degree of tumour differentiation (F)

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	Group A	Group B	Group C
Number of patients	13	26	12
Age (Median)	60	59.5	57
Sex (M:F)	09:04	24:02	10:02
Aetiology			
a)ALD	4	10	6
b)Hepatitis C	2	10	3
c)Hepatitis B	0	1	2
d)NAFLD	3	0	0
e)AIH	0	1	2
f)Haemachromatosis	1	2	0
g)Cryptogenic	1	1	1
h)PBC	2	0	0
AFP ng/ml (Mean)	12.8	1388	133.83
Incidental HCC	7	2	2
Pre-op TACE	5	19	6
Recurrent hepatitis	2	5	0
Cause of death			
a)Graft failure	1	4	1
b)Coagulopathy	1	0	0
c)MI	1	0	0
d)Sepsis	0	0	1
eAspiration pneumonia	0	0	1
f)Metastatic HCC	0	6	1
g)Gastric carcinoma	0	0	1
h)Abscess with ruptured hepatic artery	0	0	1
i)Reperfusion syndrome	0	0	1
j)Intracranial Haemorrahge	0	1	0
5 year survival estimate	92.3%	56.7%	36.4%
Median survival (months)	202	78	08
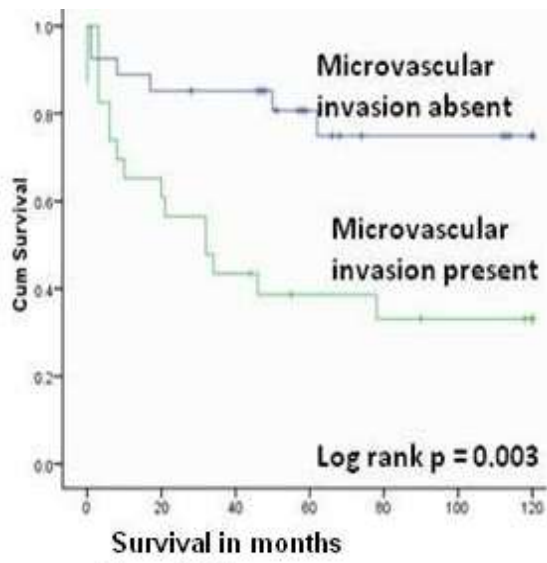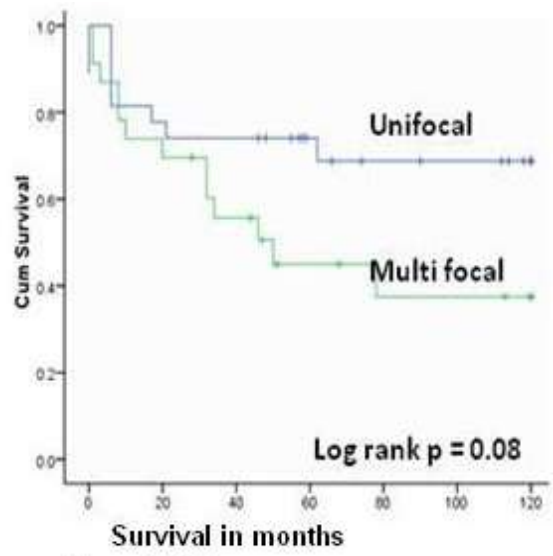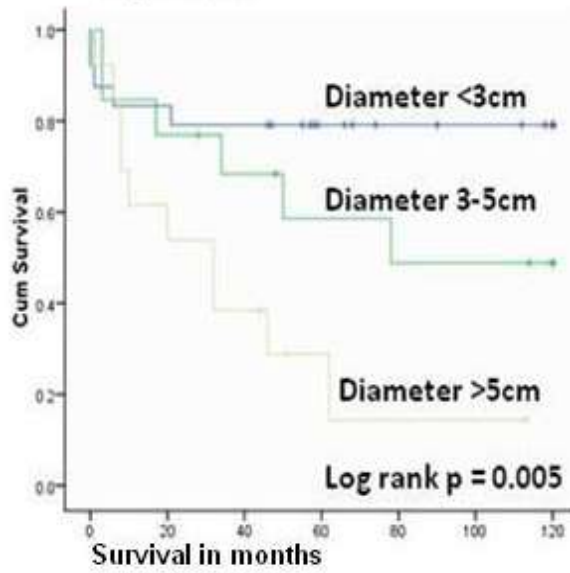
Figure 6.3 survival in comparison with microvascular invasion (A), focality of tumour (B) and tumour diameter (C)

6.3.5 The survival of our HCC patients receiving adjuvant chemotherapy was 56.7%

37 of 51 patients transplanted with an HCC had adverse pathological criteria (poorly differentiated; evidence of microvascular invasion) and adjuvant chemotherapy was administered in 26. The reasons for not giving chemotherapy included poor graft function (n = 3), sepsis (n = 5), persistent chest infection (n =2) and one perioperative death due to reperfusion syndrome. Three of the 26 who received it did not complete 6 cycles, owing to sepsis / neutropenia (n = 2) and death (n = 1). The median survival in the chemotherapy cohort was observed to be 78 months and the five year expected survival was observed to be 56.7% (figure 6.4A)..
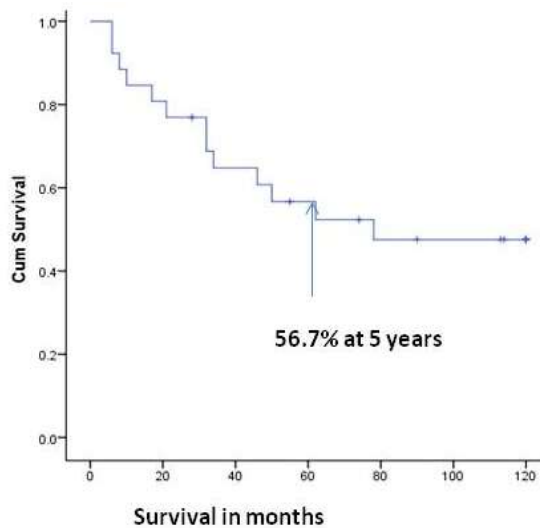
Figure 6.4A
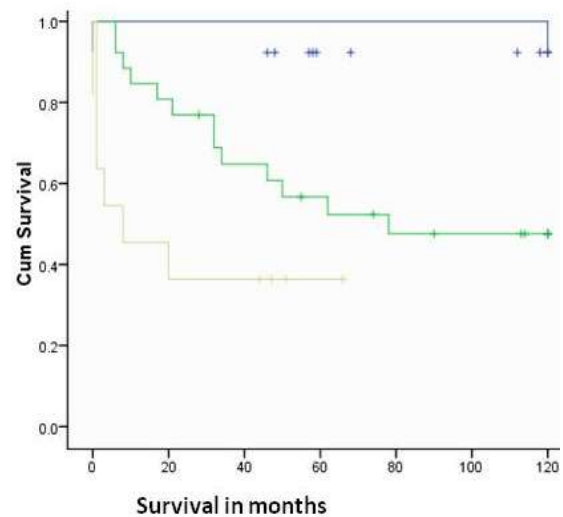
Figure 6.4B

Figure 6.4 shows the survival in the chemotherapy cohort (A) and then compared the survival versus patients not requiring chemotherapy and those which were not fit for chemotherapy (B).

6.3.6 Comparison of survival of the chemotheapy cohort with a non-chemotherapy cohort

To attempt to compare the effect of the chemotherapy, we categorised our patients into three groups. Group A patients had no adverse tumour characteristics and did not receive adjuvant chemotherapy, group B patients had adverse tumour characteristics and received chemotherapy, while group C had adverse tumour characteristics but were not fit for chemotherapy. The characteristics of the three groups are described in

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Table 6.5. The obvious difference is the mean level of AFP (more than 1000) in the patients receiving post-transplant chemotherapy.

We compared the five and ten year survival of group A with that of group B .

The estimated five year survival for patients in group A was 92.3% compared to 56.7 % for patients who received chemotherapy i.e group B (Figure 6.4B). The median survival was 202 months for patients in the no chemotherapy cohort and 78 months in the chemotherapy cohort with the log rank test showing inferior survival for the patients in the chemotherapy cohort (log rank p=0.041).

6.4 Discussion

Of all the current modalities of treatment available for the treatment of HCC, liver transplant is the only modality which deals with the cirrhosis and the liver tumour at the same time. This has been further encouraged by the fact that the 5 year survival after liver transplantation for HCC approaching that of patients undergoing liver transplant for non-malignant conditions in selected group of patients [298, 299]. There are ongoing efforts to improve the survival in the patients who undergo liver transplant for HCC.

In the retrospective review of our set of 51 patients we noted a combined 5 year survival of 61.3%. This is in congruence with the reported survival of 58 – 74% in other series [300, 301]. In our centre our protocol included the administration of adriamycin to all patients with known HCC during the anhepatic phase of the liver transplant. The aim was to kill any malignant cells shed into the systemic circulation while mobilising the diseased liver at the time of transplantation.

Reviewing the literature shows limited evidence for the role of adjuvant chemotherapy. Stone et al in 1993 reported that in a pilot study of 20 patients with unresectable tumours who underwent hepatic transplantation and received chemotherapy preoperatively, intraoperatively and postoperatively, neo-adjuvant doxorubicin favourably affects long term survival[302]. In the study the actuarial 3 year survival of the entire cohort of patients undergoing transplantation for HCC was 59% but for the patients with HCC measuring more than 5cm the 3 year actuarial survival of 63% Even in tumours larger than 5cm multi modality adjuvant chemotherapy with liver transplantation was shown to improve recurrence free interval and prolong survival[303]. Other studies have supported the role of adjuvant chemotherapy with liver transplantation for tumours that are not amenable to resection

[304, 305]. However most of these results were from small studies published in the early 1990s.

Some recent studies do not have a favourable opinion of chemotherapy due to increased side effects of the drugs, including mortality. A study on 62 patients randomised into two groups with 32 patients in the protocol and 28 in the control group. The protocol group recieved doxorubicin as adjuvant chemotherapy after transplantation and no difference in survival was noted between the two groups (overall 5 year survival was 40% in the control group and 38% in the chemotherapy group) [306] Bernal E et al in 2006 concluded that post liver transplantation chemotherapy does not avoid tumour recurrence and has fatal consequences. Furthermore, the uncertain effect of adjuvant chemotherapy on recurrence of viral hepatitis in the graft and absence of appropriate control groups warrants this drug to be used only within the confines of clinical trials[307]. However, Hsieh et al reported that the combined use of gemcitabine and cisplatin in patients where the explant did not fit the Milan criteria may in fact improve disease specific survival in this select group of patients[308]

In our cohort, we identified post operatively those patients with known adverse prognostic factors i.e poor differentiation and microinvasion. In our cohort with these features, 26 of 37 patients were deemed fit for our adjuvant chemotherapy regime. Post operative adjuvant chemotherapy was administered to nullify the adverse prognostic factors and reduce the chance of micrometastasis. The group with the known adverse factors showed a lower survival than the group which did not need chemotherapy (56.7% vs 92.3%), which was statistically significant. Though the numbers were small and the data was retrospectively collected, it can be noted that despite the addition of adjuvant adriamycin chemotherapy the survival remained poor in the group with adverse predictors for survival .

The results from our centre are also from a small non-randomised rerospective study and that that the overall results of adjuvant chemotherapy is dissapointing.No adjuvant therapy is currently advocated in HCC [309]. Our centre has also stopped the use of adjuvant chemotherapy but the encouraging results of kinase inhibitors e.g sorafenib at liver resection in a small study in 2010[310] has renewed interest in adjuvant treatment post-liver transplantation.

SUMMARY

The cohort of HCC patients that I recruited in a two and a half year period, followed up therafter for a 5 year period, has provided valuable insights in a number of areas. Firstly, in comparison to previous cohorts, it is clear that the numbers of referred patients with HCC has increased 5-10 fold in as many years. Furthermore, the underlying etiologies of chronic liver disease contributing the risk for development has changed, with steatohepatitis secondary to either alcohol or the obesity associated metabolic syndrome accounting for nearly 60% of the patients. Our sub analyses suggest that our multidisciplinary team approach to intensive patient staging and follow-up is worthwhile, as increasing numbers of patients are referred to us with early or intermediate stage disease. We have clearly shown a survival advantage to patients with tumours detected incidentally or by screening, rather than symptomatically, and one of our areas of focused research has been to try and improve the means for surveillance of HCC in at risk patients, but also to consider how we might identify the at risk population who would benefit from surveillance. At the time when we were collecting patient data, we focussed on assessing the efficacy of some of the suggested biomarkers and explored proteomic and genomic approaches to mine for novel biomarkers for HCC. Combination of alphafetoprotein and PIVKA II (Prothrombin Induced by Vitamin K Absence II) performed better that either alone but sensitivity was still low. The dismal performance of SCCA 1 (Squamous Cell Carcinoma Antigen 1) and Glypican 3 suggested that in view of the heterogenous nature of HCC it would be appropriate to select biomarkers for surveillance according to the aetiology for the background liver disease. For serum proteomics we used immunodepletion to remove abundant proteins before subjecting the serum to 2-dimensional gel electrophoresis. Of the five proteins spots showing differential expression between normal, cirrhosis and HCC we further investigated CD5L with ELISA assays. Though its ability to distinguish non-cancer from cancer individuals was poor, serum CD5L was markedly elevated in individuals with patients with NAFLD cirrhosis as compared to individuals with pre-cirrhotic disease. The proteomic strategy though shows promise in identifying future biomarkers.

The poor performance characteritistics of the CLIP and BCLC scoring systems suggests that urgency for more robust systems. Molecular biomarkers seem to be

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easily affected by human factors which precludes its use in daily practice. Adjuvant therapy post-live transplant for HCC has showed no benefit in survival.

In conclusion, these data in combination highlight the need for improved detection methods, staging systems and treatments in this increasing and changing population of individuals presenting with hepatocellular cancer.

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PAPERS

Non-invasive markers of fibrosis in non-alcoholic fatty liver disease: Validating the European Liver Fibrosis panel and exploring simple markers

Indra Neil Guha, Julie Parkes, Paul Roderick, Dipankar Chattopadhyay, Richard Cross, Scott Harris, Phillips Kaye, Alistair D Burt, Steve D Ryder, Guruprasad P Aithal, Chrisptopher P Day, William M Rosenberg

Hepatology, 2007 Feb 47(2); 455-460

AFP, PIVKAII, GP3, SCCA-1 and follisatin as surveillance biomarkers for hepatocellular cancer in non-alcoholic and alcoholic fatty liver disease.

Beale G, Chattopadhyay D, Gray J, Stewart S, Hudson M, Day C, Trerotoli P, Giannelli G, Manas D, Reeves H.

BMC Cancer. 2008 Jul 18;8:200

A Proteomic Strategy to Identify Novel Serum Biomarkers for Liver Cirrhosis and Hepatocellular Cancer in individuals with Fatty Liver Disease.

Joe Gray, Dipankar Chattopadhyay, Gary Beale, Barry King, Stephen Stewart, Mark Hudson, Christopher Day, Derek Manas, Helen Reeves

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Hepatocellular Cancer: Advances in proteomics

Dipankar Chattopadhyay, Helen Reeves

Hot Topics in Viral Hepatitis. 2011 7;21:23-29

REVIEWS

The development of targeted therapies for hepatocellular cancer

D Chattopadhyay, DM Manas, HL Reeves

Current Pharmaceutical Design; 2007 13; 3292 -3300

Tumour suppressors and oncogenes in liver tumours;

D Chattopadhyay. HL Reeves

Book chapter – Textbook of Hepatoplogy Blackwell Publications., 2007

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PRESENTATIONS

- Experience of Liver Transplant for HCC in Newcastle

  Chattopadhyay D, Reeves HLR, Manas DM

  UK and Northern Ireland Transplant meet, December, 2006

- Candidate biomarkers identified in HepG2 microarray

  Dipankar Chattopadhyay, Gary S Beale, Pamela McDonald, Stephen Stewart, Chris Day, Derek Manas, Helen Reeves

  International Liver Cancer Association meeting, October, 2007

- Candidate biomarkers of HCC and cirrhosis identified by serum proteomics in patients with NAFLD

  Dipankar Chattopadhyay, Joe Gray, Gary S Beale, , Stephen Stewart, Chris Day, Derek Manas, Helen Reeves

  International Liver Cancer Association meeting, October, 2007

- NAFLD and the changing face of hepatocellular cancer

  Debasish Das, Dipankar Chattopadhyay, Tahira Aslam, Imran Patanwala, Diane Walia, John Rose, Bryon Jaques, Derek Manas, Mark Hudson, Helen Reeves

  Bitish Society of Gastroenterology, March,2011;

  Europoean Association for Study of Liver Diseases, April, 2011;

- NAFLD related HCC is rising dramatically in the North of England
  Helen L Reeves, Janine Graham, Tahira Aslam, Dipankar Chattopadyhay, Debasish Das, Imran Patanwala, John Rose, Bryon Jaques, Derek Manas, Mark Hudson
  British Association for the Study of Liver, Annual Scientific Meeting

  September, 2011

  Accepted for the American Association for Study of Liver Diseases, Annual Meeting, November, 2011

APPENDIX

Recipes

Recipe for 10% resolving gel

Components	10%
Double distilled water	16ml
1.5M Tris(pH8.8)	10ml
10%SDS	400µl
30% Acryl BIS	13.4ml
10%APS	200 µl
TEMED	20 µl

SDS –sodium dodecyl sulphate

Acryl – acrylamide

APS = ammonium persulphate

Recipe for 4% stacking gel

	4% BIS-Tris gel
Double distilled water	12.2ml
0.5M Tris (pH6.8)	5ml
10% SDS	200 µl
30% Acryl BIS	2.3ml
10% APS	100 µl
TEMED	l

Recipe for Running buffer (stock) for SDS PAGE

10× Running buffer	
Tris base	30.3gm
Glycine	144g
SDS	10g

Recipe for transfer buffer

Transfer buffer 1L	
Tris base	3.03g
Glycine	14.4g
Distilled water	Upto 800ml
Methanol	200ml

$10 \times$ TBS buffer

Constituent	Quantity
Tris-base	61g
NaCl	90g
De-ionosed water	1000ml

Adjust pH to 7.5

Coating buffer (TBS)

20mM Tris	2.4228 gm
140 Mm NaCl	8.1816 gm
DD Water to total of 1000ml	

Substrate for ELISA

Stock solution was prepared by adding 100µg of TMB (tetramethyl benzidine) to10ml of DMSO (dimethylsulphoxide).

Solution B was prepared by adding 1.36 gm of sodium acetate to 100ml of DDW (Double Distilled Water) and pH adjusted to 6.

Then 100µl of the stock solution was added to 10ml of solution B. Then to it was added 2µl of hydrogen peroxide.

Rehydration buffer for Isoelectric Focussing

	Final concentration	Amount
Urea (FW 60.06)	8m	12g
CHAPS	2%	0.5g
Ampholye	0.5% v/v	125 μl
Bromophenol blue	0.002%	50 μl of 1% solution
Double distilled water		To 25ml

Equilibration buffer

Component	Quantity	Final concentration
Tris-HCl (pH 8.8)	10ml	50Mm
Urea (FW 60.06)	72.07g	6M
Glycerol (87% v/v)	69ml	30%v/v
SDS (FW 288.38)	4g	2% w/v
Bromophenol blue	400μl of 1% solution	0.002%
Double distilled water	Add to make total volume of 200 ml	

1mm thick 12% polyacrylamide gel recipe

Component	Volume
Protogel 30% Acryl-BIS	180ml
Tris-HCl (Ph 8.8)	112.5ml
10% SDS	4.5ml
Double Distilled Water	148.05ml

RT-mastermix

Component	Vol
10×Buffer	2.5 μl
$MgCl_2$	10 μl
DNTP	2.5 μl
Random primers	1.0 μl
RNAsin	0.5 μl
RT	0.8 μl

RT is added last

Primers for PCR

AFP

Forward primer(5' to 3')   TCC AGG AGA GCC AAG CAT TGReverse

  primer(5' to 3')   AGC AAC   GAG   AAA CGC ATT TTG

Cytokeratin 20

Forward primer(5' to 3')   CTG AAT AAA GAC CTA GCT CTC CTC AAA

Reverse primer(5' to 3')   GTG TTG CCC AGA TGC TTG TG

β-actin

Forward primer(5' to 3')   CCT GGC ACC CAG CAC AAT

Reverse primer(5' to 3')   GCC GAT CCA CAC GGA GAT CT

Standard PCR master mix for a 20 µl reaction

Component	Volume
10×buffer	2.0 µl
$MgCl_2$	1.2 µl
10mM dNTPs	0.4 µl
F Primer	0.16 µl
R Primer	0.16 µl
Taq	0.16 µl µl
Water	15.92 µl
cDNA	2.0 µl

10× TBE buffer

Component	Amount
Tris-base	108 gm
Boric acid	55 gm
EDTA (pH 8.0)	40 ml

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PIVKA-II ELISA Assay

PIVKAII Stago Diagnostics Kit components

Six 16 well strips, precipitated with the F(ab)2 fragments of anantithrombomodulin monoclonal antibody.

Calibration plasmas

Dilution buffer

Antithrombomodulin peroxidise

Reference thrombomodulin

Calibration diluents

Washing solution

Urea peroxide

Ortho-phenylenediamine (OPD) substrate

PIVKAII Assay protocol

1.   The calibration plasmas were reconstituted with the dilution buffer. The serum samples were diluted with the dilution buffer 1:10.

2.   200µl of dilution buffer was added to each well and then 50µl of the calibration plasma or of the test sample in the dilution buffer was added to the wells. The wells were filled in triplicate.The microwell plate was left to incubate for 1h at room temperature.

3.   The wells were washed five times with the washing buffer. 200µl of antibody-enzyme conjugate was added to each well.

The wells were incubated for 1h at room temperature, then washed five times with the washing solution. As soon the washing was completed, 230µl of substrate OPD/urea was added to each microwell.

The microwell plate was then incubated for exactly 5min, and then the reaction stopped by adding 50µl of 3M sulphuric acid to each microwell.

The microwell plate was then left for 10min at room temperature and the absorbance measured at 492Nm, using a micro-ELISA plate reader.

GPC-3 ELISA Assay

GPC-3 Biomosaics Kit components

1. Component 1:   Coated microtiter plate – 96 removable wells pre-coated with GPC-3 monoclonal antibody.

2. Component 2:   GPC-3 standard – vials containing lyophilised human GPC-3 protein.

3. Detector antibody: biotinylated anti-human GPC-3 antibody.

4. 400×Conjugate: Steptavidin-Peroxidase Conjugate; concentrated solution

5. Conjugate Diluent (30ml): buffer for dilution of 400× Conjugate

6. Substrate: Chromogenic substrate (TMB)

7. Sample diluent

8. 50× Plate Wash Concentrate: 50- fold concentrate solution of Tris and surfactant

9. Stop solution: 0.25N sulphuric acid

10. Plate sealers

GPC-3 Assay protocol

1.   Sample preparation: Serum sample was diluted to 1:4 using sample diluents. GPC-3 standard was reconstituted with DDW and then serially diluted with the sample diluents to obtain standard concentrations of 4050, 1350, 450, 150 and 50 pg/ml.

2.   100µl of sample diluent was pipetted into each well prior to adding samples or standards. Then 100µl of standard or the diluted samples was added in duplicate to the wells. The plates are then covered with the plate cover and incubated overnight at 2-8^0 C.

3.   The wells are washed with 1× wash buffer five times.

4.   200 µl of pre-diluted biotin conjugate anti GPC-3 was pipetted into each well.

5.   The plates were covered and incubated overnight (18 – 24 hrs) at 2-8^{0}C.The wells were then washed 5 times with 1× wash buffer.

6.   Sufficient quantity of 400× SA-HRP conjugate was diluted 1:400 in SA-HRP conjugate diluent to provide for 200 µl of 1× solution for each sample and standard well. 200µL of TMB substrate solution was added to each well and the plate

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incubated in the dark at room temperature for 30min. The wells were washed 5 times with 1× Wash solution and then emptied. .

7. 200µl of of TMB Substrate Solution was added to each well and incubated in the dark at room temperature for 30min. 100µl of Stop solution was added to each well in the same order as the TMB substrate solution. The wells were read in the spectra max at dual wavelength of 450/550nm within 30min .

Ethical approval of the project

Newcastle and North Tyneside Local Research Ethics Committees

Room G14
The Dental School
Framlington Place
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NE2 4HH

Tel: 0191 222 3581
Email: leonard.key@nhs.net

19 November 2004

Dr Helen L Reeves
Senior Lecturer and honorary consultant
gastroenterologist
University of Newcastle-upon-Tyne
Northern Institute for Cancer Research
Paul 'O Gorman Building
Framlington Place
NE2 4HH

Dear Dr Reeves,

Full title of study: The diagnostic and prognostic implications of histological pre-treatment assessment of hepatocellular tumours, in association gene and protein profiling
REC reference number: 04/Q0905/168
Protocol number:

Thank you for your letter of 17 November 2004, responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chairman.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation.

The favourable opinion applies to the research sites listed on the attached sheet. [Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed that they have no objection.]

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type: Application

An advisory committee to Northumberland, Tyne and Wear Strategic Health Authority

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Version:
Dated: 05/10/2004
Date Received: 11/10/2004

Document Type: Investigator CV
Version: Version 1
Dated: 01/07/2004
Date Received: 11/10/2004

Document Type: Protocol
Version:
Dated:
Date Received: 11/10/2004

Document Type: Covering Letter
Version: HLR
Dated: 09/10/2004
Date Received: 11/10/2004

Document Type: Summary/Synopsis
Version: version 1
Dated: 01/07/2004
Date Received: 11/10/2004

Document Type: Letter from Sponsor
Version: CM
Dated: 16/08/2004
Date Received: 11/10/2004

Document Type: GP/Consultant Information Sheets
Version: Version 1
Dated: 01/07/2004
Date Received: 11/10/2004

Document Type: Participant Information Sheet
Version: Version 2
Dated: 01/11/2004
Date Received: 18/11/2004

Document Type: Participant Consent Form
Version: Version 2
Dated: 01/11/2004
Date Received: 18/11/2004

Document Type: Response to Request for Further Information
Version: HR
Dated: 17/11/2004
Date Received: 18/11/2004

Management approval

If you are the Principal Investigator for the lead site: You should obtain final management approval from your host organisation before commencing this research.

The study should not commence at any other site until the local Principal Investigator has obtained final management approval from the relevant host organisation.

All researchers and research collaborators who will be participating in the research must obtain management approval from the relevant host organisation before commencing any research procedures. Where a substantive contract is not held with the host organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Notification of other bodies

We shall notify the research sponsor, Newcastle upon Tyne Hospitals NHS Trust that the study has a favourable ethical opinion.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC reference number: 04/Q0905/168 **Please quote this number on all correspondence**

Yours sincerely,

P.P

Professor Peter A Heasman
Chairman

Enclosures   Standard approval conditions [SL-AC1 or SL-AC2]

List of approved sites

PROFORMA FOR PATIENTS WITH HCC

COMBINED TUMOUR CLINIC:ASSESSMENT AND PROGRESS

Patient Name/

Sticky laberl

DATE											
Child-Pugh Stage									1	2	3
Encephalopathy									none	Grade1-2	Grade 3-4
Ascites									absent	mild	Mod
Bilirubin (μmol/l)									17-34	35-49	>50
Albumin (g/l)									>35	28-35	<28
PT (secs ↑d)									1-4	4-6	>6
									Grade A = 5-6; Grade B = 7-9, **Grade C ≥ 9**		
Okuda Score									0		1
Tumour Size									<50%		>50%
Albumin									>30		<30
Ascites									ABSENT		PRESENT
Bilirubin									<35		>35
									Score 0 ≡ Stage1 ≡ 28 months; Score 1/2 ≡ Stage2 ≡ 8; Score 3/4 ≡ Stage3 ≡ 1 month		
CLIP Score									0	1	2
Child-Pugh Stage									A	B	C
Tumour morphology									Uninodular + extension ≤50%	Multinodular + extension ≤50%	Massive or extension > 50%

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AFP (ng/l)								<400	>400	
Portal vein thrombosis								no	yes	
PST Score								Median survival in months: Score 0 ≡ 42.5; Score 1 ≡ 32; Score 2 ≡ 16.5; Score 3 ≡ 4.5; Score 4 ≡ 2.5; Scores 5+6 ≡ 1		

Performance StatusTest in cancer patients used to quantify functional status, is an important factor in determining prognosis (Sorensen Br J Cancer 1993)

PS 0 = normal activity; PS 1 = some symptoms but near full ambulatory; PS 2 = <50% time in bed; PS 3 = >50% time in bed; PS 4 = Bedridden.

CT / MRI scan				
Clinical comment / Other significant PMHx / Medication eg. Metformin?				
Management Plan including dates of MDT Reviews				
TACE				
RFA				

Other therapeutic Intervention - Surgery / Systemmic Chemo / irradiation				
Other inpatient admission				