Biomarker Development for Gastrointestinal and Ovarian Cancer: a Proteomic Approach

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Abstract

The development of new biomarkers for cancer patients would be advantageous in population screening for the early detection of cancers, pathological diagnosis, assessment of prognosis, tailoring treatment to individuals, and assessment of treatment response. With this in mind different proteomic approaches were used to identify biomarkers which could potentially aid prognosis and predict response in gastrointestinal and ovarian cancer.

Raf Kinase Inhibitor Protein (RKIP) was originally purified from bovine brain extracts and named phosphatidylethanolamine-binding protein (PEBP). It has subsequently been shown to be a widely expressed and highly conserved protein. Several recent studies have suggested that RKIP may suppress metastasis in melanoma, prostate, and breast cancer, as reduction or loss of RKIP expression was observed in metastatic cell lines and metastatic tissue. In this part of the project RKIP expression was assessed by immunohistochemistry in tissue microarrays (TMA) from patients with colorectal and ovarian cancer. The results confirmed the findings of earlier studies and suggest that the level of RKIP expression is significantly and inversely associated with metastatic disease and can predict the risk of metastatic relapse in patients with no evidence of metastases at presentation. The level of RKIP expression as a prognostic factor was independent of sex, age, tumour site, mitotic index, lymphovascular invasion and tumour stage.

Cytokeratin 18 (CK18) is an epithelial-specific cytokeratin that undergoes cleavage by caspases during apoptosis. Measurement of caspase-cleaved (CK18-NE) or total cytokeratin 18 (CK18) from epithelial-derived tumours could be a simple, non-invasive way to monitor or predict responses to treatment. Soluble plasma CK18-NE and CK18 were measured by ELISA from 73 patients with advanced gastrointestinal adenocarcinomas before treatment and during chemotherapy, as well as 100 healthy volunteers. Both CK18-NE and total CK18 plasma levels were significantly higher in patients compared to the healthy volunteers ($p=0.015$, $p<0.001$). The total CK18 baseline plasma levels prior to treatment were significantly higher ($p=0.009$) in patients who develop progressive disease than those who achieve partial response or stable disease and this correlation was confirmed in an independent validation set. The peak plasma levels of CK18 occurring in any cycle following treatment were also found to be associated with tumour response, but peak levels of CK18-NE did not reach significance ($p=0.01$, and $p=0.07$, respectively).

A surface-enhanced laser desorption-ionisation mass spectrometry (SELDI-MS) pilot study on serum from 8 oesophageal cancer patients and 8 healthy volunteers revealed a novel biomarker, ~4kDa, downregulated in patients ($p=0.012$). An expanded 30 tumour/normal study was performed for validation which confirmed the down-regulation of this potential biomarker ($p<0.0001$). Attempts to identify tentatively suggested that the peptide may be inter-alpha-trypsin inhibitor heavy chain H4 precursor, which was interesting as a cleavage fragment of inter-alpha -trypsin inhibitor heavy chain H4 had been previously found to be up-regulated in patients with ovarian cancer, and down-regulated in patients with breast cancer. However, it was not possible to confidently confirm this identification. In a further part of this study, haptoglobin was found to be significantly more abundant in the serum from patients with oesophageal cancer compared to healthy volunteers. It was straightforward to isolate and identify and would be amenable to immunoassay as there are good antibodies available for confirmation.

In conclusion, with the current lack of effective markers of metastatic relapse in colorectal cancer, a straightforward test like RKIP expression in the primary tumour may be a very cost-effective way to identify which patients may derive greater benefit from adjuvant treatment and closer post-operative surveillance, and in patients with advanced gastrointestinal malignancy levels of plasma CK18 are a potential marker of tumour response.

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Peer-reviewed Publications (see Appendix 1)

1. Raf Kinase Inhibitor Protein (RKIP) expression in colorectal cancer is associated with metastatic recurrence and overall survival. Fahd Al-Mulla, Suzanne Hagan, Abdulla I. Behbehani, Milad S. Bitar, Shirley S. George, James J. Going, Jorge J. Curto García, **Lucy Scott**, Nicky Fyfe, Graeme I. Murray, Walter Kolch. Journal of Clinical Oncology, 2007; 24 (36): 5672-5679.

2. Cytokeratin 18 in plasma of patients with gastrointestinal adenocarcinoma as a biomarker of tumour response. **L.C. Scott**, T.R.J. Evans, J. Cassidy, S. Harden, J. Paul, V. O'Brien, R. Brown. British Journal of Cancer, 2009; 101: 410-417.

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Author's declaration

I am the sole author of this thesis. All of the references have been consulted by me in the preparation of this manuscript. Except where otherwise stated, the work presented in this thesis was performed personally.

Definitions

µg	microgrammes
µL	microlitre
µM	micromolar
CA-125	cancer antigen-125
CarboF	carboplatin/5-fluorouracil
CK18	cytokeratin 18
CK18-NE	neo-epitope of cytokeratin 18
CEA	carcinoembryonic antigen
CF	cisplatin/5-fluorouracil
CT	computed tomography
Da	Dalton
DI	deionised
EAM	energy-absorbing matrix
ECF	epirubicin/cisplatin/5-fluorouracil
ECarboF	epirubicin/carboplatin/5-fluorouracil
ECOG	Eastern Cooperative Oncology Group
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
ESI	electrospray ionisation
FDA	Food and Drug Administration
FIGO	Cancer Committee of the International Federation of Gynaecology and Obstetrics
FOLFOX	5-fluorouracil/oxaliplatin

HPLC	high performance liquid chromatography
IHC	immunohistochemistry
MALDI	matrix-assisted laser desorption/ionisation
mL	microlitres
MS	mass spectrometry
nm	nanometre
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PVDF	polyvinylidene fluoride
pmol	picomoles
PSA	prostate specific antigen
RNA	ribonucleic acid
ROC	Receiver Operating Characteristics
RKIP	Raf kinase inhibitor protein
SCOTROC	Scottish Randomised Controlled Trials in Ovarian Cancer
SELDI-MS	surface-enhanced laser desorption and ionisation
SPA	sinapic acid
TFA	trifluoroacetic acid
TIGAR	TP53-induced glycolysis and apoptosis regulator
TMA	tissue microarray
TOF	time of flight
TPA	tissue polypeptide antigen
TPS	tissue polypeptide-specific antigen
WHO	World Health Organisation

XELOX	capecitabine/oxaliplatin

Chapter 1 Introduction – Protein and Peptide Biomarker Discovery

1.1 The Challenge

Radiological imaging of tumours is an essential part of the practice of oncology, with a crucial role in screening programmes and in the diagnosis and staging of established disease. Furthermore, the assessment of tumour size by imaging, usually with computed tomography (CT) is a key component in determining tumour response in clinical practice. However, the development of a serum or plasma biomarker to monitor treatment response would be advantageous in terms of ease of repeated analysis and use of resources compared to anatomical imaging. In addition biomarkers might give an earlier indication of potential response to treatment rather than the time lag which is necessary to observe changes in tumour size. In patients with malignancy this may avoid administering toxic treatments with little prospect of benefit and hence start alternative treatments earlier. Another area where serum biomarkers would be of value is in population screening for the early detection of cancers.

Biomarkers are defined as characteristics that are objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacological responses to a therapeutic intervention [1]. The ideal biomarker should have a high sensitivity and specificity for diagnosis; its level should correlate with disease stage and response to treatment; and it should be easily and reproducibly measured. Unfortunately, the biomarkers currently available for use in the management of solid tumours, like gastrointestinal and ovarian cancer do not fulfil all these criteria and, therefore, are not presently recommended for screening of the general population. At present the main uses of biomarkers are in determining prognosis, monitoring responses to treatment and in detection of disease recurrence. The challenge is therefore to develop the ideal biomarker.

1.2 Classes of Cancer Biomarkers

1.2.1 Screening markers

Biomarkers used for cancer screening need to be able to detect early stage disease with high sensitivity and specificity (sensitivity refers to the proportion of people with disease who have a positive test result and specificity refers to the proportion of people without disease who have a negative test result. The ideal biomarker for population screening

should have >99.9% sensitivity and specificity) [2]. However, early detection is only useful if there is an intervention that can be undertaken when early stage disease has been diagnosed, which results in improved survival outcomes for patients. In addition, widespread screening of the population is not cost-effective when considering cancers that have a low prevalence.

The biomarker that has probably been most widely used for cancer screening is prostate-specific antigen (PSA) as a screen for prostate cancer [3]. However, there is no agreement about whether screening of the general population reduces premature mortality. A UK study evaluated the combination of digital rectal examination and PSA levels as a screening tool for prostate cancer. Men with abnormal results were then referred for a transrectal ultrasound scan (TRUS) and biopsy of any suspicious areas. Of the men referred for TRUS only 6% had a biopsy taken and only 1.7% had prostate cancer [4]. These results have been supported by other trials evaluating the role of prostate cancer screening in asymptomatic men [5].

This controversy is highlighted by the results from two large clinical trials that assessed prostate-cancer mortality in screening studies which were recently reported. The first study from Europe randomised 182,000 men between 50-74 years to either a control group or a group that were offered PSA screening once every four years. The results showed that PSA screening reduced the rate of prostate-cancer death by 20%, but at a high risk of overdiagnosis [6]. The second study from the US randomised 76,693 men between 55-74 years to either a control group or a group that was offered annual PSA screening for six years and digital rectal examination for four years. The results showed that after seven to ten years of follow-up, the prostate cancer related mortality was low and did not differ significantly between the two groups [7].

It is clear that for cancer screening there is a need for much more sensitive and specific biomarkers using technologies that are reproducible, portable, cost-effective, amenable to large scale screening of populations and ideally uses readily available body fluids.

1.2.2 Diagnostic markers

Diagnostic markers are generally used to aid histopathological tumour diagnosis and classification and so allow for optimal treatment choices [8]. Where this is extremely important is in the diagnosis of adenocarcinoma of unknown primary origin (ACUP). ACUP accounts for 3% of all malignant disease, making it one of the ten most frequently

diagnosed cancers [9, 10]. However, modern chemotherapy regimens now vary greatly between different tumour types and there are targeted treatments available to treat certain cancers, and so if it is possible to accurately determine a site of origin, then there is a greater potential for tumour-specific treatments which may provide better clinical outcomes. Diagnostic markers used include CEA [8], CA19-9 [11], cytokeratin 7 (CK7) for gastrointestinal malignancy [12], CK20 and CA-125 for ovarian cancer [8], hormone receptors such as oestrogen and progesterone receptors (ER and PR) [13], and PSA for breast and prostate cancer [14], and thyroid transcription factor-1 (TTF-1) for lung cancer [15].

More recently gene-expression profiles have been developed that may potentially be of use in patients with ACUP [10, 16]. These have been used retrospectively in formalin-fixed paraffin-embedded specimens and have established putative diagnoses in patients that are compatible with clinical and pathological findings. However, prospective studies are required to assess the utility of these profiles in directing therapy.

1.2.3 Prognostic markers

Prognostic markers provide information about the malignant potential of tumours and patient survival time to facilitate further treatment choices, especially if there is more information gained regarding the risk of future metastatic spread [9]. Prognostic markers currently in use in clinical settings include hormone receptors (such as ER and PR), proliferation markers (such as Ki67), and proteases, markers of angiogenesis (such as VEGF), growth factor receptors (HER-2/neu), and p53 [9].

1.2.4 Predictive markers

Markers used to aid diagnosis can also provide predictive information about a tumour's likely response to different treatments [9]. For example, breast cancer patients with oestrogen receptor (ER) positive tumours receive treatment with either tamoxifen or aromatase inhibitors in the adjuvant and metastatic setting as clinical trials have shown a clinical benefit in patients with ER positive but not ER negative disease [9]. Another example would include the use of trastuzumab (Herceptin®) in breast cancer patients whose disease overexpresses the growth factor Her-2/neu. Trastuzumab is a monoclonal antibody that selectively binds to the HER-2 protein and clinical trials have demonstrated clinical benefit in using trastuzumab both in the adjuvant and metastatic setting in patients whose cancer overexpresses the HER-2 protein [2, 17]. These markers therefore, help to

tailor treatments to patients who will get the most clinical benefit, whilst sparing predicted non-responders the toxicities of ineffective treatments.

1.2.5 Markers of response to cytotoxic and targeted therapies

Currently, response to therapy in cancer is usually determined by radiological imaging as reduction in tumour size will generally be apparent within weeks of starting treatment. Serum biomarker measurement to monitor treatment response is advantageous in terms of ease of repeated analysis and use of resources compared to anatomical imaging, but is generally only used in the setting of advanced disease. Examples of biomarkers that are currently used to assess response in advanced disease are cancer antigen 125 (CA-125) for ovarian cancer [18], carcinoembryonic antigen (CEA) for colorectal cancer [19] and PSA for prostate cancer [8]. This use requires that the quantitative measurement of the biomarker e.g. serum concentration, should have some correlation with disease severity.

However, with the newer targeted treatments, reduction in tumour size may not occur for many months and so other measurements of tumour response are required [20]. These might include a decrease in the tumour's metabolic or proliferative activity and it would be useful to have biomarkers that could determine this either as a stand-alone test, such as a blood test that could be performed at specified time-points, or a molecular imaging technique. The main advantage of molecular imaging techniques is that they have the potential to show the level and activity of a specific molecular target *in vivo*. PET (positron emission technology) scanning gives information about disease location and can detect changes in metabolic activity within disease sites before changes are apparent on conventional CT scans [21]. However, molecular imaging relies on the development of suitable biomarkers that can be detected by available technology; are easily deliverable to the sites of disease (even crossing physiological barriers like the blood-brain barrier); accumulate within the sites of disease, but are cleared rapidly from normal tissues, and are non-toxic. The biomarkers required may also need to differentiate between disease states and even cancers arising from different sites, making this area of research very challenging.

1.3 Background to Protein and Peptide Biomarker Discovery

In 1999, the formal definition of a biomarker was created [22]. According to this definition, a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. A biomarker can serve as a clinical endpoint, surrogate endpoint or both. A clinical endpoint is defined as a characteristic or variable that reflects how a patient feels, functions or survives. A surrogate endpoint is defined as a biomarker intended to substitute for a clinical endpoint and so, a surrogate endpoint is expected to predict clinical benefit (or harm, or lack of benefit) based on epidemiological, therapeutic, pathophysiological or other scientific evidence. It was felt that the formal definition of a biomarker needed to be created in order to standardise their development and measurement, thus establishing internationally accepted markers that are easily and reproducibly measured [1].

Biomarkers in the context of cancer research are molecules occurring in body fluids or tissues that are associated with cancer. The identification or measurement of these markers is useful for diagnosis and clinical management. The ideal marker should have high sensitivity and specificity for diagnosis (i.e. be specific to the cancer type and able to reflect low tumour burden or early stage disease); should correlate with disease stage and response to treatment; and be easily and reproducibly measured. Most biomarkers have too many false positives from benign conditions to make screening feasible. Many only clearly identify malignancy once the cancer is sufficiently advanced to make this detection of limited use.

One potential way of discovering new cancer biomarkers is by proteomics. Proteomics is the complete description of all proteins encoded by the genome, called the proteome, and aims to establish biosignature profiles to discriminate between disease states. Therefore, a detailed analysis of proteins expressed by an organism may allow a greater understanding of the molecular complexities of cancer and other diseases [23]. In cancer this involves analysing the protein expression from tumours, tumour cells or extracellular fluids from cancer patients. The main advantage of this approach over gene analysis is that gene transcription levels do not necessarily correspond to protein levels and thus function within organisms as there are potentially a large number of protein products from each gene. A further advantage is that proteomics affords the potential to analyse post-translational

modifications of proteins [9]. There are more than 200 post-translational modifications that proteins can undergo that affect function, protein-protein and nucleic acid-protein interactions, stability, targeting, and half-life [1]. In addition various changes occur when a normal cell transforms to a cancerous one, ranging from differences in expression levels of proteins, differential protein modification, changes in specific activity, to aberrant localisation. All of these changes can affect cellular function and can make study of the proteome complex and challenging.

There are two main approaches for biomarker discovery in proteomics

1. A global, non-directed approach – using high-throughput technologies such as SELDI and MALDI.

2. Target-specific approaches- where known molecular targets such as antigens and antibodies are probed using cross-reacting analytes, such as antigens, antibodies or other affinity reagents.

1.3.1 Global approaches for biomarker discovery

Global approaches have only become possible due to technological advances in mass spectrometry and separation techniques, and the wealth of data gained from the human genome project. Two-dimensional gel electrophoresis was initially described thirty years ago [24]. Proteins in a 2-dimensional gel (2-D PAGE) are separated in two dimensions, the first, dependent on their isoelectric point (the pH at which a molecule carries no net electrical charge) and the second on their molecular mass. Spots of interest, for example, proteins whose intensities are significantly increased or decreased between normal and cancer samples, are then excised and identified using mass spectrometry. The main limitations with this technique are that many proteins (including potential biomarkers) are expressed at such low levels that they may escape detection and it can be difficult to ensure reproducibility due to variation in running conditions.

Mass spectrometry (MS) was developed in the early 20th century, but has undergone significant improvement over the past 20 years with the development of more sensitive, higher resolution techniques. High performance liquid chromatography (HPLC) and MS techniques have been developed that allow definitive identification and quantitative determination of compounds. The increased purity of the compounds that are introduced into the MS by HPLC allows for a greater identification capability. Further advances

include higher resolution mass spectrometers and tandem MS, which have enabled the analysis of biomolecules over 300,000 Da.

The three commonest ionisation methods used for analysis of proteins and peptides include electrospray ionisation (ESI), matrix-assisted laser desorption/ionisation (MALDI) and surface-enhanced laser desorption and ionisation (SELDI) [25, 26]. All of these ionisation methods can detect within the picomole to femtomole range, which is required for the analysis of biological samples.

MALDI and SELDI both involve spotting of samples onto a solid surface or probe, in the case of SELDI; this is called a ProteinChip (BioRad/Vermillion). MALDI requires samples to be mixed with an energy-absorbing matrix before being applied to a passive stainless steel probe. SELDI, however, uses ProteinChips that selectively bind different proteins and peptides of interest using a defined chemical chromographic characteristic (i.e. hydrophobic, ion exchange, or metal binding surfaces).

ESI creates an ion gas cloud in the source directly from the sample solution containing highly charged droplets. The droplets are then electrostatically driven through air, heat, solvents, nitrogen gas and other drying agents so that the surface charges are deposited onto the proteins and peptides. This process is a lot gentler than MALDI and SELDI and, therefore, causes less fragmentation of molecules of interest, but is less suited to high-throughput applications

These techniques generally require some form of sample fractionation, such as anion exchange chromatography, to remove the more abundant proteins which may mask the less abundant potential biomarkers within the samples and therefore help to target specific areas of proteome for biomarker discovery [1]. The concerns with sample fractionation are that the process has to be highly reproducible and there is the potential that biomarkers bound to the more abundant proteins may also be removed.

The advantages of global protein profiling are that it allows the high-throughput analysis of samples, therefore making it ideal for population screening. It can analyse readily available material, such as serum and plasma, and requires only small amounts of sample for analysis. However, it has a number of drawbacks; first the spectra generated by SELDI and to an extent MALDI do not give details of the identity of the individual proteins, but rather, give a pattern that is associated with different organs and disease states. In order to try and identify the potential biomarkers within the samples it is generally necessary to convert

proteins into their constituent peptides by digestion with a proteolytic enzyme like trypsin. Tandem MS/MS is then used to work out the sequence of the peptides by fragmenting the parent ions via collision with gas molecules. The molecular weight and sequence information obtained is then searched against suitable protein databases and peptide identities assigned based on correlations between the experimentally observed spectra and those within the database. One other drawback is that the mass spectra produced contain a large number of protein peaks from which it can be difficult to filter out the background noise, and there are also concerns about the potential lack of reproducibility and the fact that studies are generally limited to investigating only a very small fraction of the proteome at a time due to its complexity.

1.3.2 Target-specific approaches for biomarker discovery

1.3.2.1 Protein Microarrays

Protein microarray, a proteomic technology that examines precise protein-protein signalling in various disease states in an exact and high-throughput manner, has complemented 2-D PAGE MS [27]. A protein array contains a collection of immobilised protein spots. Each spot can contain a homogeneous or heterogeneous set of bait molecules. The array is interrogated with either a probe (labelled antibody or ligand) or an unknown biologic sample (e.g. cell lysate or serum sample) that contains the analytes of interest. The query molecules are tagged with a signal generating moiety which then generates a pattern of positive and negative spots. An image of the spot pattern is captured, analysed and interpreted [28]. Proteins or peptide fragments can also be attached onto the microarrays and protein-protein interactions elucidated [29]. Protein and antibody arrays are more expensive and labour intensive than DNA arrays and require a large bank of specific antibodies to known proteins. However, the specific antigen-antibody reactions allow for identification of cancer-specific antigens and more accurate diagnoses and also, the high-throughput makes it attractive as a potential screening tool.

1.3.2.2 Immunoassays

Within the field of target-specific approaches, immunoassays are one of the fastest growing technologies [30]. In its most basic form it is a biochemical test that measures the concentration of a substance within a biological fluid, using the reaction between an antigen and an antibody. The level of the unknown substance is then determined by the plotting of a standard curve. In order to detect the quantity, the antibody or antigens are

commonly labelled using enzymes, colloidal gold, radioisotopes, magnetic labels or fluorescence. The main drawbacks associated with immunoassays are that they are generally limited in the number of samples that can be examined at one time and so are not ideal for high-throughput screening. Also, they can only screen for a specific protein and so may miss unknown but significant differences between samples. However, they are routinely and effectively used in clinical practice and newer technologies such as Luminex xMAP® multiplexing technology (www.luminexcorp.com) have been developed which allow simultaneous measurement of multiple samples.

1.3.2.3 Immunohistochemistry

Immunohistochemistry is the localisation of antigens in tissue sections using labelled antibodies and is the standard method for determining the presence of markers within tissue [31]. Again, as for immunoassays, the antibodies are labelled using fluorescent dyes, enzymes, colloidal gold or radioactive elements. The type of antibody selected will depend on the tissue that is under study and the degree of sensitivity that is required.

It was first described in 1941 (Coons et al, 1941) when studies were performed using antibodies labelled with fluorescent dyes to examine tissue sections. Since then there has been advances in the sophistication of the technique and labelling of the antibodies used. This has resulted in immunohistochemistry becoming a crucial and widely used technique both in clinical diagnosis and medical research.

1.4 The Ideal Biological Fluid for Proteomic Analysis

The majority of the studies evaluating protein expression in cancer have utilised serum or plasma. The theory behind this is that organs or tissues can add to, modify, or remove proteins and peptides in the circulating bloodstream. Therefore, the plasma proteome may reflect abnormalities or pathological states affecting organs and tissues. Blood is readily accessible and medical laboratories are already equipped for its analysis making it an ideal diagnostic material. However, human plasma is thought to have tens of thousands of core proteins spanning ten to twelve orders of magnitude in protein abundance [32] and, so, there are analytical challenges posed by the complexity and depth of the plasma proteome. The data obtained following analysis of the serum proteome is very complex and bioinformatic tools can help, but the analysis of the serum proteome is reliant on a couple of presuppositions; first that the proteins or peptides shed into the serum, through either angiogenesis or invasion of surrounding tissues and vasculature, are representative of the

organs they are derived from and second, and that they are specific to the disease process affecting the organ, whether benign or malignant. In addition it is likely that specific biomarkers of disease are going to be of low relative abundance and greatly diluted within the circulation and that more abundant proteins may interfere with their detection. Therefore, as mentioned earlier some form of sample fractionation is usually necessary to increase the chances of detecting low abundance biomarkers.

This problem may to some extent, be overcome by analysis of fluids lying closer to the site of disease in which potential biomarkers will be present in higher concentrations. Examples of this include urine for diseases affecting the renal tract, nipple aspirate fluid for breast disease and cerebrospinal fluid for central nervous system disorders. The types of samples to be studied will also rely in part on the disease process of interest. Serum contains proteolytic enzymes, which may make reproducibility of results difficult due to the resulting variation in protein levels. Examining the proteomic profiles of tissue sections from non-small cell lung and brain cancer has been shown to provide prognostic information [33] and urine samples may also show differential proteomic profiles reflecting various disease states affecting the renal tract and is not subject to the same degree of proteolysis [34]

Various factors may complicate the measurement or detection of a biomarker within a human population, such as age, gender, diet and interaction with medications. These difficulties may, in part, be overcome by the use of cell lines or genetically homogenous mouse models and so these may be used as the source of material for proteomic analysis. Understandably, there are concerns about how applicable the results derived from these approaches are to humans. In fact, their main function may be to provide candidate biomarkers which may then be further assessed in human populations.

1.5 The Clinical Challenge

1.5.1 Oesophageal Cancer

The incidence of oesophageal cancer has increased over the past 30 years. Currently 7,800 cases are diagnosed in the UK each year, with more men affected than women and like most cancers, its incidence increases with age.

Unfortunately the outcomes for patients with oesophageal cancer are poor, with an overall 5-year survival rate of 8%. This is because up to two thirds of patients are diagnosed when

their disease is inoperable. For those who undergo an operation, the 5-year survival rates are up to 25%. However, recent studies have suggested that administering pre-operative chemoradiotherapy or chemotherapy alone improves patient outcomes with 2-year survival rates of up to 44% (CancerResearch UK, 2005. Cancerstats, www.cancerresearchuk.org).

At present screening for oesophageal cancer is not carried out in the UK. This is because the screening test would involve an endoscopy and the risks associated with the procedure have been judged to out way the benefits, as only a very small number of cases would be detected. Again if there was a simple blood test that could be performed in this setting which would allow targeting of endoscopies to the patients who would derive the greatest benefit and allow for earlier diagnosis of disease then survival outcomes could be improved. The potential role of serum biomarkers for the management of patients with oesophageal cancer has been looked at in several studies.

Cytokeratin expression has been studied in many different cell types over the past few decades [35]. The most universally expressed are cytokeratin 18 (CK18) and 19 (CK19) which are major components of the intermediate filament of simple epithelial cells and epithelial-derived tumours, and make up approximately 5% of the total cell protein [36]. Their expression has been documented in a number of different cancers and various antibodies have been developed for their detection [37]. There is an assay available that measures circulating soluble cytokeratin 19 (CK19), CYFRA 21-1, which is based on two monoclonal antibodies to CK19 [38]. CYFRA 21-1 has mostly been used clinically in lung, and head and neck cancers [39, 40]. In patients with oesophageal cancer high pre-operative levels of CYFRA 21-1 have been found to be associated with tumour progression and poor survival outcomes especially in patients with squamous cell oesophageal carcinoma [41, 42]. Serum squamous cell carcinoma antigen 2 (SCC-antigen) mRNA concentrations have been found to be associated with pathological changes in oesophageal cancer but are not sensitive or specific enough to be used for screening [43].

1.5.2 Gastric Cancer

In the UK around 8,000 people are diagnosed with gastric cancer each year, making it the eighth most common cancer. Although the incidence has been falling over the past eighty years, probably due to improvements in food storage and changes in diet, it tends to occur more frequently in males and with increasing age.

If gastric cancer is diagnosed and treated at the earliest stage, stage I, then the outcomes are very good with 5-year survival rates of 80%. However, unfortunately only 1% of patients are diagnosed with stage I disease; the majority of patients are diagnosed when the disease is at a much more advanced stage with 5-year survival rates of 15% or less. In fact 80% of patients are diagnosed when their disease has already spread (stage IV) and the survival rates in this group of patients are dismal, with 5-year survival rates of 5% (CancerResearch UK, 2005. Cancerstats, www.cancerresearchuk.org). It would, therefore, be extremely useful if there was a simple screening test, such as a blood test that could aid with earlier diagnosis of gastric cancer and highlight which individuals would derive most benefit from further investigations like endoscopy.

At present there are no screening tests for gastric cancer within the UK. Studies performed looking at serum biomarkers in gastric adenocarcinoma have failed to provide a biomarker that is sensitive or specific enough for population screening [44-46]. Biomarkers that have been studied include CEA, carbohydrate antigen 19-9 (CA19-9) and carbohydrate antigen 72-4 (CA72-4). CA19-9 is a monoclonal antibody raised against a colon carcinoma cell line to detect a monosialoganglioside in patients with gastrointestinal cancer [47]. It is elevated in 20-40% patients with gastric cancer. The CA72-4 assay measures a tumour associated glycoprotein (TAG-72) using two monoclonal antibodies. Raised serum TAG-72 levels have been observed in 33-59% patients with gastric cancer [48, 49]. Studies have been carried out that looking at the role of endoscopy as a screening test in gastric cancer. In one study endoscopies were performed on all patients over 40 years of age, who presented with symptoms suggestive of upper gastrointestinal pathology. The results showed that although more early cancers were discovered, 98 out of every 100 people underwent a needless endoscopy, which is not without risk. It was felt that this type of screening test was not cost-effective.

1.5.3 Colorectal Cancer

There are about 36,000 patients diagnosed with colorectal cancer in the UK each year, making it the third most frequently diagnosed cancer and the second leading cause of cancer deaths, with over 16,000 patients dying each year (CancerResearch UK, 2005. Cancerstats, www.cancerresearchuk.org). The survival rates for colorectal cancer vary widely and depend largely on the stage at which the cancer is diagnosed. The 5-year survival rates for the earliest stage, I or Dukes A, are over 80%. If, however, the cancer is diagnosed at an advanced stage where spread had occurred to distant organs, there is only a 15% 2-year survival, with a median survival of 10 months from diagnosis. Unfortunately at

present only 10% of patients are diagnosed with stage I disease, as symptoms tend to present late in the time course of the disease. If it was possible to diagnose patients when their disease was at an earlier stage then survival outcomes would be much improved. It would, therefore, be extremely useful to have a screening test that could be performed on the general population to detect patients with asymptomatic early stage disease, who would gain most benefit from further investigations and treatment.

Various different methods have been employed as screening tests for colorectal cancer including faecal occult blood (FOB) screening and techniques involving direct visualisation of the bowel such as sigmoidoscopy. Information regarding sigmoidoscopy screening suggests a 60-80% reduction in mortality from distal but not proximal cancers [50, 51]. Studies that have looked at both sigmoidoscopy and colonoscopy have shown a 50% reduction in incidence of colorectal cancer up to 6 years of follow-up [52]. One study showed an 80% reduction in cancer incidence with sigmoidoscopy which was observed after 13 years of follow-up, however, there was no reduction in mortality [53]. The interim results from a study looking at once only screening sigmoidoscopy have recently been reported [54]. This study randomised 55,736 people aged between 55-64 years to usual care or to once only flexible sigmoidoscopy with or without FOB testing. Of the 13,653 people invited to attend a screening flexible sigmoidoscopy, 8846 (65%) underwent the procedure. The incidence of colorectal cancer (this included both distal and proximal cancers) and mortality did not differ between the screened and control population, but the screen detected cancers tended to have earlier stage disease and a lower case-fatality rate although this didn't reach significance. However, within the screened group there was a significant reduction in mortality from colorectal cancer in the group who attended for screening investigations, and this is relevant as site-specific cancer mortality is considered an appropriate end-point for evaluating screening tests [55]. Endoscopic screening has been advocated in the USA for the past 10 years [56] and several countries in Europe have launched colonoscopy screening programmes [57, 58].

Trials have been reported looking at the use of annual FOB screening, one trial showed a reduction in mortality that pre-dated a reduction in incidence by 7 years [59]. At present in the UK, the NHS is carrying out a Bowel Cancer Screening Programme. As part of this screening programme, men and women aged between 60-69 years are sent out faecal occult blood (FOB) screening tests every 2 years. Individuals with positive FOB results are then offered further investigations, including colonoscopy which allows for direct visualisation of the bowel and biopsy of any suspicious lesions identified.

The screening programme is based on a pilot study which was performed in England and Scotland in 2007. The aim of the pilot study was to discover the feasibility of introducing a national screening programme for colorectal cancer based on FOB testing. The trial involved men and women aged between 50-69 years. The results showed that of the 478,250 people invited to participate, the uptake was 271,646 (56.8%). The overall rate of a positive test was 1.9%. The number of cancer cases detected was higher in the Scottish population, in men, and also increased with advancing age. The positive predictive factor was 10.9% for cancer and 35.0% for adenoma. A total of 552 cancers were discovered, of which 16.6% were polyps, 48% were stage I (Dukes A) and only 1% were stage IV (Dukes D).

The results from the pilot study were encouraging and suggest that FOB testing may be a useful method for screening the population and detecting early stage cancers. However, as can be seen from the pilot study the uptake was only 56.8%. If there was a simple blood test which could be performed as a screening test, the uptake may be higher as people tend to be regard blood tests as more acceptable. Blood is also easily accessible and clinical laboratories are well set up for its analysis.

At present there are no serum or plasma biomarkers available for colorectal cancers that are sensitive or specific enough to be used as screening tests. CEA is a member of a class of oncofetal antigens that are produced within the normal developing fetus, but only in minute amounts by normal adult cells. First described in 1965, it has become the most widely used biomarker in gastrointestinal malignancy [60] and can be measured quantitatively by immunoradiometric assay in serum, but due to its lack of sensitivity in the early stages of disease (<25%) it is unsuitable for population screening. Its main use is in the follow-up of patients after surgical resection with the aim of earlier detection of recurrence, and in monitoring responses to palliative chemotherapy in advanced disease [19].

1.5.4 Ovarian Cancer

About 6,800 women are diagnosed with ovarian cancer in the UK each year. It is the fifth commonest cancer in women after breast, lung, colorectal and endometrial cancer (CancerResearch UK, 2005. Cancerstats, www.cancerresearchuk.org).

One of the characteristics of ovarian cancer is common presentation at an advanced stage, where 5-year overall survival rates are < 30%, whereas if the disease is diagnosed at stage I

(confined to the ovary), the overall survival rates are > 90%. The current screening investigations for ovarian cancer include the biomarker, CA-125 and pelvic ultrasound scanning. CA-125 is a serum protein defined by a monoclonal OC 125 that was generated by immunizing mice with a cell line established from human ovarian carcinoma. It is elevated in about 80% of patients with advanced ovarian cancer, but only 50-60% of early-stage patients. Therefore, CA-125 has a positive predictive value (the probability that disease is really present when the test is positive) of <10%, which is increased to only 20% with the addition of ultrasound scanning [61-63]. Moreover, CA-125 can also be elevated in a number of other conditions including pregnancy, endometriosis, colorectal and pancreatic cancers. Therefore, there is a need to develop detection methods to improve the sensitivity and specificity of early-stage ovarian cancer detection.

Therefore, ovarian cancer screening is currently only used in patients who are deemed to be at high risk for the disease, including patients with *BRCA1* and *BRCA2* gene mutations [64]. These women are offered annual assessments of CA-125 and ultrasound scanning. The NCI is currently running a prospective study looking at a cohort of women at high-risk of developing ovarian cancer to determine if the rate of change of CA-125 is predictive of disease and whether there are any other serum biomarkers that can be used (www.cancer.gov/clinialtrials). Within the UK, women at high risk of ovarian cancer determined by their family history are eligible for a study offering genetic screening and annual assessments of CA-125 and ultrasound scanning. However, women at high risk of ovarian cancer due to gene mutations count for <5% of the cases of ovarian cancer and at present the screening tests are not sensitive or specific enough to be used for the general population.

1.5 Aims of the Thesis

The aims during my research were to find new biomarkers that could be used in patients with gastrointestinal and ovarian cancers to potentially aid in diagnosis, assessing prognosis and response to therapies. With this in mind a number of both global and target specific approaches were tried:

I. The first part of my research involved a collaboration with Prof Walter Kolch at the Beatson Institute looking at the expression of Raf Kinase Inhibitor Protein (RKIP) using immunohistochemistry in tissue microarrays (TMA) initially in patients with colorectal cancer and later in patients with ovarian cancer. The rationale behind this was previous work suggesting that a reduction or loss of expression of RKIP was

associated with worse clinical outcomes and the aim was to confirm these findings with a much larger cohort of patients. It would be extremely useful to have an additional prognostic biomarker especially in the management of patients with Stage II or Dukes B colorectal cancer, where the disease remains confined to the bowel wall. The role of adjuvant chemotherapy in these patients remains uncertain and so if there was a further marker that would allow greater targeting of treatment to patients who would gain the most benefit this would be very valuable. It was also decided to explore RKIP expression in a bank of TMA from patients with stage Ic to IV ovarian cancer who had participated in various chemotherapy trials, to discover whether loss or reduction of RKIP expression in these patients showed any correlation with clinical outcomes and thus help aid in decisions regarding patient management.

II. The second part involved assessing plasma and serum cytokeratin 18 (CK18) levels in patients with advanced gastrointestinal and ovarian cancers using an enzyme-linked immunosorbent assay (ELISA). The plasma/serum CK18 levels were then compared with healthy volunteers and also correlated with clinical outcome, to see whether CK18 was useful as either a diagnostic or prognostic marker. It was also decided to assess plasma CK18 levels at various time points during palliative chemotherapy in patients with advanced gastrointestinal cancer to determine whether it could be used as a marker of response to treatment, thus potentially reducing toxicity in patients where treatment was having little effect.

III. The third part of my project comprised a more global approach to biomarker discovery and involved comparing serum of patients with advanced oesophageal cancer with serum from healthy volunteers using SELDI-MS. The plan was that if there were differences observed between the serum proteomes, attempts to identify potential biomarkers would be made.

The background to each part of the project and relevant studies are discussed in greater detail within each chapter.

Chapter 2 - Materials & Methods

2.1 Materials

2.1.1 Buffers

Novex® Tricine SDS running buffer (LC1675)	Running Buffer (10x) – to make up 1000ml, add 100ml of Novex® Tricine SDS running buffer to 900ml of DI water.
Novex® Tris-Glycine transfer buffer (LC3675)	Transfer Buffer (25x) – to make up 500ml, add 40ml of Novex® Tris-Glycine transfer buffer to 50ml of methanol and 410ml of DI water.
Novex® Tricine SDS sample buffer	Sample buffer (2X) – to make up 400µl, add 200µl of Novex® Tricine SDS sample buffer to 40µl of NuPAGE® reducing agent and 160µl of DI water. Vortex the sample vigorously, centrifuge briefly and store at -20°C until use
Colloidal Blue Staining Kit (LC6025)	Colloidal Blue Staining Kit (contains Coomassie G-250) – to make up 100ml, add 55ml of DI water to 20ml of methanol, 5ml of stainer B and 20ml of stainer A. Mix well and use straight away.
IMAC Binding Buffer (K200-0002)	0.1M sodium phosphate, 0.5M NaCl pH 7.0
CM Low Stringency Binding Buffer (K200-0003)	0.1M sodium acetate, pH 4.0
U1 buffer	1M urea, 0.2% CHAPS, 50mM Tris-HCl, pH 9.0
U9 buffer	9M urea, 2% CHAPS, 50mM Tris-HCl, pH 9.0

Rehydration buffer	50mM Tris-HCl, pH 9.0
Wash buffer 1	50mM Tris-HCl, 0.1% OGP, pH 9.0
Wash buffer 2	50mM Hepes, 0.1% OGP, pH 7.0
Wash buffer 3	100mM Sodium Acetate, 0.1% OGP, pH 5.0
Wash buffer 4	100mM Sodium Acetate, 0.1% OGP, pH 4.0
Wash buffer 5	50mM Sodium Citrate, 0,1% OGP, pH 3.0
Wash buffer 6	33.3% isopropranol/ 16.7% acetonitrile / 0.1% trifluoracetic acid
Phosphate Buffered Saline (PBS)	137mM Sodium Chloride (NaCl), 8.5mM Di-sodium Hydrogen Phosphate (Na_2HPO_4), 44mM Potassium Chloride (KCl), 1.4mM Sodium Di-hydrogen Phosphate (KH_2PO_4) pH 7.4

2.1.2 Cells

CELL TYPES	DESCRIPTION	GROWTH MEDIUM
A2780/CP70	*In vitro* derived cisplatin resistant epithelial ovarian carcinoma cell line	RPMI 1640, 10% Fetal Calf Serum, and 1% glutamine
HCT-116 p53+/+ and p53-/-	Human colorectal cancer cell line. p53 null cells originate from Vogelstein.	Dulbecco's Modified Eagle's Medium, 10% Fetal Calf Serum, and 1% glutamine. The p53 null cells had geneticin added at a concentration of 0.5 mg/ml

2.1.3 Cell Culture Materials

SUPPLIER	EQUIPMENT
Harlan Sera-Lab Ltd., Crawley Down, UK	Fetal calf serum
Invitrogen Life Technologies Ltd., Paisley, UK	1X Dulbecco's Modified Eagles Medium
	200mM glutamine
	Geneticin, G148 sulphate
	RPMI 1640
	2.5% trypsin
Sigma Chemical Co., Ltd., Poole, Dorset, UK	10X Dulbecco's Modified Eagles Medium

2.1.4 Chemicals, enzymes & kits

SUPPLIER	EQUIPMENT
Invitrogen Life Technologies Ltd., Paisley, UK	Colloidal Blue Staining Kit (LC6025)
	Mark12™ Unstained Standard (LC5677)
	Novex® Tricine SDS running buffer (LC1675)
	Novex® Tris-Glycine transfer buffer (LC3675)
	Sypro Ruby Protein Gel Stain (S12000)
	Sypro Ruby Blot Stain (S11791)
Bio-Rad Laboratories Ltd, Hemel Hempstead, UK	IMAC Binding Buffer (K200-0002)
	CM Low Stringency Binding Buffer (K200-0003)
	U1 buffer (1M urea, 0.2% CHAPS, 50mM Tris-HCl, pH 9)
	U9 buffer (9M, 2% CHAPS, 50mM Tris-HCl, pH 9)
	Rehydration buffer (K100-0007)
	EAM SPA (C300-0002)
	Wash buffer 1-6 (K100-0007)
	All-in-1 Peptide Standard (C100-0005)
Fischer Scientific UK Ltd., Whitbrook Way, UK	Acetic acid (Analytical grade)
	Ethanol (Analytical grade)
	Methanol (Analytical grade)
Perbio Science UK Ltd., Unit 9, Atley Way, UK	No-weigh™ Dithiothreitol (DTT), 7.7mg DTT tubes (20291)

Peviva AB, Stromkarlsvagen 82, Sweden	M30-Apoptosense® ELISA (10010)
	M65 ELISA (10020)
Sigma-Aldrich Co Ltd., The Old Brickyard, UK	Acetonitrile (99.5%)
	Cisplatinum (II) Diammine Dichloride (1g)
	NP-40
	Sodium Dodecyl Sulphate (SDS)
	Trifluoroacetic acid (99+%)
	Urea

2.1.5 Equipment and Plasticware

SUPPLIER	EQUIPMENT
Abgene House, Blenheim Road, Epsom, Surrey, UK	Adhesive plate seals (AB-0580)
Becton Dickinson Labware, Plymouth, UK	Falcon 1059 polypropylene tubes
	Falcon 2059 polypropylene tubes
	Falcon 2098 polypropylene tubes
	Sterile plastic pack syringe needles
	18 gauge sterile syringe needles
Beatson Laboratory Technological Services Dept.	Custom-made semi-dry Western Blot System
Bibby Sterilin Ltd., Stone, Staffs, UK	60, 90, 150mm bacteriological Petri dishes
	Sterile plastic universal containers
Bio-Rad Laboratories Ltd, Hemel Hempstead, UK	PBS II ProteinChip Reader.
	96-well Bioprocessor (C503-0011)

	96-well Bioprocessor reservoir & gaskets (C5030012).
	CM10 ProteinChip array (C573-0075)
	IMAC30 ProteinChip array (C573-0078)
	NP20 ProteinChip array (C573-0043)
	ProteinChip Serum Fractionation Kit (K100-0007)
Costar Corporation, High Wycombe, Bucks, UK	24 well tissue culture plates
	96 well tissue culture plates
Edmund Buhler GmbH, Am Ettenbach 6, 72379 Hechingen, Germany	Microplate shaker Timix 2
Eppendorf AG, 22331 Hamburg, Germany	Eppendorf microcentrifuge tubes, 1.5ml (0030125.150)
	Centrifuge 5415R
Euro-DPC Ltd., Glyn Rhonwy, Wales, UK	DPC Micromix 5
DJB Labcare Ltd., 20 Howard Way, Buckinghamshire, UK	Jouan Centrifuge CR422
Jencons (Scientific) Ltd., Cherrycourt Way Ind Est, UK	Greiner 96-well, v-bottom, microplate
	Greiner Bio-one Vacuette® EDTA tubes
Millipore, 3/5, The Courtyard, UK	Microcon® Centrifugal Filter Devices, YM-10 (42421)
	Millipore Vacuum pump
	Millipore manifold basic kit
	Immobilon-P Transfer Membrane - PVDF membrane, 0.45µm pore size (IPVH00010)
	Immobilon PSQ Transfer Membrane – PVDF membrane 0.2µm pore size (ISEQ08100)

Invitrogen Life Technologies Ltd., Paisley, UK	Novex® 16% Tricine Gels (EC6695)
	Xcell*Surelock*™ Mini-Cell
Molecular Devices Ltd, 660-665 Eskdale Road, UK	Microplate reader, Spectromax plus 384
Olympus UK Ltd., 19 Colonial Way, Watford, UK	Light microscope BX40
Perbio Science UK Ltd., Unit 9, Atley Way, UK	Western Blotting Filter Paper (88600)
Syngene (UK), Beacon House, Nuffield Road, Cambridge,	Gene Genius Bioimaging System

2.1.6 Other materials

SUPPLIER	EQUIPMENT
Beatson Institute Central Services	Sterile distilled water
	Sterile phosphate –buffered saline (PBS)
Premier Brands, Adbaston, Stafford, UK	Marvel (dried skimmed milk)

2.1.7 ELISA Kit Details

2.1.7.1 M30-Apoptosense® ELISA

M30 coated microstrips (columns of 96-well format)

M30 HRP Conjugate (24x) – vial contains 0.4ml mouse monoclonal M30 antibody (anti-CK18Asp396 neo-epitope) conjugated with horseradish peroxidase (HRP) in phosphate buffer with protein stabilizers. This should be diluted with M30 Conjugate Dilution Buffer.

M30 Conjugate Dilution Buffer – vial contains 12ml of phosphate buffer with protein stabilisers for dilution of M30 HRP conjugate.

M30 Standards A to G – Standard A contains 4ml of phosphate buffered FCS. Standards B to G, 0.5ml each, contain standard material in FCS. The values of the standards A to G are 0, 75, 150, 250, 500, 750 and 1000 U/L, respectively.

M30 Control Low and High Expression – Two 0.5ml vials containing reactive components in phosphate buffered FCS. The values of the controls Low and High are 125+/-25 U/L and 650+/-100 U/L, respectively.

TMB Substrate – bottle contains 22ml of TMB (3,3'5,5'-Tetramethylbenzidine) solution.

Stop Solution – vial contains 8ml of 1.0 M sulphuric acid.

Wash Solution (10x) – vial contains 50ml of concentrated wash solution. Dilute with 450ml of DI water. Diluted buffer consists of 0.014 M phosphate buffer with 0.15 M sodium chloride and 0.1% Tween® 20

2.1.7.2 M65 ELISA

M65 coated microstrips (columns of 96-well format)

M65 HRP Conjugate (24x) – vial contains 0.4ml mouse monoclonal M5 antibody (anti-CK18) conjugated with horseradish peroxidase (HRP) in phosphate buffer with protein stabilizers. This should be diluted with M65 Conjugate Dilution Buffer.

M65 Conjugate Dilution Buffer – vial contains 12ml of phosphate buffer with protein stabilisers for dilution of M65 HRP conjugate.

M65 Standards A to G – Standard A contains 4ml of phosphate buffered FCS. Standards B to G, 0.5ml each, contain standard material in FCS. The values of the standards A to G are 0, 125, 250, 500, 750 1200 and 2000 U/L, respectively.

M65 Control Low and High Expression – Two 0.5ml vials containing reactive components in phosphate buffered FCS. The values of the controls Low and High are 200+/-20 U/L and 1000+/-100 U/L, respectively

TMB Substrate – bottle contains 22ml of TMB (3,3'5,5'-Tetramethylbenzidine) solution.

Stop Solution – vial contains 8ml of 1.0 M sulphuric acid.

Wash Solution (10x) – vial contains 50ml of concentrated wash solution. Dilute with 450ml of DI water. Diluted buffer consists of 0.014 M phosphate buffer with 0.15 M sodium chloride and 0.1% Tween® 20.

2.2 Methods

2.2.1 RAF Kinase Inhibitor Protein

To study RKIP protein expression, polyclonal rabbit RKIP (ki69) [65] was applied to formalin-fixed, paraffin-embedded sections. The RKIP antibody was raised against full-length rat RKIP protein which was expressed in *E.coli*. Sections from liver and breast paraffin blocks were used as a positive control. In addition, Auerbach's myenteric intramuscular plexuses, which have strong RKIP expression, were used as a positive internal control. The scoring system was the same as that used for the previous study [66] and corresponded to the sum of a) the percentage of positive cells (1 = <25%, 2 = 26-50%, 3 = >50%) and b) the staining intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong). The sum for each section was, therefore, anything between 0 and 6. Scores between 0 and 2 were regarded as negative, scores of 3 and 4 as weakly positive, and scores of 5 and 6 as strongly positive. Two scorers blinded to the follow-up data and recurrence status of the patients assessed each section independently.

The scores were then analysed with the clinical outcome data for the patients. Follow-up to event outcomes were analysed by Kaplan-Meier survival curves and compared by log-rank tests. My role in this collaboration was as an independent scorer of RKIP expression within the colorectal and ovarian cancer TMA, and as such, was blinded to the clinical outcome data for both patient groups.

2.2.1.1 Tissue microarray construction

Tissue microarrays were constructed as described in previous studies [67, 68]. In summary, paraffin-embedded tumour tissue blocks and matching haematoxylin-eosin (H&E)-stained slides were retrieved from the pathology archives and representative areas of tumour were marked on each H&E-stained slide. Four cores of 0.6mm^2 were taken from each donor block and arrayed on a recipient paraffin block using a precision instrument (Tissue Arrayer, Beecher Instruments, Silver Spring, MD, USA). Using a microtome, 5μm sections were cut from each TMA block and applied to aminopropyltriethoxysilane (APES)-treated slides. All sections were stained within 2 weeks of sectioning. The presence of tumour tissue on the arrayed samples was verified on an H&E-stained section.

2.2.1.2 Immunohistochemistry (IHC)

The formalin-fixed, paraffin-embedded tissue microarrays were deparaffinised in three changes of xylene, and rehydrated through graded alcohols to distilled water. RKIP protein expression was examined as described previously [69, 70] using a 1:1500 dilution of a polyclonal rabbit antibody raised against a recombinant full-length RKIP protein, for 1hr at room temperature. IHC was performed using rabbit polyclonal antibodies to p21 (Santa Cruz, 1:200, 1hr) and carbonyl reductase (1:750, overnight at 4°C, kind gift of Dr Umemoto) and mouse monoclonals to PCNA (1:10,000, cat no 2586, Cell Signaling) and TIGAR (in-house antibody, 1:200, 1hr). Antigen retrieval was performed using 0.01mol/L citrate buffer, pH 6.0 (p21, PCNA and TIGAR) or EDTA (RKIP), at 100°C for 5 minutes, followed by cooling on ice for 20min. No antigen retrieval was performed for carbonyl reductase sections. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20min and non-specific staining was blocked by incubation of sections in 10% normal goat or horse serum (Vector). Antibody binding was detected using the streptavidin-biotin method (Avidin-Biotin Complex Elite Detection kit, Vector, Peterborough, United Kingdom) and 3,3-diaminobenzidine (DAB) as chromogen (DAB peroxidase substrate kit, Vector). Slides were then counterstained in Mayer's hematoxylin. Omission of the primary antibody served as negative control. Pre-incubation of TIGAR antibody with excess peptides (raised to two regions of TIGAR), for 1hr before application to sections, served as a negative control for this protein.

2.2.2 Serum collection

2.2.2.1. Healthy volunteer samples

Subjects were eligible for this part of the study if they had no known history of metastatic disease, or any other clinically significant acute or chronic illness. A short health questionnaire was completed. Blood samples (20ml) were collected into tubes containing EDTA on one occasion from 100 healthy volunteers, between November 2004 and February 2005. Serum and plasma was separated as detailed below and stored at -70ºC. All subjects were allocated an anonymous trial number and no personal details were on the samples despatched for analysis. This study was approved by the West Glasgow Hospitals Research Ethics Committee, and all volunteers gave written informed consent.

2.2.2.2. Patient samples

Patients with a histologically or cytologically confirmed locally advanced or metastatic cancer, either colorectal adenocarcinoma receiving a fluoropyrimidine-based therapy or an adenocarcinoma of the oesophagus or stomach receiving a fluoropyrimidine and platinum-containing regimen, were eligible. All patients gave written informed consent. Twenty ml of blood were collected into tubes containing EDTA before starting chemotherapy and then prior to administration of each subsequent course of chemotherapy until discontinuation of systemic therapy. Additional 20ml samples were collected at various time-points during chemotherapy courses in selected patients, with the time-points dependent on the specific chemotherapy regimen. All samples were stored according to the trial number with no personal details. Clinical outcome data was recorded for each patient.

2.2.2.3. Colonoscopy and pre-operative serum samples

Blood samples were collected from patients undergoing colonoscopy. All patients gave written informed consent. Blood samples (20ml) were collected into tubes containing EDTA on one occasion. All subjects were allocated an anonymous trial number and no personal details were on the samples despatched for analysis. Clinical outcome data from the colonoscopy was recorded for each patient and they were categorised as, normal colonoscopy, pre-malignant adenomas and adenocarcinoma.

Blood samples were also collected from patients with operable colorectal adenocarcinoma who gave written informed consent. Blood samples (20ml) were collected pre-operatively into tubes containing EDTA on one occasion. Clinical outcome data was recorded for each patient.

2.2.2.4. Sample preparation

Plasma was separated within 2 hours of collection by centrifuging the whole blood sample at 1500g for 10min at 20°C. The supernatant was then removed, placed in a separate 15ml falcon tube and centrifuged again using the above conditions. The resulting supernatant was then aliquoted into Eppendorf tubes and immediately frozen at -70°C until analysis

2.2.3 Serum Fractionation Technique

The basic fractionation protocol used here was developed by Ciphergen (Biorad/Vermillion). The fractionation took place at room temperature. Samples were incubated on a MicroMix at a vigorous setting (amplitude 17, wave 7) unless otherwise stated. A Millipore vacuum pump was used to apply a pressure of 15mmHg.

2.2.3.1. Serum Sample Preparation

Each serum sample was centrifuged at 16 000g for 10min at 4°C. Twenty µl was aliquoted from each sample into a separate well in a v-bottom 96-well microplate (a v-bottom 96-well microplate was used as this could be easily incubated on the MicroMix). Thirty µl of U9 was added to each well and the microplate was incubated on the MicroMix for 20min.

2.2.3.2 Rehydration and equilibration of the Q HyperD F beads

Prior to commencing fractionation the Q HyperD F beads were rehydrated and then equilibrated with solution U1. This was done by adding 200µl of rehydration buffer to each well and incubating the filtration plate on the MicroMix for 60min. The filtration plate was placed on top of a waste collection plate and a vacuum was applied for 10min until all the supernatant was removed. Two hundred µl of rehydration buffer was added to each well and a vacuum was applied for 10min until the supernatant was removed. This procedure was repeated three times with the rehydration buffer and three times with U1 solution.

2.2.3.3. Fractionation

Fifty µl of each sample was taken from the v-bottom microplate and added into the corresponding well on the filtration plate. Fifty µl of U1 solution was added to each well in the v-bottom microplate and mixed 5 times to ensure that the entire sample had been removed. This solution was then added into the corresponding well on the filtration plate so that it contained 100µl of sample. The filtration plate was covered with adhesive sealing film and incubated on the MicroMix for 45min. The filtration plate was placed under a 96-well microplate which was labelled Fraction 1. A vacuum was applied for 1min until all the supernatant had been collected into the microplate. This process was repeated with the five other wash buffers with each supernatant collected into a fresh microplate.

2.2.4 Protein Array Preparation

The ProteinChip arrays were prepared using protocols developed by Ciphergen which are shown below. All chips were incubated at room temperature on the Micromix, which was set to shake vigorously (settings of amplitude 20 and wave 7).

2.2.4.1 EAM Preparation

Two hundred µl of 100% acetonitrile and 200µl of 1% TFA were added to 5mg of SPA. The mixture was then vortexed vigorously and left for 5min at room temperature. It was then centrifuged at 20 000g for 2min to pellet any particulate matter.

2.2.4.2 Preparation of the All-in-1 Peptide Standard Mix

The vial containing the standard was removed from the -20°C freezer and allowed to warm to room temperature. Twenty-five µl of resuspension solution (10 mM ammonium acetate, 25% acetonitrile, 1.25% trifluoroacetic acid) was added to the vial, flick mixed and the vortexed vigorously. A further 25µl of resuspension solution was added to the vial and the mixing steps repeated. The vial was then allowed to stand at room temperature for 10min, following which the resulting solution was divided into single use aliquots (5-10µl) in Eppendorf tubes and stored at -20°C until use.

Composition of the All-in-1 Peptide Standard

PEPTIDE	AVERAGE MOLECULAR WEIGHT (Da)	MONOISOTOPIC MOLECULAR WEIGHT (Da)
Arg8-Vasopressin	1084.25	1083.44
Somatostatin	1637.90	1636.71
Dynorphin (porcine)	2147.50	2146.19
ACTH (1-24) (human)	2933.50	2931.58
Bovine insulin β-chain	3495.94	3493.64
Human insulin	5807.65	5803.64
Hirudin, recombinant	6963.52	6958.96

2.2.4.3 Preparation of the EAM/All-in-1 Peptide Standard Mix

One hundred µl of 100% acetonitrile and 100µl of 1% TFA were added to 5mg of SPA. The mixture was vortexed vigorously and then centrifuged at 16 000g for 2min to pellet any particulate matter. 10µl of the EAM solution was pipetted into an Eppendorf tube along with 10µl of the resuspended All-in-1 Peptide mix. This was then flick mixed and centrifuged at 16 000g for 2min.

2.2.4.4 IMAC-30 ProteinChip Preparation

The ProteinChip arrays were placed in the Bioprocessor. Fifty µl of 0.1M $CuSO_4$ solution was added to each well and the solution was incubated on the Micromix for 10min. The supernatant was then removed and 200µl of DI water added to each well. This was then incubated for 1min on the Micromix. The supernatant was removed and 200µl of 0.1M sodium acetate buffer pH 4 was added to each well. This was then incubated on the Micromix for 5min. The supernatant was removed and 200µl of DI water was added to

each well. This was incubated on the Micromix for 1min. The supernatant was removed and 200µl of IMAC binding buffer was added to each well. This was incubated twice on the Micromix for 5min. The supernatant was removed from the wells and 90µl of IMAC binding buffer and 10µl of sample were added to each well. The Bioprocessor was covered with adhesive sealing film to avoid cross-contamination of the samples and incubated on the Micromix for 45min. The supernatant was removed from the wells. Each well was washed three times with 200µl of IMAC binding buffer and incubated on the Micromix for 5min. The supernatant was removed from the wells and 200µl of DI water was added twice to each well and removed immediately. The ProteinChip arrays were removed from the Bioprocessor and air dried for 15min. One µl of SPA was added to each spot and air dried for 5min. This procedure was repeated once.

2.2.4.5 CM-10 ProteinChip Preparation

The ProteinChip array was placed in the Bioprocessor. Two hundred µl of CM-10 low stringency buffer was added to each well and the solution was incubated on the Micromix for 5min. This step was repeated once. The supernatant was removed from the wells and 90µl of CM-10 low stringency buffer and 10µl of sample were added to each well. The Bioprocessor was covered with adhesive sealing film to avoid cross-contamination of the samples, and incubated on the Micromix for 45 min. Two hundred µl of CM-10 low stringency buffer was added to each well and the solution was incubated on the Micromix for 5min. This step was repeated twice. The supernatant was removed from the wells and 200µl of DI water was added to each well and then remove immediately. The ProteinChip array was removed from the Bioprocessor and air dried for 10min. 1µl of EAM was added to each spot and air dried for 5min. This procedure was repeated once.

2.2.4.6 NP20 ProteinChip Preparation for Calibration using the All-in-1 Peptide Standard Mix

The ProteinChip Array was placed in the Bioprocessor and 3µl of DI water was added to each well. The water was then blotted off using a clean laboratory wipe, being careful not to touch the actual spot area. One µl of the EAM/All-in-1 Peptide Standard was pipetted onto each pre-wet spot. The ProteinChip array was removed from the Bioprocessor and air dried for 10min.

2.2.5 Analysis of Protein Arrays

The Protein arrays were then placed in a ProteinChip Reader Model PBS II and were read using ProteinChip Software (Version 3.1).

2.2.5.1 Data Capture

Data were collected focusing on low molecular weight peptides/proteins. For the IMAC-30 ProteinChip array the mass was optimised between 1,000 to 30,000Da, with a high mass of 150,000Da. The laser intensity was set at 225 and the sensitivity at 10. For the CM-10 ProteinChip array, the mass was again optimised between 1,000 to 30,000Da, with a high mass of 150,000Da. The laser intensity was set at 240 and the sensitivity at 10.

2.2.5.2 Data Preprocessing

Ciphergen ProteinChip software (version 2.0) was used for data preprocessing. Baseline subtraction was performed, followed by normalization of traces for intensity, using the instrument-dedicated software. Peaks were then detected using the built-in peak detection algorithm, setting the signal to noise ratio to five. Peaks below 2000Da were excluded as these are often due to noise from the energy absorbing matrix [71-73]. Data were exported to Microsoft® Excel® for further analysis.

2.2.6 1-D Gel Analysis on Novex® 16% Tricine gels

2.2.6.1 Serum Sample Preparation

Fraction 1 (pH 9.0) from the serum samples used for the SELDI-MS experiments was examined on the 1-D gels (Novex® 16% Tricine gels). To prepare the samples for running on the gel, the fractionation samples were thawed on ice. Thirty µl of thawed sample was added to 30µl of 2X Novex® Tricine SDS sample buffer. The mixture was then vortexed vigorously, centrifuged briefly and placed on a heating block at 85°C for 2min. The sample was then centrifuged again briefly and either run on a gel immediately or frozen at -20°C until use.

2.2.6.2 Centrifugal Filtration of Fractionated Serum Samples

Fraction 1 (pH 9.0) from the serum samples was added to the centrifugal filter (molecular weight cut-off 10kDa) at varying volumes between 20-100µl. The samples were initially spun without using denaturing agents or detergents, but these were subsequently added at various concentrations as discussed in the text. Denaturing agents used included 10% acetonitrile, 100mM DTT, 2-8M urea, 1% NP40 and 0.5% SDS. The samples were then spun at 4°C, 14 000g for between 30-60min. The filtrate was then removed and placed in an Eppendorf tube with 2X sample buffer. This was then centrifuged briefly and placed on a heating block at 85°C for 2min. The sample was then centrifuged again briefly and either run on a gel immediately or frozen at -20°C until use. The retentate was collected by inverting the reservoir in the vial and spinning in the centrifuge at 4°C, 1000g for 3min. Thirty µl of 2X sample buffer was then added to the retentate. This was then centrifuged briefly and placed on a heating block at 85°C for 2min. The sample was then centrifuged again briefly and either run on a gel immediately or frozen at -20°C until use.

DENATURING AGENT	MECHANISM OF ACTION
10% Acetonitrile	Cleavage of salt bridges
100mM Dithiothreitol (DTT)	Disruption of disulphide bonds
2-8M Urea	Destabilisation of hydrogen bonding and hydrophobic interactions
1% NP-40	Non-ionic detergent-disrupts hydrophobic bonds
0.5% Sodium docedyl sulphate (SDS)	Anionic detergent-disrupts hydrophobic bonds and coats with negative charge

2.2.6.3 Passivation Process to Improve Recovery during Centrifugal Filtration

This involved pre-wetting of the centrifugal filters using solutions to prevent binding of target peptides to the filter membrane in an attempt to improve recovery. The solutions selected, following consultation with the manufacturer's technical support department, were 1% BSA and 5% Tween-20. The filters were inserted into the filtrate vials and the reservoirs were filled with the passivation solution. The cap was replaced on the vial and it was left to soak overnight at room temperature. The cap was then removed and the device and reservoir washed thoroughly with DI water. The filter was then placed back in the vial, filled with DI water and spun in a centrifuge at 25°C, 14 000g for 15min. The DI water was then discarded and the washing step repeated. To remove the remaining water, the reservoir was inverted in the vial and spun once at 1000g for 3min. The filter was then ready for use. If the device was not to be used immediately, it was filled with DI water, capped and refrigerated at 4°C until use.

2.2.6.4 Precipitation Protocol

This procedure was performed to increase the protein concentration prior to loading the protein sample on the gel. One hundred µl of fraction 1 (pH 9.0) from the serum samples was added to 9 volumes (1800µl) of ice cold ethanol in Eppendorf tubes. These were then incubated at -20°C overnight to precipitate the protein. The samples were then spun at 4°C, 16 000g for 15min and the supernatant removed. The short spin was then repeated and the remaining supernatant removed using a capillary tip. Five hundred µl of 70% ethanol was then added to the sample to solubilise any remaining salts and the sample vortexed vigorously until the pellet was in solution. The Eppendorf tube was then spun again 4°C, 16 000g for 5min and the supernatant removed. The short spin was then repeated and the remaining supernatant removed using a capillary tip. The Eppendorf tube was then inverted and allowed to air-dry for 5min. Freshly-prepared 2X sample buffer was then added to the Eppendorf tube and the mixture was vortexed vigorously to resuspend the pellet. This was then centrifuged briefly and placed on a heating block at 85°C for 2min. The sample was then centrifuged again briefly and either run on a gel immediately or frozen at -20°C until use.

2.2.6.5 Gel running conditions

Fifteen µl of protein sample was loaded into the appropriate wells of the 16% Tricine gel along with 5µl of unstained mark12 standard. The upper buffer chamber of the XCell *Surelock*™ MiniCell was filled with 200ml and the lower buffer chamber with 600ml of Tricine SDS running buffer. The gel was then run at the 124V for 90 min at room temperature.

Mark 12™ Standard molecular weights using Tricine gels

PROTEIN	APPROXIMATE MOLECULAR WEIGHT (kDa)
Myosin	200
β-galactosidase	116.3
Phosphorylase B	97.4
BSA	66.3
Glutamic dehydrogenase	55.4
Lactate dehydrogenase	36.5
Carbonic anhydrase	31.0
Trypsin inhibitor	21.5
Lysozyme	14.4
Aprotinin	6.0
Insulin B chain	3.5
Insulin A chain	2.5

2.2.6.6 Gel staining

The gels were removed from the holder and stained using the Colloidal Blue Staining Kit. The gel was shaken in 100ml of staining solution for a minimum of 3hr and a maximum of

12hr. The staining solution was then decanted and replaced with 200ml of DI water and the gel washed for at least 7hr. The gels were then stored in 10% acetic acid.

2.2.7 Western Blotting of Novex® 16% Tricine gels

Prior to the transfer process, the membranes were placed in 100% methanol for 1-3 seconds and then immersed in DI water for 1-2min to displace the methanol. They were then equilibrated in transfer buffer for between 15-30min (gloves and forceps were used when handling the membrane to reduce non-specific protein binding and scratches).

After electrophoresis of the gel, it was also equilibrated in a small container of transfer buffer for 5-10min, to remove the electrophoresis salts and buffers. Once both the gel and membrane were equilibrated, two pieces of filter paper were soaked in the transfer buffer and placed on the anode. A pipette was then rolled over the surface of the paper to exclude all air bubbles. The pre-wetted membrane was then placed on top of the wetted filter paper and the air bubbles rolled out again. The equilibrated gel was then placed on top of the membrane. Two further pieces of filter paper were then soaked in transfer buffer and placed on top of the gel and the air bubbles rolled out again. The cathode plate was then carefully placed on the stack and the transfer unit run at 100-200mA for 1-2hr at room temperature.

The transfer efficiency was then checked by placing the membrane in a small container of coomassie blue stain for 1min and then destaining with 40% methanol and 10% acetic acid. Alternatively, the membrane was stained with Sypro Ruby Blot Stain by allowing the membrane to completely air dry post-transfer. It was then placed in 7% acetic acid and 10% methanol for 15min. This was followed by four 5min washes of DI water. The membrane was then incubated in 50ml of Sypro Ruby Blot Stain for 15min and then washed three times for 1min in DI water. The Sypro Ruby staining was visualised using a transilluminator. The gel was also stained after transfer using coomassie blue to check for quantities of protein remaining.

2.2.8 Mass Spectrometry Analysis of Excised Gel Bands

2.2.8.1 Digestion

The gel pieces were excised and dehydrated with acetonitrile for ~10 min. A volume of 10 mM dithiotreitol (DTT) in 50mM ammonium bicarbonate sufficient to cover the gel pieces was added, and the proteins were reduced for 20 min at 56 °C. After cooling to room temperature, the DTT solution was replaced with an equal volume of 55mM iodoacetamide in 50mM ammonium bicarbonate. After 60min incubation at room temperature in the dark with occasional vortexing, the gel pieces were washed by dehydrating with acetonitrile for ~10min and rehydrating in 50mM ammonium bicarbonate / 5% acetonitrile. This wash cycle was repeated before the gel pieces were dehydrated again with acetonitrile for ~10min. To ensure complete dehydration the gel pieces were dried using a vacuum centrifuge for 30min. The dry gel pieces were partially re-swollen on ice, using 0.07µg/µL of sequencing grade trypsin. After 15min, the gel pieces were then covered with 50mM ammonium bicarbonate, 5% acetonitrile and digested at 37 °C for 1 - 2hr. Once the digest was complete, the supernatant was removed and stored. To elute the peptides from the gel pieces, they were washed twice for 20min at room temperature. Once, with 1 % formic acid and secondly with 80% acetonitrile / 0.1% formic acid, removing the supernatant after each wash. The three supernatants were combined and evaporated using a vacuum centrifuge to complete dryness. The samples were then desalted with the addition of 10uL of water and evaporated again to dryness.

2.2.8.2 LC-MS/MS

For the analysis of the unknown ~ 4 kDa peptide, HPLC chromatographic separations of the protein digests were carried out, using an Ultimate 3000 capillary LC system (Dionex) interfaced to a QSTAR XL hybrid quadrupole time of flight tandem mass spectrometer (Applied Biosystems), fitted with a microion spray source mounted on a nanospray stage. Chromatography was preformed using a nano trap column (C18, 5µm, 100 Å, 300µm i.d. x 5mm) (Dionex) and a 75µm i.d. x 15cm, PepMap (C18, 3µm, 100 Å) column (Dionex). Five µL of sample solution was loaded onto the column and eluted using a two stage gradient at a flow rate of 300nL min^{-1} with a column temperature of 30 ºC. The first stage consisted of a gradient from 95 % mobile phase A (98 % water, 2 % acetonitrile and 0.1 % formic acid) to 75 % mobile phase A and 25 % mobile phase B (80 % acetonitrile, 20 % water, 0.1 % formic acid) in 33min followed by a second stage increasing from 25 % mobile phase B to 50 % mobile phase B (50 % mobile phase A) in 10min. After the

gradient, the column was washed with 100 % mobile phase B for 6.5min and regenerated with 95 % A for 10min. During the online analysis, ions were chosen for product ion fragmentation using "Data Dependent Analysis" (DDA) through the Analyst 1.1 software, three MS/MS scans of 3 s duration were recorded per 2.5 s MS survey scan, recorded over the mass range m/z 400 – 1200. Ions were successfully chosen for fragmentation if their charge state lay between 2 to 4 and their abundance exceed 5 counts in the MS survey scan. Precursor masses were excluded using dynamic exclusion for 30s after fragmentation to limit reanalysis of identical peptides for one acquisition. For the other acquisition, three MS/MS scans of 2s duration were recorded per 1.5s MS survey scan, recorded over the mass range *m/z* 400 - 1200. Ions were successfully chosen for fragmentation if their charge state lay between 2 to 4 and their abundance exceed 5 counts in the MS survey scan. Precursor masses were excluded using dynamic exclusion for 60s after fragmentation to limit reanalysis of identical peptide.

For the analysis of the ~ 17kDa unknown protein/peptide, HPLC chromatographic separations of the protein digests were carried out, using an Ultimate 3000 capillary LC system (Dionex) interfaced to a 4000 QTRAP linear ion trap quadrupole LC/MS/MS Mass Spectrometer (Applied Biosystems) fitted with a microion spray source mounted on a nanospray stage. Chromatography was performed using direct injection onto a 75µm i.d. x 15cm, PepMap (C_{18}, 3µm, 100 Å) column (Dionex). Five µL of sample solution was loaded onto the column and eluted using a two stage gradient at a flow rate of 300nL min^{-1} with a column temperature of 30 ºC. The first stage consisted of a gradient from 95 % mobile phase A (98 % water, 2 % acetonitrile and 0.1 % formic acid) to 75 % mobile phase A and 25 % mobile phase B (80 % acetonitrile, 20 % water, 0.1 % formic acid) in 33min followed by a second stage increasing from 25 % mobile phase B to 50 % mobile phase B (50 % mobile phase A) in 10min. After the gradient, the column was washed with 100 % mobile phase B for 6.5min and regenerated with 95 % A for 30min. During the online analysis, ions were chosen for product ion fragmentation using "Data Dependent Analysis" (DDA) through the Analyst 1.4.1 software; two enhanced MS/MS scans were recorded (scan rate 4000 amu/s) per enhanced MS survey scan (mass range *m/z* 450 – 1200, scan rate 4000 amu/s). Ions were successfully chosen for fragmentation if their charge state lay between 2 to 3 and their abundance exceed 150000 counts in the MS survey scan. An enhanced resolution scan (scan rate 250 amu/s) was used to confirm the charge state of precursor ions.

2.2.8.3 Database searching

The MS/MS data was extracted from each LC-MS run using the built in Mascot™ (matrix science) script in the Analyst 1.1 software. Each MS/MS spectrum was centroided and deisotoped prior to analysis. Spectra were only rejected if less than 10 peaks were found.

Each extracted LC-MS run was submitted to an in-house Mascot™ MS/MS ion database search reporting all significant hits from the Sprot database (SwissProt, Release October 18, 2007)) compiling 285335 sequences and 104773129 residues) with the enzyme specificity set to trypsin/P, allowing for up to 2 missed cleavage. One fixed modification, Carbamidomethyl (C) and three possible variable modifications N-Acetyl (Protein), Oxidation (M), and Deamidation (NQ) were used. The peptide tolerance was set to 60ppm and a fragment mass tolerance of ± 0.1Da was also applied. The instrument type was set to Q-TOF.

For the analysis of the ~17kDa unknown peptide, the MS/MS data was extracted from each LC-MS run using the built in Mascot™ (matrix science) script in the Analyst 1.4.1 software. Each MS/MS spectrum was centroided and de-isotoped prior to analysis. Spectra were only rejected if less than 10 peaks were found.

Each extracted LC-MS run was submitted to an in-house Mascot™ MS/MS ion database search reporting all significant hits, from the Sprot database (SwissProt, Release October 18, 2007)) compiling 285335 sequences and 104773129 residues) with the enzyme specificity set to trypsin/P, allowing for up to 2 missed cleavage. One fixed modification, Carbamidomethyl (C) and four possible variable modifications N-Acetyl (Protein), Oxidation (M), Pyro-glu (N-term Q) and Deamidation (NQ) were used. The peptide tolerance was set to ± 0.6Da and a fragment mass tolerance of ± 0.6Da was also applied. The instrument type was set to ESI-TRAP

2.2.9 M30 and M65 ELISA for Cytokeratin 18 Assessment

2.2.9.1 M30-Apoptosense™ ELISA

M30-Apoptosense™ ELISA is a commercially available kit. The wells are coated with a mouse monoclonal catcher antibody that binds to an epitope on CK18. 25µl of sample are added to each well, followed by 75µl of HRP-conjugated monoclonal antibody (M30) solution, which acts as the detecting antibody by binding to the caspase cleavage site at the N-terminus of the Asp396 fragment of CK18. The samples are then incubated for 4 hours at room temperature with constant shaking (oscillation ~ 600 rpm), after which excess unbound conjugate is removed by 5 washing steps. Colour development is then achieved by the addition of 200µl of 3, 3, 5, 5'-tetramethyl-benzidine solution, followed by incubation for 20 minutes in the dark. The reaction is stopped by the addition of 50µl of 1.0M sulphuric acid and the absorbance measured in a microplate reader at 450nm. Through plotting a standard curve of known concentrations of M30 antigen (standards supplied in the kit) vs. absorbance, the amount of antigen in the controls and unknown samples can be calculated by extrapolation. The units of the M30-ELISA are defined using a synthetic peptide corresponding to the CK18 caspase cleavage site (M30 epitope) where 1 unit equals 1.24pmol of a synthesised peptide containing the M30 recognition motif according to the manufacturer.

2.2.9.2 The M65 ELISA

The M65 ELISA is another commercially available kit. Samples are reacted with the mouse monoclonal antibody "M6" against CK18, which has been immobilised to the polystyrene wells and, simultaneously, with the Horseradish Peroxidase -(HRP) conjugated monoclonal antibody "M5" directed against a different epitope on CK18. 25µl of sample are added to each well, followed by 75µl of HRP-conjugated monoclonal antibody solution. The samples are then incubated for 4hr at room temperature with constant shaking, after which excess unbound conjugate is removed by 5 washing steps. Colour development is then achieved by the addition of 200µl of 3, 3, 5, 5'-tetramethyl-benzidine solution, followed by incubation for 20min in the dark. The reaction is stopped by the addition of 50µl of 1.0M sulphuric acid and the absorbance measured in a microplate reader at 450nm. Through plotting a standard curve of known concentrations of M65 antigen (standards supplied in the kit) vs. absorbance, the amount of antigen in the controls and unknown samples can be calculated by extrapolation.

The concentration of both M30 and M65 is expressed as Units per Liter (U/L).

2.2.9.3 Cell line studies with M30-Apoptosense and M65 ELISA

An aseptic technique was maintained throughout using sterilised material in a class II microbiological safety cabinet with vertical airflow. The cell lines selected were HCT-116 colon carcinoma (p53+/+ wt), HCT-116 (p53-/-) and A2780/CP70 ovarian carcinoma cells. These particular cell lines were selected as the main aim of this part of the project was to examine cytokeratin 18 levels in plasma/serum from patients with colorectal and ovarian cancers, and so the cell line experiments were carried out as a proof of principle. The p53+/+ and p53-/- were selected to see if differences could be detected in the amount of apoptosis occurring as it would be expected that the p53-/- cells would show less apoptosis using these particular ELISAs. The HCT-116 cells were grown in DMEM, supplemented with 10% FCS and 1% glutamine. The null cells had geneticin added at a concentration of 0.5 mg/ml. The A2780/CP70 cells were grown in RPMI 1640, supplemented with 10% FCS and 1% glutamine. All three cell lines were maintained at 37ºC in 5% CO_2. On the day before addition of cisplatin, the cells were plated out at densities of 1.25 $x10^4$ and 2.5 $x10^4$ cells per well in 24-well plates containing 200µl of medium. The following day the cells received fresh medium containing various concentrations of cisplatin of 0, 1, 5, 25 µM. The cell cultures and medium were then harvested after 24 and 48hr and examined.

2.2.9.4 Total cell cultures

To allow assessment of M30 from the total cell cultures, 10 µL of 10% NP40 lysis buffer was added to each well. The plate was then placed on a rotary shaker and lysis was allowed to occur for 5min at room temperature. Duplicate 25µL samples of lysed cells and medium were then transferred to an M30-Apoptosense plate and M30 concentrations determined.

2.2.8.5 Cell culture supernatants

To allow assessment of both M30 and M65 from cell culture supernatants, the plates containing the cell cultures and medium were centrifuged at 800g for 5min at 18ºC. One hundred µl of the cell-free supernatant was then collected (care was taken not to disturb the cell monolayer) and transferred to plain 96-well microtitre plates. The remaining medium was then aspirated from the wells to leave the cell monolayer and this plate was quickly frozen at -20ºC. Duplicate 25µl samples of cell-free supernatant were transferred to an

M30-Apoptosense plate and M65 ELISA plate. The remaining supernatant samples were stored at -20ºC in plain 96-well microtitre plates.

2.2.8.6 Cell pellet lysis

To allow assessment of M30 from the cell monolayer, 50µl of lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5% NP-40, 10 mM MgCl2, pH 7.4) was added to each well of the plate and lysis was allowed to occur for 5 minutes on ice. The lysate was then aspirated and placed in an Eppendorf. The lysis procedure was then repeated. The Eppendorf tubes containing 100 µl of lysate were then centrifuged at 800g for 5min at 4ºC. Duplicate 25µl samples of supernatant were then transferred to an M30-Apoptosense plate for cleaved CK18 determination.

2.2.8.7 Cytokeratin 18 assessment in human plasma

2.2.8.7.1 Statistical Analysis

The statistical analysis for this part of the study was carried out by the Cancer Research UK Clinical Trials Unit Department of Medical Statistics. The distribution of the CK18, CK18-NE and CEA values were markedly skewed so these variables were logged in all analyses. Where there were two classifying groups (e.g. gender) the Mann-Whitney U test was use to compare these variables (or changes between variable values); when there were more than two groups (e.g. tumour site) the Kruskal-Wallis test was used. Spearman's rank correlation test was used when determining an association between either CK18 or CK18-NE and age. The replicate variability in the assays was estimated from all the replicate results available in the study.

2.2.8.7.2 Stability Studies

Plasma was collected and separated as described above. Twenty-five µl of plasma was then added to each well on the M30 and M65 ELISA and the M30 and M65 concentrations determined as described above. All samples were run in duplicate.

In order to study the stability of plasma cytokeratin 18 (CK18), 20ml of blood was collected from a healthy volunteer. Five ml was removed and the plasma separated as described above to act as a baseline sample. The remaining 15ml was spiked with 300µl of pooled high positive CK18 signal serum and aliquoted into EDTA specimen tubes. The

effects of delay in processing the sample, storage temperatures and freeze-thaw cycles were studied. For delays in processing the sample, the sample was stored at room temperature for 0, 2, 4, 6 and 24hr prior to separation of the plasma and CK18 analysis. For the effects of storage at various temperatures, the plasma was separated and stored at room temperature, 4ºC, -20ºC, and -70ºC for 24hr prior to CK18 analysis. For the effect of freeze-thawing, the plasma was separated as described above and then subject for up to 5 freeze-thaw cycles prior to CK18 analysis.

Experiments were then performed to see how stable cytokeratin 18 was in human plasma samples that may not have been frozen immediately following separation of plasma from the whole blood sample. This was in an attempt to gauge if delays in processing (as will occur in every day clinical practice) affect cytokeratin 18 concentrations. A whole blood sample was taken following written informed consent from a patient with advanced gastrointestinal adenocarcinoma and the plasma separated as described above. This was then aliquoted into Eppendorf tubes. One tube was frozen immediately at -70ºC to act as a baseline sample. The remaining tubes were stored at room temperature and frozen after 1, 2, 3, and 4 week time periods. The plasma samples were then analysed at the same time in duplicate.

Further experiments were then performed on blood samples taken from healthy volunteers to see if there was much intra-individual variation in plasma cytokeratin 18 levels from day-to-day and also if there was any diurnal variation or effects from fasting. Six blood samples were taken from the healthy volunteers at various time points 10am, 2pm, and 4pm and after fasting overnight. The samples were run in duplicate and the results for each time point averaged.

2.2.8.7.3 Healthy Volunteers and Patients with Advanced Gastrointestinal Adenocarcinoma

Plasma CK18 levels were then measured in the 100 healthy volunteers and 73 patients with advanced gastrointestinal adenocarcinomas receiving palliative chemotherapy. The objectives of this study were to compare plasma levels of caspase-cleaved and total soluble CK18 between healthy volunteers and patients with advanced gastrointestinal adenocarcinomas, and to determine if there was any correlation between changes in plasma cytokeratin 18 levels during palliative chemotherapy and clinical outcome in patients with advanced gastrointestinal adenocarcinomas, and so determine if measurement of CK18 could potentially be used as a diagnostic marker and a marker of treatment response.

2.2.8.7.4 Patients with Early Colorectal Cancer

Following on from the studies looking at advanced colorectal cancer, it was decided to examine serum cytokeratin 18 levels in patients with early colorectal cancer to see if CK18 could be used as a potential diagnostic marker in this group of patients. Serum rather than plasma was used for this part of the study as this is what had been previously collected as part of a clinical trial; however the M30-Apoptosense and M65 ELISAs can both be used with either serum or plasma.

The study included both patients undergoing colonoscopy examination for investigation of gastrointestinal symptoms and patients with operable colorectal adenocarcinoma. All patients gave written informed consent and blood samples were taken before the procedures, either colonoscopy or bowel resection, were performed. Limited clinical outcome data was collected for both patient groups.

The three groups consisted of 23 patients with normal colonoscopies, 13 patients whose colonoscopies showed polyps or pre-malignant lesions, and 23 patients with colorectal adenocarcinoma on colonoscopy or colorectal adenocarcinoma awaiting bowel resection

2.2.8.7.5 Patients with Ovarian Cancer

The SCOTROC (Scottish Randomised Trial in Ovarian Cancer)-1 trial was a randomized phase III study designed to compare efficacy, tolerability, and quality of life outcomes of docetaxel-carboplatin with paclitaxel-carboplatin as initial chemotherapy for stage Ic-IV ovarian and/or peritoneal cancers [74]. Between 1998 and 2000, 1077 women from 83 international centres were randomized to the study. Following debulking surgery, 539 were randomly assigned docetaxel-carboplatin, and 538 were assigned to the paclitaxel-carboplatin arm. The two treatment arms were well-matched with respect to patient demographics and disease characteristics. The results from the trial showed that the two treatment arms were equivalent in terms of progression-free survival and response. Blood samples were collected from patients after surgery, but prior to commencing chemotherapy (baseline samples) and at relapse (relapse samples). Plasma was separated according to protocol and stored at -70°C. CA-125 levels were also checked in all patients receiving chemotherapy and the CA-125 response assessed and classified.

The plasma samples had been stored at this centre and so were available for CK18 assessment. Initially the samples were separated into two separate cohorts, those that had

been collected from patients in Glasgow and those that had been collected at other centres involved in the study. This was due to the fact that the sample handling had been stringent at the Glasgow sites due to the proximity to the trial centre, however, other samples had been sent some distance and it was felt that it had to be ensured that the median CK18-NE and total CK18 values did not very greatly between the cohorts simply due to differences in sample handling.

The objectives of this study were to compare plasma levels of caspase-cleaved and total soluble CK18 between healthy volunteers and patients with ovarian cancer and determine if measurement of plasma CK18 could potentially be used as a diagnostic and prognostic marker.

Chapter 3 - Raf Kinase Inhibitor Protein

3.1 Background

Raf Kinase Inhibitor Protein (RKIP) was originally purified from bovine brain extracts and named phosphatidylethanolamine-binding protein (PEBP). Subsequent studies have shown that it is a widely expressed and highly conserved protein [75]. It was first identified as an endogenous inhibitor of the Raf-MAPK kinase (MEK)-ERK pathway that interfered with the phosphorylation and activation of MEK by RAF-1 [65]. Later, it was also identified as an inhibitor of nuclear factor kappa B (NFκB) transcription factor activation due to blocking of the inactivation of IκB, the inhibitor of NFκB [76]. As these pathways are often active in malignant tumours it was hypothesised that RKIP expression may have importance in the malignant behaviour of tumours and invasion [77, 78]. Several recent studies have suggested that RKIP may suppress metastasis in melanoma, prostate, and breast cancer, as reduction or loss of RKIP expression was observed in metastatic cell lines and metastatic tissue [79-81]. When RKIP was reconstituted in the metastatic cell lines by exogenous expression, the *in vitro* invasiveness and ability to form metastases in mouse models was impaired [80, 81]. Conversely, when RKIP expression was downregulated by antisense RNA, invasiveness was promoted. Mouse modelling in prostate cancer has suggested that although variations in RKIP expression do not affect the primary tumour, reduced expression is associated with the development of metastases [80].

In order to investigate if RKIP expression in human primary tumours is related to development of metastases, tissue microarrays (TMAs) of normal and cancerous tissue were evaluated for RKIP expression. As RKIP expression was found to be reduced in metastatic colorectal cancer the hypothesis that RKIP expression may predict overall survival and risk of metastatic relapse was assessed within the first part of the study in three independent cohorts of colorectal cancer patients. The aim of the second part of the study was to investigate the prognostic and predictive value of RKIP expression in tumour samples from two groups of ovarian cancer patients recruited to clinical trials of combination platinum-based chemotherapy. This study was part of a collaboration with Professor Walter Kolch's group in the Beatson Institute for Cancer Research. As well as being involved in design and analysis of the study, my role in data accrual was as an independent scorer of RKIP expression within the colorectal and ovarian cancer TMA, and as such, was blinded to the clinical outcome data for both patient groups.

3.1.1 Colorectal Cancer

Colorectal cancer is the third most commonly diagnosed malignancy, accounting for 10-15% of newly diagnosed cancers in Europe and the US with over 780,000 new cases diagnosed annually worldwide [82-85]. Colorectal cancer staging has not really changed over the past number of years, it remains largely based on clinical appearance and histopathological features [86]. Patients presenting with disease limited to the superficial bowel wall (Dukes' stage A) have a >90% 5-year survival, however patients presenting at this early stage are in the minority. Approximately 70% of patients will present with cancer that has invaded through the bowel wall, which may or may not involve regional lymph nodes. Dukes' stage B cancers do not have lymph node involvement and have a 5-year survival of 67%, whereas Dukes' stage C cancers with lymph node involvement have a 5-year survival of only 43% [87]. Surgery is the optimal treatment for patients whose disease is macroscopically operable at the time of diagnosis. The operability is gauged by the absence of metastatic disease on staging investigations, such as CT scans. Adjuvant chemotherapy is routinely offered in Dukes' stage C cancer patients with the aim to eliminate micrometastatic disease and to improve disease-free and overall survival, but the role of adjuvant chemotherapy in Dukes' stage B patients is still not clear and is being further evaluated in clinical trials.

Up to 30% of patients will present with metastatic disease (Dukes' stage D) and ultimately almost half of all patients diagnosed will develop locally advanced or metastatic disease as a result of micrometastases. Patients with advanced disease, if suitably fit, are given chemotherapy with palliative intent. However, the prognosis for patients who relapse following surgery or who present with metastatic disease is poor, with 5-year survival rates of 5% or less [88-90].

Therefore, conventional methods of staging do provide some insight into the clinical outcomes for patients, but the development of bioinformatics technology, which aims to increase knowledge of biological processes by applying computationally intensive techniques to analyse large quantities of data, and molecular analysis of an individual patient's tumour and serum may further augment the ability to predict tumour stage, clinical outcome and identify patients who may gain most benefit from other interventions such as adjuvant chemotherapy, radiotherapy, hormonal or biological therapies.

In order to investigate if RKIP expression in human primary tumours is related to development of metastases the initial assessment was performed using commercially

available TMA (Landmark High-Density Cancer Survey Tissue Microarrays, Ambion, UK). These contained 279 paraffin-embedded tissue specimens from 190 patients, including 242 matched tumour and normal specimens and 37 independent normal specimens.

The second cohort included TMA from 268 patients with colorectal cancer from the Aberdeen Colorectal Tumour Bank. All of these patients had received surgical treatment.

The third cohort included 65 patients with colorectal cancer from Glasgow and Kuwait with no evidence of lymph node or distant organ metastasis at time of surgery. This included 25 patients with no evidence of metastatic disease at time of surgery, but who subsequently relapsed and 40 patients who remained disease-free after surgery. All patients received surgical treatment and 14 of them also received six cycles of standard adjuvant chemotherapy (all of the patients receiving adjuvant chemotherapy were from Kuwait). The patients were followed up for a minimum period of 2 years (range, 2 to 9 years); with a median follow-up of 5.5 years for the survivors (84% of survivors were observed for more than 3.5 years). They were assessed clinically for evidence of disease relapse, which was then confirmed either radiologically, histologically or at post-mortem examination. Four patients were lost to follow-up. Ten patients died as a result of metastatic disease, but the date of disease recurrence had not been recorded, so the date of death in these patients was used in the disease-free analysis. Eleven of the fourteen patients receiving chemotherapy had survival data available.

3.1.2 Ovarian Cancer

Ovarian cancer is the fourth most common cancer in the UK, after breast, colorectal and lung cancer. Approximately 6,700 women are diagnosed annually, with cancers that are predominantly epithelial in origin. One of the characteristics of ovarian cancer is that it commonly presents at an advanced stage, when 5-year overall survival rates are <30%, whereas if the disease is discovered at stage I (confined to the ovary), the survival rates are >90%. The current screening investigations for ovarian cancer are only applied to high-risk groups and include the biomarker, cancer antigen 125 (CA-125) and pelvic ultrasound scanning. CA-125 is elevated in about 80% of patients with advanced ovarian cancer, but only 50-60% of early-stage patients. Therefore, CA-125 has a positive predictive value of <10%, which is increased to only 20% with the addition of ultrasound scanning [61-63]. Moreover, CA-125 can also be elevated in a number of other conditions including pregnancy, endometriosis, colorectal and pancreatic cancers. Therefore, there is a need to

develop detection methods to improve the sensitivity and specificity of early-stage ovarian cancer detection.

Carboplatin and cisplatin are the most common chemotherapeutic drugs used to treat ovarian cancer in the adjuvant and metastatic setting with response rates in the order of 65-80%; however, the majority of patients treated will eventually develop chemoresistance and die of metastatic disease. There is, therefore, a real need to develop reliable biomarkers for disease prognosis and survival in order to target adjuvant treatments to those patients who will derive the greatest benefit.

This part of the study investigated the prognostic and predictive value of RKIP expression in tumour samples from two groups of ovarian cancer patients recruited to clinical trials of combination platinum-based chemotherapy with clinical outcome data. Primary treatment for all patients consisted of surgery (total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy and peritoneal washings with cytology) and all patients were staged according to the FIGO classification (Cancer Committee of the International Federation of Gynaecology and Obstetrics, 1986). Tumours were graded and classified by a gynaecological pathologist according to the WHO criteria. Response to chemotherapy was assessed using standard criteria [74] and patients were followed up to ten years with gradually increasing intervals. Follow-up data were complete for all patients up to March 2005, with a median follow-up of 51.6 months (range, 2.8-136.5 months).

Formalin-fixed, paraffin-embedded TMA were examined from two cohorts of patients; one cohort comprised of 114 patients involved in a single clinical trial (SCOTROC-1) and a second cohort of 220 patients involved in various other clinical trials run through the Scottish Oncology Gynaecology Group The primary outcomes were overall and disease-free survival. Overall survival was defined as the time from study entry until death and disease-free survival was defined as time from study entry to first confirmed metastatic relapse. Univariate and multivariate Cox's proportional hazard models were used to analyse the effect of various clinical characteristics on patient survival.

3.2 Results

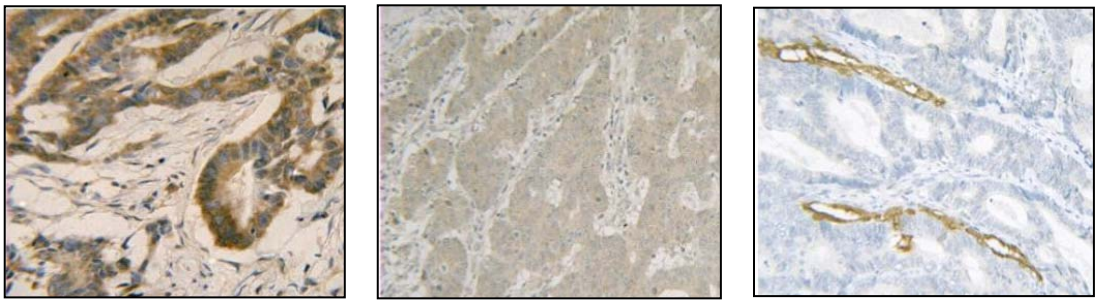
3.2.1 Colorectal Cancer

3.2.1.1. Exploratory Study Results

To study RKIP protein expression, polyclonal rabbit RKIP (Ki69) was applied to formalin-fixed, paraffin-embedded sections. Sections from liver and breast paraffin blocks were used as a positive control. In addition, Auerbach's myenteric intramuscular plexuses, which have strong RKIP expression, were used as positive internal controls (see Chapter 2.2.1). As a negative control, the RKIP antibody was either substituted with goat serum or preadsorbed with cognate antigen (i.e. the RKIP antibody was incubated with a 10 to 20 fold molar excess of purified RKIP prior to use).

To measure RKIP expression, the specificity of the RKIP antibody was validated vigorously for both western blot and immunohistochemistry. The RKIP antibody was raised against full-length rat RKIP protein which was expressed in E.coli. On Western blots, the antibody only detected RKIP, and when the antibody was preadsorbed with cognate antigen its reactivity on sections of paraffin-embedded cell lines and human tissue was strongly reduced (data not shown).

The scoring system used to assess RKIP expression was as described for previous studies [66] and represented both the area and intensity of the stain within the section. The score corresponded to the sum of a) the percentage of positive cells (1 = <25%, 2 = 26-50%, 3 = >50%) and b) the staining intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong). The sum for each section was, therefore, anything between 0 and 6. Scores between 0 and 2 were regarded as negative, scores of 3 and 4 as weakly positive, and scores of 5 and 6 as strongly positive (See Figure 3.1). Two scorers (blinded to the follow-up data and recurrence status of the patients) scored each section independently. The strength of agreement between the two scorers was calculated using linear weighted κ and found to correlate well ($\kappa>0.7$).



Positive
5-6

Weak
3-4

Negative
0-2

Figure 3.1 Representative RKIP expression scores. Scores between 0 and 2 were regarded as negative, scores of 3 and 4 as weakly positive, and scores of 5 and 6 as strongly positive

The RKIP expression in the first cohort of 279 samples was then assessed, of which 62 samples had to be excluded due to missing data and sample lifting from the slides. RKIP was found to be expressed in normal glandular epithelia of the breast, pancreas and salivary glands; renal glomeruli and tubules; transitional epithelium of the bladder; lymph and vascular endothelia; neuronal cells, hepatocytes and myocytes. When tumours derived from these tissues were examined, the RKIP expression was variable, but frequently reduced. In renal and pancreatic tumours the level of reduction of RKIP expression almost approached statistical significance compared to the tumours derived from other tissues (p=0.053, p=0.054, respectively). However, in the colorectal cancer samples, the reduction in RKIP levels was statistically significant (p=0.01) and so RKIP expression was then studied in the second, larger cohort of colorectal cancer patients.

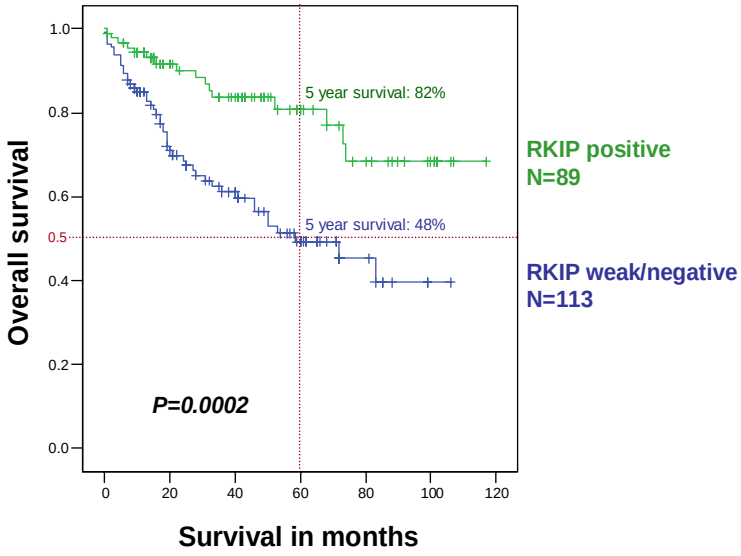
The second cohort of 268 patients had corresponding clinical data including Dukes' stage, sex, age, differentiation and tumour site (proximal colon, distal colon, or rectum). In terms of the Dukes' staging, 19.8% were Dukes' A, 38.8% were Dukes' B, and 41.1% were Dukes' C. Within this cohort there were 79 lymph node metastases that were assessed for RKIP expression, of these 67 (85%) had weak or no RKIP expression compared with 12 (15%) that expressed RKIP. In the 202 primary tumour samples, 89 (44%) were RKIP positive and 113 (56%) were RKIP negative. This suggests that the development of metastases in colorectal cancer involves a reduction or loss of RKIP expression.

Examination of the primary tumours showed a statistically significant positive correlation between RKIP expression and overall survival (p<0.001) [hazard ratio 2.84]. The mean overall survival in the 89 patients whose primary tumour scored positive for RKIP was 93 months (95% C.I.93-104), whereas reduced or no RKIP expression in 113 patients correlated with a reduced survival time of 61 months (95% C.I.52-70). As the survival curve for RKIP-positive patients never decreased below 50%, it was not possible to assess median overall survival. When a multivariate analysis was carried out, RKIP expression was found to be independent of p53 status, tumour differentiation, tumour site and B-Raf expression (other factors associated with poorer survival outcomes). This analysis also showed that reduction in RKIP expression was associated with a significantly reduced survival time comparable to the risk associated with advanced Dukes' staging.

The cohort was then stratified according to Dukes' staging and RKIP expression as Dukes' staging is the main clinical method used to predict risk of disease recurrence. The results showed that Dukes' C patients with RKIP positive primary tumours had a mean survival of 78 months (95% C.I. 57-100) versus 49 months (95% C.I. 36-62) for Dukes' C patients

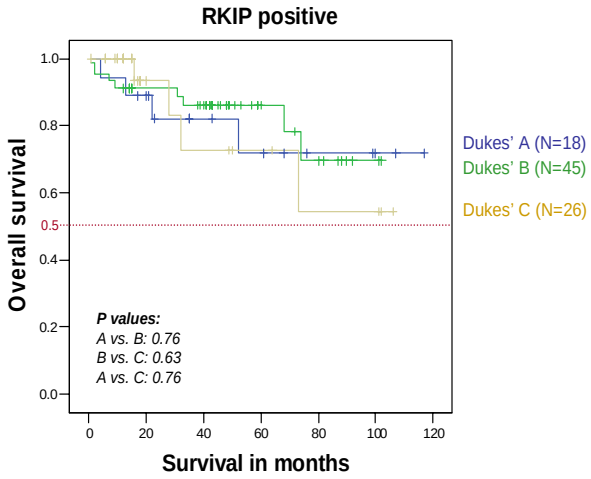
with RKIP negative primary tumours (See Figure 3.2). The mean survival of Dukes' C patients with RKIP positive primary tumours was not statistically different from the mean overall survival times of Dukes' A and B patients (92 (95% C.I. 70-113) and 85 (95%C.I. 74-95) months, respectively). These results suggest that RKIP expression in the primary tumour may independently predict overall survival and help to identify a high-risk population with reduced or no RKIP expression that may derive greater benefit from adjuvant therapies in colorectal cancer.

A)



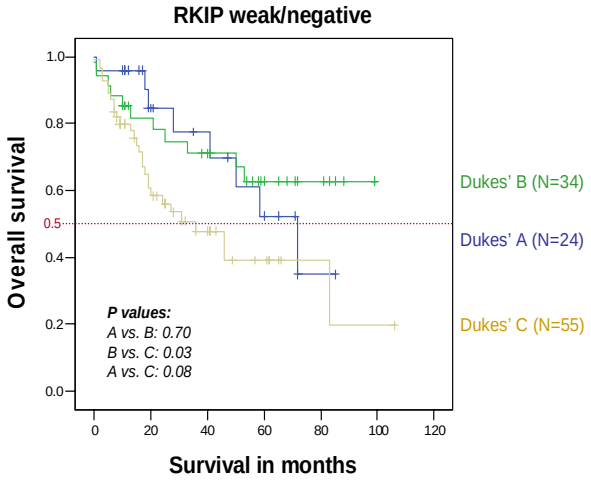
RKIP expression	Survival Time	Standard Error	95% Confidence Interval
Positive	Mean: 93 months Median: not applicable	5	83 – 104
Weak/negative	Mean: 61 months Median: 59 months	5 13	52 – 70 34 – 83

B)



Dukes' Stage	Survival Time	Standard Error	95% Confidence Interval
A	Mean: 92 months	11	70 - 113
	Median: not applicable		
B	Mean: 85 months	5	74 - 95
	Median: not applicable		
C	Mean: 78 months	11	57 - 100
	Median: not applicable		

C)



Dukes' Stage	Survival Time	Standard Error	95% Confidence Interval
A	Mean: 59 months	7	46 - 72
	Median: 72 months	14	44 - 100
B	Mean: 70 months	7	56 - 84
	Median: not applicable		
C	Mean: 49 months	7	36 - 62
	Median: 36 months	10	17 - 55

Figure 3.2. Kaplan-Meier plots of overall survival and mean survival of the Aberdeen cohort of colorectal cancer patients in relation to Raf kinase inhibitor protein (RKIP) expression. (A) All evaluated patients. (B, C) Relationship of RKIP expression to Dukes' stages

In the third cohort of 65 early-stage colorectal cancer patients, loss or reduced RKIP expression was significantly associated with metastatic recurrence (p=0.004) and reduced disease-free survival (p=0.004). The 5-year disease-free survival was 79% (95% CI 62.9-95.9) for RKIP positive tumours vs. 31% (95% CI 1.9-59.6) for weak positive RKIP tumours vs. 47% (95% CI 25.2-69.1) for RKIP negative tumours. The risk of metastatic relapse was not found to correlate with sex, age, tumour site or differentiation, mitotic and apoptotic indices, lymphovascular invasion, or depth of invasion. Again when a multivariate analysis was performed looking at the above variables, reduction or loss of expression of RKIP was the most significant independent prognostic factor (p=0.002, hazard ratio, 2.843, S.E. = 0.331).

The median survival of patients with reduction or loss of RKIP expression was 4.57 and 3.46 years, respectively compared with more than 8 years in patients with RKIP positive primary tumours. Kaplan-Meier plots (see Figure 3.3) show that patients with RKIP positive tumours had stable disease-free survival rates of 90% between 2 to 4 years after surgery and 80% after year 4. In contrast, patients with reduced or no RKIP expression in the primary tumours had a steady decline in disease-free survival over the observation period. These results suggest that the level of RKIP expression is significantly and inversely associated with metastatic disease and can predict the risk of metastatic relapse in patients with no evidence of metastases at presentation. The level of RKIP expression as a prognostic factor was independent of sex, age, tumour site, mitotic index, lymphovascular invasion and tumour stage.

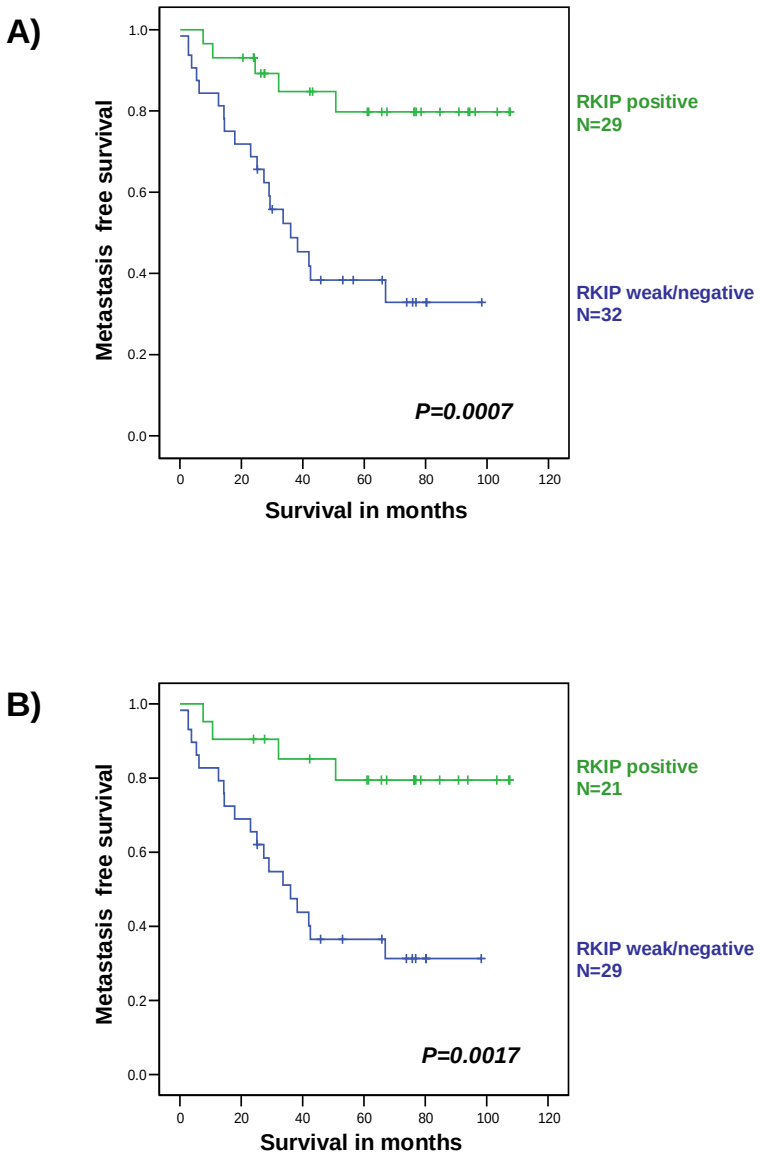


Figure 3.3. Kaplan-Meier plots of disease-free survival of patients with early stage colorectal cancer in relation to Raf kinase inhibitor protein (RKIP) expression. (A) Effect of RKIP expression on disease-free survival (metastasis-free survival) in 61 patients without stratification. (B) Data limited to patients who received surgery alone (Dukes' A and B1). Blue lines, patients with weak and negative RKIP expression; green lines, RKIP-positive patients. *P* values represent the log-rank test.

The level of RKIP expression was then correlated with mitotic index, tumour differentiation, vascular endothelial growth factor production, tumour blood vessel counts, p53 expression and apoptotic index in primary tumours. There was a positive correlation between RKIP expression and tumour differentiation in the third cohort (p=0.003). However, this was not seen with the second cohort of patients and so, RKIP expression does not seem to be simply a marker of differentiation, but is an independent marker of prognosis, whereas differentiation was not linked to survival in either of the patient cohorts. RKIP expression also correlated positively with apoptotic index (p=0.024). Tumours with loss of or reduced RKIP expression had mean apoptotic indices of 6.3 and 11.3, respectively compared with RKIP positive tumours with an apoptotic index of 15.

In this study the relationship between RKIP expression and response to adjuvant chemotherapy was not addressed. Recent data [[79]] has suggested that certain chemotherapeutic drugs can induce RKIP expression, sensitising cells to apoptosis, and our study did show a positive correlation between RKIP expression and apoptosis. A follow-up study looking at the potential for RKIP to act as a predictive marker of response to adjuvant chemotherapy in early-stage colorectal cancer would, therefore, be very interesting. If this demonstrated that RKIP expression acted as a predictive marker there is the possibility that it could then be used to direct adjuvant treatment to patients who would stand to gain most benefit, and the potential for development of novel targeted drugs that may increase RKIP expression and thus response to chemotherapy.

3.2.1.2. Validation Study Results

Following on from these results, RKIP expression was assessed in a further cohort of 209 patients with Dukes B colorectal cancer treated at St. Vincent's Hospital, Dublin between 1990 and 2002 as an independent validation set. This cohort of patients has been followed up for 14 years, 118 (56%) were male and 91 (44%) were female . The TMA consisted of 222 samples of colorectal cancer tissue with matched normal tissue present in 193 cases. There were four cores of normal and tumour tissue present in each case that had been chosen by a pathologist as being representative of the tumour as a whole. All cases were reviewed by a pathologist before inclusion in the TMA.

The results of the RKIP scoring showed that 97 (43.7%) of tumours were strongly positive, 100 (45%) of tumours were weakly positive and 14 (6.3%) were negative. Eleven of the tumours were excluded due to sample lifting from the slide (see Table 3.1). The intensity of RKIP staining was significantly correlated with disease-specific survival (p=0.007) [see

Figure 3.4] and negative RKIP expression was associated with poorly differentiated tumours. There was no significant association between RKIP positivity and tumour size, lymphovascular invasion, peritoneal involvement or tumour stage. In a multivariate analysis RKIP expression level was found to be an independent prognostic factor along with peritoneal invasion and lymphovascular invasion. When these three prognostic factors were combined a sub-group comprising 25% of the patients were identified whose 5-year survival was similar to the of patients with node-positive (stage III or Dukes C) disease.

RKIP expression	Number of samples
Negative (0-2)	14 (6.3%)
Weakly positive (3-4)	100 (45.0%)
Strongly positive (5-6)	97 (43.7%)
Excluded samples	11 (5.0%)

Table 3.1. Breakdown of Raf kinase inhibitor protein (RKIP) expression in tissue microarrays (TMA) from patients included in the Dublin cohort**.**

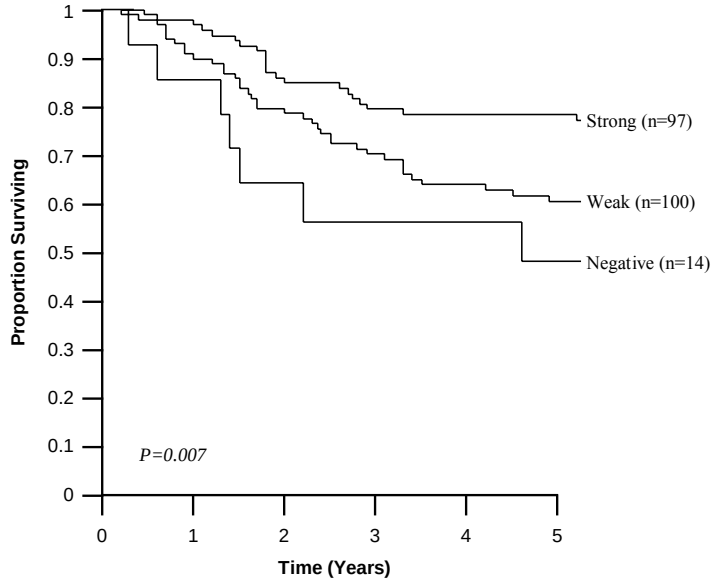


Figure 3.4. Kaplan-Meier plots of disease-specific survival of patients with early stage colorectal cancer in relation to Raf kinase inhibitor protein (RKIP) expression (Dublin cohort).

To further validate these results, RKIP expression in TMA from 1034 colorectal cancer patients were studied (kindly provided by Dr. Nik Zeps, Western Australia Research Tissue Network). The patient characteristics are shown in Table 3.2. The slides were stained for RKIP protein and scored using a semi-quantitative system combining both staining intensity and percentage area stained as described previously. Using this system RKIP expression was again divided into 3 groups; negative, weakly positive and strongly positive.

		Number of cases (% of total)
Gender	Male	510 (49)
	Female	524 (51)
Dukes' Stage	A	13 (1)
	B	629 (61)
	C	386 (37)
	D	6 (1)

Table 3.2 Patient Characteristics of 1034 patients with Colorectal Cancer in the Australian Cohort.

The results showed that of the 1034 samples, 119 could not be scored due to sample lifting from the slides, leaving 915 for analysis. Of the 915, 53 were RKIP negative, 384 were weak RKIP positive and 478 were strongly RKIP positive (see Table 3.3).

There was no difference in survival seen between the RKIP negative and weakly positive group owing to the small size of the negative group, and so these groups were therefore combined and compared to the strongly positive RKIP group. There was a statistically significant difference in both overall (p=0.0007) and disease-specific (p=0.0024) survival between the 2 groups, with low RKIP expression correlating with a worse prognosis (Figure 3.3). The median survival of RKIP positive patients was 107.6 months, compared with 62.5 months in RKIP negative patients [hazard ratio 0.74 (95% CI = 0.62-0.89). The RKIP positive patients also had improved 5-year survival (62% v 51.3%) and 10-year survival (44.6% v 33.7%). It is of note from this mature survival data that the survival curves do come together eventually about 170 months from diagnosis.

RKIP expression	Number of samples
Negative (0-2)	53 (5.1%)
Weakly positive (3-4)	384 (37.1%)
Strongly positive (5-6)	478 (46.2%)
Excluded samples	119 (11.5%)

Table 3.3. Breakdown of Raf kinase inhibitor protein (RKIP) expression in tissue microarrays (TMA) from patients included in the Australian cohort.

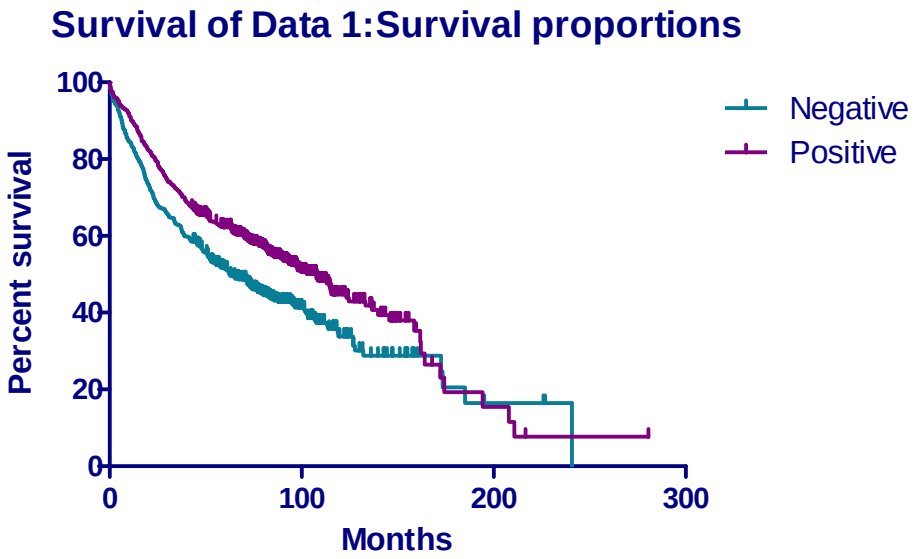


Figure 3.5: Kaplan-Meier plot demonstrating the difference in survival in 915 patients with colorectal cancer, depending on RKIP expression levels

In univariate analysis RKIP expression levels correlated with grade and lymph node status and weak/negative RKIP expression was associated with a worse prognosis (hazard ratio, 0.74, 95% C.I. 0.621-0.881, p=0.001). In a multivariate analysis RKIP expression level and lymph node status were shown to give independent prognostic information, again weak/negative RKIP expression was associated with worse prognosis (hazard ratio, 0.709, 95% C.I. 0.563-0.893, p=0.004). The conclusion to be drawn from this study is that RKIP is a useful prognostic marker which may aid in risk-stratifying patients with colorectal cancer.

3.2.1.3. Conclusions

In summary, the results from exploratory studies and subsequently confirmed in validation studies show that there is a statistically significant relationship between reduced RKIP expression, metastatic recurrence and overall survival. These associations are independent of other clinical/pathological features. These observations may be explained partly by recent data showing that RKIP regulates the spindle checkpoint in cells implying that its loss could result in chromosomal instability [91, 92]. This would in turn affect the aggressiveness of the tumour and response to adjuvant therapy, independent of other prognostic factors such as Dukes' staging. However, with the current lack of effective markers of metastatic relapse in colorectal cancer, a straightforward test like RKIP expression in the primary tumour may be a very cost-effective way to identify which patients may derive greater benefit from adjuvant treatment and closer post-operative surveillance.

3.2.2 Ovarian Cancer

The aim of this part of the study was to investigate the prognostic and predictive value of RKIP expression in tumour samples from two groups of ovarian cancer patients recruited to clinical trials of combination platinum-based chemotherapy. RKIP expression was initially determined in the first cohort of TMA from 114 epithelial ovarian cancer patients. Corresponding clinical data was available including age, tumour grade, stage and differentiation. Out of the 114 samples, 79 were suitable for assessment of RKIP expression. The others were deemed unsuitable either because of specimen lifting from the slide or absence of tumour tissue within the section. The scoring system used is described above. For the statistical analysis, samples were grouped 0-4 equalling RKIP weak/negative and > 4 equalling RKIP positive. In total 41 (51.9%) of patient samples had reduced or loss of RKIP expression and 38 (48.1%) of patients were RKIP positive. RKIP

expression was found to correlate significantly with progression-free survival, with RKIP positive patients having significantly greater progression-free survival than those with reduced or no RKIP expression (see, Figure 3.5, p=0.025), in fact, the hazard ratio associated with RKIP >4 for this cohort was 0.516 (95% CI 0.219-0.919). However, there was no correlation with overall survival. RKIP expression was also compared to CA-125 response and clinical/radiological response data, but was not found to be significantly associated with either response.

Carbonyl reductase expression has previously been shown to be a prognostic marker for epithelial ovarian cancer [66] and so the expression of this marker was compared to RKIP expression. In this cohort carbonyl reductase was not found to be significantly associated with progression-free survival, overall survival, CA-125 response or clinical/radiological response.

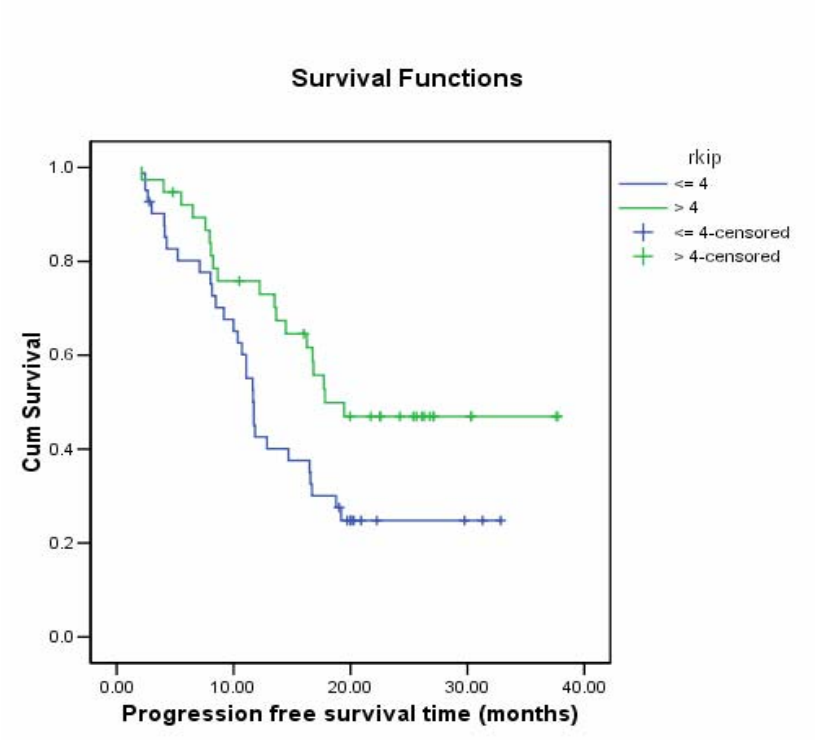


Figure 3.6. Demonstrates the difference in progression-free survival between Raf kinase inhibitor protein (RKIP) positive and negative epithelial ovarian cancers in the SCOTROC 1 trial (p=0.025)

RKIP expression in the second cohort of 216 patients was then examined. From this cohort 37 samples were unsuitable for analysis due to sample lifting from the slide, leaving 179 samples. Of these 179 samples, 83 (46.4%) were RKIP positive and 96 (53.6%) were RKIP weak positive or negative. Cox regression analysis was again used to assess whether RKIP expression correlated with progression-free or overall survival. However, in this cohort of patients the exact opposite result was observed, the hazard ratio associated with RKIP >4 for the non-SCOTROC-1 cohort was 1.627 (95% CI 1.183- 2.238). RKIP expression did not correlate with either progression-free or overall survival and there was no relationship between RKIP expression and either CA-125 or radiological response in this cohort.

So in the first cohort of patients involved in the SCOTROC-1 trial, RKIP positive patients had a greater progression-free survival, but in the second cohort of non-SCOTROC-1 trial patients, the reverse was true (this difference was real [$p<0.001$]). It is difficult to explain the discrepancy between the results from these two cohorts. The results from the first cohort are in accord with those observed in the previous studies of colorectal cancer patients, but the second cohort shows completely conflicting results. The staining methods used for the two cohorts were identical; there were no significant differences between the two cohorts in the distribution of disease stage, patients' age and performance status, and presence of residual disease. One potential explanation is that the paraffin blocks used to prepare the non-SCOTROC-1 TMA were older than the SCOTROC-1 blocks, having been stored in some cases for up to twenty years. In that case it would be expected that the RKIP staining may in fact be weaker and demonstrate more variability for the older blocks as has been shown in other studies [93], however, in the current study this was not the case. A further possibility would be if the non-specific background stain in the non-SCOTROC-1 TMA had increased over time so that many tumours become false positive which then could explain the reversed risk association.

Another possibility considered was that all the SCOTROC1 patients were treated with combination chemotherapy of carboplatin and paclitaxel while some of the non-SCOTROC trials involved single agent carboplatin and this could be of importance especially if RKIP is involved in spindle control. However, when univariate and multivariate analyses were performed and stratified for type of chemotherapy, this was not found to be significant. As yet, there is not a satisfactory explanation for the discrepancy in the results for the two ovarian cancer cohorts.

Chapter 4 - Cytokeratin 18

4.1 Background

4.1.1 Type I Cytokeratins

As can be seen from the previous chapter, RKIP has some utility as a prognostic biomarker in colorectal cancer, identifying patients with primary bowel cancer at higher risk of metastatic relapse and poorer survival outcomes. However, the results from the ovarian cancer part of the study show conflicting results and highlight the need for alternative more reliable biomarkers for diagnosis, prognosis and monitoring treatment outcomes in patients with cancer.

There are five different groups of intermediate filament proteins: type I (acidic cytokeratins, 40-56kDa [CK9-CK20]); type II (basic cytokeratins, 53-68kDa [CK1-CK8]); type III (vimentin, desmin, glial fibrillary acidic protein and peripherin); type IV (neurofilaments, nestin and internexin); type V (nuclear lamin). The intermediate filament cytoskeleton of mammalian epithelial cells is generated from pairs of type I and type II cytokeratins [94, 95].

Cytokeratin expression has been studied in many different cell types over the past few decades [35, 96, 97]. The most universally expressed are cytokeratin 18 (CK18) and 19 (CK19) which are major components of the intermediate filament of simple epithelial cells and epithelial-derived tumours, and make up approximately 5% of the total cell protein [36]. Their expression has been documented in a number of different cancers and various antibodies have been developed for their detection [37].

Type I cytokeratins are cleaved during apoptosis and reorganise into granular structures. This reorganisation of the cytoskeleton is thought to be important for apoptosis to proceed. CK18 is cleaved at the sequence VEVD/A located within the non-helical L1-2 linker region (after Asp238) by caspase 6 [98] and also at the sequence $DALD_{396}$ which lies within the C-terminus by caspase -9 [99]. The $DALD_{396}$ sequence is unique to CK18 and so can be potentially exploited to form antibodies to allow for its detection.

4.1.2 Type I Cytokeratins as Tumour Markers

Cytokeratins have been used as serum biomarkers in patients with epithelial malignancies for some time. The three most commonly used in the clinical setting are tissue polypeptide antigen (TPA), tissue polypeptide-specific antigen (TPS) and CYFRA 21-1. TPA was

originally identified in human carcinoma tumours and cell lines [100], but it was not until many years later that the antibodies defining TPA were found to stain cytoskeletal intermediate filaments in HeLa cells and TPA was found to consist of a mixture of CK8, CK18 and CK19 [97]. It has been used for some time as a serological marker in epithelial cancers such as breast, lung, colorectal, head and neck, and bladder cancer [101-106].

The marker for TPS relies on the use of a specific monoclonal antibody M3 that binds an epitope at residues 322-340 in CK18 [107] and has been used as a tumour marker in various epithelial cancers such as breast, ovarian, gastrointestinal and prostate malignancies [108-111].

The assay that measures circulating soluble CK19, CYFRA 21-1, is based on two monoclonal antibodies to CK19 [112]. CYFRA 21-1 has mostly been used clinically in lung, and head and neck cancers [39, 40].

However, as with other tumour markers currently used in clinical practice, these cytokeratin markers are not recommended for diagnosis of early disease due to insufficient sensitivity and specificity, but may have some value in detecting disease recurrence and in predicting response to chemotherapy, especially when used in combination with other markers.

4.1.3 Origin of Circulating Cytokeratins

TPS and CYFRA 21-1 are generally considered to be markers of cell proliferation. Externalization of TPA has been observed after cell division resulting in a rise in TPA detected in cell culture medium [113]. However, it is unlikely that this physiological process can account for the levels of TPA detected in the serum of cancer patients. The cytokeratins detected in the circulation comprise complexes of partially degraded polypeptides that are unlikely to be exported from cells, but more likely to be released as part of the dying process [35]. Previous studies looking at TPS and CYFRA 21-1 have shown that induction of apoptosis results in increased levels of extracellular TPS and CYFRA 21-1, but conversely inhibition of apoptosis, or promotion of necrosis did not [114].

A monoclonal antibody, M30, has been developed that recognises a neo-epitope of CK18-NE (CK18-Asp396 cleavage product) exposed after caspase-mediated cleavage during apoptosis, but not intact CK18. The antibody in the form of immunohistochemistry (M30

Cytodeath™) has been validated as a marker of apoptosis for a few years and has been used effectively in patient studies [115-117]. As cytotoxic drugs potentially induce apoptosis with different kinetics, the ideal assay should measure the accumulation of the products of apoptosis to avoid multiple time-point sampling. It has been shown that the more recently developed M30-ELISA measures accumulated CK18 cleavage products following apoptosis in medium and cell extracts [118].

Following cytotoxic treatments, cells receiving an apoptotic stimulus may not necessarily undergo apoptotic cell death, but rather undergo necrosis [119]. As a result of the loss of cell membrane integrity during necrosis, intracellular proteins are released into the extracellular space. The M65-ELISA uses two monoclonal mouse antibodies specific for epitopes on CK18 to measure total (both caspase-cleaved and un-cleaved) soluble CK18. The two ELISAs can be used in conjunction to calculate the relative proportion of caspase-cleaved CK18 to total CK18 in medium, cell extracts and plasma [118] and, thus, the primary mechanism of epithelial cell death can potentially be deduced.

The theory that cytokeratins could be released from cells following necrosis due to disintegration of the plasma membrane or as part of the apoptotic process was studied using the M30 antibody [120]. The HCT-116 colorectal carcinoma cell line was selected and cells were treated with agents reported to induce either apoptosis or necrosis. The M65 and M30 ELISA were then used to measure levels of extracellular CK18 and caspase-cleaved CK18. The results showed that necrosis resulted in increased levels of CK18 molecules that were not cleaved by caspases (with no accompanying increase in caspase-cleaved CK18), but that the apoptosis-inducing agents resulted in increased levels of caspase-cleaved CK18.

However, the situation *in vivo* may be very different as levels of caspase-cleaved or uncleaved CK18 may not be truly representative of the predominant mode of cell death occurring. This is because apoptosis and necrosis may not result in equally efficient release of cytokeratins into the circulation, and apoptotic bodies tend to be cleared to some extent by macrophages and other phagocytes whereas necrotic cells are not. Also, the half-life of the different CK18 species within the circulation is unknown, and so, the ratios of caspase-cleaved to uncleaved CK18 will only be an approximation of the type of cell death that is actually occurring [121].

There is also some debate as to whether it is possible to detect differences in circulating levels of cytokeratins in cancer patients against a background of normal physiological cell-

turnover. However, there have been several studies (discussed in the next section) showing elevated levels of both caspase-cleaved and uncleaved CK18 in patients with tumours, and also in patients with septic shock [122], liver, and renal graft-versus-host disease [123-127], and myocardial infarction [128]. One theory to explain why this might happen is that the amount of apoptosis ongoing as part of the disease process overwhelms the clearing ability of the macrophages resulting in the presence of apoptotic bodies within the circulation. These apoptotic bodies may be subsequently broken down within the circulation (secondary necrosis) releasing their contents [129]. Another theory relates to the close proximity of tumour cells to blood vessels due to tumour-induced angiogenesis [130]. This means that some apoptotic bodies may gain access to the circulation before encountering macrophages. So, although there are increased levels of caspase-cleaved CK18 detected in the circulation, the source may not be clear. It may be as a result of apoptosis within the tumour and therefore representative of the mode of tumour cell death, or as a result of secondary necrosis of apoptotic bodies within the circulation.

The possible source of CK18 detected within the circulation of patients with cancer has been studied to try and establish if the increased levels are derived from the tumour itself. One study looked at different modes of cell death using both the M30-Apoptosense and M65 ELISA [120]. Serum samples were collected from pelvic blood vessels and peripheral venous sites of 37 patients undergoing surgery for endometrial cancer and 19 patients undergoing surgery for benign endometrial conditions. Higher levels of caspase- cleaved CK18 (CK-NE) were observed in the local serum collected from patients with cancer than those with benign disease 178 vs. 125 U/L (p=0.009), and also in the peripheral serum samples, 178 vs. 145 U/L (p=0.01). Total CK18 levels were similarly higher in the local serum of cancer patients than those with benign disease, 1303 vs. 333 U/L (p<0.0001) suggesting that the origin was from the tumour.

4.2 Studies using the M30-Apoptosense and M65 ELISA

4.2.1 Cytokeratin 18 as a Tumour Marker

A recent study quantified CK18-NE levels in the sera of 201 patients with breast cancer and compared it with 82 healthy subjects using the M30-ELISA. The results showed that patients with primary cancer had higher levels of CK18-NE than healthy controls (p=0.0001). Patients with recurrent cancer had higher levels than healthy controls and patients with primary cancer (p<0.0001 and p=0.008, respectively). Patients with

oestrogen-receptor negative cancer had higher levels than those with oestrogen-receptor positive cancer. Also, in patients with recurrent cancer, there was some correlation between CK18-NE levels and number of involved organs and performance status (p=0.041 and p=0.014, respectively) [131]. The results from this study showed that CK18-NE could discriminate between patients with breast cancer and healthy volunteers and also that elevated serum levels of CK18 may have some prognostic value in terms of number of involved organs and patient performance status.

4.2.2 Cytokeratin 18 as a Biomarker of Treatment Response

4.2.2.1 Breast Cancer

Cytotoxic chemotherapy may induce apoptosis (and necrosis) in cancer cells and, therefore, measurements of circulating apoptotic products could be a simple, non-invasive way to monitor responses to treatment. A number of studies have been performed evaluating serum CK18 as a biomarker of treatment response in patients with cancer. Changes in the levels of circulating CK18-NE were analysed during the chemotherapy treatment with cyclophosphamide, epirubicin and 5-flourouracil or docetaxel of 32 patients with recurrent breast cancer [118]. Blood samples were collected on days 1, 3, and 6 of each treatment cycle. An index was calculated based on the pre-treatment level observed in each patient and the maximum levels observed during chemotherapy. All the patients had pre-treatment levels within the stated normal range (<200 U/L). The results showed that increases in serum CK18-NE of at least 50% were significantly associated with clinical response (p=0.0001). In 57% of responders, increases of 50% or more in the level of CK18-NE were seen, versus 5.6% of non-responders. This suggests that increases in the serum level of CK18-NE during chemotherapy may correlate with clinical response in patients with breast cancer.

A recent study evaluated the use of CK18 as a biomarker for monitoring chemotherapy – induced cell death in breast cancer [132]. In this study, both CK18-NE and total CK18 were assessed using the ELISA and drug-induced release of CK18 examined from both breast carcinoma cells and tissue. Serum CK18 levels were then determined in 61 patients with breast cancer receiving either docetaxel or cyclophosphamide/epirubicin/5-flourouracil (CEF) chemotherapy. The results confirmed that CK18-NE was released from cell and tissue cultures to the extracellular compartment. In terms of the serum studies, docetaxel was found to induce increased levels of CK18-NE, indicating that the primary mode of cell death was apoptosis. In contrast, CEF induced increased levels of total CK18,

indicating that the primary mode of cell death in these patients was necrosis. Also, the level of increase of total CK18 at 24hr post-treatment was correlated with clinical response to CEF chemotherapy (p<0.0001). The results from this study show that different chemotherapy regimens can result in different forms of cell death within the same tumour type and again that levels of CK18 may predict treatment outcomes.

4.2.2.2 Colorectal Cancer

Two studies have recently been published evaluating CK18 in patients with colorectal cancer. The first studied pre- and post-operative serum levels of CK18-NE in 31 patients [133]. It also assessed serum levels of CK18-NE in 10 patients receiving combination chemotherapy with oxaliplatin/capecitabine. The results showed that peri-operative levels of CK18-NE correlated significantly with tumour recurrence (p=0.016), but that increases in CK18-NE observed during chemotherapy did not correlate with response. The second study measured pre- and post-operative plasma levels of CK18-NE and total CK18 in 49 patients with colorectal cancer and correlated the levels with patient and tumour characteristics, and survival outcomes [134]. The results showed that peri-operative plasma levels of both CK18-NE and total CK18 were correlated with disease stage and were predictive of disease-free survival independent of tumour stage. Also the ratio of plasma CK18-NE/total CK18 which decreased with tumour progression, was also predictive of disease-free survival

4.2.2.3 Prostate Cancer

Changes in serum CK18 have also been evaluated in patients with prostate cancer. One study [120] looked at the pattern of increases of serum CK18-NE and total CK18 during cytotoxic chemotherapy treatment in 25 men with hormone-refractory prostate cancer and correlated the results with alterations in the level of prostate-specific antigen (PSA). Currently PSA is the most widely used serum biomarker in patients with prostate cancer to monitor response to treatment, and help detect early disease recurrence. The patients classified as PSA non-responders i.e. had a <50% decrease in PSA level during treatment, were also classified as CK18-NE non-responders (<2-fold increase of M30 U/L). Whereas the patients classified as PSA-responders (>90% decrease in PSA during treatment) were also classified as CK18-NE responders. They concluded that increases in total CK18 were not always paralleled by increases in CK18-NE; again signifying that apoptosis was not the dominating mode of cell death. This suggests that it may be important to monitor both

types of cell death (necrosis and apoptosis) during treatment, but that changes in serum CK18-NE levels may help to predict treatment responses in this group of patients.

Another study looked at levels of CK18-NE and total CK18 in the sera of 82 patients with hormone refractory prostate cancer receiving palliative chemotherapy [135]. Serum samples were collected on days 1, 3, 5, and 7 of each cycle of treatment and pre- and post-treatment levels were compared. The results showed that both docetaxel and vinorelbine caused significant increases in CK18-NE, usually between days 5 to 7 of each treatment, and that the amplitude of docetaxel-induced increases in CK18-NE was associated with baseline PSA and CK18 serum levels in this patient group suggesting a tumour origin. The conclusions from this study were that serum CK18-NE could be used to assess apoptosis *in vivo* and that the clinical efficacy of these chemotherapy treatments was due to induction of apoptosis.

4.2.2.4 Lung Cancer

Circulating CK18 levels have also been evaluated in patients with lung cancer. One study compared serum levels of CK18-NE between 60 patients with lung cancer, 22 patients with benign lung disease and 32 healthy volunteers. The results showed a statistically significant difference between these groups ($p<0.001$). They also looked at baseline serum levels of CK18-NE in terms of predictive power of survival and found that patients with CK18-NE levels >43.8 U/L had significantly shorter median survival ($p=0.013$; hazard ratio: 3.9) (95% CI=1.3–11.4). Changes in serum CK18-NE in 18 patients receiving palliative chemotherapy were then measured at baseline and then days 1and 2 of treatment. The results showed a four-fold increase in CK18-NE at 48 hours ($p<0.001$). The conclusions from this study were that measuring serum CK18-NE might be used as a novel biomarker for prediction of survival as well as for monitoring the efficiency of chemotherapy in lung cancer patients [136].

4.2.3 Cytokeratin 18 as a Biomarker in Early Clinical Trials

Many of the newer targeted treatments are not myelotoxic and so traditional endpoints, such as bone marrow toxicity, used in early clinical trial evaluation may be less relevant. Therefore, greater emphasis will be placed on the measurement of biological responses as surrogate markers of activity for potential trial endpoints, and potentially allowing intra-patient dose escalation to biologically active dose levels. Studies have been performed to evaluate the use of CK18 as a potential pharmacodynamic biomarker (or surrogate marker

of the effect that the drug is having on the body). One study validated the use of M30-Apoptosense ELISA as a potential pharmacodynamic marker for evaluating the clinical efficacy of an antisense compound targeted to the X-linked inhibitor of apoptosis protein (XIAP) within a phase I trial [137]. Independent quality control data were achieved through the treatment of X-G4 cells (XIAP knockdown cells) with staurosporine (a potent protein kinase C inhibitor that induces apoptosis) and collection of media for analysis. The measurements on assay precision over time and between kits were within the manufacturer's acceptance criteria of 10%. Also, the M30 antigen levels appeared to be stable in plasma stored -80°C for at least 6 months, and had a half-life of 80-100 hours in plasma stored at 37°C. The results demonstrated that CK18-NE appeared to be a relatively stable marker, which is desirable for a marker to be used as part of a clinical trial. Previous studies by our group have also demonstrated that disease stabilisation was associated with CK18-NE plasma levels in patients with advanced solid tumours treated in a phase 1 clinical trial of the novel hydroxamate histone deacetylase inhibitor, belinostat [138].

Therefore, measurement of circulating CK18 may be of use in assessing newer targeted anti-cancer treatments in clinical trials in addition to potentially acting as a real time marker of clinical response to cytotoxic therapy allowing for earlier adjustments of dosage or drug as required.

My aims in this part of the project were to:

1. Perform cell-line studies using colon and ovarian carcinoma cell lines treated with cisplatin to determine the mode of cell death in gastrointestinal and ovarian cancer cells receiving a cytotoxic stimulus, and to test the proof of principle using the M30-Apoptosense and M65 ELISA.

2. Measure serum and plasma CK18 levels from healthy volunteers to determine a normal range.

3. Measure serum CK18 levels in patients with early colorectal cancer and compare these to patients with pre-malignant polyps and healthy volunteers.

4. Measure plasma CK18 levels patients with advanced gastrointestinal malignancy (both pre-treatment and in patients receiving palliative chemotherapy) and in patients with ovarian cancer to determine the utility of CK18 as a potential biomarker for diagnosis, prognosis and assessing treatment outcomes in these patient groups.

4.3 Cell Line Studies

4.3.1 Results

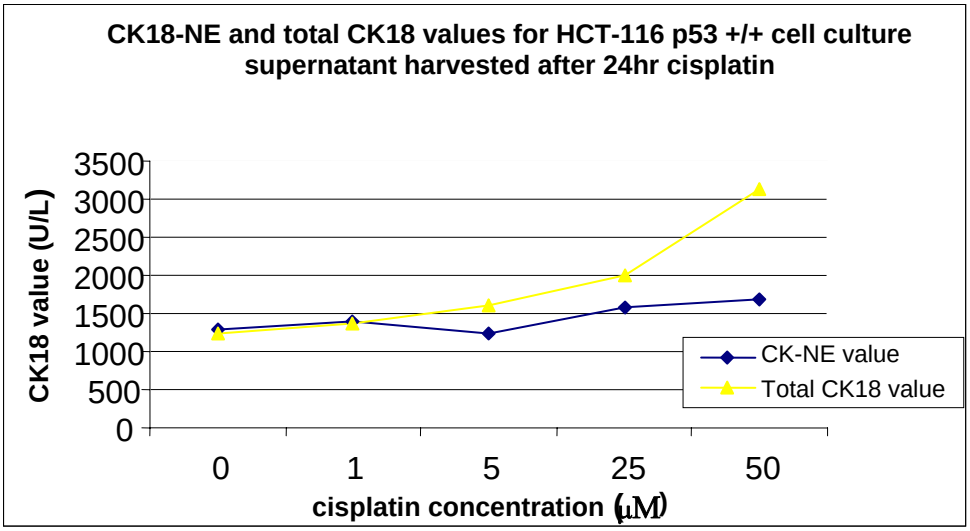
My aim in this part of the project was to perform cell-line studies using colon and ovarian carcinoma cell lines treated with cisplatin to determine the mode of cell death in gastrointestinal and ovarian cancer cells receiving a cytotoxic stimulus, and to test the proof of principle using the M30-Apoptosense and M65 ELISA. HCT-116 p53+/+, HCT-116 p53-/- colon carcinoma and A2780/CP70 ovarian carcinoma cells were grown in culture (see Materials and Methods section 2.2.9.3). The cells were then plated and treated with various concentrations of cisplatin, as described in the materials and methods section (see section 2.2.9.3). They were then harvested after 24 and 48 hours of cisplatin exposure. The total CK18 and CK18-NE were determined from the cell culture supernatant (see Table 4.1A & B, Figures 4.1A & B)) and the ratio of CK18-NE (M30) to total CK18 (M65) calculated for the HCT-116 colon carcinoma cell lines (see Table 4.2). The CK18-NE (M30) values were then determined for the cell pellet lysis (see Table 4.3 and Figure 4.2) and total cell culture lysis (see Table 4.4 and Figure 4.3). The samples were run in duplicate and the values expressed are the mean of the two measurements (see Materials and Methods section 2.2.8.4-2.2.8.6 for definitions of cell culture supernatant, cell pellet lysis and total cell culture lysis).

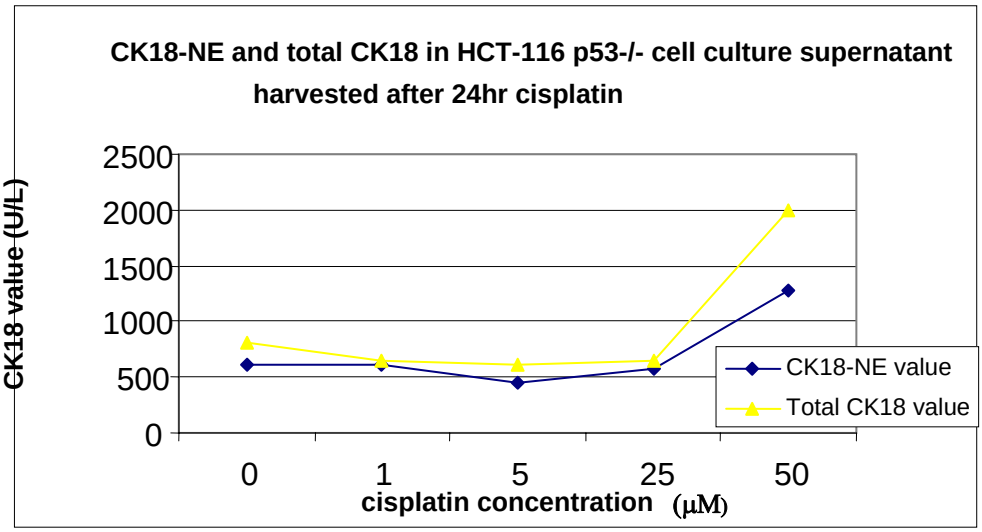
	cisplatin conc. µM				
cell line	0	1	5	25	50
A2780/CP70	*	*	*	*	49 U/L
HCT-116 p53 +/+	1232 U/L	1370 U/L	1606 U/L	2005 U/L	3143 U/L
HCT-116 p53 -/-	811 U/L	648 U/L	610 U/L	651 U/L	2003 U/L

Table 4.1 A. Table showing total CK18 (U/L) concentration in cell culture supernatant harvested at 24hr from 2.5 x10^4 plates. This demonstrates increasing concentrations of total CK18 detected with increasing cisplatin concentrations in both HCT-116 cell lines. *level undetectable

	cisplatin conc. µM				
cell line	0	1	5	25	50
A2780/CP70	*	*	*	*	*
HCT-116 p53 +/+	1294 U/L	1390 U/L	1234 U/L	1589 U/L	1671 U/L
HCT-116 p53 -/-	615 U/L	615 U/L	451 U/L	576 U/L	1275 U/L

Table 4.1 B. Table showing CK18-NE (U/L) concentration in cell culture supernatant harvested at 24hr from 2.5 x10^4 plates. This demonstrates increasing concentrations of CK18-NE detected with increasing cisplatin concentrations in the HCT-116 p53 -/- cell line. *level undetectable.





Figures 4.1A & B. Total CK18 and CK18-NE levels in cell culture supernatant from HCT-116 p53+/+ and HCT-116 p53-/- cells harvested 24 hours after cisplatin treatment. This demonstrates higher levels of apoptosis and cell death at lower cisplatin concentrations in the HCT-116 p53+/+, as would be expected given the central role of p53 in apoptosis.

Cisplatin concentration (μM)	M30:M65 ratio	
	HCT-116 +/+ cells	HCT-116 -/- cells
0	1	0.8
1	1	1
5	0.7	0.7
25	0.7	0.8
50	0.5	0.6

Table 4.2. Table showing the ratio of M30 to M65 in colon carcinoma cell lines

	cisplatin conc. μM				
cell line	0	1	5	25	50
A2780/CP70	55 U/L	65 U/L	50 U/L	91 U/L	170 U/L
HCT-116 p53 +/+	1021 U/L	931 U/L	947 U/L	1686 U/L	1698 U/L
HCT-116 p53 -/-	583 U/L	475 U/L	538 U/L	830 U/L	1556 U/L

Table 4.3. Table showing CK18-NE (U/L) concentrations in cell pellet lysate harvested at 24hr from 2.5 x10^4 plates. This demonstrates increasing CK18-NE levels with increasing cisplatin concentrations especially in the colon carcinoma cell lines**.**

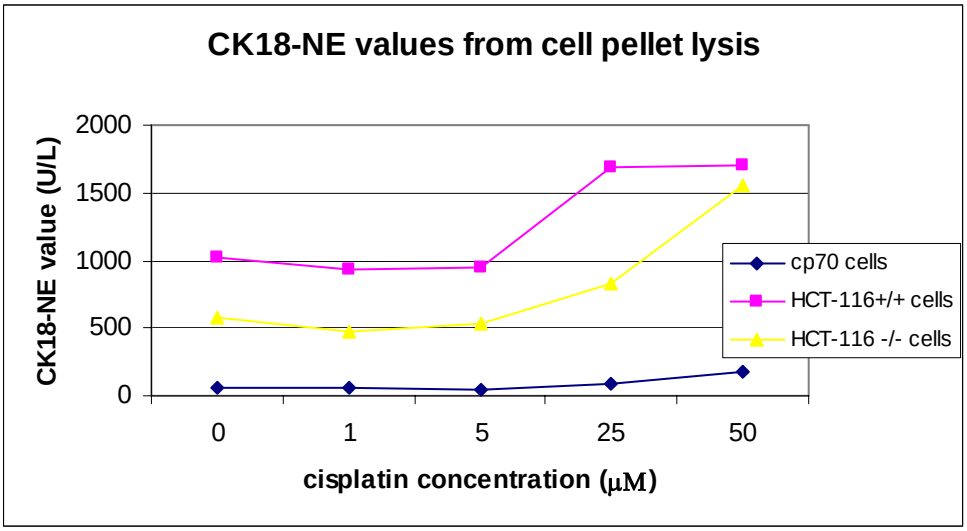


Figure 4.2. CK18-NE values from cell pellet lysis in both colon carcinoma and ovarian carcinoma cell lines harvested 24 hours after cisplatin treatment. This demonstrates very low levels of apoptosis occurring in the A2780/CP70 ovarian carcinoma cell lines even at high cisplatin concentrations. It also shows greater apoptosis in the HCT p53+/+ colorectal carcinoma cell lines compared to the HCT p53-/-, as would be expected, even in cells cultured without cisplatin in the medium. However as can be seen at higher cisplatin concentrations, there is convergence of the CK-18 NE values for both HCT-116 cells lines, demonstrating that the higher cisplatin concentration in the medium overcomes the inherent resistance to apoptosis within the p53 null cells.

	cisplatin conc. µM				
cell line	0	1	5	25	50
A2780/CP70	*	*	*	171 U/L	167 U/L
HCT-116 p53 +/+	1670 U/L	1597 U/L	1555 U/L	1722 U/L	1790 U/L
HCT-116 p53 -/-	999 U/L	832 U/L	823 U/L	1171 U/L	1530 U/L

Table 4.4. CK18-NE (U/L) in total cell culture lysate harvested at 48hr from 1.25 x104 plate.***level undetectable**

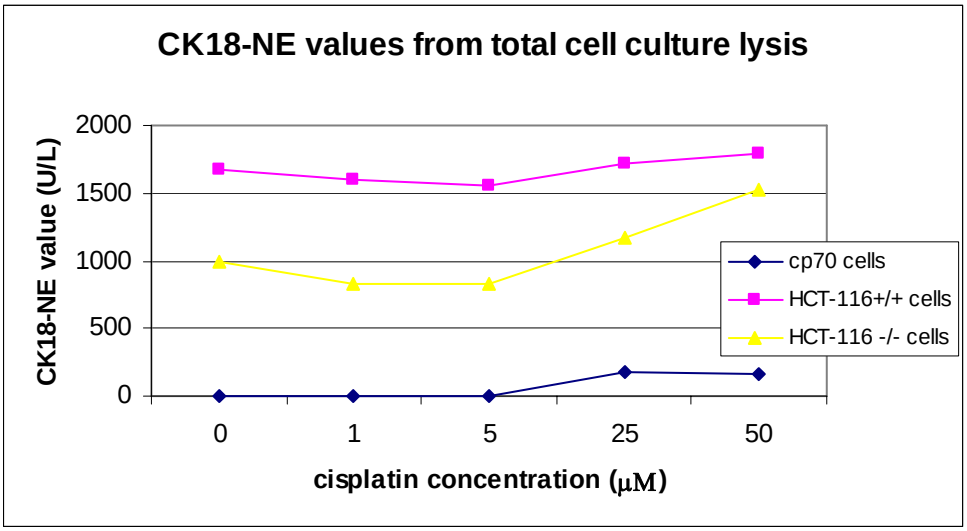


Figure 4.3. CK18-NE levels in from total cell culture lysate in both colon carcinoma and ovarian carcinoma cells 48 hours after cisplatin treatment. This demonstrates very low levels of apoptosis occurring in the CP70 ovarian carcinoma cell lines even at high cisplatin concentrations. It also shows greater apoptosis in the HCT p53+/+ colorectal carcinoma cell lines compared to the HCT p53-/-, as would be expected given the central role of p53 in apoptosis.

4.3.2 Conclusions

The results confirm that the M30-Apoptosense and M65-ELISAs measure both CK18-NE and total CK18 released from colon and ovarian carcinoma cells following incubation with cisplatin. In the culture supernatant from the HCT-116 cell lines, the low ratio of M30 to M65 of 0.5 and 0.6 (for the HCT-116 p53+/+ and HCT-116 p53-/- cells respectively) at the maximum cisplatin concentration of 50 μM, indicates that the primary mode of cell death is necrosis, but the correspondingly high ratio of M30 to M65 of 1.0 at decreased cisplatin concentrations shows the primary mode of cell death is apoptosis (see table 4.2).

In the HCT-116 colorectal carcinoma cell line, there were increased levels of apoptosis demonstrated by higher concentrations of CK18-NE in the cell culture supernatant (see Table 4.1B, Figure 4.1), cell pellet lysis (see Table 4.3, Figure 4.2) and total cell culture lysis (see Table 4.4, Figure 4.3) in the p53 wild-type cells compared to the null cells, as was to be expected given the central role of p53 in cell apoptosis. The results also demonstrated greater apoptosis in the HCT p53+/+ colorectal carcinoma cell lines compared to the HCT p53-/- in cells cultured without cisplatin in the medium (see Figure 4.2). However as can be seen at higher cisplatin concentrations, there is convergence of the CK-18 NE values for both HCT-116 cells lines, demonstrating that the higher cisplatin concentration in the medium overcomes the inherent resistance to apoptosis within the p53 null cells. In the A2780/CP70 ovarian carcinoma cell line, the inherent cisplatin resistance was apparent with low levels of cell death observed at cisplatin concentrations lower than 50 μM (see Figures 4.2 & 4.3).

The results from the cell line studies confirm that the M30 and M65 ELISAs detect CK 18 released from cancer cells undergoing cytotoxic treatment and can give some indications as to the primary mode of cell death.

4.4 Cancer versus normal control samples

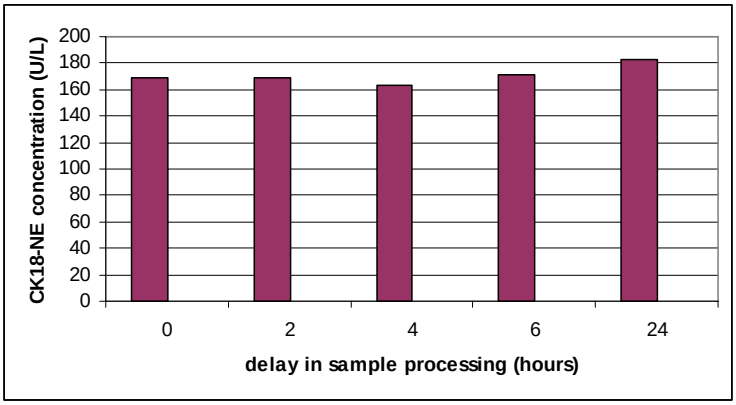
4.4.1 Stability Studies

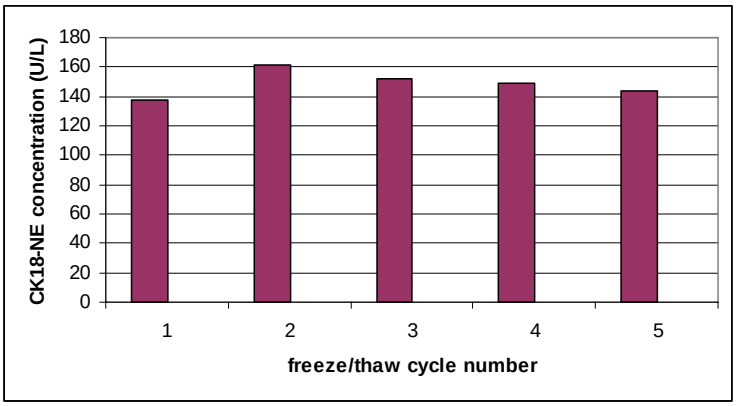
The next aim was, therefore, to study CK 18 levels in human plasma from healthy volunteers, patients with gastrointestinal and ovarian cancer and patients with advanced gastrointestinal cancer receiving palliative chemotherapy to determine the utility of CK18 as a potential diagnostic and prognostic biomarker. As a prelude to this, I evaluated some

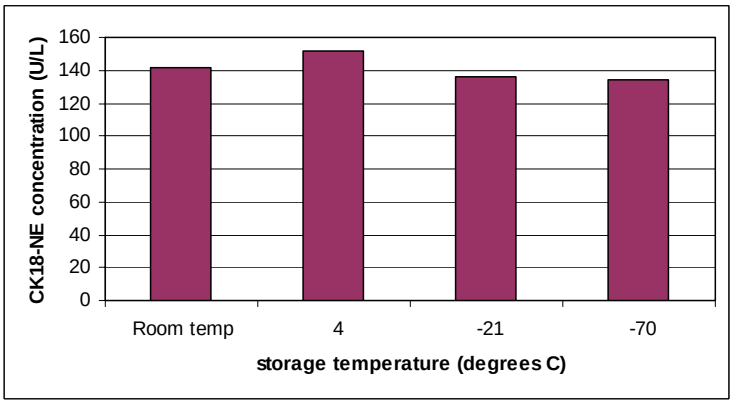
of the characteristics of the M30 and M65 epitopes in spiked serum samples to assess the issue of stability, and different storage conditions on site.

Experiments were initially performed using the M30-Apoptosense ELISA to check the stability of CK18-NE in blood samples stored under various conditions: in samples with a delay in processing, and in samples that had been frozen and thawed. A 20ml venous blood sample was taken from a healthy volunteer. This was spiked with a sample of pooled high CK18-NE positive signal serum from a patient sample (supplied by Dr Plumb). The effects of a delay in processing the whole blood sample stored at room temperature were examined (sample processed at 0, 2, 4, 6, and 24 hours). The effects of storing plasma extracted from the sample at room temperature, 4ºC, -20ºC, and -70ºC for 24 hours prior to CK18-NE determination were examined. Finally the effect of 4 freeze-thaw cycles on the CK18-NE levels was also studied.

EFFECT	VARIABLE
Delay in processing (whole blood)	0, 2, 4, 6, 24hr
Sample storage temperature (plasma)	Room temperature, 4ºC, -20ºC, and -70ºC
Freeze-thaw cycles	Up to 4 freeze-thaw cycles







Figures 4.4 A-C Results of plasma stability studies. (A) Shows the variation in plasma CK18-NE during a delay in sample processing from 2 to 24hr. The samples were stored at room temperature. The values varied by 8% from a minimum of 162.7 U/L to a maximum of 182.5 U/L. (B) shows the variation in plasma CK18-NE from up to 4 freeze/thaw cycles. The samples were stored at -70°C prior to analysis. The values varied by 11% from a maximum of 161.3 U/L to a minimum of 143.6 U/L. (C) shows the variation in plasma CK18-NE in samples stored at a range of temperatures prior to analysis. The values varied by 11 % from a maximum of 151.5 U/L to a minimum of 134.5 U/L**.**

The results showed that CK18-NE appears to be quite stable as there was little variation (up to 11%) in the levels detected no matter how long the blood sample was kept prior to processing, what temperature the plasma was stored at and how many freeze-thaw cycles the plasma had undergone (Figure 4.4 A-C). This variation was very close to the assay precision of 10% stated within the manufacturer's acceptance criteria.

Experiments were then performed to see how stable CK18 was in human plasma samples that may not have been frozen immediately following separation of plasma from the whole blood sample. This was in an attempt to gauge if delays in processing (as will occur in every day clinical practice) affect CK18 concentrations. A whole blood sample was taken following written informed consent from a patient with advanced gastrointestinal adenocarcinoma and the plasma separated as described above. This was then aliquoted into Eppendorf tubes. One tube was frozen immediately at -70ºC to act as a baseline sample. The remaining tubes were stored at room temperature and frozen after 1, 2, 3, and 4 week time periods. The plasma samples were then analysed at the same time in duplicate.

The results showed that for both CK18-NE and total CK18 there was a steady decline in concentration over the time period. For CK18-NE this fell from a baseline level of 382.2 U/L, to week 1 - 354.6 U/L, week 2 – 303.8 U/L, week 3 – 255 U/L, week 4 – 200.2 U/L. This represents a 48% decrease from baseline. For total CK18 this fell from a baseline level of 957.6 U/L, to week 1 – 791.5 U/L, week 2 – 619.4 U/L, week 3 – 563.2 U/L, week 4 – 495.2 U/L. This represents a 51% decrease from baseline (see Figure 4.5).

The conclusion to be drawn from this part of the study is that there is a steady decline in plasma CK18 levels in plasma stored at room temp that can be detected even after one week, and so with clinical samples destined for CK18 assessment the aim should be to have the plasma separated and frozen within 24 hours of the blood sample being taken, which is eminently achievable, and so within the normal operation of the clinic we did not envisage variations in CK18 due to e*x vivo* handling issues to influence our interpretation of the data.

A recent study was published which examined methods to increase the robustness of these assays for routine clinical use [139]. Matched plasma and serum samples were collected from 31 lung cancer patients and 18 controls. The conclusions drawn were that a greater than 4-hour delay in processing lead to a significant increase in CK18-NE ($p<0.0001$) which was minimized by incubating the sample on ice. Both serum and plasma were fairly resistant to processing variations and that the values between serum and plasma correlated

well, although CK18-NE tended to be lower in serum ($p<0.0005$). Prolonged storage (-80 °C) led to an increase in CK18-NE of 12% at 6 months and 34% at 1 year).The study also reported less variation between duplicate measurements made using serum and concluded that this was the preferred medium as it was more resistant to variations in sampling handling.

Further experiments were then performed on blood samples taken from healthy volunteers to see if there was much intra-individual variation in plasma CK18 levels from day-to-day and also if there was any diurnal variation or effects from fasting. Six blood samples were taken from the healthy volunteers at various time points 10am, 2pm, and 4pm and after fasting overnight. The samples were run in duplicate and the results for each time point averaged.

The results showed that there was not a large amount of intra-individual variation with the average CK18-NE ranging from 94.7 to 153.6 U/L and total CK18 ranging from 285.3 to 384.1 U/L (see Figure 4.6).

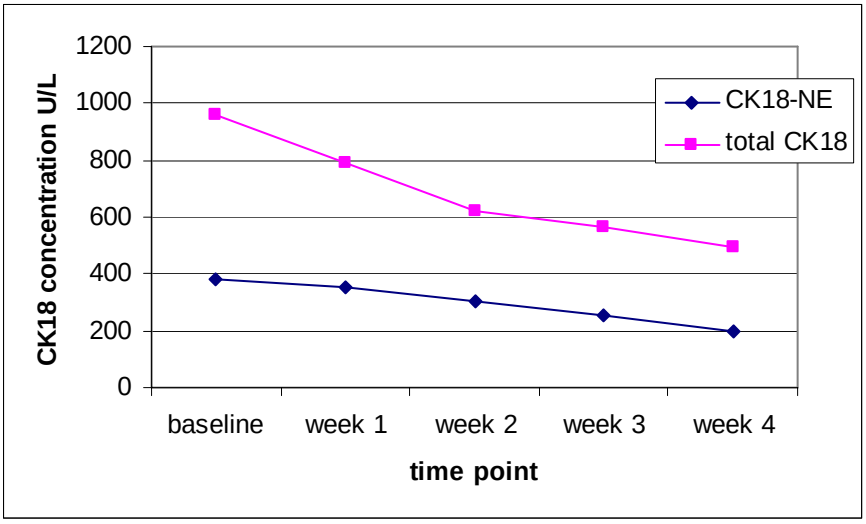


Figure 4.5. Demonstrates decrease in CK18-NE and total CK18 concentrations in plasma samples stored at room temperature for up to 4 weeks prior to analysis. The results show that for both CK18-NE and total CK18 there is a steady decline in concentration over the time period. For CK18-NE this falls from a baseline level of 382.2 U/L, to week 1 - 354.6 U/L, week 2 – 303.8 U/L, week 3 – 255 U/L, week 4 – 200.2 U/L, representing a 48% decrease from baseline. For total CK18 this falls from a baseline level of 957.6 U/L, to week 1 – 791.5 U/L, week 2 – 619.4 U/L, week 3 – 563.2 U/L, week 4 – 495.2 U/L representing a 51% decrease from baseline

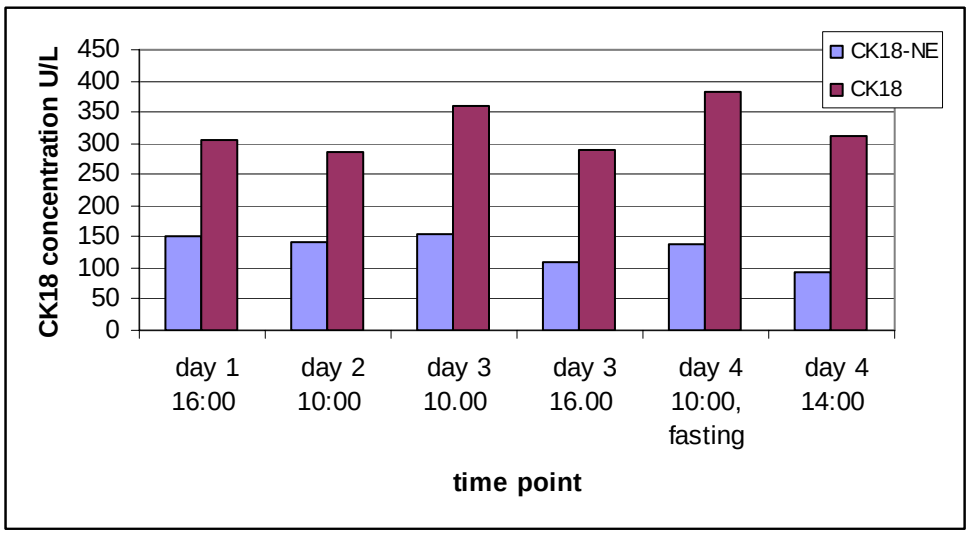


Figure 4.6. Bar chart summarising intra-individual variation in CK18-NE and total CK18 plasma levels

4.4.2 Plasma Cytokeratin 18 Levels in Healthy Volunteers

The CK18-NE values within 100 normal healthy controls were then assessed using the M30 Apoptosense ELISA (the manufacturers of this assay quote the normal range ≤180 U/L). Within the population studied there was a wide range of values, from 51 to 849 U/L, with a median of 121 U/L (see Figure 4.7). The values were independent of age (p=0.80) and gender (p=0.21). The range in females was 51 – 849 U/L and that in males was 79 – 616 U/L). The total soluble CK18 levels were also determined for the 100 normal healthy volunteers using the M65 ELISA and as for the CK18-NE, there was a wide range in total soluble CK18 levels (161 - 899 U/L), with a median of 312 U/L. As for CK18-NE, the range in females was greater compared to the males (167-889 U/L vs. 161-630 U/L). Again these values were independent of age (p=0.45) and gender (p=0.06).

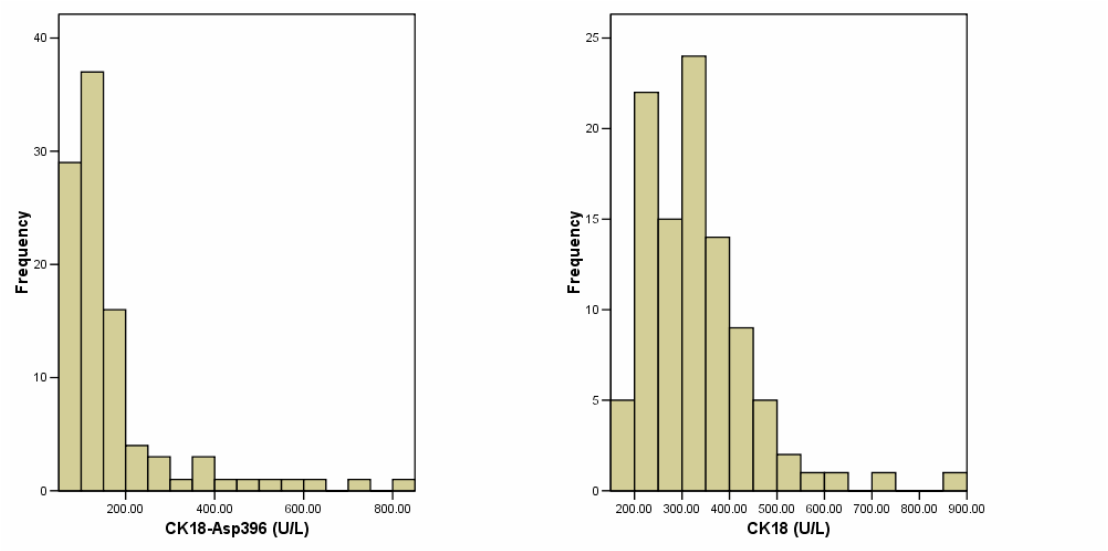


Figure 4.7.CK18-Asp396 (CK18-NE) and CK18 plasma levels in healthy volunteers. Histograms showing the range of plasma levels of CK18-Asp396 (CK18-NE) and CK18 in the healthy volunteers and the frequency of their occurrence. Plasma CK18-Asp396 (CK18-NE) ranges from 51-849 U/L with a median value of 121 U/L and CK18 ranges from 161-899 U/L with a median value of 312 U/L.

4.4.3 Serum Cytokeratin 18 in Early Colorectal Cancer

Following on from the studies looking at plasma CK18 levels in healthy volunteers, it was decided to examine CK 18 levels in patients with early colorectal cancer to see if CK18 could be used as a potential diagnostic marker in this group of patients. Serum rather than plasma was used for this part of the study as this had been previously collected as part of a clinical trial and compared with serum from the healthy volunteers; however the M30-Apoptosense and M65 ELISAs can both be used with either serum or plasma.

The study included both patients undergoing colonoscopy examination for investigation of gastrointestinal symptoms and patients with operable colorectal adenocarcinoma. All patients gave written informed consent and blood samples were taken before the procedures, either colonoscopy or bowel resection, were performed. Limited clinical outcome data was collected for both patient groups.

The three groups consisted of 23 patients with normal colonoscopies, 13 patients whose colonoscopies showed polyps or pre-malignant lesions, and 23 patients with colorectal adenocarcinoma on colonoscopy or colorectal adenocarcinoma awaiting bowel resection. The results showed there was no significant difference in the median serum CK18-NE or total CK18 between the 3 different groups. However, as the groups were quite small it was decided to measure the serum CK18-NE and total CK18 levels in the blood samples collected from the 100 healthy volunteers and compare this to patients with pre-malignant polyps shown on colonoscopy, and the group of patients with early colorectal cancer. When the median serum CK18-NE and total CK18 were compared between the healthy volunteers and the patients with pre-malignant polyps, the CK18-NE level was significantly higher in patients with polyps (p=0.043), but there was no statistically significant relationship for total CK18 (p=0.214). However, when median serum CK18-NE and total CK18 levels were compared between the healthy volunteers and the patients with early colorectal cancer, both CK18-NE and total CK18 were significantly higher in patients with early colorectal cancer (p=0.044, and p=0.005, for CK18-NE and total CK18, respectively) {Figure 4.8}.

The results are interesting as they suggest that serum CK18-NE levels may differentiate between patients with pre-malignant polyps and healthy volunteers, and that both serum CK-NE and total CK18 may differentiate between healthy volunteers and patients with

early colorectal cancer, and thus could potentially be used as an early diagnostic biomarker. However, the sample sizes used for this part of the study were very small and it was not possible to expand the study as the samples had been collected as part of an earlier clinical trial. Also, there was only very limited clinical data collected on the groups of patients, so, it is difficult to draw any definite conclusions. It is also likely that a diagnostic test based on these results would have low sensitivity and specificity due to a substantial overlap in the distribution of the values.

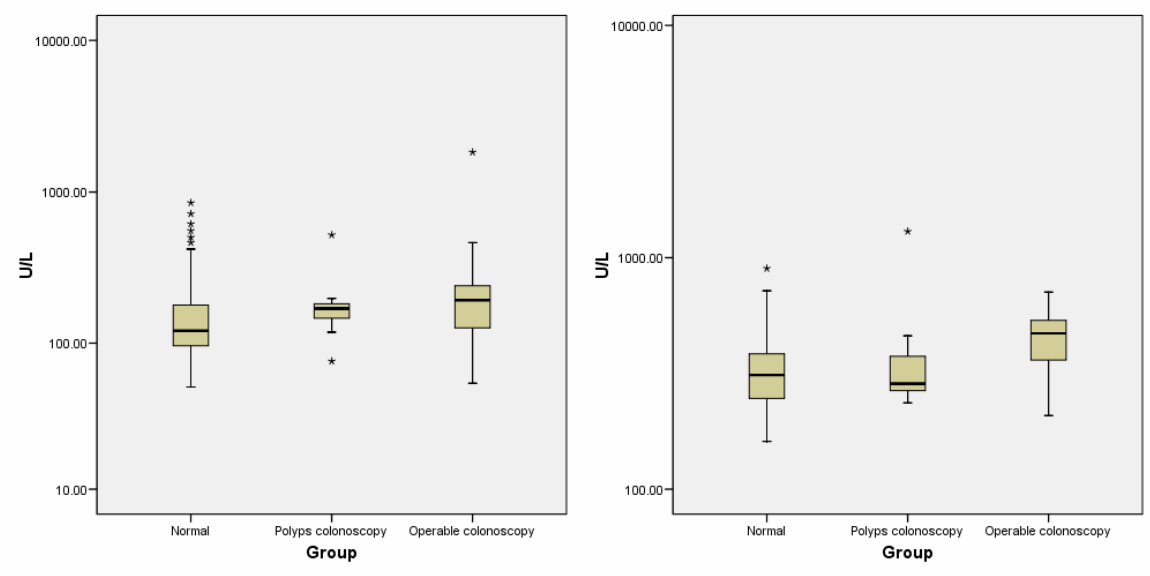


Figure 4.8. Box plots showing the CK18-NE on the left and total CK18 serum levels in the healthy volunteers, patients with pre-malignant polyps on colonoscopy and patients with early colorectal cancer**.**

4.4.4 Plasma Cytokeratin 18 Levels in Advanced Gastrointestinal Cancer Patients

The CK18-NE and CK18 levels were then assessed in the 73 patients with advanced gastrointestinal adenocarcinoma, including 18 patients with oesophageal adenocarcinoma, 19 patients with gastric adenocarcinoma, and 36 patients with adenocarcinoma of the colon or rectum (see Table 4.5). Thirty-two of the patients with gastric or oesophageal adenocarcinoma were treated with combination chemotherapy comprising epirubicin, cisplatin, and 5-FU (ECF), 3 were treated with a combination of cisplatin and 5-FU (CF), and 2 with carboplatin and 5-FU (CarboF). Thirty-three of the patients with colorectal cancer were treated with xeloda (capecitabine) monotherapy, and 3 with infusional 5-FU and folinic acid (modified "de Gramont" regimen).

Receiver operating characteristics (ROC) curves for CK18-NE and CK18 distinguished between patients with advanced gastrointestinal malignancy and healthy volunteers (Figure 4.10). Pooled data for all 73 patients demonstrated that CK18-NE has a sensitivity of 27% at a specificity of 90% in distinguishing patients with advanced gastrointestinal malignancy and healthy volunteers. Similarly, CK18 has a sensitivity of 71% at a specificity of 90% in distinguishing between patients with advanced gastrointestinal malignancy and healthy volunteers. These results suggest that CK18 may be a better biomarker than CK18-NE in distinguishing between plasma from patients with cancer and healthy volunteers, but that both markers may have limited use as a diagnostic marker.

The median CK18- NE value was 207 U/L (range 35 - 2535 U/L) and the median CK18 value was 717 U/L (range 206 - 7747 U/L). When these results were compared to the healthy volunteers, the pre-treatment plasma levels of both CK18- NE and CK18 were significantly higher in the plasma samples of patients with gastrointestinal adenocarcinoma compared with plasma samples from healthy volunteers (see Figure 4.9 A & B). These significantly higher plasma levels of both CK18- NE and CK18 were also demonstrated when plasma samples from the different tumour types were compared with plasma samples from the healthy volunteers ($p=0.015$ for patients with gastric cancer and $p <0.001$ for patients with oesophageal and colorectal cancer) (See Table 4.6).

Table 4.5. Table summarising patient demographic data. * ECF = epirubicin/cisplatin/5-fluorouracil, CF = cisplatin/5-fluorouracil, CarboF = carboplatin/5-fluorouracil

Characteristic		Number of patients
Age	Median – 68 years	73
	Range – 24-88 years	
Gender	Male	41
	Female	32
Primary Tumour Site – Colorectal		36
	Oesophageal	18
	Gastric	19
Disease Extent	Locally Advanced	11
	Metastatic	60
	Unknown/Adjuvant	2
Chemotherapy	Capecitabine	33
	Modified de Gramont	3
	ECF*	32
	CF*	3
	CarboF*	2
Chemotherapy Cycles	Median – 4 cycles	
	Range – 1-12 cycles	
Clinical Outcome	Partial Response	16
	Stable Disease	25
	Disease progression	27
	Non evaluable & missing	5

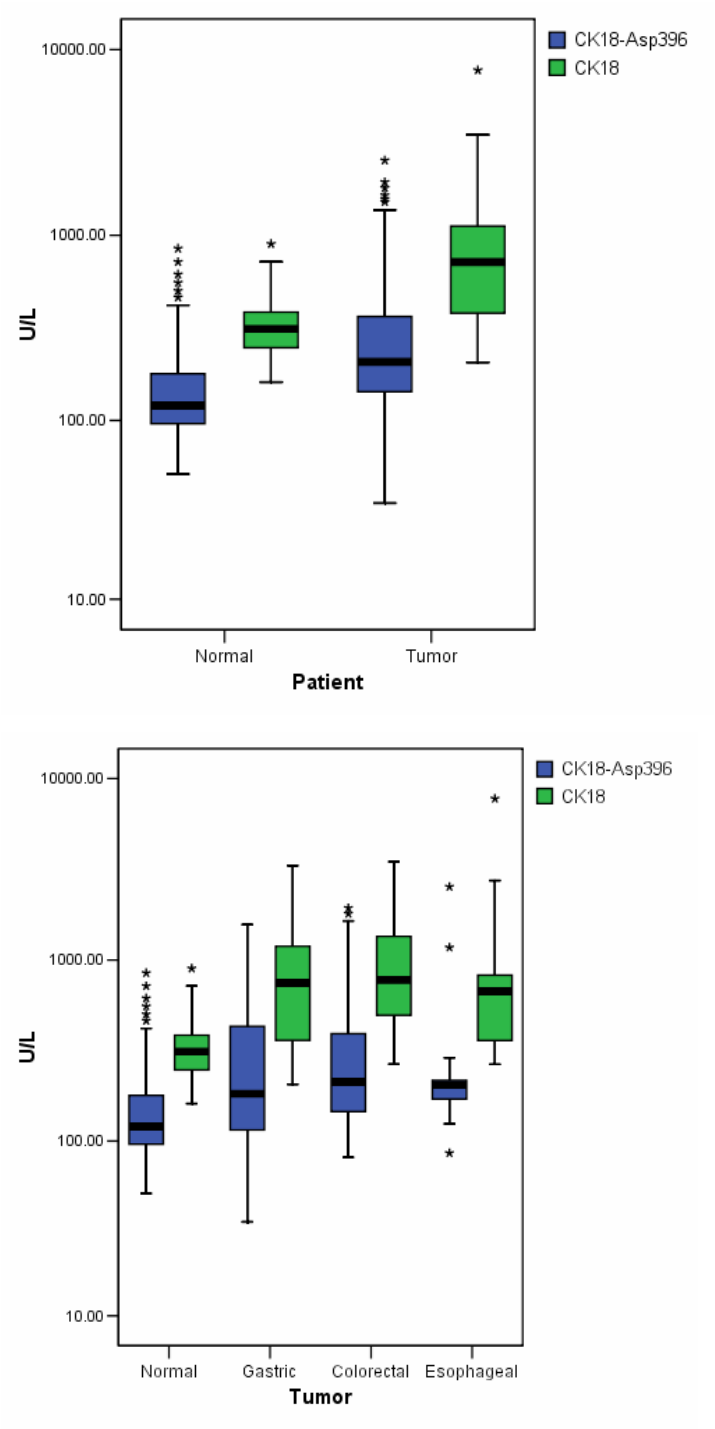


Figure 4.9 A & B. Plasma CK18-Asp396 (CK18-NE) and CK18 levels in patients versus healthy volunteers, and in different tumour types versus healthy volunteers. A, Box plot demonstrating significantly higher plasma CK18- NE and CK18 levels in patients with advanced gastrointestinal adenocarcinomas compared to healthy volunteers (p <0.001). B, Box plot demonstrating significantly higher baseline CK18- NE and CK18 plasma levels in patients with advanced oesophageal (p<0.001, for CK18- NE and CK18), colorectal (p<0.001 for CK18- NE and CK18) and gastric (p=0.015, p<0.001, for CK18- NE and CK18, respectively) cancer compared to healthy volunteers. U/L is defined according to the manufacturer's brochure where 1 unit equals 1.24pmol.

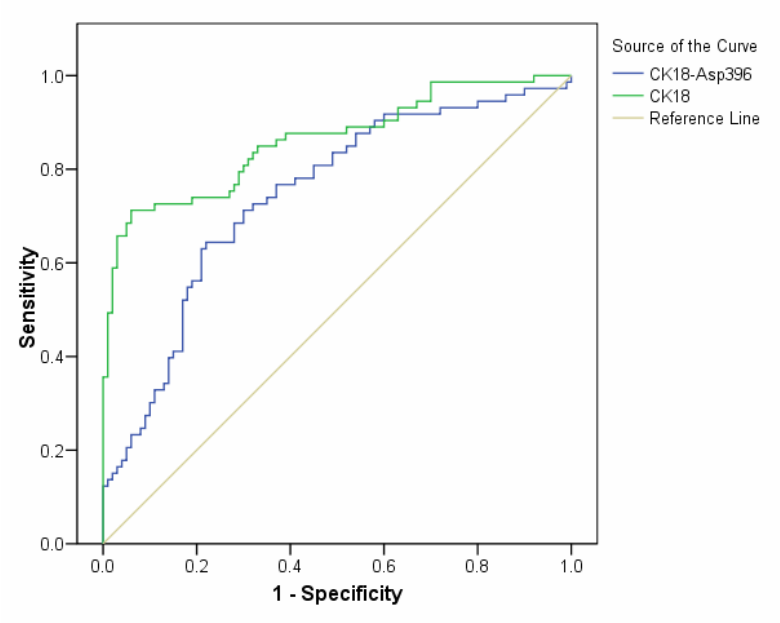


Figure 4.10. ROC curves for CK18-NE and CK18 distinguishing between patients with advanced gastrointestinal malignancy and healthy volunteers

	CK18-NE median value & range (U/L)	CK18 median value & range (U/L)	p-value for CK18-NE between tumour & healthy volunteer	p-value for CK18- between tumour & healthy volunteer
Healthy volunteers	**121 (51-849)**	**312 (161-899)**	**-**	**-**
Gastric cancer	**183 (35-1569)**	**746 (206-3313)**	**p=0.015**	**p<0.001**
Oesophageal cancer	**204 (86-2535)**	**672 (266-7747)**	**p<0.001**	**p<0.001**
Colorectal cancer	**213 (81-1936)**	**780 (267-3482)**	**p<0.001**	**p<0.001**

Table 4.6 Table summarising the median and range of CK18-NE and CK18 plasma levels in healthy volunteers and cancer patients

4.4.5 Plasma Cytokeratin 18 Levels in Advanced Gastrointestinal Cancer Patients receiving Palliative Chemotherapy

4.4.5.1 Analysis of Plasma Cytokeratin 18 at Baseline Prior to Chemotherapy

The plasma CK18 levels from the 73 patients with locally advanced or metastatic gastrointestinal adenocarcinomas receiving palliative chemotherapy were then analysed at various time-points throughout their chemotherapy treatment (see Materials and Methods section 2.2.2.2). Plasma was not available for all time points during treatment (70.5% of samples were from day 1 of each chemotherapy cycle); however, baseline pre-treatment plasma samples were collected from all patients undergoing chemotherapy.

Objective tumour response data was available for 68 out of the 73 patients, and included 16 patients with partial responses, 25 patients with stable disease and 25 patients with progressive disease. A further 2 patients had rapid clinical disease progression which occurred before radiological disease assessment and were deemed to have progressive disease. Five others were non-evaluable as 4 had missing scan data and 1 patient was receiving adjuvant treatment. Patients' case notes were also reviewed to document the timing and grade of epithelial toxicity observed during chemotherapy using standard clinical methods. This was then correlated with changes in plasma total CK18 and CK18-NE. As can be seen from just plotting out the values of CK18-NE and total CK18 at various time-points, patients with clinical responses tended to have higher peaks in plasma CK18-NE, total CK18 or both observed during treatment, than in patients with progressive disease for all tumour types (see Figure 4.11).

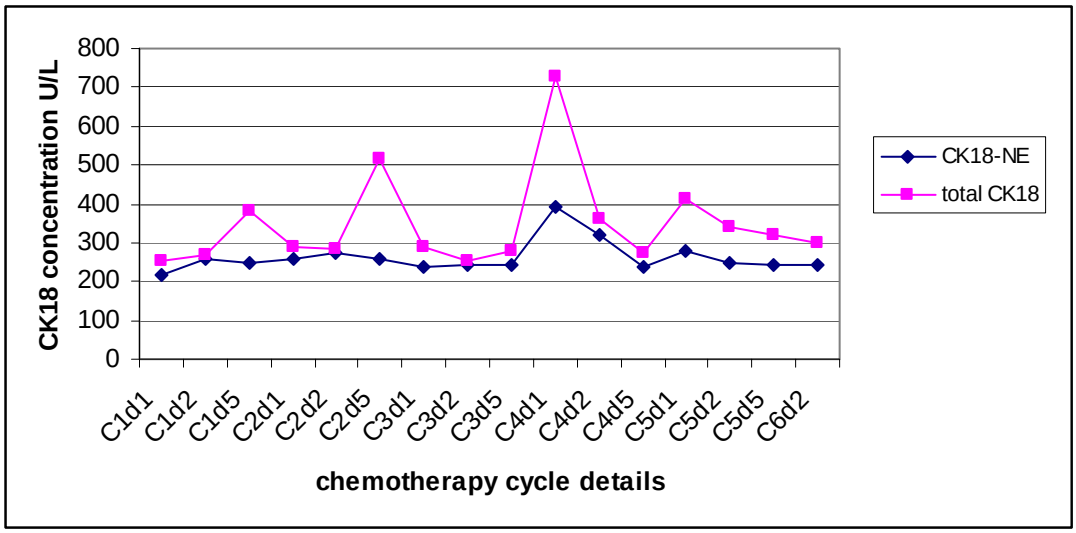


Figure 4.11 A. Patient with oesophageal cancer and partial response documented on CT scanning. 'C', corresponds to the chemotherapy cycle number and 'd' to the day within that cycle. This demonstrates a peak in plasma total CK18 at Cycle 4 day 1 of treatment**.**

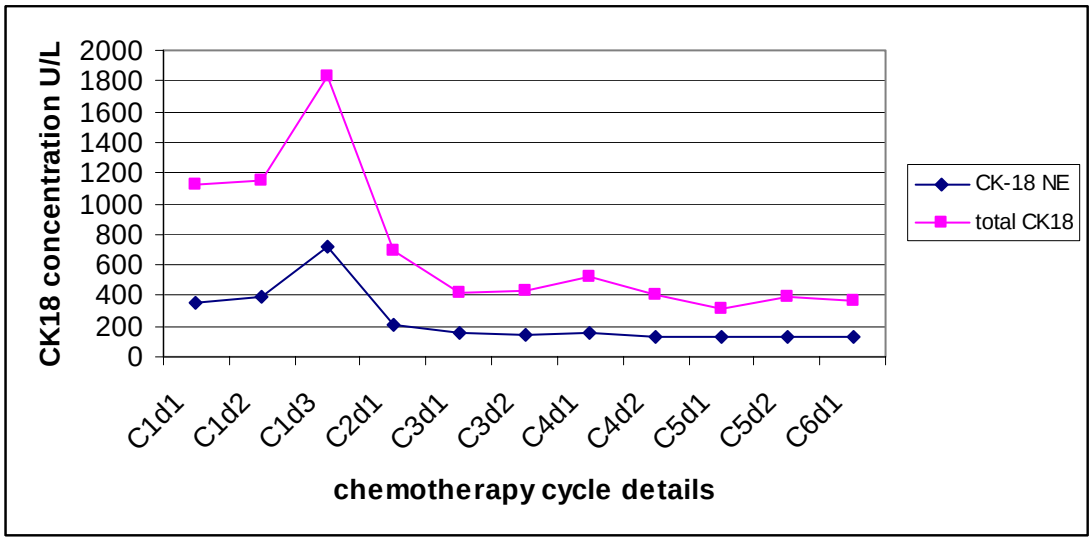


Figure 4.11 B. Patient with gastric cancer and partial response documented on CT scanning, C', corresponds to the chemotherapy cycle number and 'd' to the day within that cycle. This demonstrates peaks in both total CK18 and CK18-NE at cycle 1 day 3 of treatment.

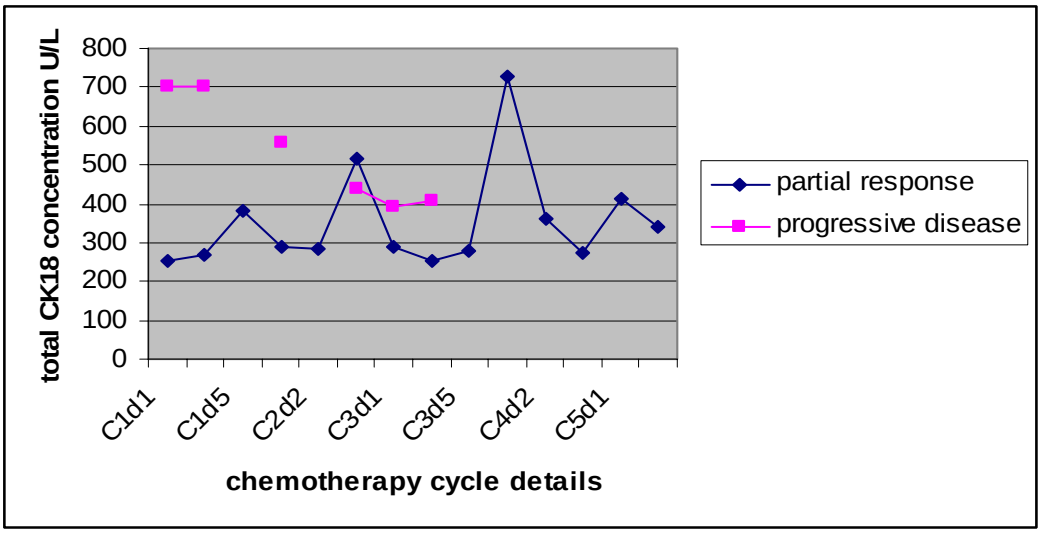


Figure 4.11 C. Figure demonstrating the variation in total CK18 in two patients with oesophageal cancer during palliative chemotherapy. Note the peak in CK18 in the patient whose disease responded to chemotherapy compared to the patient whose disease progressed during treatment.

Baseline plasma CK18-NE and total CK18 levels were significantly higher in the patients with metastatic disease compared with those with locally advanced disease (p=0.014 and p=0.011, respectively). Also, the CK18-NE and total CK18 median plasma levels at baseline were higher (289 U/L vs. 194 U/L for CK18-NE, and 1021 U/L vs. 618 U/L for total CK18) in patients who subsequently developed progressive disease during treatment (n=27) compared to patients who subsequently developed partial response or stable disease (n=41), although was only statistically significant for total CK18 (p=0.125, p=0.009, for CK18-NE and total CK18, respectively, (see Figure 4.12). There is an overall sensitivity of 22% at a specificity of 90% for CK18-NE and an overall sensitivity of 19% at a specificity of 90% for CK18 baseline plasma levels in distinguishing between patients who will subsequently progress through chemotherapy and those who will have partial response/stable disease with treatment.

Carcinoembryonic antigen (CEA) is produced within the normal developing fetus, but only in minute amounts by normal adult cells. It was first described in 1965 and has become the most widely used biomarker in gastrointestinal malignancy [60], but due to its lack of sensitivity in the early stages of disease it is unsuitable for population screening. Its main use is in the follow-up of patients after surgical resection with the aim of earlier detection of recurrence, and in monitoring responses to palliative chemotherapy in patients with advanced colorectal cancer [19] In 32 of the 36 patients with colorectal cancer, serial measurements of serum CEA were taken at each cycle of chemotherapy and the fall in serum CEA correlated with clinical outcomes. The results demonstrate that fall in CEA level was associated with response category (p=0.03) with the major difference between the patients achieving a partial response and those who developed progressive disease (p=0.008). Therefore, in patients with colorectal cancer baseline plasma total CK18 may give an earlier indication of which patients are more likely to respond to palliative chemotherapy than the crucial fall in serum CEA observed with each cycle of treatment.

Various patient demographic factors, including age, gender and baseline disease extent (either locally advanced or metastatic disease at commencement of chemotherapy) were then analysed with treatment outcome to chemotherapy, but no correlation was found [p=0.514, p=0.149, and p=0.934, for age, gender and disease extent, respectively]. Similarly, there was no significant association between baseline plasma CK18-NE and total CK18 levels and patient's age (p=0.345, p=0.112 for CK18-NE and total CK18, respectively) and gender (p=0.519, p=0.257 for CK18-NE and total CK18, respectively). In addition, a straightforward visual inspection of the data revealed no obvious association between the timing of epithelial toxicity experienced and the occurrence of peak values of

plasma total CK18 and CK18-NE, although the sampling for CK18 measurement was within the first 2 days of each cycle of treatment while epithelial toxicity tended to occur mid-way through treatment cycles, although epithelial damage at the cellular level in normal tissues may well occur at the same time as in the cancer but become clinically apparent later during the treatment cycle.

4.4.5.2 Analysis of Plasma Cytokeratin 18 Levels as a Pharmacodynamic Marker of Response

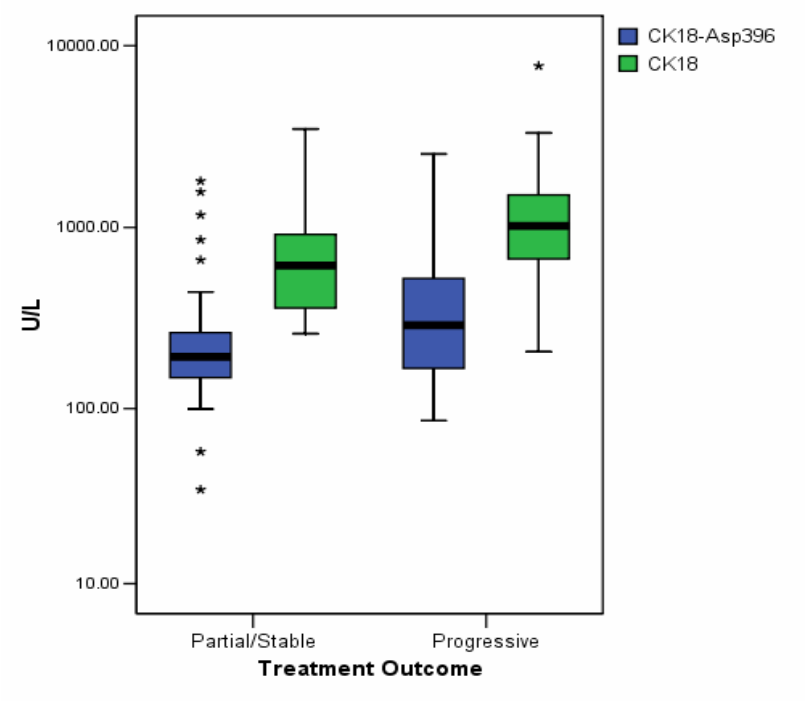
In order to evaluate plasma CK18 as a potential pharmacodynamic marker of response to palliative chemotherapy in patients with advanced gastrointestinal adenocarcinomas further analyses were performed on the data. The data were first examined to see if there was any correlation between patients who achieve a decrease in plasma CK18-NE and total CK18 between days 1 and 2 of the first cycle of chemotherapy and between day 1 levels of the first 2 cycles of treatment, and clinical outcome (partial response/disease stabilisation versus progressive disease), whilst accounting for the variability in the replicate CK18-NE and total CK18 levels (see Materials & Methods section 2.2.8.7). Sixty-two point five per cent of patients, who achieved a partial response with treatment, had a decrease in CK18-NE compared to 29.6% of patients who developed progressive disease and 48% of patients with stable disease. For plasma total CK18 the equivalent figures for patients achieving a decrease were 62.5% for partial responders, 52% for patients with stable disease and 44.4% for patients with progressive disease. There was no statistically significant association between response category and decrease in either CK18-NE or total CK18 plasma levels (p=0.051 and p=0.347, respectively), although the CK18-NE was verging on significance.

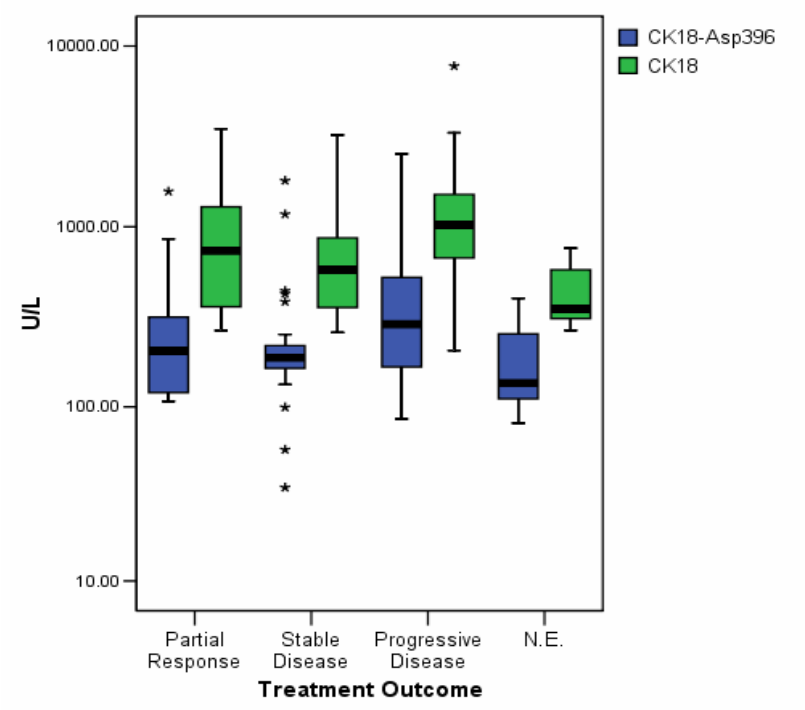
The plasma levels of CK18-NE and CK18 were examined for each patient and the maximum level (or peak level) observed during treatment, defined as the maximum level that had been observed for each patient during any cycle of treatment, was compared with tumour response. Peak levels of CK18 were found to be associated with tumour response, but peak levels of CK18-NE did not reach significance ($p = 0.01$, and $p = 0.07$, respectively (Figure 4.13 A & B).

Comparison of the different chemotherapy regimens (ECF and capecitabine) and tumour response is confounded by comparing responses between the two different tumour groups (upper gastrointestinal cancer and colorectal cancer). Chi-squared test analysis of the relationship between the chemotherapy regimens and response (partial response/stable

disease vs. progressive disease) found no relationship between chemotherapy regimen and tumour response. Also, from the analysis the association between baseline plasma CK18-NE, total CK18 and response does not appear to be greatly affected when the chemotherapy regimen is allowed for.

In summary, plasma levels of CK18 at baseline are significantly higher in patients with progressive disease compared to patients with partial response/stable disease (Figure 4.12), and peak plasma levels of CK18 observed during treatment are associated with treatment response. Differences in plasma CK18-NE and CK18 levels do not significantly associate with the patient's age, gender or the extent of disease at baseline.





Figures 4.12. Baseline plasma levels of CK18-NE and total CK18 correlated with treatment outcome. Box plot demonstrating baseline CK18-NE and total CK18 plasma levels in patients who developed progressive disease through chemotherapy compared to those who achieved a partial response/stable disease. The total CK18 level is significantly higher in patients with progressive disease (p=0.009, Mann-Whitney), [N.E. stands for non-evaluable treatment outcome].

4.4.5.3 Validation Study

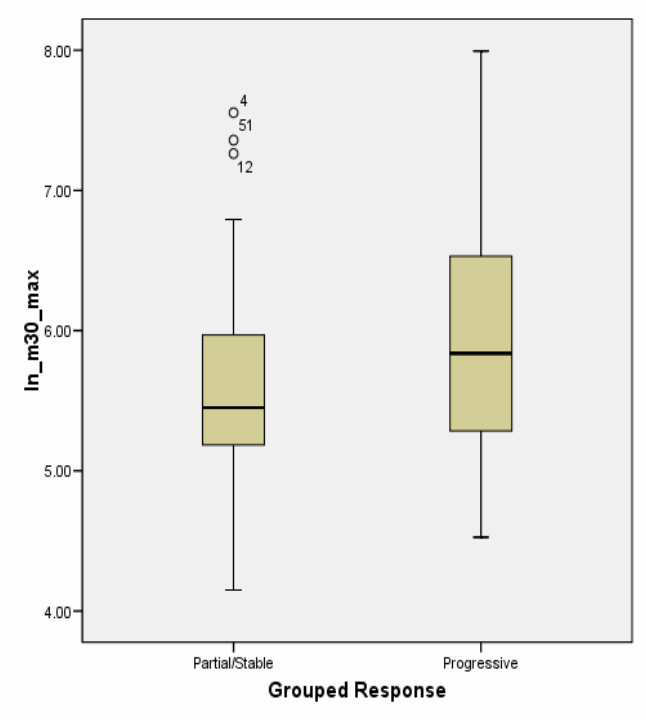
A validation study was then carried out to see if the baseline plasma levels of CK18-NE and total CK18 showed a reproducible correlation with treatment outcome in 53 patients with advanced gastrointestinal malignancy. This included 25 patients with colorectal cancer, 15 with gastric cancer and 13 with oesophageal cancer. Twenty-five of the patients with gastric or oesophageal adenocarcinoma were treated with combination chemotherapy comprising epirubicin, cisplatin, and 5-FU (ECF), 1 was treated with a combination of cisplatin and 5-FU (CF), 1 with carboplatin and 5-FU (CarboF) and 1 with epirubicin, carboplatin, and 5-FU (ECarboF). Fifteen of the patients with colorectal cancer were treated with xeloda (capecitabine) monotherapy, 2 with infusional 5-FU and folinic acid (modified "de Gramont" regimen), 5 with combination chemotherapy comprising capecitabine and oxaliplatin, 1 with oxaliplatin and 5-FU (FOLFOX) and 2 with capecitabine, oxaliplatin and cetuximab.

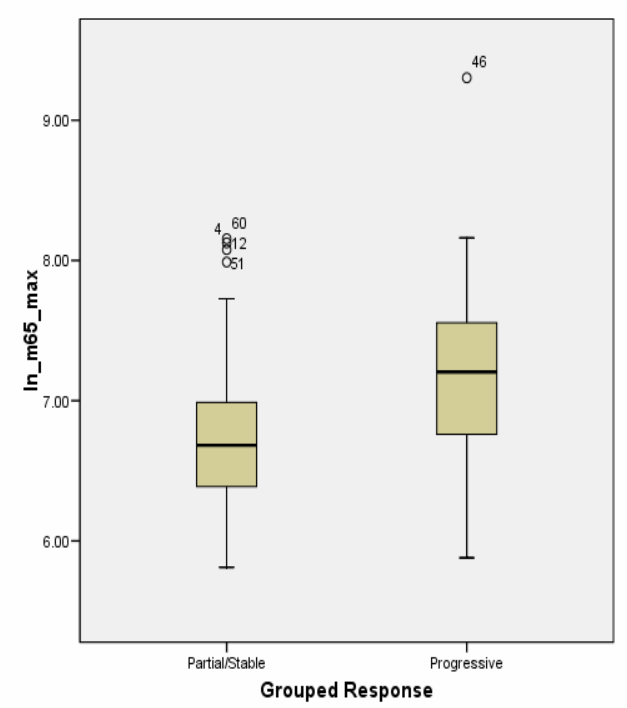
Objective tumour response data was available for all of the 53 patients, and included 8 patients with partial responses, 24 patients with stable disease and 12 patients with progressive disease. A further 9 patients had rapid clinical disease progression which occurred before radiological disease assessment and were deemed to have progressive disease (see Table 4.7).

The validation group showed overlapping range and similar median values for CK18-NE and CK18 as the original test set (median 158 U/L, range 56 – 18786 U/L for CK18-NE and median 660 U/L and range 235 – 19702 U/L for CK18). In the validation group, baseline plasma levels of both CK18-NE and CK18 were again significantly associated with treatment outcomes (partial response/stable disease vs. disease progression: $p = 0.028$, $p = 0.003$, respectively).

Table 4.7. Table summarising patient demographic data for the validation study. * XELOX = capecitabine/oxaliplatin, FOLFOX = oxaliplatin/5-fluorouracil, ECF = epirubicin/cisplatin/5-fluorouracil, CF = cisplatin/5-fluorouracil, CarboF = carboplatin/5-fluorouracil, ECarboF = epirubicin/carboplatin/5-fluorouracil

Characteristic		Number of patients
Age	Median – 70 years	53
	Range – 41-86 years	
Gender	Male	31
	Female	22
Primary Tumour Site – Colorectal		25
	Oesophageal	11
	Gastric	17
Disease Extent	Locally Advanced	14
	Metastatic	39
Chemotherapy	Capecitabine	15
	Modified de Gramont	2
	XELOX*	5
	FOLFOX*	1
	XELOX/Cetuximab*	2
	ECF*	26
	CF*	1
	CarboF*	1
	ECarboF	1
Clinical Outcome	Partial Response	8
	Stable Disease	24
	Disease progression	21





Figures 4.13 A & B. Peak plasma levels of CK18-NE (M30) and total CK18 (M65) correlated with clinical response (partial response/stable disease vs. progressive disease). Box plot demonstrating peak plasma levels of CK18-NE and total CK18 in patients who developed progressive disease through chemotherapy compared to those who achieved a partial response/stable disease. The peak CK18 level is significantly associated with response (p=0.01).

4.4.6 Conclusions

This is the first report, to our knowledge, documenting that the mean plasma CK18 level is high in patients with advanced gastrointestinal malignancy compared to healthy volunteers. The groups were not age-matched, but levels of CK18 do not correlate with age. It is of note that the range of both plasma CK18-NE and CK18 was wide in the healthy volunteers. Alcohol intake is known to increase caspase-cleaved CK18 values in serum as alcohol may cause apoptosis of liver cells [140]. Other studies have also shown that viral illness, chronic hepatitis and sepsis will increase levels of caspase-cleaved CK18 detected by the M30 Apoptosense ELISA [122, 123, 141]. Thus the wide variation in caspase-cleaved CK18 values in healthy volunteers could potentially be explained by intercurrent viral illness or alcohol consumption. A short health questionnaire had been completed by all the healthy volunteers participating in the study and details were recorded regarding alcohol intake. Using a cut-off of > or < 10 units of alcohol consumption per week, the effect of average weekly alcohol intake on CK18-NE and total CK18 plasma levels in the 100 volunteers was assessed using the Mann-Whitney test. There was no significant association found between alcohol consumption and either CK18-NE or total CK18 (p=0.905, p=0.980, for CK18-NE and total CK18, respectively). However, this may be due to under-reporting of alcohol intake on the health questionnaires.

The group of healthy volunteers was not followed up long-term and so it is unknown whether they subsequently developed any pathology to account for the variation in levels observed. As there is a significant overlap in plasma CK18-NE levels between the healthy volunteers and cancer patients it may be challenging to draw conclusions in individual cases.

After our sample collection from healthy volunteers had been completed, it was reported that elevated serum levels of caspase-cleaved CK18 may be an indicator of myocardial damage [128]. However, healthy volunteers were ineligible if they had significant past or current illnesses. Although it was feasible that the patients with gastrointestinal cancer could have developed myocardial damage during the course of the study, these chemotherapy regimens are used with caution in patients with significant cardiac co-morbidities and we believe that it is unlikely that myocardial damage accounts for the levels of plasma CK18 observed.

The results suggest that the plasma CK18 level prior to commencing chemotherapy may predict outcome with treatment in patients with advanced gastrointestinal adenocarcinoma.

The patients with comparatively higher baseline levels of plasma total CK18 tended to have higher levels of disease progression through chemotherapy compared to patients with lower baseline levels. This may be a reflection of the extent of disease present and potential for access to the circulatory system, as patients with metastatic disease had higher baseline levels of total CK18 and CK18-NE than those with locally advanced disease. However, when baseline disease extent as determined by CT scanning was correlated with treatment outcome a statistically significant association was not found. This suggests that baseline plasma CK18-NE and total CK18 may not just give an indication of tumour burden, but also the amount of cell death that is occurring, whether this is as a result of chemotherapy or part of the ongoing disease process.

In conclusion, the results from this study suggest that measuring baseline and peak plasma levels of CK18 in patients receiving palliative chemotherapy for advanced gastrointestinal malignancy may help predict individual outcomes to therapy. However, a larger prospective clinical study is required to validate these results.

4.5 Plasma Cytokeratin 18 in Ovarian Cancer

4.5.1 Background

As discussed previously, ovarian cancer is the fourth most common cancer in the UK, after breast, colorectal and lung cancer, and there is a need to develop detection methods to improve the sensitivity and specificity of early-stage ovarian cancer detection.

The SCOTROC (Scottish Randomised Trial in Ovarian Cancer) 1 trial was a randomized phase III study designed to compare efficacy, tolerability, and quality of life outcomes of docetaxel-carboplatin with paclitaxel-carboplatin as initial chemotherapy for stage Ic-IV ovarian and/or peritoneal cancers [74]. Between 1998 and 2000, 1077 women from 83 international centres were randomized to the study. Following debulking surgery, 539 were randomly assigned docetaxel-carboplatin, and 538 were assigned to the paclitaxel-carboplatin arm. The two treatment arms were well-matched with respect to patient demographics and disease characteristics. The results from the trial showed that the two treatment arms were equivalent in terms of progression-free survival and response. Blood samples were collected from patients after surgery, but prior to commencing chemotherapy (baseline samples) and at relapse (relapse samples). Plasma was separated according to protocol and stored at -70°C. CA-125 levels were also checked in all patients receiving chemotherapy and the CA-125 response assessed and classified.

The plasma samples had been stored at this centre and so were available for CK18 assessment. Initially the samples were separated into two separate cohorts, those that had been collected from patients in Glasgow and those that had been collected at other centres involved in the study. This was because sample handling had been stringent at the Glasgow sites due to the proximity to the trial centre, however, other samples had been sent some distance and it was felt that it had to be ensured that the median CK18-NE and total CK18 values did not vary greatly between the cohorts simply due to differences in sample handling.

The aims for this part of the study were:

1. To assess if there was a difference between baseline plasma levels of CK18 in patients with ovarian cancer and healthy volunteers, i.e. a diagnostic marker

2. To assess if plasma CK18 was a stable epitope that could be examined in samples from patients with ovarian cancer that had been subject to variations in sample handling, previous freeze-thaw cycles and prolonged storage at -70°C.

3. To assess if post-surgery, but pre-chemotherapy plasma levels of CK18 (baseline levels) in ovarian cancer patients could predict for clinical outcomes in terms of progression-free and overall survival, and also, CA-125 and radiological responses, i.e. a prognostic marker.

4. To assess if plasma CK18 levels at the time of first disease relapse in ovarian cancer correlated with clinical outcome.

4.5.2 Results

4.5.2.1. Comparison between Plasma Cytokeratin 18 levels in Healthy Volunteers and Baseline Levels in Patients with Ovarian Cancer

The baseline CK18 plasma levels were compared between the 170 patients with ovarian cancer and the 100 healthy volunteers. The results showed that the total CK18 plasma levels were significantly higher in the patients compared to the healthy volunteers ($p<0.001$) [see Figure 4.14], however, CK18-NE did not show a significant association ($p=0.418$).

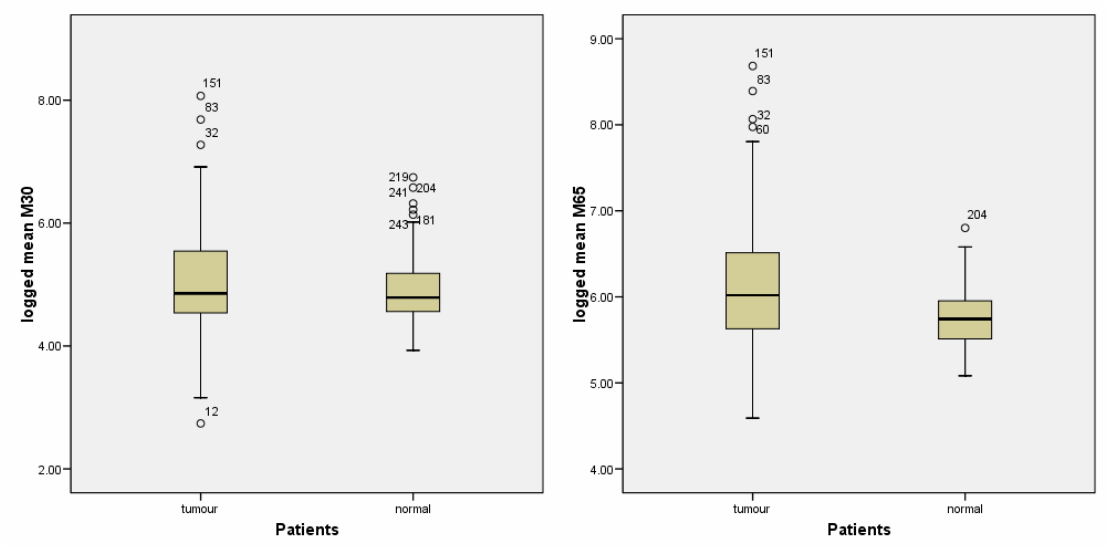


Figure 4.14. Box plots showing CK18-NE and total CK18 plasma levels between healthy volunteers and ovarian cancer patients. The total CK18 (M65) levels are significantly greater in patients compared to the healthy volunteers, $p<0.001$

4.5.2.2. Correlation between Baseline Plasma Cytokeratin 18 levels in Patients with Ovarian Cancer and Clinical Outcomes

There were 70 baseline plasma samples available within the Glasgow cohort for CK18 assessment. The results showed that although there was not a significant correlation between CK18-NE, total CK18 and progression-free survival (p=0.064 and p=0.34, respectively), there was a statistically significant association between baseline plasma CK18-NE, total CK18 and overall survival (p=0.046 and p=0.003, respectively). There was no association between baseline CK18 plasma levels and either CA-125 response or clinical/radiological responses.

The non-Glasgow cohort comprised 100 randomly selected plasma samples. The results for this cohort showed that there was a statistically significant association between baseline plasma CK18-NE, total CK18 and progression-free survival (p<0.001 for both CK18-NE and total CK18), and overall survival (p<0.001 for both CK18-NE and total CK18). This difference in association with progression-free survival within the two groups of patients was surprising, but one potential explanation is that the differences observed could be due to variances in sample handling between the two patient populations. As for the Glasgow cohort, there was no association between baseline CK18 plasma levels and either CA-125 response or clinical/radiological responses in this cohort. When the median values for CK18-NE and total CK18 between the two cohorts were compared there were no significant differences between them, and so for further statistical analyses the two cohorts were combined into one group of 170 patients.

On the basis of these results a multivariate Cox regression analysis was then carried out looking at baseline CK18-NE and total CK18 plasma levels in relation to residual disease after surgery, FIGO stage and ECOG performance status in comparison to overall survival. Residual disease was classified into three categories; none or microscopic, macroscopic <2cm, and macroscopic >2cm. The FIGO stages were classified into three groups; Ic and II, III, and IV. The ECOG performance status was also classified into three groups; 0, 1, and 2. The results showed that baseline plasma CK18-NE was not independently statistically significant (p=0.432), however residual disease after surgery and ECOG performance status were both significant (p=0.005, p=0.015, respectively) [see Table 4.8]. The results for total CK18 were along similar lines, as baseline plasma total CK18 was not independently statistically significant (p=0.083), however residual disease after surgery and ECOG performance status were both significant (p=0.017, p=0.014, respectively) [see Table 4.9]. The CK18 levels were then correlated with each of the factors studied in the

multivariate analysis using the Kruskall-Wallis test (see Materials & Methods section 2.2.8.7). Both the baseline CK18-NE and total CK18 plasma levels were significantly associated with residual disease ($p<0.001$), FIGO stage ($p=0.003$, $p<0.001$, for CK18-NE and total CK18, respectively), and the total CK18 plasma level was also significantly associated with performance status ($p=0.002$) [see Table 4.10, Figure 4.15]. There was no association between baseline plasma levels of either CK18-NE or total CK18, and histology of the primary ovarian tumour (serous, mucinous, clear cell, endometrioid, anaplastic, papillary and other) which may suggest that the plasma CK18 levels measured are a feature of tumour vascularisation and access to the peripheral circulation rather than the histological type.

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
CK18-NE (logged)	0.127	0.162	0.617	1	0.432	1.136	0.827	1.560
Residual disease after surgery	-	-	10.750	2	0.005	-	-	-
Residual disease after surgery (1)	-1.291	0.421	9.388	1	0.002	0.275	0.120	0.628
Residual disease after surgery (2)	-0.621	0.293	4.498	1	0.034	0.538	0.303	0.954
FIGO stage	-	-	1.884	2	0.390	-	-	-
FIGO stage (1)	-0.575	0.487	1.393	1	0.238	0.563	0.217	1.462
FIGO stage (2)	-0.378	0.313	1.455	1	0.228	0.685	0.371	1.266
ECOG performance status	-	-	8.361	2	0.015	-	-	-
ECOG performance status (1)	-0.208	0.420	0.244	1	0.621	0.813	0.357	1.851
ECOG performance status (2)	0.607	0.332	3.339	1	0.068	1.835	0.957	3.518

Table 4.8. Multivariate Cox Regression analysis of plasma CK18-NE and residual disease after surgery, FIGO stage and ECOG performance status in patients with ovarian cancer in comparison to overall survival. The results showed that baseline plasma CK18-NE was not independently statistically significant (p=0.432), however residual disease after surgery and ECOG performance status were both significant (p=0.005, p=0.015, respectively).

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Total CK18 (logged)	0.344	0.198	2.998	1	0.083	1.410	0.956	2.080
Residual disease after surgery	-	-	8.196	2	0.017	-	-	-
Residual disease after surgery (1)	-1.174	0.428	7.529	1	0.006	0.309	0.134	0.715
Residual disease after surgery (2)	-0.515	0.300	2.952	1	0.086	0.597	0.332	1.075
FIGO stage	-	-	0.636	2	0.727	-	-	-
FIGO stage (1)	-0.329	0.516	0.406	1	0.524	0.720	0.262	1.978
FIGO stage (2)	-0.245	0.325	0.565	1	0.452	0.783	0.414	1.481
ECOG performance status	-	-	8.526	2	0.014	-	-	-
ECOG performance status (1)	-0.122	0.424	0.083	1	0.773	0.885	0.386	2.030
ECOG performance status (2)	0.658	0.329	4.005	1	0.045	1.931	1.014	3.678

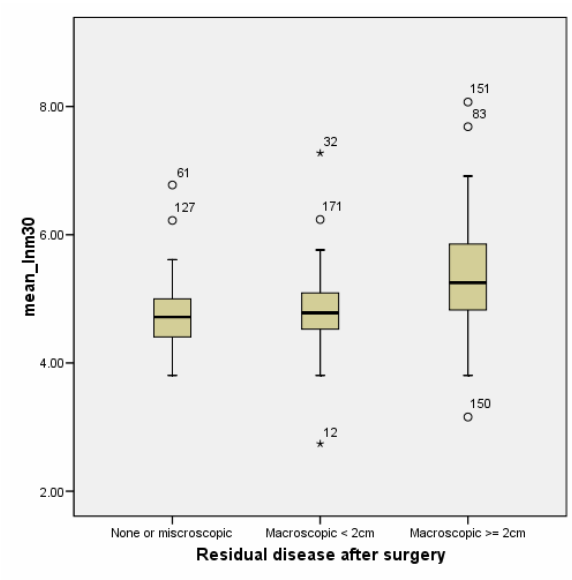
Table 4.9. Multivariate Cox Regression analysis of plasma total CK18 and residual disease after surgery, FIGO stage and ECOG performance status in patients with ovarian cancer in comparison to overall suvival. The results showed that total CK18 was not independently statistically significant (p=0.083), however residual disease after surgery and ECOG performance status were both significant (p=0.017, p=0.014, respectively).

		N	p-value
mean CK18-NE (logged)	**None or microscopic**	45	< 0.001
	Macroscopic <2cm	63	
	Macroscopic >= 2cm	62	
Mean total CK18 (logged)	**None or microscopic**	45	< 0.001
	Macroscopic <2cm	63	
	Macroscopic >= 2cm	63	

		N	p-value
mean CK18-NE (logged)	**Ic and II**	32	0.003
	III	114	
	IV	24	
Mean total CK18 (logged)	**Ic and II**	32	< 0.001
	III	114	
	IV	25	

		N	p-value
mean CK18-NE (logged)	**0**	53	0.168
	1	86	
	2	31	
Mean total CK18 (logged)	**0**	53	0.002
	1	86	
	2	31	

Table 4.10. Kruskal-Wallis Test for a) residual disease after surgery, b) FIGO stage, and c) ECOG performance status in patients with ovarian cancer. The CK18-NE and total CK18 plasma levels were then correlated with each of the factors looked at in the multivariate analysis. Both the baseline CK18-NE and total CK18 plasma levels were significantly associated with residual disease (p<0.001), FIGO stage (p=0.003, p<0.001, for CK18-NE and total CK18, respectively) and the total CK18 plasma level was also significantly associated with performance status (p=0.002).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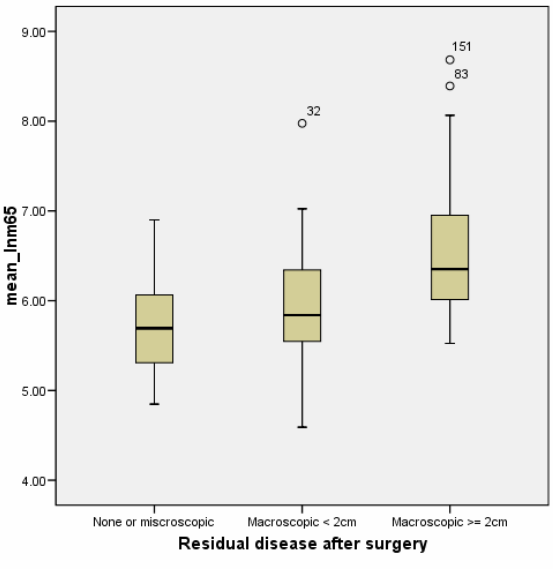
171
151
83
150
12
None or miscroscopic
Macroscopic < 2cm
Macroscopic >= 2cm
Residual disease after surgery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None or miscroscopic
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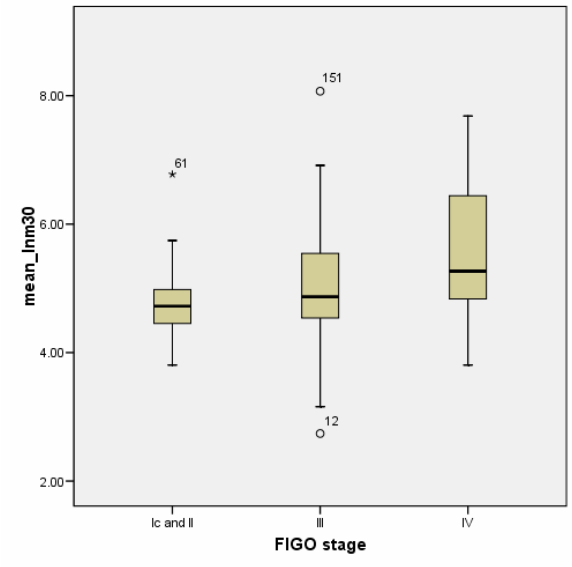
Residual disease after surgery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Ic and II
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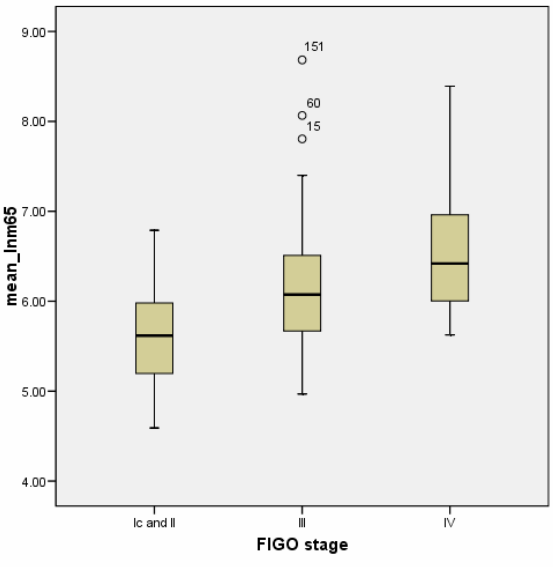
IV
FIGO stage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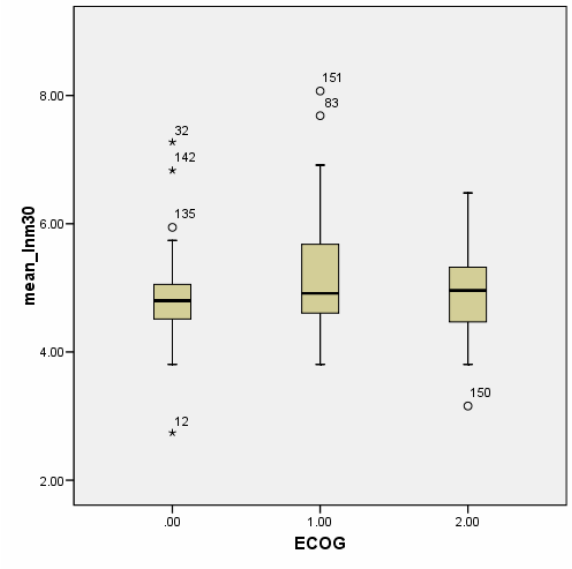
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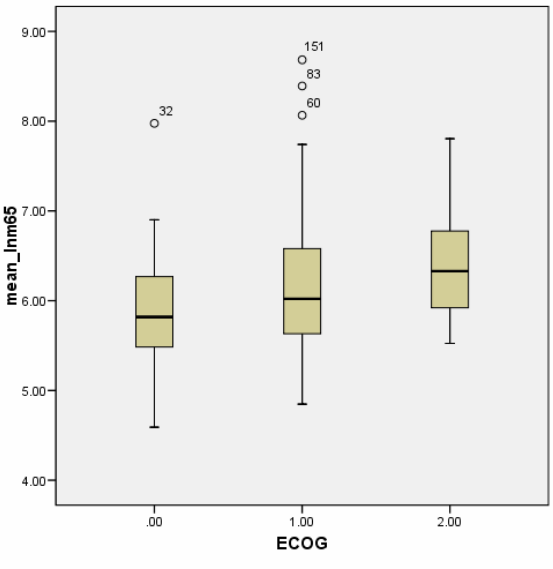
ECOG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ECOG

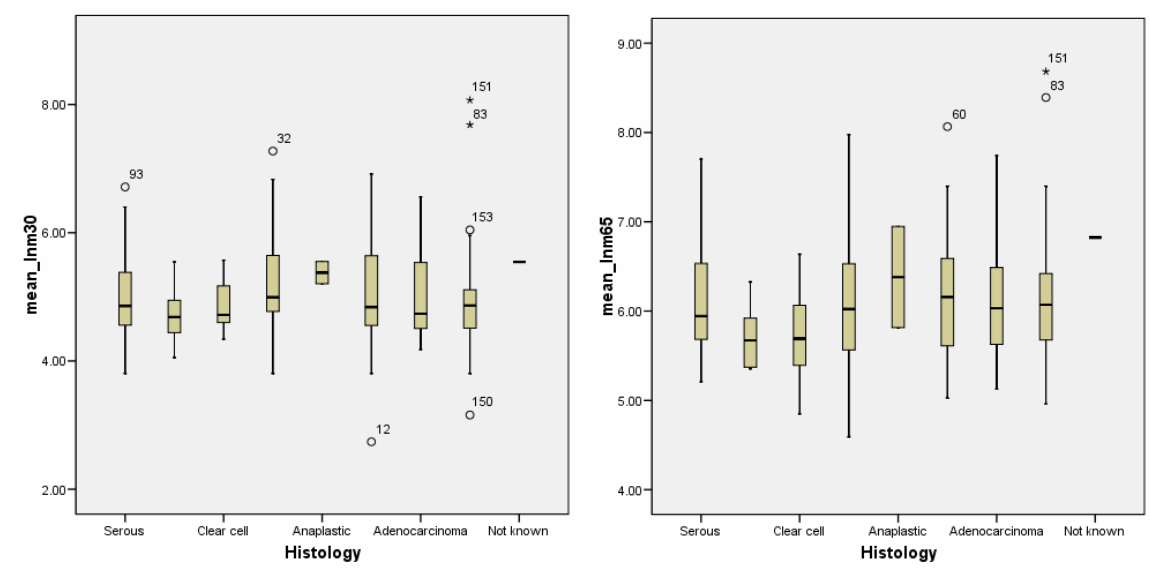

Figure 4.15. Box plots showing baseline CK18-NE and total CK18 plasma levels in relation to A) residual disease after surgery, B) FIGO stage, C) ECOG performance status, D) histology of primary ovarian tumour. The baseline CK18-NE (M30) and total CK18 (M65) plasma levels were significantly associated with residual disease (p<0.001), FIGO stage (p=0.003, p<0.001, for CK18-NE and total CK18, respectively) and the total CK18 plasma level was also significantly associated with ECOG performance status (p=0.002).

4.5.2.3. Correlation between Baseline and Relapse Plasma Cytokeratin 18 levels in Patients with Ovarian Cancer and Absolute Values of CA-125

It was then decided to look to see if there was any association between plasma CK18 levels and absolute values of CA-125 taken at baseline and at first relapse. This analysis was carried out as CA-125 is currently the most widely used biomarker in ovarian cancer to monitor treatment responses, early detection of recurrence, and also to assist in diagnosis, although it is not sufficiently sensitive or specific to be used alone or in screening programmes. Spearman's Rank Correlation was used to see if there was an association between baseline and relapse values of plasma CK18-NE and total CK18, and CA-125 (see Table 4.11).

The results showed that there was a statistically significant correlation between the baseline CK18 plasma levels and absolute CA-125 levels in the 169 patients with absolute CA-125 levels available ($p<0.001$ for CK18-NE and total CK18). They also showed a statistically significant correlation between the CK18 plasma levels and absolute CA-125 levels in 52 patients at first relapse ($p=0.013$, and $p=0.001$ for CK18-NE and total CK18, respectively).

	n	Correlation Coeff (ρ)	p-value
mean CK18-NE	169	0.323	< 0.001
mean total CK18	169	0.439	< 0.001

	n	Correlation Coeff (ρ)	p-value
mean CK18-NE	52	0.346	0.013
mean total CK18	52	0.452	0.001

Table 4.11. Spearman's Rank Correlation a) baseline plasma CK18-NE, total CK18 and CA-125, b) first relapse plasma CK18-NE, total CK18 and CA-125 in patients with ovarian cancer. The results showed that there was a statistically significant correlation between CK18 plasma levels and absolute CA-125 levels in the 169 patients at baseline (p<0.001 for CK18-NE and total CK18). They also showed a statistically significant correlation between the CK18 plasma levels and absolute CA-125 levels in 52 patients at first relapse (p=0.013, and p=0.001 for CK18-NE and total CK18, respectively).

4.5.2.4. Correlation between Plasma Cytokeratin 18 Levels at First Disease Relapse in Patients with Ovarian Cancer and Clinical Outcomes

Based on the results suggesting that there was a correlation between baseline plasma CK18-NE and total CK18 levels and survival outcomes, it was decided to look at matched baseline plasma CK18-NE and total CK18 levels with samples taken at first relapse. A small cohort of 54 matched relapse plasma samples was assessed initially to see if there was an association between the CK18 plasma levels at relapse and survival outcomes. To begin with, the CK18-NE and total CK18 levels at relapse were compared with the matched baseline samples to see if there was any significant difference between them, but this was not the case, as neither showed a statistically significant difference (p=0.968 and p=0.416, for CK18-NE and total CK18, respectively). When the relapse plasma levels were then correlated with survival outcomes, there was a statistically significant association between plasma CK18-NE, total CK18 and progression-free survival (p=0.019 and p=0.044, respectively). However, there was no association with overall survival (p=0.308 and p=0.995, respectively). As for the baseline sample cohort, there was no association between relapse CK18 plasma levels and either CA-125 response or clinical/radiological responses in this cohort.

On the basis of the above results, it was decided not to expand the relapse sample cohort any further as the results were not statistically significant and it was probable that the CK18 plasma levels would not come out as a significant independent factor on multivariate analysis.

4.5.3 Conclusions

In summary, the results from this part of the study are interesting as they show that plasma total CK18 is significantly higher in patients with ovarian cancer than healthy volunteers (p<0.001). When the baseline levels were correlated with survival outcome measures, both CK18-NE and plasma CK18 were significantly associated with overall survival within both cohorts (Glasgow and non-Glasgow) of patients studied, however within the larger non-Glasgow cohort of 100 patients, the baseline plasma levels of CK18-NE and total CK18 were also significantly associated with progression-free survival (p<0.001). The difference in the results between the two patient cohorts may potentially be due to sample handling differences. In a further small cohort of 54 Glasgow patients additional plasma samples taken at relapse showed a significant association between plasma CK18-NE, total

CK18 and progression-free survival (p=0.019, p=0.044, respectively), but not overall survival.

When a multivariate analysis was carried out, the baseline CK18-NE and total CK18 were significantly associated with residual disease after surgery and FIGO stage. The plasma CK18 levels were then studied with CA-125, and a significant correlation was found both with the baseline samples (p=<0.001, for both CK18-NE and total CK18) and the samples taken at relapse (p=0.013, p=0.001 for CK18-NE and total CK18 respectively). Taken with the results of the multivariate analysis this would tend to suggest that CK18 may be acting as an additional marker of tumour burden.

In conclusion, measuring plasma CK18 levels in patients with ovarian cancer may help to give some indication of who will have better survival outcomes. However, in this case, the plasma CK18 level may be a reflection of the disease burden.

Chapter 5 - Serum Biomarker Discovery using Proteomic Approaches

5.1 Background

The incidence of oesophageal cancer has increased over the past 30 years for reasons that are unclear. About 7,800 new cases are diagnosed in the UK each year, making it the ninth commonest cancer (Cancer Research UK National Statistics). It tends to affect more males than females. Two thirds of patients are diagnosed when their disease is locally advanced (inoperable) or metastatic. In these patients the prognosis is dismal with 5-year survival rates of 8%. At present population screening programmes are not carried out for oesophageal cancer, as this would entail an invasive procedure (endoscopy and biopsy) with inherent risks. So, it would be extremely useful if there was a simple blood test capable of detecting early oesophageal cancer that could be performed as an initial screening tool, which may then pinpoint which patients should proceed to endoscopy and biopsy.

Mass spectrometry (MS) is an analytical technique for determining the composition of samples or molecules. It can also be used to discover the composition of chemical compounds and peptides. The main benefit of using MS for potential biomarker discovery is that it is high-throughput and thus ideal for screening large numbers of clinical samples. MS/MS techniques which provide amino acid sequence data are becoming increasingly more widely used because of the unambiguous identification of proteins and the fact that they can give information about protein structure.

The general principle behind the technology involves ionising chemical compounds to generate charged species, which are accelerated along a vacuum tube. The three commonest ionisation methods used for analysis of proteins and peptides include electrospray ionisation (ESI), matrix-assisted laser desorption/ionisation (MALDI) and surface-enhanced laser desorption and ionisation (SELDI) [25]. All of these ionisation methods can detect within the dynamic range, which is required for the analysis of biological samples.

ESI creates an ion gas cloud in the source directly from the sample solution containing highly charged droplets. The droplets are then electrostatically driven through air, heat, solvents, nitrogen gas and other drying agents so that the surface charges are deposited onto the proteins and peptides. This process causes less fragmentation of molecules of interest than MALDI and SELDI, but is less suited to high-throughput applications.

MALDI and SELDI both involve spotting of samples onto a solid surface or probe, in the case of SELDI; this is called a ProteinChip (BioRad/Vermillion). MALDI requires samples to be mixed with an energy-absorbing matrix before being applied a passive stainless steel probe. The analyte is then embedded in a solid state matrix crystal on the probe. The energy absorbing matrix then converts laser energy to thermal energy which facilitates the desorption/ionisation process. SELDI uses ProteinChips that selectively bind different proteins and peptides of interest using a defined chemical chromographic characteristic (i.e. hydrophobic, ion exchange, or metal binding surfaces). Consequently in SELDI, the sample-presenting surface plays an active role in the extraction, presentation, structural modification, amplification and/or ionisation of a given sample.

In both MALDI and SELDI the sample is then put in a vacuum chamber and the crystal is hit with a laser, causing the proteins to desorb and ionise, producing ionised protein molecules in the gas phase. The ions are accelerated down a flight tube and a detector at the end of the tube records the time of flight. A deflector is also used to mask the peaks due to the matrix ions and reduce the risk of detector saturation. MALDI and SELDI are often coupled to time-of-flight (TOF) analyzers, which calculate mass from the time taken for ions to travel from the source to the detector when the same kinetic energy is applied, using the formula KE = 1/2 mv^2 (where $v = d/t$). Given the time of flight, the known length of the tube and the voltage applied (V); the mass-to-charge ratio (*m/*z value) of the protein can be derived. M/z values are directly related to the mass of the corresponding molecules (mass relation to charge is 1, therefore m/z equals 1).

The data obtained following analysis of the serum proteome is challenging to analyse due to the complexity and dynamic range. Bioinformatic tools can help with this, but the analysis of the serum proteome is reliant on a couple of presuppositions; first that the proteins or peptides shed into the serum, through either angiogenesis or invasion of surrounding tissues and vasculature, are representative of the organs they come from, and second that they are specific to the disease process affecting the organ.

The spectra generated using these approaches do not give details of the identity of the individual proteins or their abundance; rather they give a pattern that is associated with different organs and disease states. Mass spectra generated from a training set of samples are analysed by pattern-recognition algorithms to identify diagnostic signature patterns comprising a subset of key mass-to-charge (*m/*z) species and their relative intensities. Mass spectra from unknown samples are then subsequently classified by the similarity to the pattern found in the mass spectra used in the training set.

In choosing the type of sample to be studied using mass spectrometry approaches like SELDI, serum is obviously an attractive option as it is readily attainable with minimal discomfort to the patient. However, due to the large numbers of proteins and peptides that are expressed within it, rapid high resolution techniques are required to unravel the complexities and identify biomarkers that will be significant and reproducible when applied to larger patient populations. Also, serum contains proteolytic enzymes, which may make reproducibility of results difficult. The types of samples to be studied also depends in part on the disease process of interest; examining the proteomic profiles of lung and brain tissue sections from patients with non-small cell lung and brain cancer has been shown to provide prognostic information [33], and urine samples may also show differential proteomic profiles reflecting various disease states affecting the renal tract. One of the benefits of using urine is that it is not subject to the same degree of proteolysis as serum [34].

In choosing which approach to use for serum biomarker discovery, we decided to use SELDI-MS initially to determine if there was a difference in the serum proteome between patients with oesophageal adenocarcinoma and healthy volunteers, because when the project was conceived there had been several interesting studies published using SELDI as a platform for novel biomarker discovery, with some success in distinguishing patients with breast, lung, prostate and ovarian cancers from normal controls. There was no data published describing the use of SELDI in upper gastrointestinal cancers, and so we decided to see if we could discover novel biomarkers that would help aid earlier diagnosis in this particular patient group who have notoriously poor treatment outcomes, usually as a result of advanced disease at presentation. The attraction with SELDI was that as a high-throughput technology it would theoretically allow for rapid population screening using easily accessible material, such as serum or plasma.

There are limitations associated with the SELDI technique. Retrospective analysis of published studies has highlighted the fact that many of the supposed differences between sample groups could in fact be due to artefact (as discussed in the next section). Therefore, it is crucial to have quality assurance and control specifications, as well as detailed sample handling and processing protocols. The differentially expressed peptides are not identified; the samples are classified into sample groups on the basis of unique proteomic patterns, so it is especially important to ensure that any differences observed are real and not due to experimental artefact.

Also, the stringent procedures required to reduce the risk of experimental artefact may potentially make everyday use of SELDI in the clinical setting difficult. Therefore, we also decided to examine samples collected using alternative methods (gel-based technologies) as a way to validate the results from the SELDI, and to establish whether there were other markers of tumour response within serum samples that were easily detected using less stringent sample handling and preparation protocols than the SELDI and, therefore, may be more applicable to everyday clinical practice.

5.2 Studies using the SELDI-MS technique

SELDI technology has been used to determine patterns of protein expression in a number of different types of cancer including prostate [142], ovarian [143, 144], breast [146] [See Table 5.1], lung [147], pancreatic [149], colorectal [150], gastric [151], nasopharyngeal [152], and hepatocellular cancer [153] and also different biological fluids including serum [144], plasma [155], urine [156], human tears and nipple aspirate [158]. However, the majority of the studies evaluating protein expression in cancer have involved the use of serum or plasma. The theory behind this approach is that each organ and tissue is perfused by blood, which can add to, modify, or remove circulating proteins and peptides. Therefore, the serum proteome may reflect abnormalities or pathological states of organs and tissues. Non-malignant conditions have also been studied including Alzheimer's disease [159] and renal allograft rejection [34].

Early studies using SELDI looked at development of biomarkers in malignant disease. One study aimed to identify molecular markers associated with the pathologic progression of prostate cancer using laser capture microdissection and tissue proteomics [160]. Fifteen hundred samples including matched normal, prostatic intraepithelial neoplasia and frankly malignant cells were microdissected and analysed. The results showed that there was a specific and reproducible protein phenotype associated with each cell type and that the analysis would be feasible in the clinical setting.

An influential paper describing the use of serum proteomics to distinguish between pathological states in the clinical setting was published in *The Lancet* in 2002 [144]. The paper described the generation of serum proteomic patterns using SELDI mass spectrometry to find a unique signature that would differentiate women with ovarian cancer from benign disease controls. An initial training set was derived from 50 controls and 50 women affected with ovarian cancer. A unique proteomic pattern was then determined by an iterative searching algorithm and used to classify an independent masked

set. This method correctly identified all of the 50 cancer samples, including 18 early stage tumours, and 63 out of 66 control samples were also identified. However, there were concerns expressed by the group that the mass spectrometers used were low resolution and not designed for routine clinical use. Also, the results were not reproducible over time and tended to vary between machines [144].

In June 2003 doubts were first raised regarding the results of the Petricoin study when biostatisticians reanalysed a data set that had been posted on-line the previous year. Although they also identified many differences between the serum proteomic patterns of the normal controls and the patients with cancer, on re-analysis these seemed to be experimental artefact. Their major concern was that most of the differences observed were in proteins with *m/z* values of < 500, which may be less reliable as they tend to include values generated by matrix ions, experimental noise or errors in measurement [161].

The published results were also reanalysed by another group. They again found that the results were not reproducible, which was concerning as the reproducibility of the proteomic patterns is critical to the success of a potential screening programme, especially as this approach does not provide an explanation to support the diagnosis. They felt that the mass calibration and experimental protocols had varied across the experiments. Their conclusions were that many of the differences between the sample groups described in the original experiments could be due to sample processing artefact and not due to true changes within the serum proteome [162].

They produced guidelines to ensure reproducible results. Suggestions included using some form of baseline correlation (or subtraction) as the baselines of different spectra could be variable between different instruments, and also on the same instrument on different days (the baseline signal is due to a mixture of chemical noise from the matrix molecules and electronic noise and tends to be proportionally larger in the low *m/z* region). They also suggested that standard protocols should be drawn up to eliminate technological differences from being interpreted as biological differences. Careful experimental design was a necessity, e.g. by randomising the samples, to ensure that factors like differences in machine calibration, chip quality and variations in the reagents did not cause artefact. Also, the results should be carefully calibrated and revalidated after every change in protocol and samples tested using both versions of the protocol to ensure that the results are confirmed.

Following the paper published in the *Lancet*, Petricoin and his group further refined their methods [163]. They analysed a total of 248 serum samples from both healthy women and

preoperative samples from women diagnosed with epithelial ovarian cancer. The samples were prepared with a Biomek 2000 robotic liquid handler and a control reference sample was randomly applied to one spot on each array as a quality control for process integrity, sample preparation and mass spectrometry function. The samples were then analysed using a standard protocol on both a high-resolution hybrid quadropole time-of-flight mass spectrometer (QSTAR pulsar *I,* Applied Biosystems, Inc., Framingham, MA, USA) and the lower resolution Protein Biological System II time-of-flight mass spectrometer (PBS-II, Biorad/Vermillion).

The results showed that the mass spectra from the QqTOF MS led to proteomic patterns with a higher level of sensitivity and specificity than those from the PBS-II. The QqTOF MS showed 100% sensitivity and specificity in identifying samples from unaffected patients to those suffering from ovarian cancer. This included the correct identification of serum samples taken from all 18 stage I ovarian cancer patients. They excluded spectra from the analysis, which were felt to be of poor quality. Their conclusions were that high-resolution MS yielded superior classification patterns; the main source of error occurred during the acquisition of the mass spectra, which could be reduced by quality assurance and control specifications; and that the distinct proteomic patterns discovered required validation in a larger scale clinical trial.

Following the study by Petricoin and Liotta in 2002, a new proteomic test for early detection of ovarian cancer known as OvaCheck™ was developed. This test measures breakdown products of proteins in blood serum and looks for patterns that may indicate disease. However, several groups have reanalysed the data from the study and there are now doubts about the potential reliability of OvaCheck™. Professional bodies, such as the Society of Gynecologic Oncologists, feel that further clinical validation is required, before this test becomes available to the general public. OvaCheck™ is currently undergoing clinical trials in preparation for FDA regulatory review (Correlogic Systems www.correlogic.com).

MARKER	CANCER	SENSITIVITY	SPECIFICITY
PSA	Prostate	65%	35%
SELDI multi-marker profile	Prostate	83%	97%
CA15.3	Breast	23%	69%
SELDI multi-marker profile	Breast	93%	91%
CA-125	Ovarian	35%	98%
SELDI multi-marker profile	Ovarian	82%	92%

Table 5.1. Table summarizing sensitivity and specificity of the SELDI multi-marker profiles compared to the currently available tumour markers in prostate, breast and ovarian cancer (BioRad/Vermillion**)**.

5.2.1 Is identification of potential biomarkers crucial?

The confidence in a biomarker is greatly increased if it makes biological sense. It also allows results to be confirmed by other methods including enzyme-linked Immunosorbant Assay (ELISA) antibody depletion of antigen and Western Blotting. Identification of the biomarkers also gives an insight to the disease on a molecular level and may allow for mapping out of protein interaction pathways and possible development of treatments to a specific molecular target.

In a recent study serum proteomic expressions were analysed on 153 patients with invasive epithelial ovarian cancer, 42 with other ovarian cancers, 166 with benign pelvic masses and 142 healthy women [164]. The SELDI technique was used for protein expression profiling. Three potential biomarkers were discovered, two of which were down-regulated in the cancer group and one was up-regulated. The potential biomarkers were purified and identified; the down-regulated peptides were apolipoprotein A1 and a truncated form of transthyretin, and the up-regulated peptide was a cleavage fragment of inter-alpha-trypsin inhibitor heavy chain H4. The identified biomarkers were identified as acute phase reactants suggesting they are by-products of the host response to the tumour.

The discriminatory power of the markers was confirmed with samples from five centres through both cross- and independent validation. The biomarkers were then combined with CA-125 in a multivariate predictive model and were found to significantly improve on the sensitivity of CA-125 alone (74% vs. 65%) whilst maintaining a relatively high specificity (97%). Two of the markers were also evaluated using immunoassays and provided preliminary analysis of their tumour site specificity. The levels of apolipoprotein A1 were also studied in breast and colon cancer patients and were found to be unaffected; the levels of transthyretin were not altered in the serum of breast or prostate cancer patients. This study concluded that the identified biomarkers demonstrated the potential to improve the detection of early stage ovarian cancer.

A further study using SELDI-MS examined 94 urine samples, including samples from patients with transitional cell carcinoma (TCC) of the bladder, patients with urogenital disease and healthy donors. Multiple protein changes were reproducibly detected in the TCC group, including 5 potential biomarkers. One of these markers was also present in the cancer cells and identified as α-defensin. Defensins are a small family of peptides with antimicrobial, cytotoxic and anti-tumour activities [165, 166].When the protein clusters and potential biomarkers were combined in analysis, the sensitivity of detecting TCC was

87% with a specificity of 66%. Compared with voided urine or bladder-washing cytology, the combined analysis provided a sensitivity of 78% for detecting low-grade TCC versus 33% for cytology. The study concluded that this proteomic approach could potentially result in the development of a sensitive urinary TCC diagnostic test [156].

Patients with high-risk breast cancer, defined by standard prognostic factors, currently have a 30-50% chance of developing metastatic disease despite receiving adjuvant chemotherapy. Therefore, a study was performed looking at early post-operative serum from 81 women with high-risk breast cancer using SELDI-MS. The aim was to determine if proteomic profile differences could help predict relapse, thus allowing better tailoring of adjuvant treatment to individuals [167]. The results showed that there were protein peak differences that varied according to clinical outcome. This allowed a multi-protein model to be built which correctly predicted the outcome in 83% of the patients. The 5-year metastasis-free survival was 83% vs. 22% (p=0.0001) in the good prognosis vs. the poor prognosis group. Components of the multi-protein model were identified as haptoglobin, C3a complement fraction, transferrin, apolipoprotein C1 and apolipoprotein A1. The conclusion was that the post-operative serum protein pattern might have important prognostic value in high-risk early breast cancer, although these results have not been validated by a prospective trial.

Again the markers identified by this study were well-known host-response proteins part of a complex systemic response. This is not really surprising as the blood samples were taken in the early post-operative period, but these proteins may still have a role to play in tumour metastasis due to increased release of growth factors, angiogenesis and potential negative effects on immune surveillance. Haptoglobin is mainly synthesised by the liver and has been shown to be up regulated in solid tumours [168-170]. It has a role in angiogenesis, tissue remodelling and cell migration [171]. Transferrin signalling possibly has a role in increasing the metastatic potential of solid tumours and promoting angiogenesis [172, 173]. Post-translational modification of abundant host proteins may also occur by specific tumour enzyme processes.

A further study looking for novel biomarkers for breast cancer compared sera from 152 breast cancer patients and compared it with 129 healthy controls using SELDI-TOF MS [174]. There were ten peak clusters that were found to discriminate between the cases and controls. These clusters were identified as, inter-alpha-trypsin inhibitor heavy chain 4 fragments, a fibrinogen fragment and a tentative identification of C3a des-arginine anaphylatoxin.

Without the identification of the host-response proteins the understanding that the biomarkers were all components of this pathway would not have been appreciated. It also provides some evidence of a biological basis as to why the levels of these particular proteins should vary between patients with cancer and healthy volunteers.

5.3 Results

5.3.1 SELDI-MS analysis of human serum samples-pilot study

My aims in this part of the study were:

i. To perform a pilot study to discover if there were differences in the serum proteome between healthy volunteers and patients with oesophageal adenocarcinoma.

ii. If there were differences between the serum proteome to then perform a 30 healthy volunteers x 30 patients with oesophageal adenocarcinoma study to validate the results.

iii. To identify any potential biomarkers discovered.

Initially a pilot study was carried out comparing 8 serum samples from patients with metastatic oesophageal cancer and 8 healthy control samples. Serum was chosen rather than plasma as the clotting factors in plasma tend to interact with the sample binding surface. The sample collection procedure was stringent to try and reduce the risk of artefact (See Materials and Methods 2.2.2.4). All samples were processed by a standard operating procedure (SOP) and serum fractionation was carried out prior to sample analysis to reduce the likelihood that abundant proteins such as albumin would interfere with the binding of potential biomarkers. The serum fractionation was performed via anion exchange chromatography by stepwise pH gradient elution according to established protocol. Fractionation was carried out as more abundant proteins, like albumin, can block the binding of less abundant proteins (potential biomarkers) to the ProteinChip array. However, as potential biomarkers may be bound to albumin, both fractionated and unfractionated samples were examined. A 1-D gel was run and stained with Coomassie blue to validate the fractionation process (see Figures 5.1 . A & B).

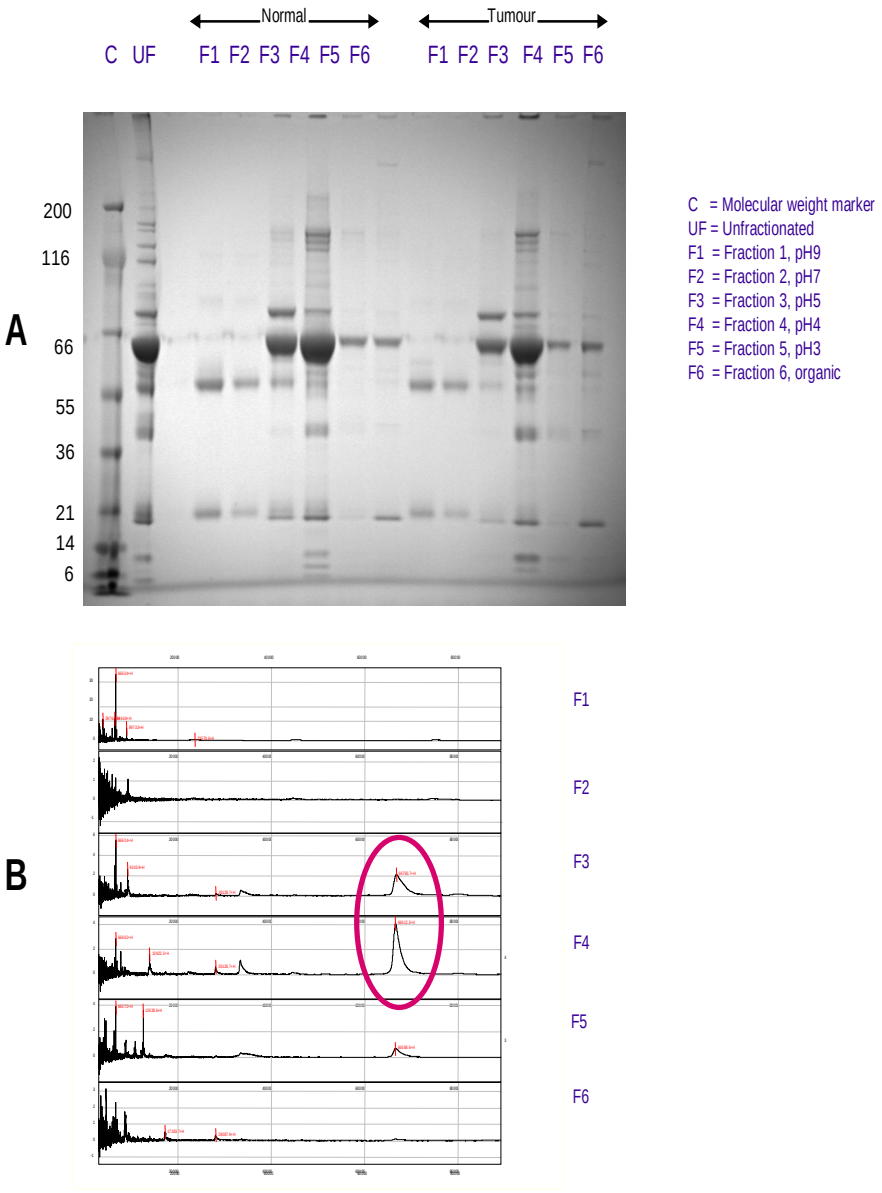

Figure 5.1. A) Gel stained with Coomassie blue demonstrating representative sample fraction of human serum**. B**) Mass spectra demonstrating sample fractionation with albumin peak in fractions 3 & 4 (characteristic peak at mass = 66kDa)

Optimisation experiments were then carried out with both fractionated and unfractionated samples on CM10 (cationic exchange surface) and IMAC30 (metal ion binding surface) ProteinChip arrays. These demonstrated capture of different protein subsets on the two different chip surfaces (see Figure 5.2).

The pilot study revealed that a novel biomarker *m/z* ~ 4 kDa was downregulated (p=0.012) in 6 out of the 8 oesophageal tumour samples in fraction 1, pH9, on the CM10 ProteinChip (see Figure 5.3).

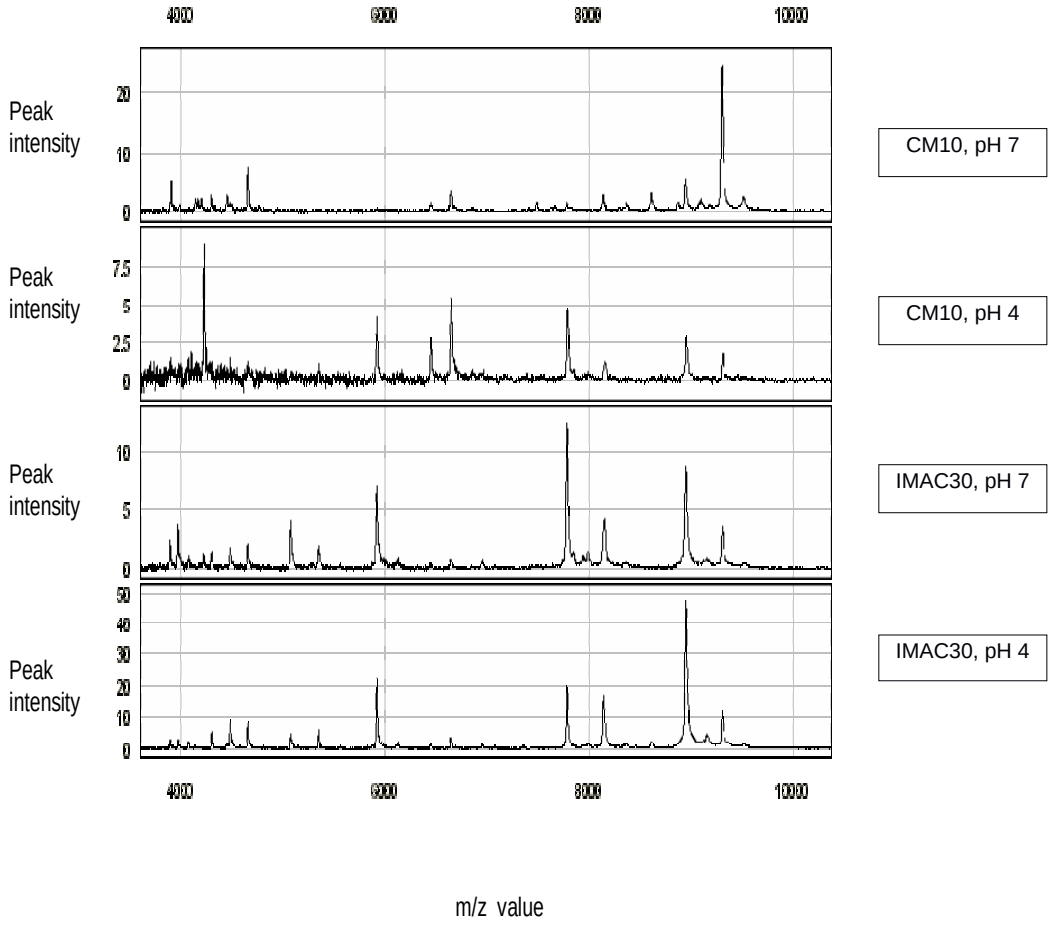

Figure 5.2. Comparison showing same sample on 2 different chip surfaces at different binding pH, different subsets of proteins captured.

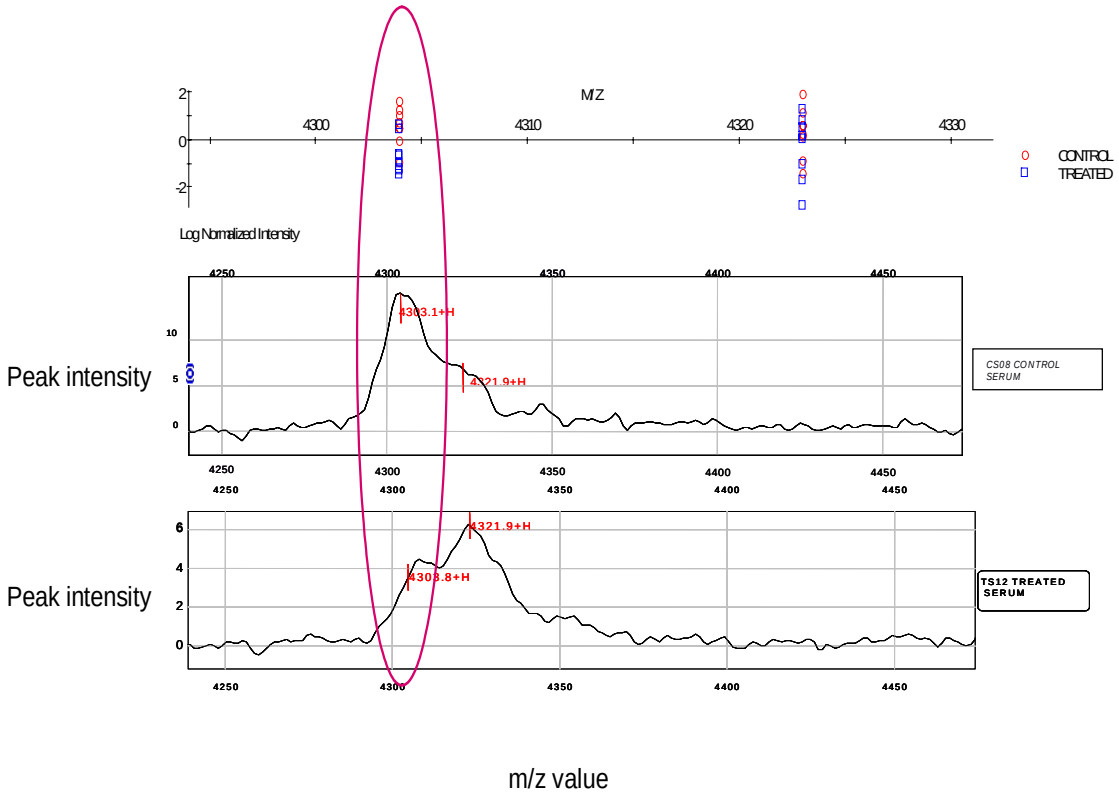

Figure 5.3. Representative spectra from the pilot study of 8 fractionated serum samples from patients with advanced oesophageal cancer and 8 healthy volunteers showing down-regulation of a ~4kDa peptide in 6 out of the 8 oesophageal cancer samples in fraction 1, pH9, on the CM10 ProteinChip surface (p=0.012).

5.3.2 SELDI-MS analysis of human serum samples-30x30 study

In order to further investigate the results from the pilot study, a further study was performed using the serum from 30 patients with advanced oesophageal cancer and 30 healthy volunteers. The serum samples were fractionated as previously described and fraction 1 was examined using both the IMAC and CM10 ProteinChip arrays according to the conditions established using the pilot study.

The results from this larger validation study also confirmed down-regulation of the ~4kDa protein/peptide within fraction 1 of the serum from patients with advanced oesophageal cancer compared to the healthy volunteers on the CM10 ProteinChip array ($p<0.0001$).

In an attempt to identify the potential biomarker, the unfractionated and fractionated serum samples were run on 1-D gels with the aim of obtaining a concentrated sample of the unknown protein of interest that could then be sent for further analysis (and identification) using mass spectrometry (MALDI-MS) or alternative methods (See Figure 5.5).

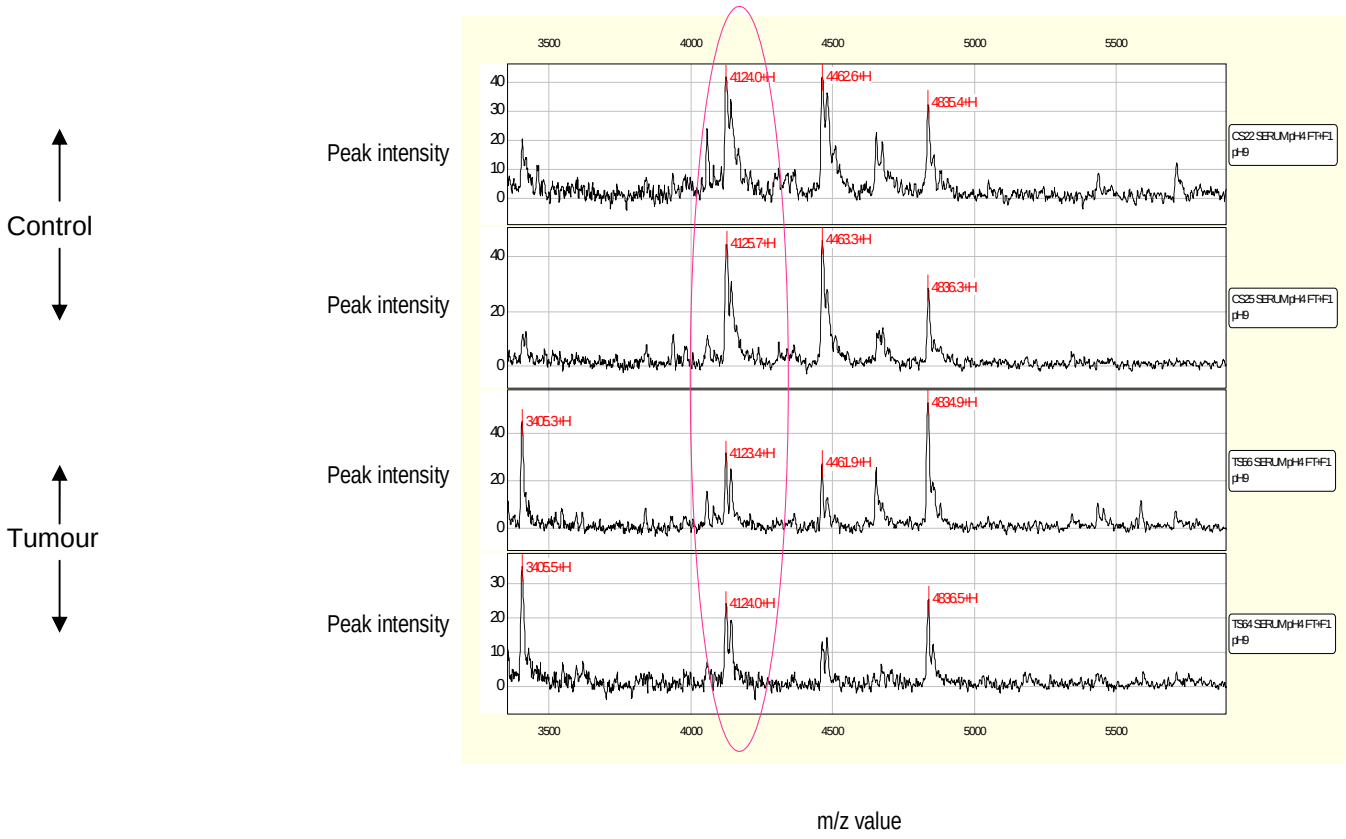

Figure 5.4. Representative spectra from the 30x30 healthy volunteers versus patients with advanced oesophageal cancer. This again confirms down-regulation of ~4kDa peptide in fraction 1, pH9, on the CM10 ProteinChip surface ($p<0.0001$).

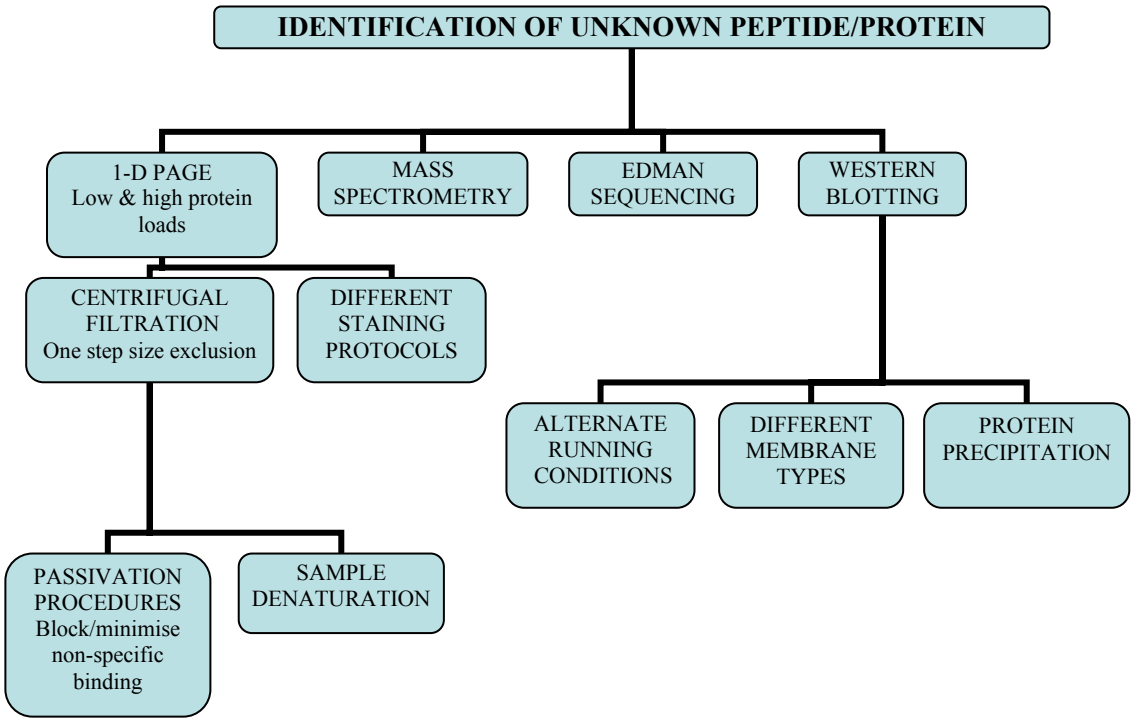

Figure 5.5. Flow chart summarising the various approaches attempted to identify the unknown peptide/protein down-regulated in the serum of patients with advanced oesophageal adenocarcinoma compared to the healthy volunteers. Low and high protein loads are loosely defined as 10 µL of fraction 1 and 50 µL of fraction 1**.**

5.4. Polyacrylamide Gel Electrophoresis (PAGE) on 16% acrylamide Tricine gels.

Polyacrylamide gel electrophoresis (PAGE) is a powerful and widely used method to evaluate complex mixtures of proteins and peptides. Therefore, we ran serum samples from the patients with advanced oesophageal cancer and the healthy volunteers on 1-D gels to see if the ~4kDa peptide of interest could be visualised, and that being the case excise the band to see if the protein/peptide could be identified. Tricine gels were used as these are more suitable for resolving low molecular weight proteins and peptides. The tricine system was developed by Schaegger and von Jagow in 1987. In this system tricine replaces glycine in the running buffer allowing more efficient stacking and destacking of low molecular weight proteins resulting in greater resolution of lower molecular weight peptides (Schaegger & von Jagow, 1987).

Initially 16% Tricine gels were run with 10µl of fraction 1 control/tumour serum and 5µl of unfractionated serum (See Materials and Methods 2.2.6). The gel was stained with Coomassie blue and demonstrated a band visible ~4kDa. The decision was then made to repeat the gel, but to filter the serum samples using a centrifugal filter (molecular weight cut off [MWCO] 10kDa, Microcon® Centrifugal Filter Devices) prior to electrophoresis, to see if this would improve further MS analysis by removing higher molecular weight proteins.

In order to prepare the filtered sample to run on the gel, 20µl of unfractionated control/tumour serum was added to the centrifugal filter. The samples were spun at 4°C, 14,000g for 60min. The filtrate was then removed and placed in an Eppendorf tube with 20µl of sample buffer. Ten µl of the filtered unfractionated buffered serum sample was loaded onto the gel and the gel run at the manufacturer's recommended conditions (see Materials & Methods 2.2.6.5). The gel was then stained with Coomassie blue. There were no bands visible in the filtered samples, but bands were visible in the unfiltered controls. The conclusions drawn were that either the filters were holding back the protein/peptides of interest or that the Coomassie wasn't sensitive enough to detect the minute amounts of protein (Coomassie sensitivity 20ng BSA). The plan was then to repeat this experiment using Sypro Ruby to stain the gel as its sensitivity is greater than that of Coomassie blue at 1-2ng of BSA. Other benefits with the Sypro ruby stain is that it stains a number of different classes of protein, and it is also compatible with subsequent analysis of proteins

using Edman-based sequencing and mass spectrometry. When the experiment was repeated, there were still no bands visible within the filtered samples. Therefore, the experiment was repeated using increased concentrations of protein (by loading larger volumes of serum to the centrifugal filter) and using the rapid fixation/staining step for Sypro Ruby stain as this is supposed to increase the sensitivity of detection to 0.25-1ng of BSA.

The filtration step was repeated, however this time, 40µl of both unfractionated serum and fraction 1 serum were added to the centrifugal filter (20ul had been previously used). For the fractionated samples 30µl of filtrate was added to 15µl of sample buffer and for the unfractionated samples 25µl of filtrate was added to 40µl of sample buffer (to make up a less concentrated sample because of very intense staining seen at higher concentrations). Ten µl of the fractionated samples and 5µl of the unfractionated samples (previously used 10µl, but the concentration was quite strong) was then loaded onto the gels which were run at the manufacturer's recommended conditions. Following the rapid fixation/staining with Sypro Ruby, there were bands ~4kDa visible in the unfiltered, but not the filtered samples. The conclusions drawn were that an alternative ultrafiltration protocol was needed as either aggregates were forming over the filtration membrane preventing filtration, or the ~4kDa protein/peptide was potentially bound to a larger protein and thus not passing through the filter.

The experiments were then repeated using unfiltered control/tumour fractionated and unfractionated samples, but increasing the protein loading onto the gel to see if this improved the detection. Fifteen µl of fraction 1 control/tumour serum and 10µl of unfractionated serum were, therefore, loaded onto the 16% tricine gels (10µl and 5µl respectively had previously been used). This was followed by the basic staining protocol using Sypro Ruby. This demonstrated bands clearly visible ~4kDa using a number of different control and tumour samples (See Figure 5.6). It was then decided to repeat this experiment using Coomassie blue staining, the rationale being that if the bands of interest were visible using Coomassie (despite its decreased protein sensitivity), then there should be sufficient protein within the samples for successful MS analysis. The repeat experiment did show bands ~4kDa, which were excised and sent for MS analysis (see Figure 5.7). This was repeated with several other gels and the gel pieces excised to ensure that there was sufficient material for analysis.

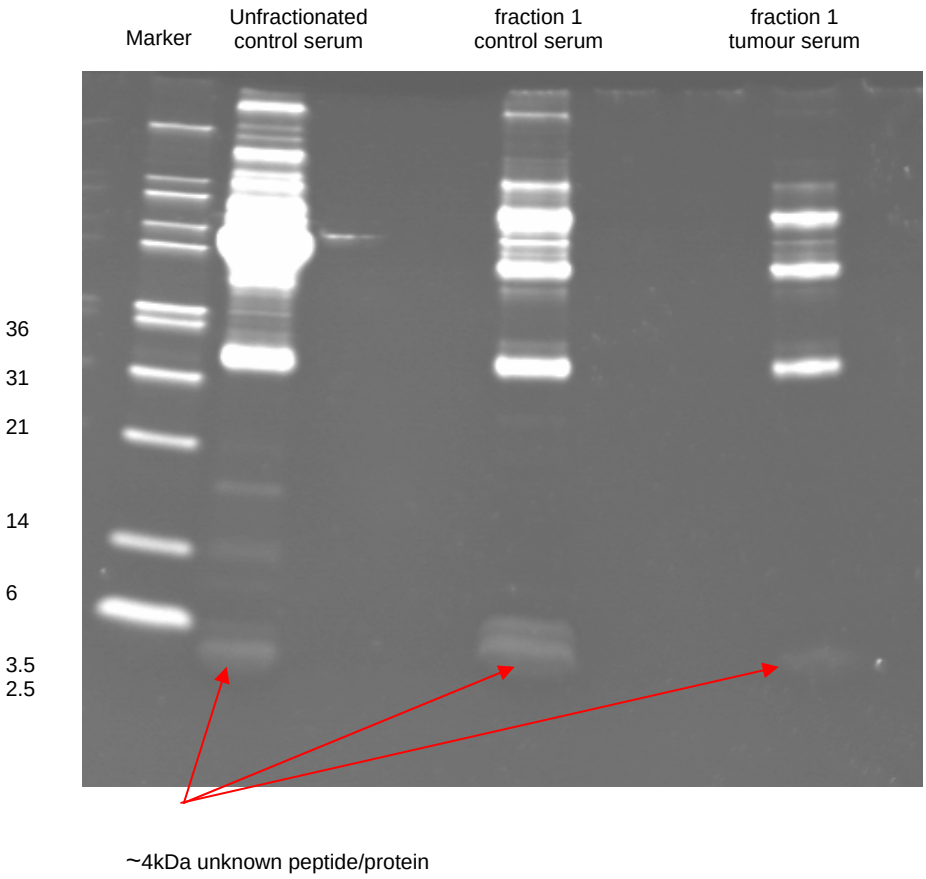

Figure 5.6. 16% Tricine gel stained with Sypro Ruby showing potential 4kDa biomarker in fraction 1 of the fractionated serum from the healthy volunteer and patient with oesophageal carcinoma.

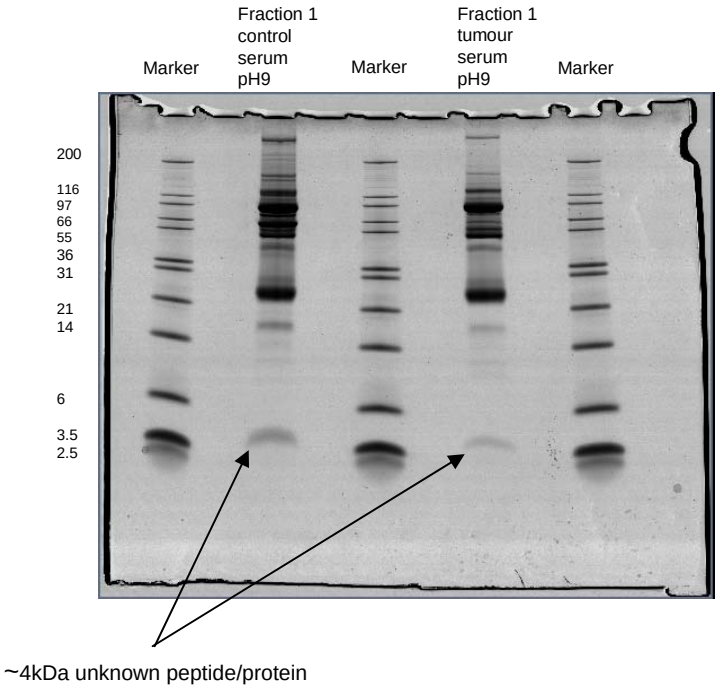

Figure 5.7. 16% Tricine gel stained with Coomassie blue showing potential 4kDa biomarker in fraction 1 of the fractionated serum from the healthy volunteer and patient with oesophageal carcinoma. Bands highlighted were excised and sent for further analysis.

5.4.1 MS analysis of excised gel pieces

The MS analysis was hindered due to a number of technical difficulties and although there was a peak demonstrated at 4.2kDa, the mascot search identified this as the trypsin used for the digest. One of the reasons this may have occurred was as a result of the reduction/alkylation step used to block potential cysteine residues prior to the in-gel digest, and so, the plan was to repeat the MS analysis, but to omit the reduction/alkylation step and use chymotrypsin instead of trypsin for the digest as it is less specific. A number of further gels were, therefore, run and the gel pieces excised and sent for MS analysis. Again identification was not possible. Further gel bands were provided, analysis from which showed only keratins and polymers, which were probably related to the plastics from the Eppendorf tubes reacting with the ethanol in the storage solution. The plan was then to repeat the analysis, but reduce the storage time for the samples prior to analysis. One analysis gave a potential identification as inter-alpha-trypsin inhibitor heavy chain H4 precursor, which is interesting as this has been identified in some other studies. However, the confidence of this potential identification was low and further samples were provided to try and confirm this.

It was then decided to re-run the gels, excise the bands of interest and send them to the proteomics department in Dundee, as they have a different (Orbitrap MS) mass spectrometry instrument. Unfortunately, Dundee was not able to identify the protein of interest either. The full results did show increased levels of Apoliprotein C-1 in the control samples compared to the tumour samples, but they advised that the protein concentration in the control samples was generally higher than that in the tumours anyway and this may explain the difference. Also, it was felt that the Apoliprotein C-1 was probably a fragment and not the full length precursor, and so confidence in this potential identification was low. The low confidence in potential identifications is an important problem with this type of study especially when the unknown peptides/proteins are of very low mass. This is because the lower the mass, the fewer residues are present to allow for a confident identification.

5.4.2 Edman sequencing

Due to the difficulties experienced in trying to identify the protein/peptide using MS analysis, a different approach was considered – Edman or protein sequencing. Edman degradation is a method of sequencing amino acids in a peptide where the amino-terminal residue is labelled and cleaved from the peptide without disrupting the peptide bonds between the other amino acid residues. Trifluoroacetic acid (TFA) is used to cleave the

first amino acid and leaves the new amino terminus available for the next degradation cycle. The cleaved amino acid is then selectively extracted into an organic solvent and treated with acid to form a more stable amino acid derivative. This is then transferred to a reverse phase C-18 column for detection by high pressure liquid chromatography (HPLC). The process is repeated sequentially to provide the N-terminal sequence of the protein/peptide.

The advantage of this technique is that it requires only 10-100 picomoles of peptide for the sequencing process. The disadvantages are that peptides being sequenced in this way cannot have more 30 residues (the process is able to accurately sequence up to 30 amino acids with about 98% accuracy per amino acid), and as the degradation proceeds from the N-terminus of the protein, it will not work if the N-terminal amino acid has been chemically modified or if it is concealed within the protein. In such cases, internal sequencing can be performed by sequencing the peptide fragments resulting from enzymatic or chemical digestion of a specific protein.

Sample purity is one of the most critical factors for successful sequencing. Samples should ideally contain only one protein or peptide component and be free of reagents that interfere with the degradation and sequencing process. Samples can be purified by techniques such as HPLC, and SDS-PAGE and Western Blot. SDS-PAGE and Western Blotting are especially useful if the protein of interest is a component of a complex mixture, such as serum, and so we used Western Blotting as a source of material. Assumptions that can be made when preparing samples using electrophoresis and electroblotting are that only 80% of the peptide of interest is actually concentrated into the band of the gel and only 50% of the protein transfers to the PDVF-membrane and remains there after staining and destaining (Max Planck Institute for Molecular Genetics).

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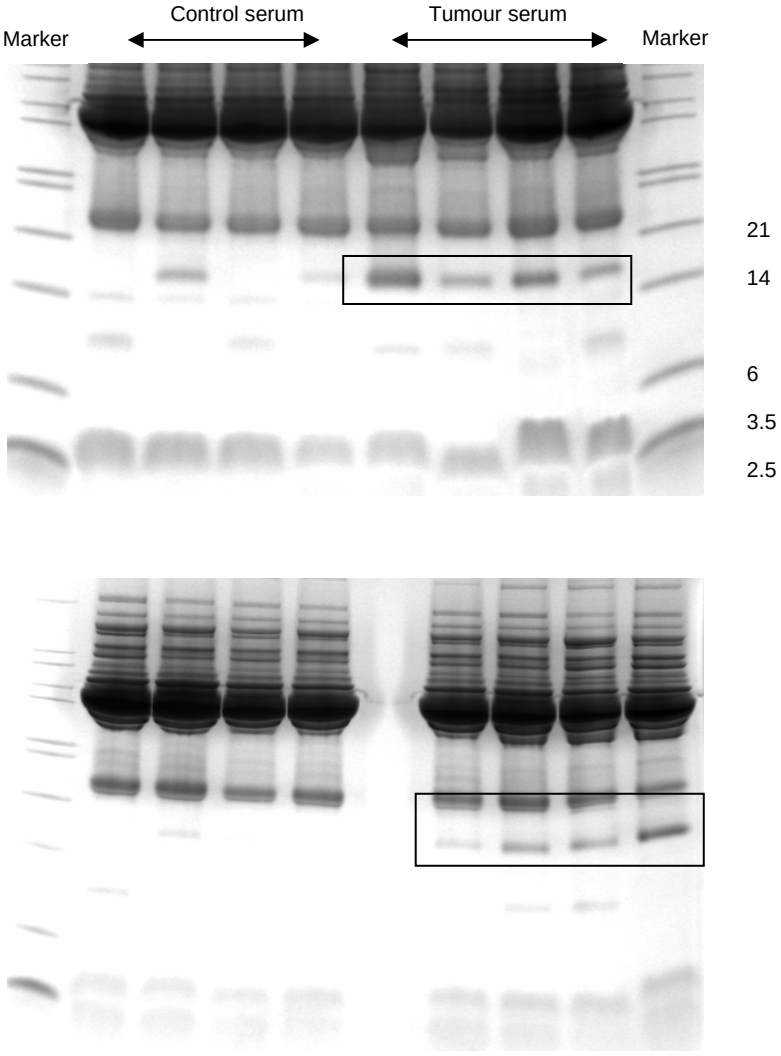

Figure 5.8. 16% Tricine gels stained with Coomassie blue showing greater abundance of protein ~17kDa within the oesophageal cancer serum samples compared to the healthy volunteers.

5.5 Western Blotting in preparation for Edman sequencing

To try and optimise the protein/peptide identification from Edman sequencing, a 16% Tricine gel was loaded with 15µl of fractionated control/tumour samples and run as before (124V for 90min). Tris-Glycine (1x) transfer buffer was prepared with 10% methanol and PVDF transfer membrane (0.45µm pore size) was used. A semi-dry Western Blot was performed, run at 200mA for 75min and the membrane stained with Coomassie blue for 1 minute and destained with 40% methanol/10% acetic acid. The plan was to excise the membrane piece containing the protein band of interest and send this to Dundee for Edman sequencing. However, the results showed that there were no bands visible on either the membrane or the gel at the 4kDa mark. The conclusion drawn was that the protein had passed straight through the membrane, probably due to its very low molecular weight.

The experiment was, therefore, repeated, but the protein concentration loaded onto the gel was increased and the transfer time decreased. A 16% Tricine gel was loaded with 30µl (previously 15µl) of fraction 1 control/tumour sample and the transfer time reduced to 45min (from 75min). Again following staining the band was not visible on both the gel and the membrane. The conclusion was that the pore size on the membrane was too large and that further blotting should be performed with 0.2µm PVDF membrane.

We, therefore, performed western blotting using immobilon-PSQ 0.2µm PVDF transfer membrane to see if the smaller pore size improved the protein transfer. The tricine gel was loaded with 30ul of fraction 1 control/tumour sample with a transfer time of 15min (reduced from 45min) using tris-glycine transfer buffer. The results showed that the band remained on the gel indicating that the transfer time was not long enough, and so the experiment was repeated with an increased transfer time of 45min. The results showed that the insulin control had transferred to the membrane, but the protein of interest had not. When the gel was stained there were still faint bands present ~4kDa in the samples and insulin control indicating that the protein of interest had not transferred efficiently between the gel and the membrane.

The experiment was, therefore, repeated again, however the amount of methanol in the transfer buffer was increased to 20% from 10%, the membrane and gel were both soaked in transfer buffer for 10min prior to blotting and the transfer time was increased from 75 to 90min. Unfortunately, again the results showed that the protein of interest had not

transferred and in fact there seems to be very inefficient transfer of all proteins between the gel and membrane using this method.

We then tried changing the transfer buffer to Nupage transfer buffer (20% methanol) from tris-glycine transfer buffer with the hope that this would improve transfer, and also reduce interference if protein sequencing was attempted. The gel and membrane were soaked in transfer solution for 15min with agitation prior to blotting and the transfer time was increased to 2hr. However, the transfer was much less efficient than that using the tris-glycine transfer buffer with a lot of protein (including the peptide of interest) left on the gel following staining.

We, therefore, increased the concentration of fraction 1 sample:sample buffer (30µl:10µl) and loaded all of this onto the gel to try and get the maximum possible concentration of the protein of interest; the gel time was to be decreased from 90min to 45min, to see if this concentrated the ~4kDa protein over a smaller area; and the transfer time was increased to 2hr. The results showed that the 4kDa band had disappeared from the gel, but was not present on the membrane. A possible explanation for this is that the Commassie stain was not sensitive enough to detect the low concentrations of protein. Therefore, the plan was to repeat the experiment, but this time stain the membrane with Sypro Ruby Protein Blot (which is compatible with MS analysis and Edman sequencing). However, staining with the Sypro Ruby confirmed that the protein of interest had not transferred to the membrane.

The other option was to precipitate the protein from the fraction 1 serum samples to try and maximise the concentration of protein loaded onto the gel and then repeat the transfer (See Materials & Methods 2.2.6.4). Fifty µl of the precipitated protein sample was then run on a 16% Tricine gel for 90min, which was then stained with Coomassie blue. The results showed that the precipitation step did not improve recovery of the protein of interest and suggested that the peptide may be soluble in 70% ethanol.

If this precipitation step had worked, then another option may have been to collect all the fraction 1 filtrations performed using the centrifugal filter together and try to precipitate the protein from this as theoretically this should contain a pure, concentrated sample of the protein ~4kDa, with all the more abundant proteins of >10kDa removed, however, unfortunately this was not the case.

It was then decided to re-visit the centrifugal filtration as it was felt if this was successful it would provide a sample that could be used for both MS analysis and Edman sequencing. It

was decided to use a denaturing agent in case the ~4kDa protein was bound to a larger protein and that this was preventing filtration. Two M urea was initially selected as a denaturing agent as it would not interfere with MS analysis. One hundred µl of fraction 1 control/tumour sample was added to the centrifugal filter (molecular weight cut off 10kDa) with 25µl of 2M urea. The samples were spun at 24°C, 14,000g for 25min. The retentate was collected by spinning at 1,000g for 3min. The filtrate sample was prepared by adding 30µl of filtrate to 30µl of sample buffer; 40µl of sample buffer was added to the retentate. Thirty µl of buffered filtrate and 15µl of buffered retentate were then loaded onto a 16% Tricine gel. The results from the gel showed that the band of interest was not present in the filtrate but still lay within the retentate.

This experiment was then repeated with 8M urea and 10% acetonitrile as denaturing agents. Again the gel showed that the band of interest remained within the retentate. A possible reason was that the protein of interest may potentially be linked by disulphide bonds preventing filtration. It was, therefore decided to add 100mM dithiothreitol (DTT) to the 8M urea as a denaturing agent. The gel showed a faint suggestion of a band ~4kDa in the filtrate sample, but this was not conclusive. So, 1% NP40 was added to 100mM DDT, in case the protein was very hydrophobic and this was preventing passage through the centrifugal filter. Again the gel showed only a very faint band ~4kDa in the filtrate sample, which was not a high enough concentration to send for either MS or Edman sequencing (Figure 5.9).

Due to the difficulties experienced with the centrifugal filtration process, it was decided to prepare samples of insulin and BSA to act as positive and negative controls to check whether the filters were working correctly. The sample was also prepared with and without heating (to 85°C) to see if this resulted in protein aggregation and thus difficulties in the filtration process. The results showed that the centrifugal filter was holding back the BSA within the retentate as expected, and that heating the sample didn't appear to result in protein aggregation. The results for the insulin control showed that although the insulin was present in the filtrate and not the retentate i.e. the centrifugal filter was allowing it to pass through, the concentration of protein appeared much lower than for the non-filtered controls indicating the filter was preventing passage of most of the insulin through into the filtrate and that there must be some binding to the filter membrane (Figure 5.10).

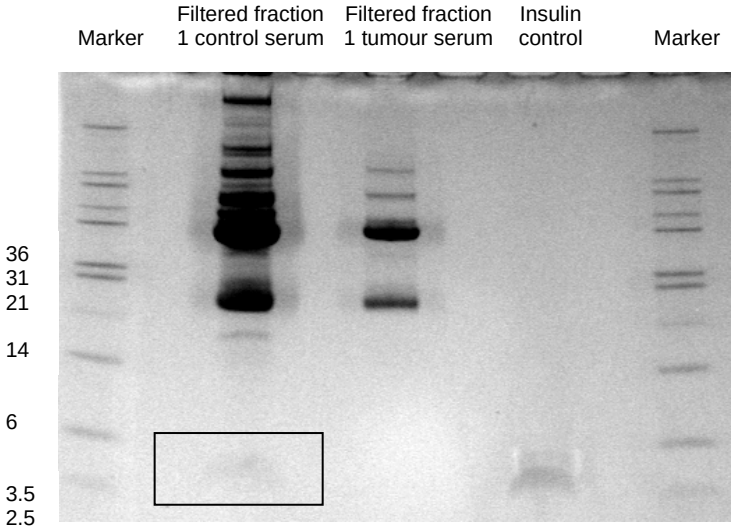

Figure 5.9.16% Tricine gel stained with Coomassie blue. Centrifugal filters (molecular weight cut-off 10kDa) underwent passivation process with 1% BSA and 5% Tween. Fractionated serum samples were denatured with NP40 and DTT, filtered and run on the gel using standard conditions. Note the faint band present at ~4kDa in fraction 1 of the healthy volunteer serum. Insulin MW 5 kDa served as a positive control.

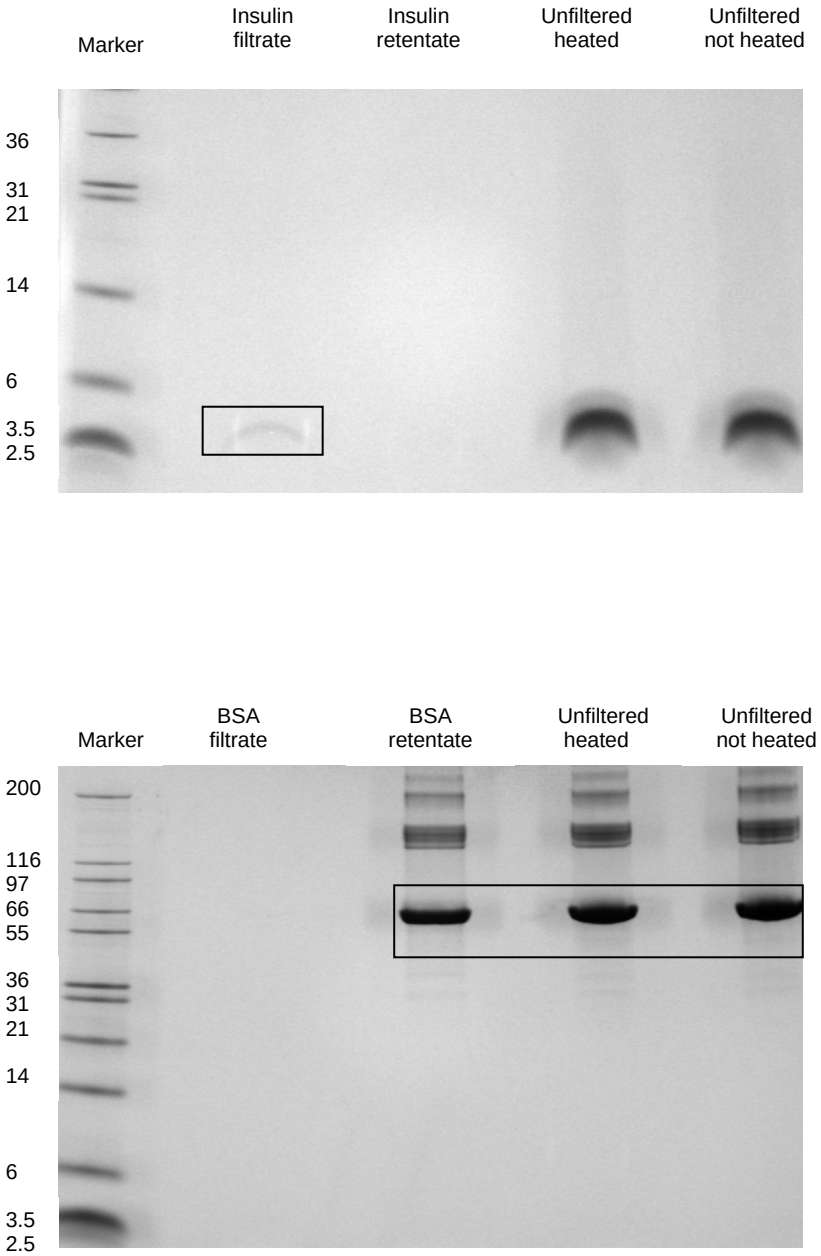

Figure 5.10. 16% Tricine gels stained with Coomassie blue demonstrating the results of the control experiments using insulin and BSA (MW 66kDa) to check the function of the centrifugal filters with a molecular weight cut-off of 10, 000Da. Insulin is present within the filtrate and not the retentate showing it is in fact being filtered through the centrifugal filter, however it is present at much lower concentrations than the unfiltered control samples indicating a degree of binding to the filter membrane . BSA is present within the retentate, but not the filtrate showing that it is being retained by the filter membrane.

On the advice of Millipore, a passivation protocol to prevent binding of target peptides to the filter membrane was then tried with the centrifugal filters that involved pre-soaking the filters using various solutions. The solutions chosen were 1% BSA and 5% TWEEN-20. The filters were soaked overnight with the passivation solution, washed thoroughly with dH2O, and the sample of insulin then filtered as per the normal protocol. The results showed that although the insulin was present in the filtrate and not the retentate, the concentration was much lower than for the unfiltered control, and so, the conclusion drawn was that the passivation process had not made much difference to the protein recovery.

The passivation process was applied to the fraction 1 control/tumour samples to see if there was improved recovery of the protein of interest. Again the filters were soaked with 1% BSA and 5% TWEEN-20. They were then loaded with samples containing 1% NP40 and 100mM DDT as denaturing agents and using insulin as a control. The results showed that the protein of interest still remained within the retentate. The plan was to repeat the process using 0.5% SDS (ionic detergent) in addition to 100mM DDT as denaturing agents, but to omit the passivation process as this did not seem to result in improved recovery. The results showed that once again the peptide of interest remained within the retentate. Possible reasons for this could include the DTT solution which had been made previously, aliquoted and stored at -20°C until use. It could also be the particular filter type was not performing very efficiently. The possibility that the peptide was retained in detergent micelles was excluded as the concentration of NP40 used was below the critical micellar concentration.

5.6 Haptoglobin

On visual inspection of the Tricine gels there seemed to be a difference in the protein concentration between the control and tumour samples in fraction 1 observed from the gels (with decreased protein concentration in the tumour samples), so we decided to run all the unfractionated control and tumour samples that had been used in the 30x30 SELDI-MS study on 16% tricine gels to see if there was a marked variation in protein concentration between the control and tumour samples. Ten µl of unfractionated serum was added to 100µl of sample buffer and 10µl of this was loaded onto the gel. The results did not show large observable differences in protein concentration. However, there did seem to be greater abundance of some proteins in the tumour samples compared to the normal controls, especially a band ~17kDa which was clearly visible in 26/29 (90%) tumour samples and 23/32 (72%) control samples (Figure 5.8). This gel band was excised and sent

for MS analysis. It was identified as haptoglobin, an acute phase reactant. This is not really surprising as a lot of acute phase reactive proteins are up-regulated in patients with cancer as discussed previously. However, in contrast to the ~4kDa unknown peptide, it was easily detected and identified. The results also suggest that differentially expressed proteins which are not identified as acute-phase reactants may provide a disease-specific biomarker

5.7 Conclusions

In summary, the SELDI-MS results were encouraging and suggested that there was a ~4kDa peptide that was significantly down-regulated in the serum of patients with oesophageal carcinoma. However, despite much effort identification was not made although there was a tentative suggestion that it may be an inter-alpha-trypsin inhibitor heavy chain H4 precursor, which was interesting as a cleavage fragment of inter-alpha -trypsin inhibitor heavy chain H4 had been previously found to be up-regulated in patients with ovarian cancer and down-regulated in patients with breast cancer.

Other techniques that might have been tried to potentially identify the peptide included using an alternative make of centrifugal filter (Nanosept® omega 10K MWCO) and repeating the filtration process. The protocol for the Nanosept® filters recommended diluting the sample to 500ul with TRIS-HCl pH7 to try and prevent membrane fouling, this was not tried with the Millipore centrifugal filters and so it is not known whether this would have made a difference to the protein filtration.

A search was also performed for a commercially available antibody to inter-alpha-trypsin inhibitor heavy chain H4 precursor to see if this could be used to confirm down-regulation of the protein of interest within the patient serum. However, this was not available. Non-commercial routes were not tried and it may have been an option to develop either an in-house or commercially made custom antibody

On the other hand, haptoglobin was found to be significantly more abundant in the serum from patients with oesophageal cancer compared to healthy volunteers. It was straightforward to isolate and identify and would be more amenable to immunoassay. A recent study compared the sera from 39 patients with breast cancer and compared it with 40 healthy controls using 2-DE separations coupled with MALDI [175]. Proteins that were found to be upregulated in patients with breast cancer included alpha1-antitrypsin precursor and a haptoglobin precursor. It may be that haptoglobin would be a better biomarker to take forward for future studies.

Chapter 6 - General Discussion

6.1 Background

Gastrointestinal and ovarian cancers are unfortunately commonly diagnosed at an advanced stage when the chance of cure and responses to treatment are low resulting in decreased survival outcomes. It would therefore be very beneficial if biomarkers existed that were sensitive and specific enough to allow for diagnosis of these cancers at an earlier stage. If these markers could be detected in blood samples that would potentially increase uptake of this as a screening test as blood is readily accessible, so most people find blood testing acceptable, and clinical laboratories are equipped for its analysis.

It would also be of benefit to have additional biomarkers of prognosis that would allow for greater tailoring of cancer treatment in individuals. This is of importance in patients with early colorectal cancer which remains confined to the bowel wall. At present the role of adjuvant chemotherapy within this patient group had not been firmly established and so it would be very helpful to have additional prognostic information that would identify patients who would gain most benefit from adjuvant treatment.

At present responses to chemotherapy are generally assessed radiologically at certain time-points during chemotherapy treatment such as midway through and at the end of treatment. This unfortunately means that some patients will receive chemotherapy that is not effective but may be associated with toxicity. If there were biomarkers available that could help assess responses to chemotherapy at an earlier stage, patients could potentially be changed to alternative treatments that may be more effective. There is the prospective that biomarkers measured at baseline, prior to patients commencing treatment, may help to predict which patients are likely to respond to particular chemotherapy regimens and so help to tailor treatment to individuals.

Newer targeted treatments that have been developed tend to have cytostatic rather than cytotoxic effects (at least initially) and so it can be challenging to assess responses to treatment using conventional radiological techniques as it may take months to observe tumour shrinkage on CT. In these patients it would be invaluable to have a biomarker that could act as a surrogate marker of tumour response again to help avoid the potential toxicities of ineffective treatments.

6.2 Aims of the project

My aims during my research were to find new biomarkers that could be used in patients with gastrointestinal and ovarian cancers to potentially aid in diagnosis, assessing prognosis and response to therapies.

The first part of my research involved looking at the expression of RKIP using immunohistochemistry in TMA initially in patients with colorectal cancer and later in patients with ovarian cancer. The rationale behind this was previous work suggesting that a reduction or loss of expression of RKIP was associated with worse clinical outcomes and we wanted to confirm these findings with a much larger cohort of patients. It was also decided to explore RKIP expression in a bank of TMA from patients with stage Ic to IV ovarian cancer to discover whether loss or reduction of RKIP expression in these patients showed any correlation with clinical outcomes and thus help aid in decisions regarding patient management.

The second part of my research comprised assessing plasma and serum CK18 levels in patients with advanced gastrointestinal and ovarian cancers using an ELISA. Plasma CK18 levels were also assessed at various time points during palliative chemotherapy in patients with advanced gastrointestinal cancer to determine whether it could be used as a marker of response to treatment.

The third part of my project comprised a more global approach to biomarker discovery and involved comparing serum of patients with advanced oesophageal cancer with serum from healthy volunteers using SELDI-MS. The plan was that if there were differences observed between the serum proteomes, attempts would be made to identify potential biomarkers

6.3 Results & Future Aims

6.3.1 Prognostic marker in colorectal cancer – RKIP

The results from this part of the study confirmed the findings of earlier studies, namely that loss of or reduced expression of RKIP in patients with colorectal cancer was associated with worse outcomes with an increased potential for metastatic spread and reduced overall survival. One theory as to why this may occur is that RKIP has a role in regulating the spindle checkpoint in cells and so its loss may result in chromosomal instability. This may

in turn affect metastatic potential and response to treatment. With the current lack of effective markers of metastatic relapse in colorectal cancer, a straightforward test like RKIP expression in the primary tumour may be a very cost-effective way to identify which patients may derive greater benefit from adjuvant treatment and closer post-operative surveillance.

In contrast the results from the ovarian cancer part of the study were conflicting. Within the first cohort there was a correlation seen between reduced RKIP expression and decreased progression-free survival, which is in accord with the results seen in colorectal and other cancers. However, the results from the second larger cohort were completely contradictory with reduced RKIP associated with improved survival outcomes. As yet there is not a satisfactory explanation for this observation, although technical problems related to the long-term storage of the paraffin-embedded tissue samples are suspected.

The future aims for this part of the project are to examine the methylation status of fresh frozen samples taken from the Dublin colorectal cancer cohort using pyrosequencing to see if epigenetic silencing is responsible for the reduction in RKIP expression observed within the tumour samples. Gene silencing by promoter methylation has been reported in some studies [176, 177], but negative results have also been observed [178] and it will be interesting to see if the results from this part of the project confirm promoter methylation.

6.3.2 Biomarkers of response in advanced gastrointestinal malignancy- CK18

The results from the early colorectal cancer part of the study are interesting as they suggest that serum CK18-NE levels may differentiate between patients with pre-malignant polyps and healthy volunteers, and that both serum CK18-NE and total CK18 may differentiate between healthy volunteers and patients with early colorectal cancer, and thus could potentially be used as an early diagnostic biomarker. However, the sample size was small and as the samples had been collected as part of an earlier clinical trial, it was not possible to either expand or perform a validation study. However, it would be interesting to see if these results held up in a larger prospective clinical trial.

The results from the advanced gastrointestinal cancer part of the study showed that both CK18-NE and total CK18 plasma levels were significantly higher in patients compared to the healthy volunteers. The total CK18 baseline plasma levels prior to treatment were

significantly higher in patients who developed progressive disease than those who achieved partial response or stable disease and this correlation was confirmed in an independent validation set. The peak plasma levels of CK18 occurring in any cycle following treatment were also found to be associated with tumour response. In conclusion, levels of plasma CK18 were found to be a potential marker of tumour response in patients with advanced gastrointestinal malignancy, however a larger prospective clinical trial would confirm if plasma CK18 could be used as a prognostic marker in advanced gastrointestinal cancer.

The results from the ovarian cancer part of the study were interesting, they showed that baseline total CK18 plasma levels were significantly higher in the patients compared to the healthy volunteers ($p<0.001$) and that there was a statistically significant association between baseline CK18-NE and total CK18 plasma levels and overall survival. The baseline CK18-NE and total CK18 plasma levels were then examined in relation to residual disease after surgery, FIGO stage and ECOG performance status but were not found statistically significant as an independent marker in the multivariate analysis. It was felt that on the basis of these results there was not a lot to be gained by taking this part of the project on any further. The fact that elevation of both CK18-NE and total CK18 plasma levels correlated with residual disease, FIGO stage and absolute CA-125 plasma levels would tend to suggest that they may be acting as markers of disease burden in ovarian cancer. However, there is the possibility that plasma CK18 could also characterise a more aggressive type of ovarian cancer associated with worse clinical outcomes, possibly either due to higher rates of cell turnover or increased conditioning of the blood with CK18 from highly vascularised tumours.

6.3.3 Diagnostic markers – downregulated peptide in advanced oesophageal cancer.

The SELDI-MS results were encouraging and suggested that there was a ~4kDa peptide that was significantly down-regulated in the serum of patients with oesophageal carcinoma ($p<0.0001$). However, one of the limitations of using SELDI-MS is that it does not identify the peptides. The MALDI-MS did tentatively suggest that the peptide may be inter-alpha-trypsin inhibitor heavy chain H4 precursor, which was interesting as a cleavage fragment of inter-alpha -trypsin inhibitor heavy chain H4 had been previously found to be up-regulated in patients with ovarian cancer and down-regulated in patients with breast cancer. However, the confidence of the identification was low and we were unable to

reproduce this result as attempts at the protein/peptide identification were hampered in part by its low mass. It may be that the peptide is bound to more abundant proteins, or the reason for its down-regulation in the serum of patients with advanced oesophageal cancer is alternative post-translational modifications which affect its ionisation. This would make sense as authors of the previous studies in ovarian cancer have made no reference to difficulties in isolating or identifying inter-alpha-trypsin inhibitor heavy chain H4 precursor.

Other techniques which could have been applied would have centred on immunological detection of inter-alpha-trypsin inhibitor heavy chain H4 precursor or alternative ultrafiltration devices with reduced capacity for non-specific binding (see Chapter 5.6).

This part of the project was very challenging due to the difficulties experienced in trying to prepare a sample of the protein of interest that was pure and concentrated enough to permit identification by either mass spectrometry or Edman sequencing. Due to the poor prognosis of the majority of patients diagnosed with oesophageal adenocarcinoma, it would obviously be extremely useful to have a serum biomarker that could potentially distinguish between patients with cancer and healthy volunteers, maybe allowing for earlier diagnosis, whilst disease is potentially operable. However, the identity of the ~4kDa peptide found to be down-regulated in patients with advanced oesophageal cancer in this study has proved elusive, and without an identity it is difficult to provide a biological answer for its down-regulation or to use other approaches to confirm the findings of this study. The ~4kDa peptide does appear to have unusual properties which mean that it may not be the best biomarker due to difficulties in its isolation and detection, and due to the limitations associated with the SELDI-MS, it is unlikely that this technique is practical enough to be adopted in routine clinical practice due to the stringent protocols required for sample handling and processing

On the other hand, haptoglobin was found to be significantly more abundant in the serum from patients with oesophageal cancer compared to healthy volunteers. This makes biological sense because, as mentioned previously, haptoglobin is an acute phase reactant and has been shown to be up regulated in solid tumours [168-170]. It has a role in angiogenesis, tissue remodelling and cell migration [171]. Haptoglobin was straightforward to isolate and identify and would be more amenable to immunoassay as there are good antibodies available for confirmation. It may be that this would be a better biomarker to take forward for future studies, although it may not be specific and may need to be part of a multi-marker profile.

In conclusion, the need for more effective and reliable biomarkers for the diagnosis, prognosis and prediction of clinical outcomes in cancer patients is clear and the search for non-invasive and reproducible laboratory assays has been intensive over the past number of years. The overriding aim is to create ideal biomarkers to optimise the management of patients with cancer ensuring that they receive accurate diagnoses, and treatment tailored to individuals ensuring they receive the most effective and least toxic therapies available.

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