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**THE ROLE OF THE Raf/MAP Kinase PATHWAY IN THE
DEVELOPMENT OF ANDROGEN-INSENSITIVE
PROSTATE CANCER**

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ABBREVIATIONS

ASPC – androgen-sensitive prostate cancer

AIPC - androgen-insensitive prostate cancer

GnRH gonadotropin-releasing hormone

PSA – prostate specific antigen

MAB – maximum androgen blockade

AR – androgen receptor

DBD – DNA-binding domain (of the androgen receptor)

LBD – ligand-binding domain

NTD – N-terminal domain

ARE – androgen response element

AF-1/2 – activation function-1/2 region (of the androgen receptor)

pRaf (ser338) – activated Raf-1 phosphorylated at the serine 338 residue

pRaf (ser259) – inactivated Raf-1 phosphorylated at the serine 259 residue

pMAP Kinase – activated phosphorylated p42/44MAP Kinase

EGFR – epidermal growth factor receptor

EGFR vIII - variant III epidermal growth factor receptor

AP-1 – activated protein-1

DIIT - dihydrotestosterone

HSP – heat-shock protein

SRC-1 – steroid receptor co-activator

CBP - cyclic AMP binding protein

IL-6R – interleukin-6 receptor

STAT3 - signal transducer and activator of transcription 3

JAK – janus kinase

PIAS - protein inhibitor of STAT3

COX-2 – cyclo-oxygenase-2

PI3K - phosphatidylinositol 3-kinase

PIP3 - phosphatidylinositol 3,4,5-triphosphate

PDK 1/2 – 3-phosphoinositide-dependent protein kinase 1/2

PTEN - phosphatase and tensin homologue deleted on chromosome 10

MEK – MAP kinase kinase

PKA – protein kinase-A

CRD - cysteine-rich domain (of Raf-1)

Grb-2 - growth factor receptor bound protein-2

SOS - guanine-nucleotide exchange factor “son of sevenless”

IHC – immunohistochemistry

TURP – transurethral resection of the prostate

TRUS – transrectal ultrasound

MREC - Multiple Research Ethical Committee

LREC – Local Research Ethical Committee

SUOG – Scottish Urological Oncological Group

TMA –tissue micro-array

ICCC – interclass correlation coefficient

Rb - retinoblastoma tumour suppressor protein

VEGF – vascular endothelial growth factor

JE – Joanne Edwards

LMcG – Lianne McGlynn

NSK – Sarath Krishna

KG – Ken Grigor

KP – Katherine Pirret

SUMMARY

Prostate cancer is the second most commonly diagnosed cancer in the Western world and the second most common cause of male cancer-specific death. Most patients either present with extracapsular or advanced disease, or develop it following radical therapy for localised disease. The mainstay of treatment for advanced prostate cancer has not changed significantly for over 50 years and is based on androgen-deprivation therapy.

Hormonal or androgen-deprivation therapy aims to inhibit androgen production or action, thereby reducing stimulation of the androgen-receptor (AR). This in turn prevents the activation of androgen-regulated genes, which normally result in on-going growth, survival and inhibition of apoptosis. Initial response rates to androgen-deprivation therapy are high, however within an average of 18-24 months following commencement of treatment patients relapse with androgen-insensitive disease (AIPC). This indicates reactivation of androgen-dependent gene transcription, resulting in renewed tumour growth. Clinically, AIPC manifests itself in local tumour progression, distant metastasis and, ultimately, death within 12-18 months. This remains a significant clinical problem as it contributes to the majority of the morbidity and mortality associated with advanced prostate cancer.

The progression to AIPC involves disruption to cell growth, differentiation and apoptosis despite on-going androgen suppression. These result from alterations to the androgen signalling pathway and other sub-cellular molecular pathways. The development of novel therapies to treat AIPC however has been hampered in the past by limited information regarding these underlying molecular causes. A profound understanding of the various molecular pathways involved is crucial as specific targets are needed for molecular-based

cancer therapy. The use of patient material to define such targets is clearly required and although their exact nature is still poorly understood, interactions with growth factors and their receptors are clearly involved. Such potential targets include the type 1 receptor tyrosine kinases (EGFR and HER2) and their downstream pathways.

We hypothesised that the Raf/MAP Kinase cascade to be one such pathway, based on previous gene array data from our laboratory. This revealed amplification of members of the Raf/MAP Kinase pathway along with HER2 with the development of AIPC, which suggested that the Raf/MAP Kinase pathway promoted the development of clinical AIPC. The purpose of this M.D. was to further investigate the role of this signal cascade in AIPC by determining if these previously identified genetic changes had functional consequences in terms of protein expression. The role of the Raf/MAP Kinase pathway was investigated using clinical, archival material which consisted of paired, matched histological tissue specimens obtained before commencement of androgen deprivation therapy and after the development of androgen-escape. The expression levels and activation status of Raf-1 and MAP Kinase (2 critical members of the MAP kinase cascade) were determined in these matched ASPC and AIPC tumours using immunohistochemistry. Protein expression was then correlated with time to biochemical relapse, time to death from relapse and overall survival. Correlations were also made with the type 1 tyrosine kinase receptors, EGFR and HER2 (known to activate the pathway), and the known downstream targets, AR and AP-1, in the same cohort of patients.

In doing so, increased protein expression of the pathway was seen to significantly impact on patient survival. Patient tumour specimens which displayed a rise in Raf-1 expression had a significant reduction in time to biochemical relapse ($p < 0.0005$). Furthermore, rising nuclear expression of MAP Kinase was strongly associated with shortened time to death

from ($p=0.0255$) relapse resulting in reduced overall survival ($p=0.0068$). Correlations also existed with other critical members of the pathway *in vivo*. Correlations were seen between type 1 receptor tyrosine kinases and members of the Raf/MAP Kinase pathway before and after the development of AIPC, whilst further significant correlations developed with the c-fos and c-jun (components of the transcription factor AP-1) with the development of AIPC.

From this data, the following can be concluded. Raf-1 and MAP Kinase appear to be involved in the development of AIPC, as their expression significantly impacts on known clinical parameters – namely, time to biochemical relapse and overall survival. This indicates a potential prognostic role for the MAP Kinase cascade in AIPC. In addition, positive correlations with other proteins may indicate that a type 1-tyrosine kinase receptor/Raf-MAP Kinase /AP-1 axis forms a functional pathway which plays a role in the development of clinical AIPC. This would imply that members of the Raf/MAP Kinase pathway may act as a therapeutic target in selected patients.

The data provides, for the first time, clinical evidence to support our hypothesis that the Raf/MAP Kinase pathway is utilised in AIPC and plays a role in promoting its' development and progression. Further analysis of this pathway, and its' role in AIPC using clinical material, are clearly warranted. These findings lead us to hypothesise further that expression of Raf-1 and MAP Kinase would yield useful predictive information for patients at risk of AIPC and that targeting of the type 1-tyrosine kinase receptor/Raf-MAP Kinase /AP-1 axis in this sub-group of patients may yield a novel and exciting therapeutic avenue in the future.

INTRODUCTION

1.1 Prostate Cancer – Background

1.1.1 Incidence and Natural History

Prostate cancer is the second most commonly diagnosed cancer in the Western world. Prostate cancer incidence in the United Kingdom is approximately 25,000 cases per year and is the second most common cause of male cancer-specific death (2003) (Trachtenberg and Blackledge, 2002) (Debes and Tindall, 2004). At diagnosis, localised or organ-confined disease may be treated and potentially cured with radical surgery or radiotherapy. However many patients present with extracapsular or advanced disease (be it locally or regionally advanced or metastatic disease), the management of which has not significantly changed for over 50 years (Linja et al., 2001). Furthermore, approximately a third of patients who undergo radical therapy for localised disease will eventually relapse with advanced disease. The mainstay of treatment for patients with advanced disease is based on androgen-deprivation therapy, and this approach has remained unchanged ever since Huggins and Hodges first demonstrated the hormonal dependence of prostate cancer in 1941 (Huggins and Hodges, 2002). Their work revealed, for the first time, the growth suppressive effect of reducing endogenous androgen levels, by surgical castration, in patients with prostate cancer. Prostatic growth and development is dependent on androgen, in the form of testosterone and dihydrotestosterone. Hormonal or androgen-deprivation therapy aims to inhibit androgen production or action, thereby reducing stimulation of the androgen-receptor (AR). This in turn prevents the activation of androgen-regulated genes, which normally result in on-going growth, survival and inhibition of apoptosis. Initially this is effective in reducing tumour growth in 70% - 80% of men (androgen sensitive prostate cancer or ASPC), however all patients eventually develop resistance to therapy and relapse with androgen insensitive prostate cancer (AIPC) for which there are only limited, palliative treatment options.

The development of androgen escape remains a significant clinical problem, as it leads to disease progression and metastasis. This in turn contributes to the majority of the morbidity and mortality associated with prostate cancer (Sciarra et al., 1999) (Dorkin and Neal, 1997). The development of novel therapies to treat or prevent the onset of AIPC has been hampered in the past by limited information regarding its' underlying causes. Such treatments are, however, required urgently as the incidence of prostate cancer is forecast to increase by 20% within the next 10 to 25 years (Beemsterboer et al., 1999).

1.1.2 Current Challenges & Treatment Options

1.1.2.1 Current Challenges in Prostate Cancer

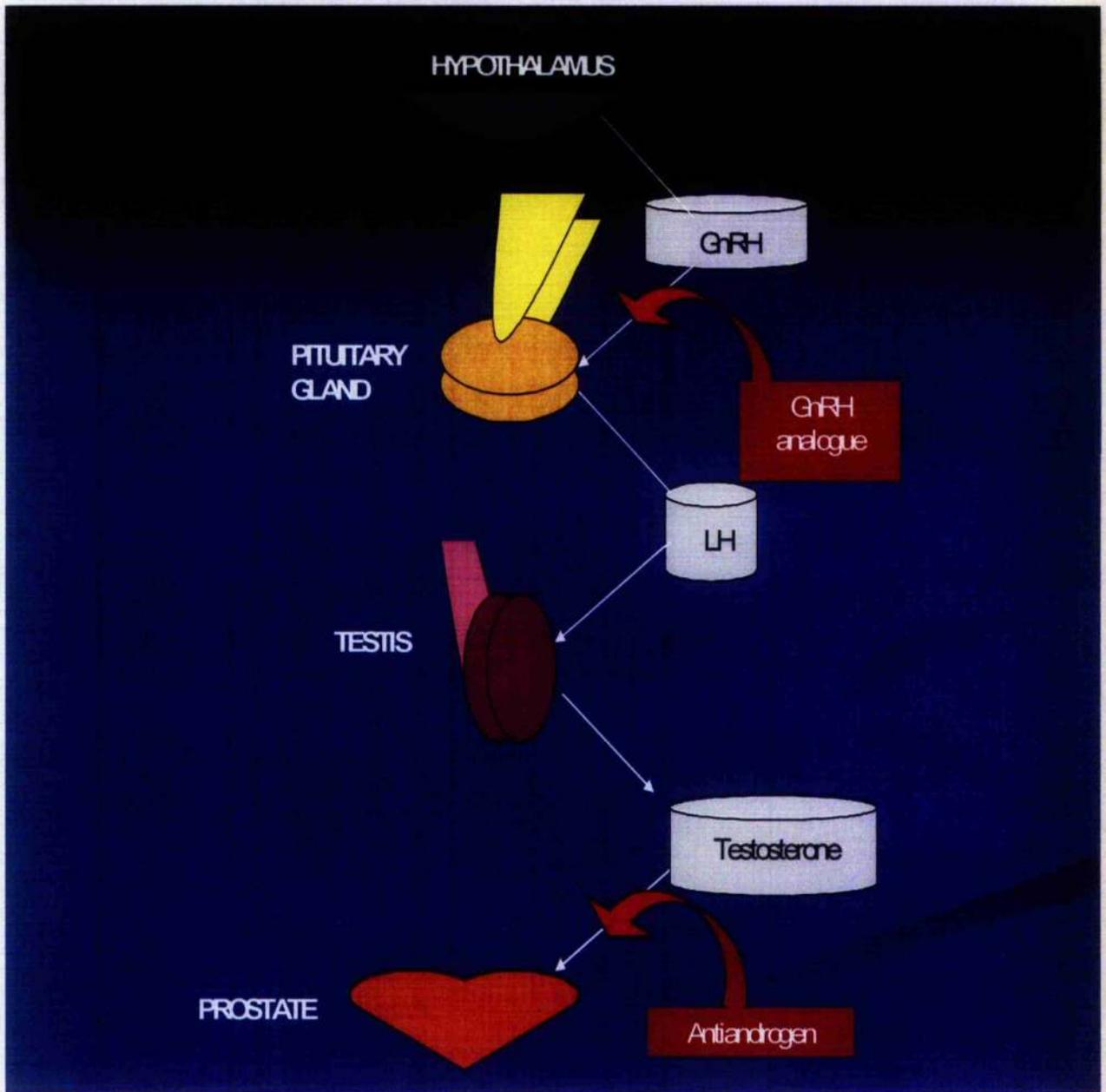
Various difficulties exist with regard to the understanding of the biological course of prostate cancer as well as its' therapeutic management. The development of clinically progressive disease does not always occur within the lifetime of every patient with histological evidence of cancer (Culig et al., 2002). As a result, while some patients have a high risk of dying of prostate cancer, the remainder live with microscopic but not clinical evidence of disease, eventually succumbing to death from other causes. The detection and diagnosis of prostate cancer as a result of screening is hindered a failure to differentiate effectively between these patients, thereby reducing mortality (Parker, 2003). One of the goals of current research is to develop new ways of identifying patients with clinically aggressive and life-threatening disease, and, thereafter, deciding what form of treatment should be administered - whether curative (by radical surgery or radiotherapy) or not.

Another controversy lies in the management of advanced or non organ-confined disease. Androgen-deprivation therapy relies on the suppression of androgen production and/or function, thus preventing activation of AR-mediated signalling and gene transcription (see **Figure 1**). Inhibition of testicular androgen production may be achieved surgically (bilateral orchidectomy) or chemically, using gonadotropin-releasing hormone (GnRH) agonists. The latter induces castrate levels of testosterone by down-regulating pituitary GnRH receptors (and therefore gonadotropin hormone production) through constant stimulation. In addition, the action of androgen may be blocked at a peripheral level using androgens, which inhibit ligand binding to AR and subsequent activation. Androgens may be steroidal (cyproterone acetate) or non-steroidal (hydroxyflutamide or bicalutamide – which have the advantage of possessing no hormonal activity). Either form of therapy may be used alone in the treatment of locally advanced disease, however overall survival in localised, high grade or metastatic disease prostate cancer is improved only with medical or surgical castration (Wirth et al., 2004). Long-term adjuvant androgen-deprivation therapy has also been shown to increase local control and survival when used in conjunction with radical radiotherapy in locally advanced disease (Gottschalk and Roach, III, 2004) (Bolla et al., 2002).

Initial response rates to androgen-deprivation therapy are high, however within an average of 18-24 months following commencement of treatment patients relapse with “androgen-escape” or androgen-insensitive/independent disease (AIPC). This indicates reactivation of androgen-dependent gene transcription, resulting in renewed tumour growth. Clinically, AIPC manifests itself in local tumour progression, distant metastasis and, ultimately, death within 12-18 months (Trachtenberg and Blackledge, 2002). Serum levels of the kallikrein, prostate-specific antigen (PSA) – an androgen-regulated gene product

which acts as a biochemical indicator of disease progression and tumour load – subsequently rise (Gao et al., 1997) (Stamey et al., 1987). PSA is therefore used as a clinical marker of this event, as well as in the initial diagnosis of prostate cancer. The development of AIPC results in considerable patient morbidity and mortality, with features such as spinal cord compression, pathological fractures and hypercalcaemia (secondary to bone metastasis), as well as obstructive renal failure (secondary to bladder outlet obstruction).

Figure 1. Androgen-Axis with Therapeutic Sites of Action



1.1.2.2 Current Treatment Options for AIPC

Maximum Androgen Blockade

Currently, second-line strategies involving hormonal therapy have been explored in a bid to delay the progression of (if not prevent or cure) androgen-insensitive disease itself.

Maximal androgen blockade (MAB) consisting of both forms of androgen-deprivation

therapies was first proposed over 20 years ago (Labric et al., 1985). The principal behind such an approach is to nullify the effects of the 10-30% of circulating androgen produced by the adrenal glands, which are not counteracted by GnRH analogue treatment alone. However its' therapeutic benefit still remains unproven, with the majority of trials showing only a modest survival benefit at best. It has been suggested that this may be related to varying trial designs (including differing outcome measures) as well as differing efficacies between non-steroidal androgens themselves (Klotz, 2001). Despite this, it is thought that MAB has the potential disadvantages of increased drug toxicity, although this too remains under review (Pether and Goldenberg, 2004) (Damber, 2004). Interestingly, following the development of AIPC, withdrawal of androgen treatment has been shown to result in a temporary decline in PSA in up to a third of patients, with a subsequent clinical improvement (Kelly and Scher, 1993). The underlying molecular mechanisms behind this "androgen-withdrawal" phenomenon are not as yet clear, however they may also be related to the subsequent development of AIPC (Miyamoto et al., 2004). These mechanisms will be discussed in more detail in the next section.

Chemotherapy

As the proliferation rate of prostate cancer is comparatively low, with respect to other cancers, chemotherapy was initially thought to be of limited value in advanced stages of disease progression (Culig et al., 2002) (Zelivianski et al., 2003). Long-term therapy with adriamycin and taxol, for example, leads to resistance to apoptosis (Culig et al., 2002). Despite this, several clinical studies have sought to determine the potential role of chemotherapy in advanced AIPC (Culine and Droz, 2000). Whilst not curative, chemotherapy has been shown to give significant palliative benefit in symptomatic patients in terms of pain control and quality of life, especially in combination with

corticosteroids. Moreover, recent trials with combination chemotherapy, in particular with the taxol-based chemotherapeutic agents paclitaxel and docetaxel, have yielded promising results with regards to PSA levels and survival benefits (Culine and Droz, 2000). Paclitaxel in combination with estramustine results in a PSA decrease of greater than 50% in 65% of patients, although with no overall survival benefit (Smith et al., 1999). A phase II trial using docetaxel in combination with mitoxantrone resulted in a similar PSA response in 62% of patients (Heidenreich et al., 2003) and in 84% of patients when combined with estramustine (Petrylak et al., 1999). Following on from this, 2 recent phase III trials have confirmed a modest survival benefit with docetaxel combined with estramustine (Petrylak et al., 2004) or prednisolone (Lannock et al., 2004) - the first time large-scale randomised trials have demonstrated a survival benefit with any form of chemotherapy in AJPC. The molecular basis for taxol-based chemotherapy is well-characterised. Taxanes, like other chemotherapeutic agents such as estramustine, inhibit microtubule disassembly by binding to tubulin and arresting cells at the G2/M phase (Debes and Tindall, 2004). In addition, docetaxel can lead to phosphorylation of bcl-2, increasing free bax and activating apoptosis via the caspase cascade.

Despite these developments, any modest survival benefit of such chemotherapeutic regimes must be balanced with its' toxicity, in particular neutropenia, which clearly impacts on and compromises the quality of life for patients who have a limited life expectancy in any case.

Other Hormonal Treatments

The use of oral oestrogen (stilboestrol) therapy for medical castration has long been superseded due to cardiovascular mortality. Such effects may be circumvented however by parenteral oestrogen administration which has been shown to have equal efficacy as MAB in terms of survival and cardiovascular side-effect profile (Hedlund and Henriksson, 2000).

In contrast to analogues, GnRH antagonists (e.g. abarelix) bind to pituitary GnRH receptors, blocking GnRH release. A recent phase III trial comparing it with MAB (leuprolide and bicalutamide) revealed a more rapid reduction of testosterone to castration levels with no androgen flare. However, no actual improvement in survival has been demonstrated and as a result there remains a significant question regarding its' cost-benefit ratio.

Therefore, current treatments for AIPC are mainly palliative with no effective curative options for what is clearly a serious clinical problem. Prostate cancer is predominantly a disease of the elderly, however current demographic trends forecast a 60% rise in the male population over the age of 50 in the next 15 years, with a resulting 20% rise in the incidence of prostate cancer itself (Beemsterboer et al., 1999) (Boyle P, 4 A.D.). In addition, there is an increasing trend for patients to be diagnosed earlier through screening programmes. The risks of developing AIPC will therefore increase in younger patients as androgen-deprivation therapy is being increasingly used as adjuvant therapy with radical treatments. There are a lack of effective treatment options that improve survival and/or prevent complications due to disease progression. The clear challenge facing clinicians and scientists is therefore to develop novel therapies that improve survival, with minimal

adverse effects that may compromise on patient quality of life. This will have a major impact on the provision of future healthcare resources.

1.1.3. Future Management

In order to develop effective novel therapeutic approaches for the future, it is crucial to understand the molecular mechanisms underlying the development of prostate cancer and AIPC. This has not been straightforward, with the failure to establish a reliable primary culture model to study androgen-responsiveness being one particular difficulty (Culig et al., 2002) (Linja et al., 2001).

Nevertheless, progress has been made recently. Prostatic cells are known to be regulated by androgens, with the testosterone-AR complex / axis playing a key role in determining the expression of genes controlling cellular growth, and differentiation, as well as apoptosis. In the last 10 to 15 years there has been a great deal of published research dealing with the crucial role of this AR signalling pathway in controlling normal cell development, as well as its' role in prostate carcinogenesis. This has led to a greater understanding of its' participation, and in particular its' dysregulation, in the progression of the disease including the development of AIPC. The AR signalling pathway may well therefore contain potential therapeutic targets, and this possibility will be explored in the next section.

However, the microenvironment of prostate cells and the AR signalling pathway is also influenced by external regulatory factors such as growth factors and cytokines. Androgens and AR-signalling are a central component of this process, interacting with such regulatory factors. These factors may also be responsible for setting up paracrine and

autocrine feedback loops leading to dysregulated and possibly androgen-independent growth via activation of downstream signal cascades, such as the p42/44 MAP kinase pathway. An imbalance between these finely balanced growth stimulatory and inhibitory mechanisms is thought to be the major factor driving the neoplastic process in general. It is therefore not surprising that the development of AIPC is associated with a significant increase in growth factor expression, which is believed to fuel androgen-independent growth (Culig et al., 1994). Preventing the activation of these pathways provides a possible new avenue for the treatment of AIPC and will also be discussed in a later section.

Long-term androgen-deprivation leads to recovery in growth and increased sensitivity to low doses of androgens. It has therefore been hypothesised that, whilst in the early phase of prostate carcinogenesis cells possess a degree of androgen-dependence, disease progression is marked by acquired resistance to treatment possibly due to genetic mutations. Such changes result in cells becoming susceptible to chronic autocrine stimulation from growth-promoting signalling pathways, independent of androgen-control. Increased or dysregulated oncogene activation resulting in the altered expression and function of growth factors, growth factor-receptors and loss of tumour suppressor gene products may therefore form the basis of AIPC development. An alternative theory however proposes that androgen-independent cells co-exist with androgen-dependent cells from the start, and subsequently undergo "selection pressure" during androgen-deprivation therapy (Miyamoto et al., 2004) (Linja et al., 2001) (Isaacs, 1999). This has led to the clonal selection theory of AIPC which results from intrinsic resistance. As a result, the use of intermittent androgen suppression - with cycles of hormone therapy given only during rises in PSA and withdrawn upon stabilisation - is currently under

evaluation as a means of delaying AIPC development (Pether and Goldenberg, 2004). This technique would theoretically allow androgen-sensitive cells to repopulate between cycles of therapy, whilst offering equal therapeutic efficacy and a reduction in the long-term drug side-effects and therefore improvement in quality of life. The results of definitive, randomised trials are, however, awaited.

As a result of our current understanding at a molecular level, prostate cancer is now thought to be a heterogeneous disease, both histologically and biologically. This is due to complex molecular interactions between androgens, growth factors, receptor and intracellular proteins. A multi-disciplinary approach to its' investigation encompassing endocrinology, biochemistry, molecular genetics and histopathology will therefore provide a better understanding of the disease. It seems likely that the use of combined conventional and molecular therapies will form the basis of future treatment of advanced prostate cancer (Culig et al., 2002). The development of novel therapies may depend on the "molecular profiling" of patients i.e. matching specific targets with the individual. For such an approach to be successful, it will be essential to use patient material to validate such targets or pathways in terms of their actual function in clinical AIPC development. It is this premise that provides the basis of this thesis.

1.2 The Molecular Basis of AIPC

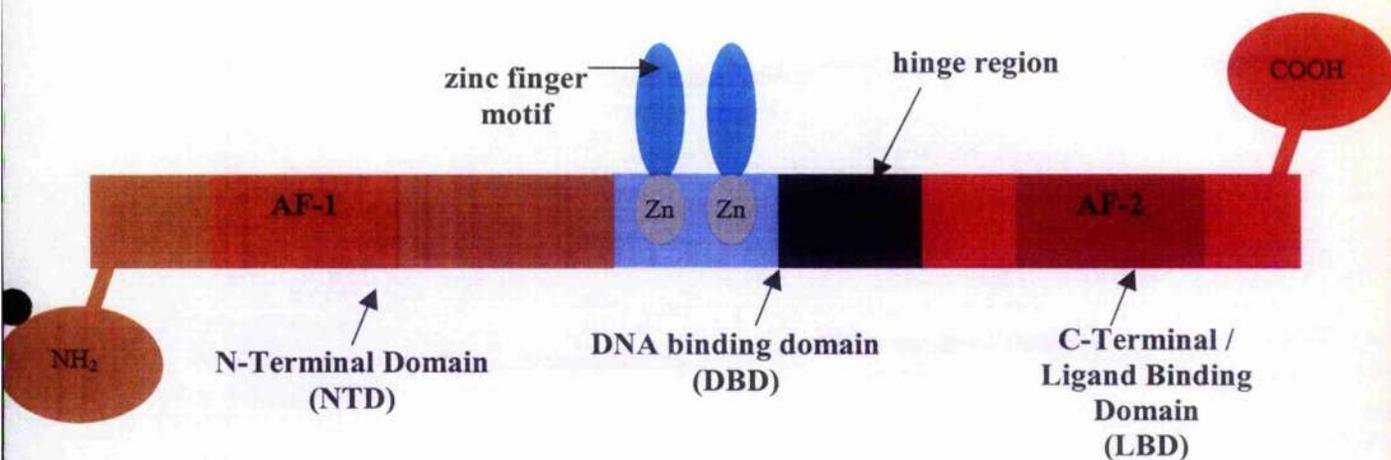
1.2.1 The Androgen Receptor

1.2.1.1. Structure and signalling of the Androgen Receptor

The androgen receptor (AR) is a steroid receptor and a ligand-activated transcription factor. It is involved in the regulation of male sexual development, maintenance of libido, and the growth and functioning of accessory sexual organs such as the prostate gland (Culig et al., 2002) (Trachtenberg and Blackledge, 2002). At the molecular level it consists of 3 domains (Culig et al., 2002) (Edwards and Bartlett, 2004) (**Figure 2**). The DNA-binding domain (DBD) contains a hinge-region and 2 zinc finger motifs that bind to specific androgen-responsive elements (AREs) of androgen-regulated genes. The hinge region contains specific sequences involved in receptor dimerisation and translocation to the nucleus (Lee and Chang, 2003). Phosphorylation at these sites may result in inactivation of the AR (Rochette-Egly, 2003). The C-Terminal or ligand-binding domain (LBD) is critical for the binding of hormone and the subsequent ligand-dependent activation of the AR. In addition, it is involved in the regulation of transcription via the activation function-2 (AF-2) region, and interacts with co-activators (following a conformational change triggered by ligand-binding) (Rochette-Egly, 2003). The N-Terminal domain (NTD) contains the activation function-1 (AF-1) region, which is also involved in the regulation of transcriptional activity, and is the target of multiple protein kinase pathways (Lee and Chang, 2003) (Ueda et al., 2002b) (Edwards and Bartlett, 2005). AF-1 contains numerous putative ligand-dependent and independent phosphorylation sites consisting of a variable number of polyglutamine or glycine repeat sequences. It also interacts with p160 co-regulatory proteins, independently of hormone-binding, allowing bridging with the AF-2 domain (Rochette-Egly, 2003). Inhibition of this communication by phosphorylation may reduce ligand-binding, inhibiting AR function

(Rochette-Egly, 2003). Phosphorylated NTD may also allow targeting of the AR for enzymatic degradation (Rochette-Egly, 2003).

Figure 2. Structure of the AR

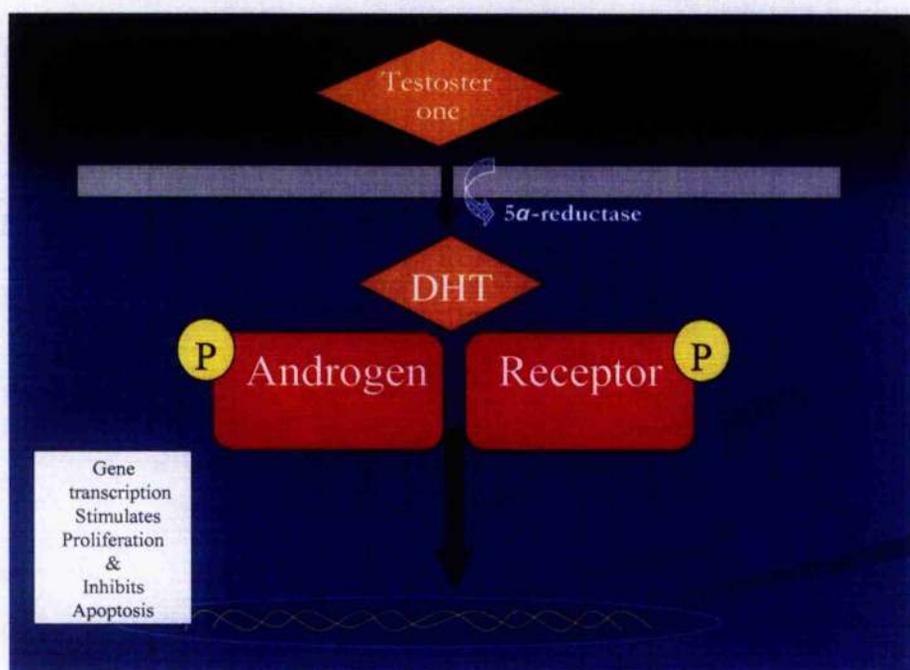


The functional status of the AR, in particular its' transcription ability, is closely associated with its' phosphorylation status (Grossmann et al., 2001) as well as its' homodimerisation ability (Culig et al., 2002). While AR agonists and antagonists are known to differentially phosphorylate the AR, identifying which other cellular protein/regulatory kinases that also act on the AR, and their respective phosphorylation sites is crucial to unravelling the molecular details of the AR-signalling pathway.

The primary ligand for the AR is dihydrotestosterone (DHT), which has a 10 times higher affinity than testosterone as a ligand (Suzuki et al., 2003) (see **Figure 3**). Testosterone (produced by testicular Leydig cells) is converted to DHT by 5 α -reductase type 2 upon entry into prostatic epithelial cells. In the absence of ligand, the AR is normally bound to several heat shock proteins (HSPs), which protect it from degradation, maintaining a dormant state and preventing activation (Culig et al., 2002) (Edwards and Bartlett, 2005).

Upon binding to ligand, a conformational change occurs allowing dissociation of HSPs (Ueda et al., 2002b). The AR is then phosphorylated, which stabilises the ligand–receptor complex, and forms a homodimer (Lee and Chang, 2003) (Edwards and Bartlett, 2005). The complex subsequently translocates from the cytoplasm to nucleus to act as a transcription factor (Wang et al., 1999) but as such is exposed to protease degradation. The AR initiates gene transcription by binding to specific AREs (consensus nucleotide sequences that are specific for the AR in promoter regions of target genes) (Lee and Chang, 2003). Upon binding, co-activators (eg SRC-1, CBP) also interact with the AR to enhance transcription by bridging various transcription factors to aid formation of a stable and functional pre-initiation complex (Rochette-Egly, 2003). The formation of this complex at the initiation site facilitates entry of transcription machinery, such as RNA polymerase II, allowing commencement of specific gene transcription (Rochette-Egly, 2003; Suzuki et al., 2003).

Figure 3. Interaction Between Androgen and the AR Pathway



1.2.1.2. Genetic Modifications of the AR in AIPC

Downregulation of the AR–signalling pathway with hormone therapy could potentially explain the development of AIPC on a molecular level. Using immunohistochemical studies, however, the AR has been consistently demonstrated in prostate tumours even after the development of androgen-insensitive disease (Craft et al., 1999) (Grossmann et al., 2001) (Edwards et al., 2003a) (Hobisch et al., 1995). AR expression and activity is known to increase after long-term hormone-therapy and its' expression continues with the development of metastatic AIPC (Culig et al., 2002).

There is therefore strong evidence to support a crucial role for the AR–signalling pathway with the development of AIPC. As a consequence of this, the majority of research into the mechanisms of androgen-escape has focused on how alterations and aberrant stimulation of the AR at a molecular level can re-activate AR signalling, with or without ligand-binding. Two hypotheses have emerged as a result - the first implicating genetic modifications (mutations and amplification) of the AR, and, more recently, a second focusing on post-translational modification of the AR, specifically the role of cell signalling pathways in altering the activation status of the AR protein itself.

Several studies have focused on mutations of the AR binding domain in AIPC and amplifications of the AR gene leading to increased protein expression. These changes may lead to the abnormal functioning of the AR signalling pathway, despite continued androgen-blockade. This may subsequently allow increased sensitivity of the AR to low levels of circulating androgen produced by the adrenals or the AR may indeed respond to antiandrogens as well as other steroids (Culig et al., 2002) (Edwards and Bartlett, 2005) (Chen et al., 2004;Culig et al., 1999).

Several AR mutations have been characterised (Grossmann et al., 2001) (Gottlieb et al., 1998). Point mutations, in particular those involving the hormone-binding domain, can alter ligand binding affinity as well as transcriptional activity in cell-line studies - both resulting in cellular proliferation (Linja et al., 2001) (Wallen et al., 1999) (Culig et al., 1998) (Grossmann et al., 2001) (Culig et al., 2002) (Edwards and Bartlett, 2005). Mutations in the N-terminal are more common in AIPC tumours (Wallen et al., 1999) and may allow binding of co-activators, with subsequent receptor activation in the absence of ligand binding (Edwards and Bartlett, 2005). Point mutations are not common in the early stages of prostate cancer, but are known to be more prevalent in cases of metastatic disease and AIPC (Lee and Chang, 2003) (Marcelli et al., 2000) (Culig et al., 2002) (Suzuki et al., 1996). Alterations to the AR can also have paradoxical short-term effects. Certain point mutations, in particular, are thought to lead to the "antiandrogen-withdrawal syndrome" (Kelly and Scher, 1993) (Suzuki et al., 1996) (Miyamoto et al., 2004).

Amplifications of the AR have been consistently linked with androgen-escape (Bubendorf et al., 1999) (Linja et al., 2001) (Edwards et al., 2004) (Visakorpi et al., 1995). Up to a third of androgen-independent tumours contain amplification of the AR gene, a phenomenon seen much less frequently in primary tumours (Bubendorf et al., 1999) (Edwards and Bartlett, 2005) (Linja et al., 2001) (Koivisto et al., 1997) (Edwards et al., 2003a). Not surprisingly, increased levels of AR expression with the development of AIPC have also been reported (Linja et al., 2001) (Trapman and Cleutjens, 1997) (Suzuki et al., 2003) (Hobisch et al., 1995) and often in association with gene amplification (Koivisto and Rantala, 1999) (Edwards et al., 2003a). Studies using paired androgen-sensitive and insensitive human prostate xenografts have confirmed that increased AR expression occurs consistently with the development of androgen-insensitive disease, and

can also sensitise the AR to low levels of androgen and antiandrogen (Chen et al., 2004). AR protein expression has also been shown to correlate with tumour grade, stage and disease-free survival, as well as predicting patient response to therapy (Trapman and Cleutjens, 1997) (Koivisto and Rantala, 1999) (Edwards et al., 2003a). One study revealed improved response to MAB in patients with AR amplification compared to those without (Palmberg et al., 1997).

AR mutations and gene amplification are not however seen in all AIPC tumours (Edwards et al., 2003a) (Culig et al., 2002). Whilst mutations are more commonly found in *in vitro* models of AIPC, they appear to be a relatively infrequent occurrence in clinical metastatic disease and AIPC (albeit more frequent when compared with clinical ASPC) (Linja et al., 2001) (Suzuki et al., 2003). Their presence may simply be secondary to an unstable genome resulting from advanced disease rather than a reflection of mutations promoting disease (Edwards and Bartlett, 2005). In addition, these receptors generally cannot bind androgen or have decreased transcriptional activity (Culig et al., 2002). Furthermore, mutations are also too infrequent to account for the majority of patients who develop the antiandrogen-withdrawal syndrome. Patients who do not possess AR mutations eventually develop androgen-independent disease suggesting mechanisms other than AR mutations result in tumour progression (Lee et al., 2002). As a result there is little clinical evidence currently linking specific mutations with the development of AIPC, although the possibility of mutations resulting in at least a small sub-group of patients developing androgen-insensitive disease cannot be ruled out (Edwards and Bartlett, 2005).

Amplification of the AR gene does not always have functional consequences in terms of protein expression. Furthermore, mutations as well as amplifications in ASPC tumours do

not predict poor response to treatment (Edwards and Bartlett, 2005). AR amplification has not been shown to correlate with time to relapse or disease progression once AIPC is established (Culig et al., 2002) (Edwards et al., 2003a). Not every patient responds or behaves in a similar fashion to androgen-deprivation therapy and it is now clear therefore that tumours may utilize more than one survival mechanism in order to develop androgen-independence. Our laboratory has demonstrated amplification of AR prior to androgen therapy in a patient who had a full clinical response to androgen-deprivation, and whose AR expression fell once AIPC developed (despite on-going amplification) (Edwards et al., 2003a). This suggests that AR amplification does not necessarily negate a positive response from therapy and therefore may not be essential for the development of androgen-escape (Edwards and Bartlett, 2005). In another study using matched pairs of ASPC and AIPC patient specimens, AR amplification, whilst seen more frequently, was still present in only 20% of cases of AIPC (Edwards et al., 2003a). Although 80% of tumours with AR amplification also had raised AR expression, AR expression was also seen in 35% of cases with no amplification.

This supports the role of alternative mechanisms resulting in increased AR expression in the absence of amplification, such as up-regulation of mRNA, protein stabilisation or decreased protein degradation (Gregory et al., 2001b) (Linja et al., 2001) (Edwards and Bartlett, 2005). AR expression in animal models has been shown to induce AIPC, independently of increased gene copy number (Chen et al., 2004). Conversely, AR downregulation has also been demonstrated in cell-lines showing metastatic properties (Culig et al., 2002) and in some AIPC patients (Linja et al., 2001). Hypermethylation of the AR gene promoter region, which is seen more frequently in AIPC, has been forwarded as a mechanism of inhibiting AR gene transcription and protein expression despite

amplification and may occur in up to 15% of tumours (Linja et al., 2001) (Suzuki et al., 2003) (Culig et al., 2002). Correlation between AR gene amplification and expression of the androgen-dependent gene, PSA (which one might expect in AIPC even with androgen deprivation) has been noted in some studies but not others, reflecting the complex nature of androgen-dependent gene expression (Linja et al., 2001).

Therefore evidence in the literature, including our own data, questions the role of androgen-receptor modification at the genetic level in the development of androgen-escape. Whilst they may remain clinically relevant, the occurrence of both amplifications and mutations are too infrequent to account for the majority of cases of AIPC (Chen et al., 2004). There must be alternative mechanisms or pathways regulating AR activation. It is not surprising that, unlike breast cancer where oestrogen and progesterone receptor status have prognostic and diagnostic relevance, AR status has not achieved this in prostate cancer (Culig et al., 2002).

1.2.1.3. Post-Translational Modifications of the AR in AIPC

Recent findings have therefore increased our understanding of the role of the AR in AIPC. As a result, studies have now shifted to the analysis of AR post-translational modifications. In particular, expression and function of signal transduction pathways and/or co-activators can activate the AR independent of androgens.

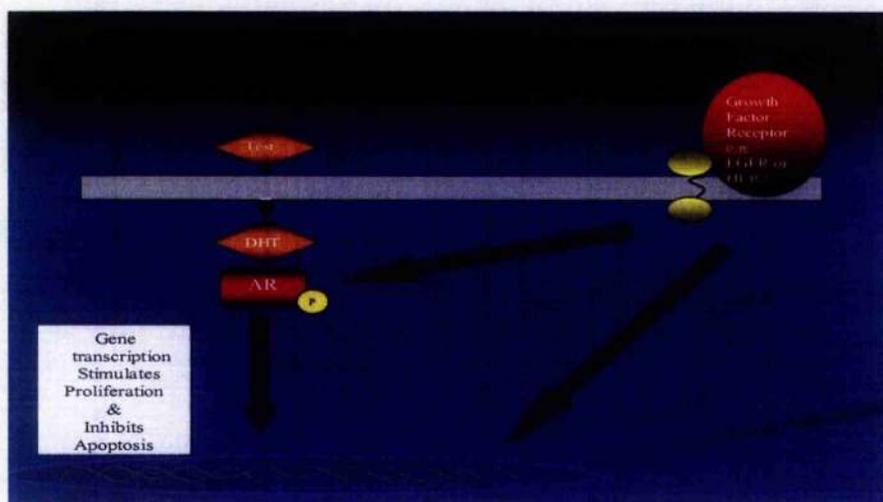
The entire sequential cascade of steps initiated upon ligand-binding to the AR is inherently dependent on diverse but key phosphorylation events. Protein phosphorylation plays a key role in regulating steroid hormone activity, such as DNA binding and activation of transcription (Zhu and Liu, 1997) (Rochette-Egly, 2003). Phosphorylation of the AR was

first described in 1990 (Edwards and Bartlett, 2005). Binding of androgen initiates hyperphosphorylation by various protein kinases at multiple and specific serine residues in the N-terminal AF-1 (and LBD AF-2) region (Rochette-Egly, 2003). This protects the AR from proteolytic degradation, leading to increased stability of the ligand-receptor complex and, importantly, increases transcriptional activity (Wen et al., 2000) (Zhou et al., 1995) (Blok et al., 1998) (Lee and Chang, 2003) (Rochette-Egly, 2003). Protein kinase activity on the AR, and its' co-activators, therefore acts synergistically with hormone/ligand to achieve maximal AR transcriptional activity (Rochette-Egly, 2003).

However AR phosphorylation and activity is not just dependent on receptor or ligand concentration nor even ligand binding, as other mechanisms may allow androgen-independent phosphorylation of the AR (Edwards and Bartlett, 2005) (see **Figure 4**). Phosphorylation at different, but specific, consensus serine and proline peptide sequences can be mediated by a variety of cytokines and growth factors which act as ligands for tyrosine-kinase receptors (Rochette-Egly, 2003). These in turn stimulate several intracellular protein kinase signal transduction cascades, which enter the nucleus to act on the AR (Rochette-Egly, 2003). Cross-talk with these signalling pathways may promote the activation of the AR even in the absence of androgens (Rochette-Egly, 2003) (Craft et al., 1999) (Edwards and Bartlett, 2005). For example, *in vitro* studies have already demonstrated that activation of the MAP Kinase and Akt signal transduction cascades can result in phosphorylation of the AR (Chen et al., 2004) (Abreu-Martin et al., 1999) (Yeh et al., 1999) (Wen et al., 2000) (Bakin et al., 2003) (Rochette-Egly, 2003). This results in AR-mediated transcription with or without androgen. MAP Kinase can phosphorylate the NTD AF-1 region of the AR at serine 515 while Akt phosphorylates at serine 210, both aiding recruitment of co-activators. In addition, the LBD also contains sites that act as

targets for ligand-independent phosphorylation by intra-cellular kinases (Rochette-Egly, 2003). The resulting increase in the transcription of target genes and their growth stimulatory effects may therefore be a mechanism of AIPC development.

Figure 4. Interaction of Signal Transduction Pathways with the Androgen Receptor



Phosphorylation and activation of the AR can therefore occur via cross-talk with growth factor and cytokine-mediated signalling pathways. *In vivo*, this may allow the AR to be activated in the absence of androgens, to other steroids, or at the very least sensitised to the low levels of circulating adrenal androgens that are present during androgen-deprivation therapy (Chen et al., 2004) (Rochette-Egly, 2003). The AR gene itself contains AREs implying a self-regulating, but potentially positive feedback mechanism upon over-activation of the AR-pathway, which would result in its' own upregulation (Chen et al., 2004).

AR activation may therefore be affected in many ways, at multiple levels, and with differing results. However, there is no evidence as yet that phosphorylation of the AR by other pathways can induce homodimer formation, or that ligand-independent activation always leads to cell proliferation. Furthermore, the on going and rising expression of PSA in AIPC may suggest that, despite AR suppression, AR-independent mechanisms can occur and are responsible for tumour growth. The AR-signalling pathway might in fact be bypassed completely by activation of regulatory molecules capable of effecting the transcription of androgen-dependent genes e.g. AP-1, p53, bcl-2, and neuroendocrine factors (Grossmann et al., 2001).

The alteration in the expression and function of signalling cascades involved in AR regulation could therefore result in tumour proliferation with or without involvement of the AR-signalling pathway. Targeting of these signalling pathways may serve as a future therapeutic strategy, for example through the use of monoclonal antibodies directed at growth factor receptors, inhibition of receptor tyrosine kinase activity or via the specific inhibition of cell signalling molecules (such as MAP Kinase or Akt) (Agus et al., 1999) (Morris et al., 2002) (Wen et al., 2000) (Lee et al., 2002). However much of the data involving their role in the molecular basis of AIPC has resulted from *in vitro* studies, and their presence in clinical AIPC remains undefined (Chen et al., 2004). It is therefore vital to determine the clinical importance of these signal transduction cascades with reference to AIPC patients using clinical material (Grossmann et al., 2001). To this effect, our laboratory has already identified 2 members of the MAP kinase pathway (hRas and Raf-1) whose genes are amplified more commonly in androgen-insensitive compared to androgen-sensitive prostate cancer (Edwards et al., 2003b).

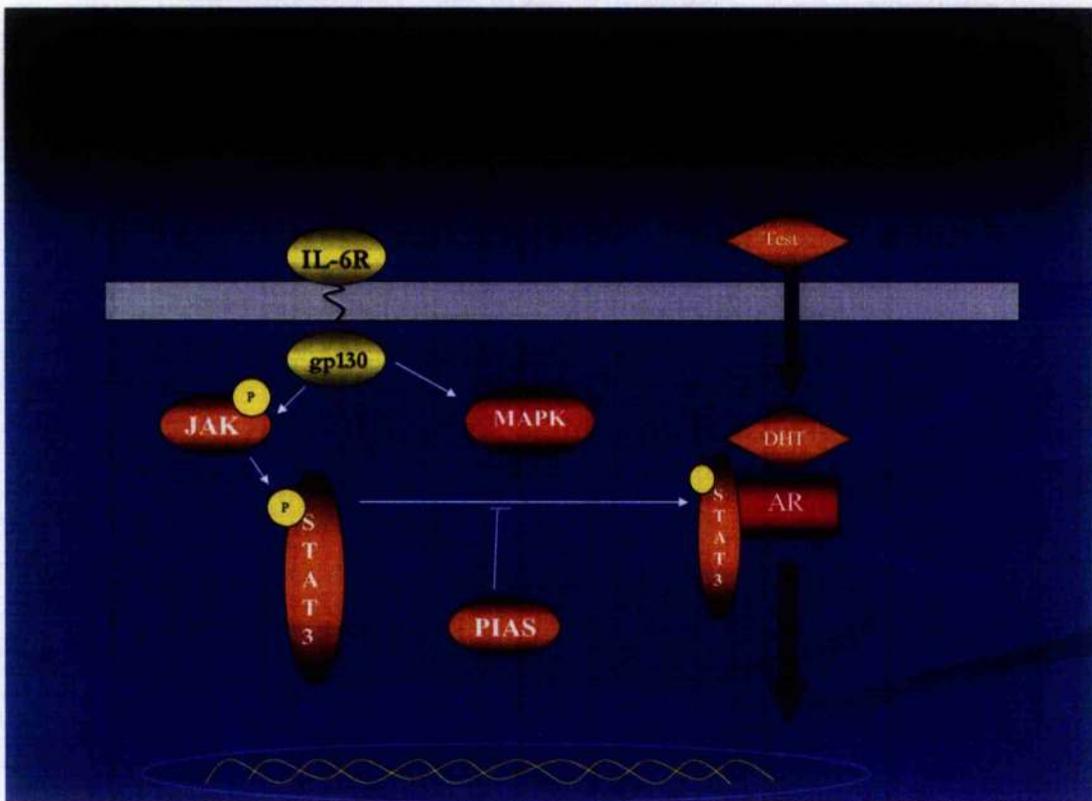
1.3 Signal Transduction Pathways associated with AIPC

1.3.1 IL-6 and the JAK/STAT Pathway

Among the various pathways known to regulate AR activity, the IL-6 cascade is thought to play an important role. IL-6 was originally described as a regulator of the immune and inflammatory responses, but is now known to influence tumour cell growth in an autocrine/paracrine manner (Ueda et al., 2002b). The IL-6 receptor (IL-6R) contains the signal transducer gp130 which, upon ligand-binding, dimerises and activates the Janus kinases (JAK). The signal transducer and activator of transcription 3 (STAT3) protein is the main target of IL-6R (Culig et al., 2002) (Ueda et al., 2002b) (Grossmann et al., 2001) and, upon tyrosine phosphorylation by JAK, migrates to the nucleus and initiates transcription (Ueda et al., 2002b) (Figure 5). It has already been suggested that activation of the JAK/STAT3 pathway via IL-6R plays an important role in ligand-independent activation of the AR, and may be a factor in the development of AIPC (Ueda et al., 2002b) (Chen et al., 2002). Activated STAT3 has been shown to bind the AR, independently of ligand, and subsequently transcribe androgen-regulated genes in LNCaP cells (Ueda et al., 2002b) (Chen et al., 2002). Expression of PIAS (protein inhibitor of STAT3) is known to reduce AR activity (Chen et al., 2002). Whilst the PSA gene does not contain STAT3 binding sites, so cannot be transcribed by it directly, STAT3 has been shown to directly interact with amino acids 234-558 of the AR (Ueda et al., 2002b). Furthermore, the AR can be activated by IL-6R by phosphorylation at the NTD, resulting in increased PSA expression and cellular proliferation, independently of androgen, in LNCaP and DU-145 cell lines (Ueda et al., 2002b) (Hobisch et al., 1998). This may be mediated via STAT3 or MAP Kinase. Subsequent addition of low amounts of androgen synergistically increases PSA expression (although not proliferation), further implicating IL-6 in clinical AIPC development. IL-6R can activate MAP Kinase directly through JAK

(Ueda et al., 2002b), or indirectly via a gp130-mediated interaction and activation of HER2 (Qiu et al., 1998). Furthermore, MAP Kinase may in turn activate STAT3 (Ueda et al., 2002b), indicating a critical role for MAP Kinase in merging or focusing signal cascades into a common pathway (Figure 5). Finally, in AR negative cells, IL-6R has also been shown to interact with the PI3K/Akt pathway (Chung et al., 2000).

Figure 5. The IL-6/JAK/STAT Pathway and its' interaction with the AR and MAP Kinase



In vitro models of AIPC have also demonstrated upregulation of IL-6R mRNA and protein expression (Hobisch et al., 2000) (Culig et al., 2002). Both stimulatory and inhibitory effects on LNCaP cell growth have been reported (Hobisch et al., 1998) (Culig et al., 2002) (Ueda et al., 2002b), which may be attributable to the number of cell passages (Culig et al., 2002). The clinical relevance of IL-6R, however, remains unclear. IL-6 and IL-6 receptor are expressed in benign and malignant prostatic tissue. Serum IL-6 is elevated in AIPC, while protein expression of IL-6R is upregulated in prostate cancer

tissue specimens (Culig et al., 2002) (Drachenberg et al., 1999) (Grossmann et al., 2001) (Edwards and Bartlett, 2005) (Ueda et al., 2002b) implying that autocrine or paracrine loops are being formed (Hobisch et al., 2000). This may involve the transcription factor NFkB, which positively regulates the secretion of IL-6 and promoter activity of the IL-6 gene.

1.3.2 Type 1 Receptor Tyrosine Kinases – The EGF-Receptor Family of Growth Factor Receptors

The epidermal growth factor receptor family of type 1 receptor tyrosine kinases has 4 members – EGFR (erbB1 or HER 1), HER2 (erbB2 or neu), HER3 (erbB3), and HER4 (erbB4). In addition, there exist mutated forms such as the constitutively-active form of the EGFR - variant III EGFR (EGFR vIII). Increased expression of EGFR, HER-2 and EGFR vIII has all been described in prostate cancer (Craft et al., 1999) (Olapade-Olaopa et al., 2000).

Upon binding of ligand, EGFR dimerises and activates downstream signal transduction pathways. EGF has been shown to activate AR in the absence of androgen in DU-145 cells (Culig et al., 1994) although contradictory findings have also been reported, which may reflect differences in cell-line differentiation (Ueda et al., 2002b). Both ligand-dependent and independent effects on AR activity have been reported, however EGF is a less potent stimulator of the AR than androgen (Gupta, 1999). EGFR may be upregulated by androgenic hormones (Culig et al., 2002). Interestingly, progressive loss of EGFR has been noted with the progression of prostate cancer (Olapade-Olaopa et al., 2000). This may be a result of EGFR vIII overexpression, resulting in dysregulated growth independent of ligand (Edwards and Bartlett, 2005). Consistent with this, it has been

shown that EGF vIII expression is markedly increased in prostate cancer specimens (Olapade-Olaopa et al., 2000).

HER2 contains intrinsic tyrosine kinase activity, and is thus able to activate downstream pathways in the absence of ligand (Wen et al., 2000). HER2 amplification has been shown in several cancers including AIPC, and is associated with a poor prognosis. Only low-level heterogeneous HER2 amplification, however, is observed in prostate cancer (Ouyang et al., 2001) (Kaltz-Wittmer et al., 2000) (Bartlett et al., 2005). Amplification of the HER2 gene has been implicated with other endocrine neoplasms including breast and ovarian cancers (Slamon et al., 1989). HER2 status predicts failure of anti-oestrogen therapy, and is associated with decreased survival in node positive breast cancer (Leitzel et al., 1995). Overexpression has also been linked to neovascularisation in breast cancer (Ross et al., 1997) and has been shown to induce cyclo-oxygenase-2 (COX-2) expression in colorectal cancer (Vadlamudi et al., 1999). The use of herceptin, a monoclonal antibody directed at preventing HER2 activation, is now well established clinically in advanced breast cancer in combination with chemotherapy (Craft et al., 1999) (Solit and Agus, 2001).

As a result of its' successful translational-based research in breast cancer, HER2 activity has been studied extensively with relation to the development of AIPC (Yeh et al., 1999) (Craft et al., 1999) (Solit and Agus, 2001). Overexpression and/or gene amplification of HER2 has been noted in clinical AIPC as well as in the pre-malignant prostatic intraepithelial neoplasia (PIN), suggesting a role in prostatic carcinogenesis (Grossmann et al., 2001) (Morris et al., 2002) (Signoretti et al., 2000) (Ross et al., 1997) (Kuhn et al., 1993) (Mellon et al., 1992). Gene amplification and increase copy number has also been

shown to correlate with high Gleason score, poorer prognosis and androgen resistance in several studies but not others (Craft et al., 1999) (Sadasivan et al., 1993) (Shi et al., 2001) (Wen et al., 2000) (Ross et al., 1997) (Solit and Agus, 2001). Consistent with other reports, work in this laboratory using matched ASPC and AIPC tumours demonstrated that, although amplification of HER2 is not associated with hormone relapse, increased protein expression significantly impacts on patient survival (Bartlett et al., 2005). In support of this, it has been demonstrated that HER2 expression increases in the progression from androgen-sensitive to androgen-insensitive disease in both cell-line studies and xenograft studies, reversing growth arrest and allowing ligand-independent growth via the AR (with a concomitant increase in PSA expression) (Craft et al., 1999) (Agus et al., 1999) (Yeh et al., 1999). In addition, synergistic growth in the presence of low levels of androgen has also been noted. Serum levels of HER2 extracellular domain have also been correlated with AIPC (Arai et al., 1997), and may be related to variable tumour protease activity (Solit and Agus, 2001).

Interestingly, unlike EGFR, HER2 activation of the AR cannot be blocked by the antiandrogen bicalutamide, which acts at the ligand-receptor interface. This indicates a different and more distal site of interaction between HER2 and the AR compared with EGFR, and provides a mechanism for AIPC development (Craft et al., 1999) (Yeh et al., 1999). HER2 cross-talk with the AR may involve a signal transduction pathway resulting in post-translational modification of the AR as discussed earlier (perhaps in combination with activation of a co-activator or accessory protein involved in the transcription complex) (Craft et al., 1999) (Yeh et al., 1999). HER2 is known to activate the MAP Kinase pathway (Ben Levy et al., 1994) and its' overexpression can stimulate proliferation of LNCaP cells via MAP Kinase (Craft et al., 1999). Concurrent PSA

expression falls upon addition of the MAP Kinase inhibitors (Yeh et al., 1999). Interestingly, simultaneous addition of ARA-55 and ARA-70 increases AR transactivation synergistically with HER2 (Yeh et al., 1999). This study therefore suggests a role for HER2 in promoting ARA-AR interaction, via MAP Kinase. HER2 may also be a central component of IL-6 signalling via MAP Kinase in prostate cancer, as has been demonstrated in LNCaP and DU-145 cell lines (Qiu et al., 1998). Although MAP Kinase inhibition decreases, it does not completely block PSA expression via HER2-AR activation, implying that HER2 can act via other signal cascades. In support of this, HER2 (and EGFR) are both known to activate the PI3K/Akt pathway, promoting tumourigenesis via the AR in the absence of androgen (Zhou et al., 2000) (Wen et al., 2000).

As a result of their possible role in advanced prostate cancer, type 1 receptor tyrosine kinases may provide a novel target for antibody therapy such as Herceptin and Iressa (1701). A recent phase II study using a bispecific antibody against HER2, involving 25 patients with HER-2 positive hormone refractory prostate cancer, has yielded promising results (James et al., 2001). A phase Ib/II trial of the anti-EGFR monoclonal antibody cetuximab, in combination with the doxorubicin, produced stable disease in 38% of androgen independent prostate cancer patients (Slovin et al., 1996). Studies investigating androgen independent prostate cancer xenograft models have reported positive results with herceptin especially in combination with paclitaxel (Agus et al., 1999).

The current data therefore supports the hypothesis that signal transduction cascades, and such as the Raf/MAP Kinase pathway, can act as mediators for the modulation and ligand-independent activation of the AR by HER2 in patients with AIPC. However there is no data as yet to support this using clinical material.

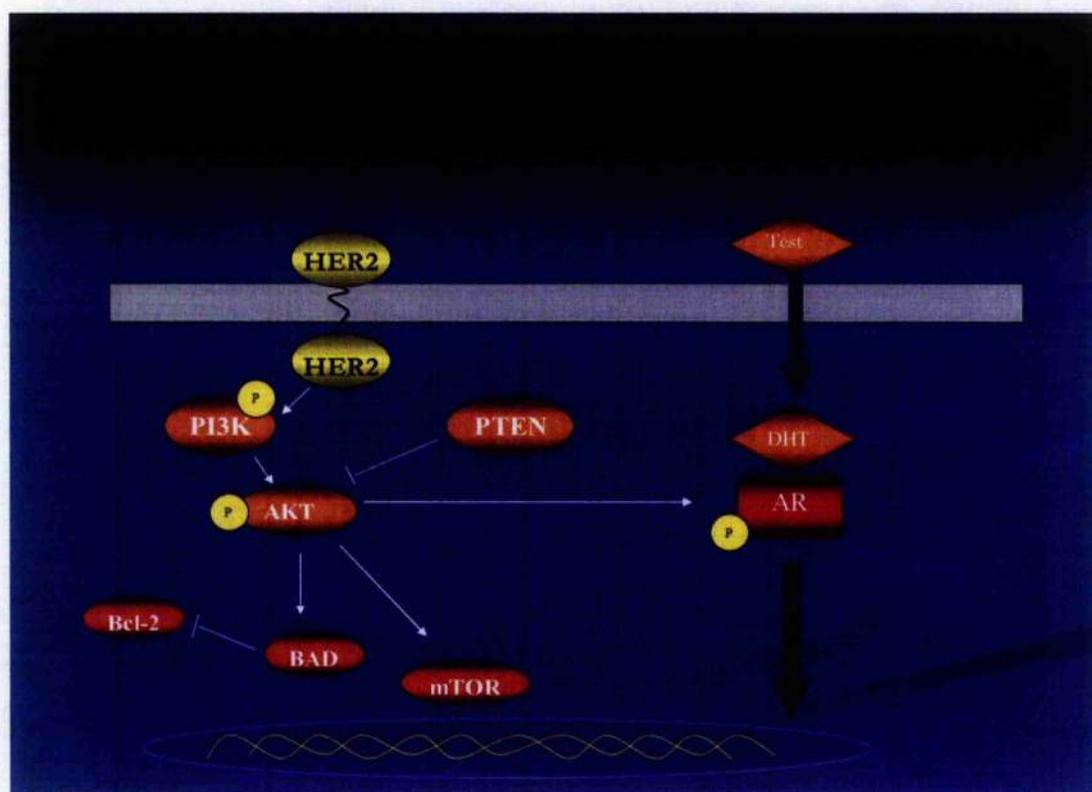
1.3.2.3 PI3K/Akt Pathway

The PI3K/Akt pathway plays a key role in the control of cell proliferation and survival. Overexpression of the pathway has therefore been implicated in prostatic carcinogenesis. Constitutive activation of PI3K (phosphatidylinositol 3-kinase) or Akt can induce cellular transformation (Davies et al., 1999). PI3K phosphorylates phosphatidylinositol to phosphatidylinositol triphosphate (PIP3), which binds and contributes to the activation of the serine-threonine kinase Akt (or protein kinase-B). Akt subsequently migrates to the cell membrane at which point phosphorylation, by the phosphoinositide-dependent protein kinases 1 and 2 (PDK1 and PDK2), results in its' complete activation. Akt goes on to inhibit pro-apoptotic proteins (such as BAD) and activate proteins involved in cell proliferation (such as mTOR) (Grossmann et al., 2001) (Gao et al., 2003) (see **Figure 6**).

Inactivation of PTEN (phosphatase and tensin homologue deleted on chromosome 10), an inhibitor of PI3K, by mutations and deletions is known to occur in advanced prostate cancer (Culig et al., 2002) (Fenci and Woenckhaus, 2002) (Li et al., 2001). The subsequent activation of PI3K/Akt phosphorylates BAD, leading to increased bcl-2 activation (a known inhibitor of apoptosis) (see **Figure 6**) (Grossmann et al., 2001). Overexpression of bcl-2 has also been implicated in AIPC development, perhaps serving as another mechanism to bypass the AR pathway (Grossmann et al., 2001) (Chen et al., 2004). However, PTEN is also known to cause regression and inhibition of AR activity via inhibition of PI3K, thereby inducing apoptosis and suppression of PSA (Li et al., 2001). Overexpression of bcl-2 in LNCaP cells blocks PTEN-induced apoptosis but not growth suppression, implying other regulatory effects of PTEN and PI3K/Akt (Davies et al., 1999). Interestingly, AR activity may be reduced by phosphorylation at serine 210 in the NTD - the site of action of Akt - disrupting co-activator binding (Edwards and

(Bartlett, 2005). This may lead to diminished expression of the pro-apoptotic androgen-regulated gene, p21/WAF1. The PI3K/Akt pathway has therefore been shown to promote prostate cancer cell survival and growth both via and independently of the AR (Lin et al., 2001) (Sharma et al., 2002).

Figure 6. The PI3K/Akt Pathway



Whilst withdrawal of androgen triggers apoptosis in androgen-sensitive tumours, this does not occur in AIPC (Grossmann et al., 2001). Upregulation of Akt appears to compensate for this in LNCaP cells (Gao et al., 2003) and Akt activity is also known to be greater in AIPC tumour cell lines (Graff et al., 2000) (Nakatani et al., 1999). Its' expression increases in high-grade tumours, correlating with serum PSA (Fenci and Woenckhaus, 2002) (Liao et al., 2003). Akt can increase AR expression in LNCaP cells (Manin et al., 2002) and activate PSA expression in the absence of androgen (Wen et al., 2000). It has

been suggested, therefore, that androgen-deprivation may promote AIPC by stimulating a HER2/PI3K/Akt pathway, resulting in cell survival and indeed cellular proliferation (Wen et al., 2000) (Lin et al., 2001).

This data would imply that the PI3K/Akt pathway may have a significant role to play in the development of AIPC, either through the prevention of apoptosis or by stimulation of androgen-regulated genes possibly via the AR. PI3K is known to be activated by IL-6 as well as EGFR and HER2 (Grossmann et al., 2001) (Wen et al., 2000) and Akt can also crosstalk with the MAP Kinase pathway, a point which will be discussed later. In support of this, our laboratory has also demonstrated gene amplification of PI3K and Akt as well as members of the MAP Kinase pathway with the development of AIPC (Edwards et al., 2003b). Further studies are therefore required to determine the full clinical role of the PI3K/Akt pathway in the development of AIPC.

1.4 The Raf/MAP Kinase Pathway Signal Transduction Cascade

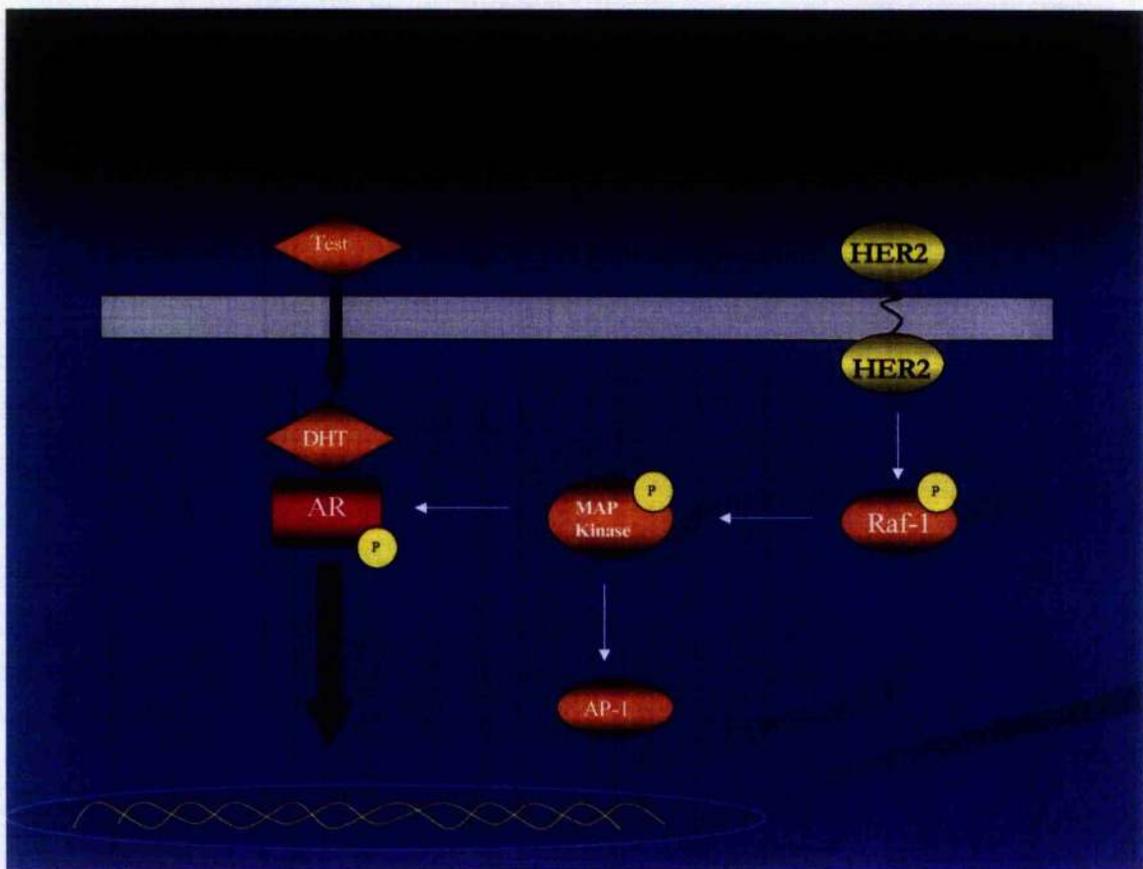
1.4.1 Background Role in Carcinogenesis

The Raf/MAP Kinase pathway is a signal transduction cascade that plays an important role in cellular proliferation, differentiation and apoptosis (Zhu and Liu, 1997). Several growth factors are known to stimulate the pathway via receptor tyrosine kinases. Once activated the membrane-bound GTPase, Ras, phosphorylates the Raf family of serine/threonine kinases, of which Raf-1 is ubiquitously expressed (Weinstein-Oppenheimer et al., 2000). When activated, by phosphorylation at the serine 338 position, Raf translocates to the membrane where it activates the MAP Kinase kinases (or MEKs). This results in a cascade of successive phosphorylation steps, culminating in activation of p42/44 MAP Kinase at specific tyrosine and threonine sites (Weinstein-Oppenheimer et al., 2000) (see **Figure 7**). Several other protein kinase pathways, once activated, are also known to converge on the MAP Kinase cascade (Rochette-Egly, 2003). In turn, MAP Kinase has a variety of downstream targets including other protein kinases, receptors for hormones and growth factors, and nuclear transcription factors. Given its' role in cellular development, disruption to the pathway is thought to result in pathological changes such as carcinogenesis.

Alterations to any member of the Raf/MAP kinase pathway have therefore been linked with the progression of several cancers (Weinstein-Oppenheimer et al., 2000) (Khleif et al., 1999) (Ito et al., 1998) (Oka et al., 1995). MAP Kinase is known to activate the oestrogen receptor in breast cancer cells upon stimulation by oestradiol or EGF (Kato et al., 1995) (Bunone et al., 1996). Prostate cancer cell-line studies have also revealed increased cell proliferation via type 1 receptor tyrosine kinase activation of the MAP Kinase pathway (Bell et al., 2003). Up-regulation may lead to androgen-independent

activation of the AR via phosphorylation of the AF-1 domain as mentioned previously (Feldman BJ and Feldman D, 2001) (Rochette-Egly, 2003). Furthermore, in vitro studies have demonstrated that activation of the Raf/MAP Kinase pathway may increase prostate cancer cell growth both via and independently of the AR, further implicating it in the development of AIPC (Abreu-Martin et al., 1999) (Bakin et al., 2003) (Yeh et al., 1999) (Zhu and Liu, 1997).

Figure 7. The Raf/MAP Kinase Pathway



Most solid tumours demonstrate a link between Ras mutation and MAP Kinase activation (Edwards and Bartlett, 2005). Mutated Ras has been linked with increased levels of activated MAP Kinase and the development of androgen-independent growth in

LNCaP cells (Bakin et al., 2003) (Voeller et al., 1991). Activated MAP Kinase and MEK are also differentially expressed during the progression of prostate cancer in a transgenic mouse model (Uzgare et al., 2003). Raf and MEK are expressed in both non-metastatic and metastatic prostate cancer cells (Weinstein-Oppenheimer et al., 2000) (Fu et al., 2003). Increased MAP Kinase activity is known to be elevated in androgen-insensitive cell-lines and in clinical AIPC (Abreu-Martin et al., 1999) (Gioeli et al., 1999). Intriguingly, the constitutively active form of MAP Kinase also induces Raf-1 activation in cell-line studies, forming the positive feedback loop required for chronic autocrine stimulation (Weinstein-Oppenheimer et al., 2000).

MAP Kinase can also increase transcription of androgen-dependent genes, independently of androgen, via phosphorylation of the AR or AR cofactors (Abreu-Martin et al., 1999) (Bakin et al., 2003) (Ueda et al., 2002b) (Rochette-Egly, 2003) (Franco et al., 2003). MAP Kinase is known to activate the AR NTD by phosphorylation of serine 515 independent of androgen (Yeh et al., 1999) (Rochette-Egly, 2003). Inhibition of MAP Kinase in cell-line studies is known to abrogate IL-6 and protein kinase-A (PKA) activation of the AR, suggesting a crucial role in the convergence of both pathways towards the AR NTD (Ueda et al., 2002b). Hydroxyflutamide has been shown to activate the Raf/MAP Kinase pathway independently, stimulating cell proliferation, possibly via a member of the EGFR receptor family (Lee et al., 2002). Activation of the pathway has therefore been linked with the pathogenesis of the androgen-withdrawal syndrome, by stimulating androgen-independent cell growth in response to antiandrogens. Data has also revealed increased activation of the MAP Kinase pathway with the clinical progression of prostate cancer (Lee et al., 2002) (Gioeli et al., 1999) (Magi-Galluzzi et al., 1997) (Magi-Galluzzi et al., 1998). Taken together, the MAP Kinase pathway

appears to play a crucial role in cross-talking with the AR-signalling pathway and modulating its' response to ligands. It may also function as a surrogate for ligand-activation during androgen withdrawal, resulting in the progression to AIPC.

The biological response of cells to activation of the Raf/MAPK pathway is dependent on their environment, which may explain its' role in proliferation and differentiation as well as growth arrest (Zimmermann and Moelling, 1999) (Kolch, 2000). This varies from the type, duration and strength of external stimuli (e.g. growth factors/cytokines, androgen, temperature, osmolarity, and genotoxic stress in the form of irradiation or chemotherapy) as well as the particular cell type studied (Zhu and Liu, 1997) (Abreu-Martin et al., 1999) (Zimmermann and Moelling, 1999). For example, in the absence of androgen, MAP Kinase kinase kinase (MEKK) activates transcription of androgen-regulated genes to induce apoptosis, not cell growth, via the AR-signalling pathway in LNCaP cells (Zhu and Liu, 1997) (Abreu-Martin et al., 1999). Addition of androgen increases this transcription synergistically. Therefore MAP Kinase activation can effect apoptosis upon androgen withdrawal. This may be mediated by AR-induced caspase activation or via direct stimulation of JNK (c-jun N-terminal kinase) (Zhu and Liu, 1997) (Abreu-Martin et al., 1999) (Magi-Galluzzi et al., 1997). Interestingly, antiandrogen treatment does not completely abrogate apoptosis or transcription of the PSA gene, which may be the result of a more distal site of MEKK activation on the AR pathway or may imply bypassing of the AR entirely. MEKK is also capable of inducing PSA expression 2-fold in the absence of the AR, which further supports this hypothesis.

1.4.2 Downstream Effectors of the Raf/MAP Kinase Pathway

As discussed earlier, the MAP Kinase cascade can be activated by a variety of growth factor and cytokine receptors, of which the type 1 receptor tyrosine kinases are the most commonly studied in prostate cancer. Similarly, activation of MAP Kinase can have a variety of consequences. MAP Kinase is capable of direct phosphorylation of the AR *in vitro*, resulting in its' activation. MAP Kinase may in fact effect AR post-translational modifications indirectly via other signal transduction cascades (Zhu and Liu, 1997) (Abreu-Martin et al., 1999). Members of the MAP Kinase cascade are capable of interacting with several other signalling molecules, such as the AR co-activators (e.g. ARAs and GRIP), in order to form large multi-protein complexes involving the AR itself and possibly aiding its' activation (Abreu-Martin et al., 1999) (Bevan and Parker, 1999) (Fujimoto et al., 2001). The AR co-activator SRC-1 (steroid receptor co-activator) contains 2 potential phosphorylation sites for MAP Kinase (Suzuki et al., 2003) (Rowan et al., 2000). Activation of SRC-1 may therefore be an important route for androgen-independent AR activation through its' interaction with the AR NTD and LBD (Ueda et al., 2002a). As mentioned earlier, SRC-1 expression increases after hormone therapy and tumour recurrence (Gregory et al., 2001a) and it has been shown to enhance androgen-independent activation of the AR in LNCaP cells (Edwards and Bartlett, 2005). Whether these events occur in prostate cancer cells *in vivo* is not yet known, but MAP Kinase may well act on the AR directly via phosphorylation and indirectly via activation of co-activators.

Simultaneous phosphorylation of steroid receptors as well as other transcription factors, resulting in differing transcription events, has been described (Rochette-Egly, 2003). MAP Kinase can function through transcription factors such as AP-1 (activated protein-1 - a heterodimer formed between c-fos and phosphorylated c-jun), Elk-1, c-myc as well as

NF- κ B. These may co-operate with the AR resulting in activation of androgen-dependent genes (Bubulya et al., 2001) (Sato et al., 1997) (Wise et al., 1998). MEKK can target c-jun N-terminal kinase (JNK) which is required for AP-1 activity *in vitro*. In addition, c-jun can interact with the NTD and hinge region of the AR, promoting homodimerisation and increasing its' transactivation ability, even in the absence of ligand (Wise et al., 1998) (Bubulya et al., 2001). C-jun can also prevent the inhibitory effects of the AR co-repressor p53 (Lee and Chang, 2003), which may be another mechanism by which MAP kinase activates the AR to effect androgen-independent growth (Zhu and Liu, 1997).

The PSA promoter site itself contains binding sites for various transcription factors, including AP-1, possibly allowing for AR-independent transcription via the MAP Kinase pathway. The MAP Kinase pathway is known to stimulate AP-1 transcriptional activity in both AR-positive and negative cells (Abreu-Martin et al., 1999). Activation of the natural PSA promoter in the absence of androgen may occur as a result of such ARE-independent effects, as use of an artificial template in cell-line work required ligand-binding activation of the AR (Abreu-Martin et al., 1999). Activated MAP Kinase therefore may stimulate prostate cancer cell growth independently of the AR *in vivo* via activation of transcription factors such as AP-1 (Weinstein-Oppenheimer et al., 2000). Work in our own laboratory has already linked increased AP-1 expression with androgen-resistance and decreased patient survival (Edwards et al., 2004).

1.4.3 Raf-1

Raf-1 is ubiquitously expressed and is regarded as the main Raf molecule. As a result it has become the most studied and best characterised member of the MAP Kinase cascade (Dhillon et al., 2003). Raf-1 connects with a variety of upstream extracellular signals

(through cell surface receptors) integrating and relaying their signals downstream to effect nuclear transcription via other members of MAP Kinase cascade (Dhillon et al., 2002a). It acts as the sole entry point to the MAP Kinase cascade, via its' activation of MEK (Dhillon et al., 2002b) (Tzivion et al., 1998). The Raf/MAPK pathway is therefore regarded as a linear pathway due to the narrow range of substrates each member of the pathway has i.e. Raf-1 activates MEK only and MEK only activates MAP kinase (Dhillon et al., 2003).

Inactive Raf-1 in the cytoplasm is bound to the HSP90, HSP50, Cdc37 and 14-3-3 proteins (Tzivion et al., 1998). Its' activation depends predominantly (but not entirely) on receptor activation of GTP-bound-Ras which binds to the N terminal domain of Raf-1 - primarily to the Ras-binding domain (RBD) of Raf-1, but also to the Raf-1 cysteine-rich domain (CRD) via its' farnesylated tail (Dhillon et al., 2002a) (Abraham et al., 2000). Raf is the main effector of Ras (Dhillon et al., 2003), and Ras is activated itself at the site of receptor tyrosine kinases by binding to GTP. This is catalysed through activation of 2 proteins, Grb-2 (growth factor receptor bound protein-2) and SOS (guanine-nucleotide exchange factor "son of sevenless"). Upon binding to Ras, Raf translocates from the cytoplasm to the plasma membrane where a multistep activation process takes place. This involves phosphorylation of the serine 338 and tyrosine 341 residues (Dhillon et al., 2002a). Only a small fraction of Raf-1 is actually activated, however, and subsequently released from the membrane back into the cytoplasm (Dhillon et al., 2002a) (Dhillon et al., 2002b) (Tzivion et al., 1998).

In resting cells, the phosphorylation status of 3 serine residues in the N-terminal (regulatory) domain of Raf-1 – serines 43, 259, 621 - play a crucial role in the regulation

of Raf-1 activation and its' downstream signalling ability (Dhillon et al., 2002b). Serine 259 is the main inhibitory phosphorylation site and as such plays a pivotal role in Raf-1 activation (Dhillon et al., 2002b), mediating binding of the 14-3-3 protein which results in Raf-1 inactivation (Tzivion et al., 1998). This in turn interferes with phosphorylation of the serine 338 activation site (an essential activating site of Raf-1 in the kinase domain), and possibly disrupts the interaction with Ras (Zimmermann and Moelling, 1999) (Dhillon et al., 2002a) (Dhillon et al., 2002b). The physical presence of a phosphate group at serine 259 is necessary for this inhibition (Dhillon et al., 2002b). Conversely, Ras-mediated dephosphorylation of serine 259 reduces binding to 14-3-3, selectively (i.e. does not interfere with the serine 621 site, which is the other binding point of 14-3-3). This promotes membrane translocation, from its' inactive state in the cytoplasm, and making it accessible for further dephosphorylation and activation by the enzyme phosphatase, PP2A (Dhillon et al., 2002a). Dephosphorylation on serine 43 and 621 in resting cells, which is also essential for Raf-1 function (Abraham et al., 2000) (Tzivion et al., 1998), is possibly mediated by phosphatase PP2A, which binds Raf-1 at the cell membrane. This in turn prevents formation of inactive 14-3-3 complexes (Abraham et al., 2000). Ras binding also induces the subsequent phosphorylation of serine 338, at the cell membrane (Dhillon et al., 2002a).

Raf activity is therefore dependent on phosphorylation of serine residues and the integrity of the 14-3-3 dimer. Binding of the 14-3-3 protein stabilises inactive as well as active conformations, as its' displacement can lead to deactivation of Raf-1 kinase activity and complete loss of catalytic function (Tzivion et al., 1998). The inhibitory effect of serine 621 may also be reversed by binding of 14-3-3 (Tzivion et al., 1998) (Dhillon et al., 2002b). Dephosphorylation of serine 259, in response to Ras-stimulation, is also essential for and corresponds to peak activation of Raf-1 by facilitating its' membrane translocation

and further enhancing its' interaction with Ras (Dhillon et al., 2002a). Dephosphorylation of serine 259 therefore enhances the catalytic activity of Raf-1, increasing coupling to and activation of MEK. PP2A-mediated dephosphorylation prevents further formation of inactive 14-3-3 complexes, increasing Raf-1 activation (Abraham et al., 2000). This may be aided further through increased turnover of the Raf-1/MEK complex through an allosteric effect and binding of modulating proteins (e.g. Raf-1 inhibitory protein or RKIP) (Dhillon et al., 2002a). Once phosphorylated at the membrane site, MEK dissociates to the cytosol (Dhillon et al., 2002a). Mutations to the serine 259 site (to alanine, aspartate or glutamine) results in a constitutively active Raf-1 resistant to phosphorylation. This increases kinase activity because of enhanced binding to Ras (Zimmermann and Moelling, 1999) (Dhillon et al., 2002a) (Dhillon et al., 2002b). Mutated serine 259 is unable to bind 14-3-3 and also facilitates phosphorylation of serine 338 to a greater degree.

Interestingly, whilst effecting cell proliferation in cell-line studies, mutated serine 259 fails to induce the malignant transformation and differentiation associated with enhanced activation of the MAP Kinase pathway (Dhillon et al., 2003). Mutations to serine 259 therefore play a crucial role in governing the biological outcome of Raf-1 activation by altering its' ability to activate the normal MAP Kinase pathway, perhaps indicating the existence of an alternative Raf-1 effector pathway separate to MAP Kinase. In support of this, Raf-driven inhibition of apoptosis has been noted to be independent of MAP Kinase (Dhillon et al., 2003). This may be aided by upregulation of RKIP, which is known to disrupt Raf-1/ MAP Kinase coupling and also regulate cell transformation at multiple levels (e.g. suppression of NFkB).

Phosphorylation of serine 338 acts synergistically with phosphorylation of the remaining activation site, tyrosine 341 (Dhillon et al., 2002a). Phosphorylation of these activating sites is essential to but not sufficient for full activation of Raf-1 (Dhillon et al., 2002b), possibly combining to adjust Raf-1 activation to its' proper level (Dhillon et al., 2002a). Phosphorylation of serine 338 and dephosphorylation of serine 259 show close correlation with the catalytic activity of Raf-1, possibly co-regulated by PKA (Dhillon et al., 2002a). Hyperphosphorylation of serine 259 as well as serine 43 and 621 may be mediated by PKA, whose expression correlates with the level of inhibition of Raf-1 activity (Dhillon et al., 2002b). PKA therefore acts as a negative regulator of Raf-1, preventing a binding site for 14-3-3 and interfering with its' binding to Ras (Dhillon et al., 2002b) (Abraham et al., 2000). This provides the sole mechanism by which PKA crosstalks with and inhibits gene expression via the MAP Kinase pathway (Dhillon et al., 2002b). Mutant serine 259, interestingly, is resistant to PKA phosphorylation (Dhillon et al., 2002b).

Akt has also been shown to antagonise Raf-1 activity by direct phosphorylation of serine 259 – providing another example of cross-talk between the pathways (Zimmermann and Moelling, 1999). Interestingly, this work, carried out on a breast cancer cell-line, demonstrated a proliferative response to PI3K-Akt pathway with transient activation of the Raf/MAPK pathway. The prolonged activation of Ras however inhibited growth. (Zimmermann and Moelling, 1999). It is already accepted that autocrine growth factors can stimulate multiple pathways, such as the Raf/MAP Kinase and PI3K/Akt pathways. Raf-mediated transformation may be induced when regulatory cross-talk with other signal cascades is disrupted, leading to the defective inhibition of Raf activity.

1.5 Hypothesis and Statement of Aims

Although the Raf/MAP Kinase pathway has been implicated in the development of AIPC through cell-line and xenograft models (Bakin et al., 2003) (Abreu-Martin et al., 1999), its' precise role in the development of clinical AIPC is still poorly understood. Earlier studies using clinical tissue have revealed increased activated MAP kinase expression in only 2 and 4 patients respectively (Lee et al., 2002) (Gioeli et al., 1999). If chronic autocrine stimulation of the pathway by growth factors results in progression to androgen-independent disease, one would predict increased expression and activation of members of the pathway (Gioeli et al., 1999). The further elucidation of its' role would clearly have therapeutic implications as *in vitro* studies suggest inhibition of the pathway, perhaps in conjunction with hormone therapy or chemotherapy, may provide a novel method of treatment in AIPC (Bakin et al., 2003) (Zelivianski et al., 2003).

We hypothesised, therefore, that the Raf/MAP Kinase pathway promotes the development of clinical AIPC. The purpose of this M.D. was to further investigate the role of this signal cascade in AIPC by determining if the genetic changes we have previously identified had functional consequences in terms of protein expression. We therefore proposed to investigate the role of the Raf/MAP Kinase pathway in clinical, archival material consisting of paired, matched histological tissue specimens obtained before commencement of androgen deprivation therapy and after the development of androgen-escape. We investigated expression levels and activation status of Raf-1 and MAP Kinase, 2 critical members of the MAP kinase cascade, in these matched ASPC and AIPC tumours, using immunohistochemistry (IHC). We then correlated this expression with time to biochemical relapse, time to death from relapse and overall survival. We also correlated expression with that of the type 1 tyrosine kinase receptors, EGFR and HER2

(known to activate the pathway), and the downstream targets, AR and AP-1, in the same cohort of patients.

Project Aims

- To develop a pre-existing database of matched tissue specimens from 49 to 65 patients.
- To develop a tissue micro-array system and analyse its' use in the study of AIPC.
- To determine protein expression of the Raf-1, including its' active and inactive, phosphorylated forms, in AIPC specimens using immunohistochemistry.
- To determine protein expression of p42/44 MAP Kinase, including its' active, phosphorylated form (pMAP Kinase), in AIPC specimens using immunohistochemistry.
- To correlate protein expression with time to biochemical relapse, time to death from relapse, and overall survival
- To correlate expression with known upstream regulators and downstream targets, including EGFR, HER2, AR, and AP-1.

MATERIALS AND METHODS

2.1 PATIENT RECRUITMENT AND TISSUE RETRIEVAL

- EXPANSION OF ANDROGEN-ESCAPED DATABASE

The database used for the study consisted of matched pairs of tissue obtained sequentially from patients before androgen deprivation therapy and following the development of androgen escape. Patients were retrospectively selected for inclusion in the study if they had a full biochemical response to androgen deprivation therapy (as defined by falling serum PSA levels of at least 50%), but subsequently developed biochemical relapse (as confirmed by a sustained rise in PSA despite maximal treatment). Recruitment criteria therefore required the availability of androgen-sensitive or "naïve" tissue (i.e. prior to initial successful treatment). These sections originated from either a transurethral resection of the prostate (TURP) or from diagnostic transrectal ultrasound (TRUS) biopsies. Following the development of biochemical relapse, androgen-insensitive tissue was obtained from palliative TURP, carried out to relieve clinical outflow symptoms. This database originally consisted of 49 pairs, however this was expanded to 65 patients.

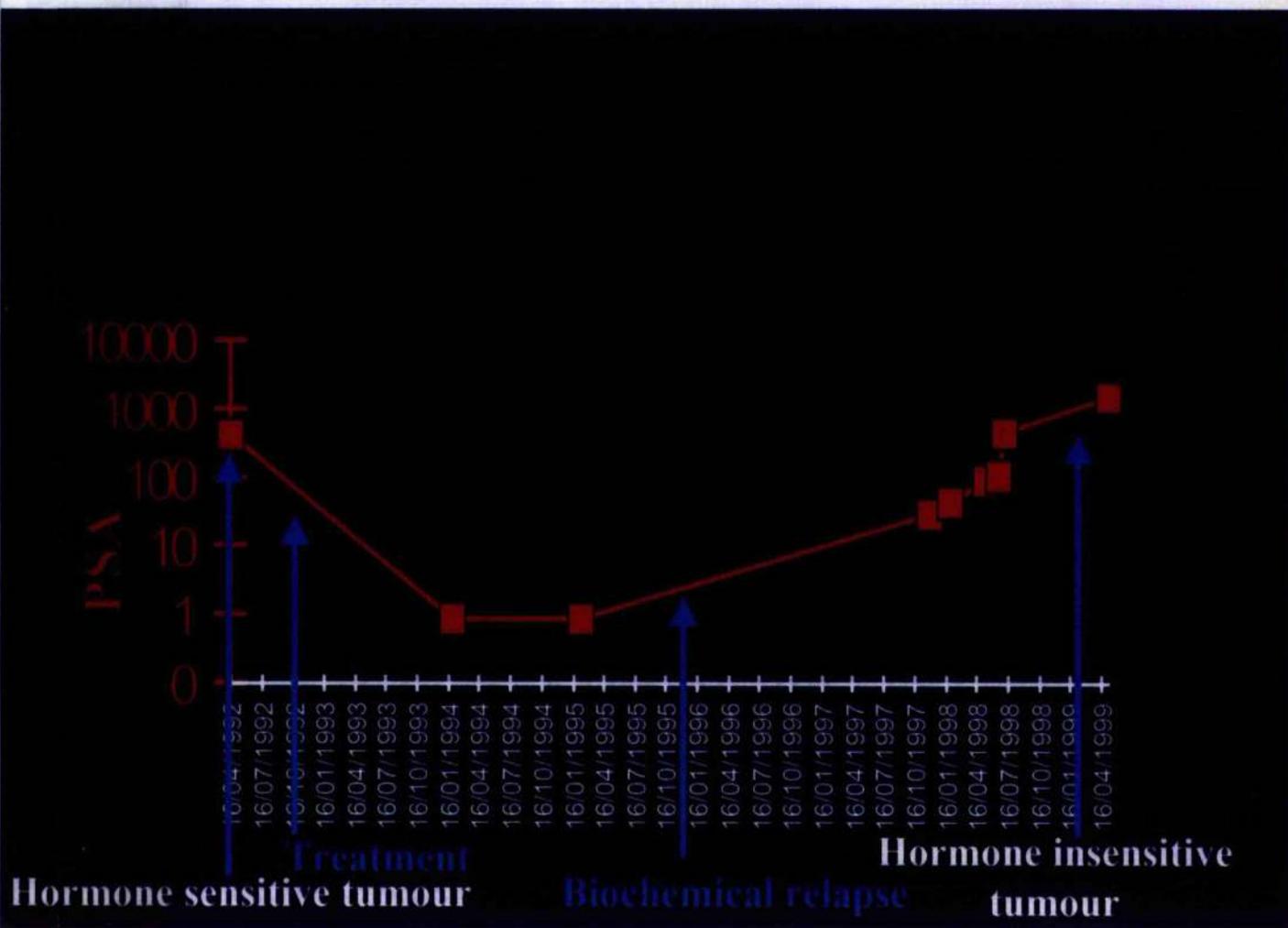
Twenty-one patients were recruited from Ayr Hospital after an extensive review of pathological records. From this search, patients with evidence of 2 or more separate prostatic biopsies were identified. Thereafter an extensive case note review was performed to identify those patients who satisfied the recruitment criteria as described above. The two sequential, paraffin-embedded tumour specimens from each patient (one taken before and one after the development of AIPC), were then retrieved from the pathological archives.

In order to develop this cohort we received ethical approval from the Multiple Research Ethical Committee (MREC), the relevant Local Research Ethical Committees (LREC), as well as support from the Scottish Urological Oncological Group (SUOG), to enable us to recruit patients from throughout Scotland. LREC approval was obtained from the following NHS trusts: Ayrshire and Arran, Glasgow Royal Infirmary, Lanarkshire, Lothian, and Stirling. Ethical approval also included permission for the construction of tissue micro-arrays (TMAs) (see **section 2.4**). Once paraffin blocks were sectioned, the remaining tissue was returned to the hospital archives.

Full clinical follow-up was available for every patient in the database, including treatment information, survival statistics and PSA profiles throughout therapy (an example of which can be found in **Figure 1** - see **Appendix 1** for full clinical, pathological, and biochemical data of entire patient cohort). Detailed pathological data on stage, the presence of metastasis and Gleason grade, where available, was also documented upon review of case notes. All tumour sections were then made anonymous by removal of patient identification including hospital and pathology numbers. Each tissue section was subsequently assigned a number, for coding purposes. Time to biochemical relapse, time to death from relapse and overall survival times were calculated from the date of diagnosis to the date of death (where available) or date of last follow-up (censored observations). The proportion of patients surviving was then calculated according to the method of Kaplan-Meier and any significant differences were identified using the log rank test. Furthermore, in order to determine correlations with MAP kinase pathway, data on PSA, AR, HER2, EGFR, and AP-1 expression were also made available for each tumour specimen from previous work carried out in the laboratory on the same tissue cohort.

Figure 1.

Example PSA profile for patient illustrating points where tumour samples were retrieved.



2.2 PREPARATION OF TISSUE SECTIONS

Formalin-fixed tissue embedded in paraffin blocks, was retrieved from the archives. Prior to sectioning, tissue blocks were cooled to -10°C (Tissue Tec[®] cooler). Four to Five micrometer thick sections were then cut from the blocks using a Leica[®] RM 2135 microtome. Ribbons of cut sections were then floated onto a waterbath, heated to 45°C , in order to flatten out any tissue folds (leading to artefacts), before application onto aminopropyltriethoxysilane-treated (sialinised) slides. Slides (BDH 1.0mm thick, clear glass) were sialinised in order to create greater adherence with the tissue, according to the following protocol:

- Slides placed in acetone for 5 minutes, followed by 2% silane (v/v 8ml in 400ml acetone).
- Slides then washed in running tap water for 5 minutes, before being bathed in tap water for a further 20 minutes, before dried overnight in a fume hood.

Sections were then ready for immunohistochemical staining (see **Section 2.5**)

2.3 CREATION OF TISSUE MICRO-ARRAYS (TMAs)

2.3.1 INTRODUCTION

Tissue micro-arrays (TMAs) are a well-recognised method facilitating the rapid analysis of multiple tissue specimens (Camp et al., 2000). In order to assess their usefulness and accuracy in determining protein expression in prostate cancer, TMAs of our dataset, were created and stained. Their histoscores were then compared and correlated with the standard histological scores of the corresponding full tissue sections.

2.3.1 HAEMATOXYLIN AND EOSIN STAINING

In order to identify tumour areas suitable for selection into the array, paraffin sections from each patient required staining with haematoxylin and eosin, using the following standard protocol:

- Sections dewaxed in 2 separate washes of xylene for 2 minutes each.
- Sections rehydrated in graded alcohol solutions – 2 washes of 2 minutes each in 99% alcohol; 1 minute wash in 90% alcohol; 1 minute in 70% alcohol – after which the sections were washed in running tap water.
- Sections then placed in a staining dish containing haematoxylin for 2 minutes, then washed in running tap water.
- Sections then destained in acid alcohol solution for a maximum of 10 seconds, before being washed in running tap water once again.
- Sections then placed in a staining dish containing eosin for 2 minutes, then washed in running tap water.
- Sections then dehydrated in graded alcohol solutions in reverse, followed by 2 washes in xylene.
- Sections then mounted in DPX, before application of cover slips

2.3.2 IDENTIFICATION OF TUMOUR AREAS

Areas of tumour to be cored were then confirmed by a Consultant Pathologist (KG). Where possible, areas were subclassified into predominantly high and low differentiation, based on their Gleason grade. Grade 3 (single, separate, more irregular glands or rounded masses of cribriform or papillary epithelium) was used as low, and grade 5 (almost absent, few tiny glands or signet ring cells) as high (Gleason, 1992). Most tumours contained a mixture of both high and low areas of terms of tissue differentiation.

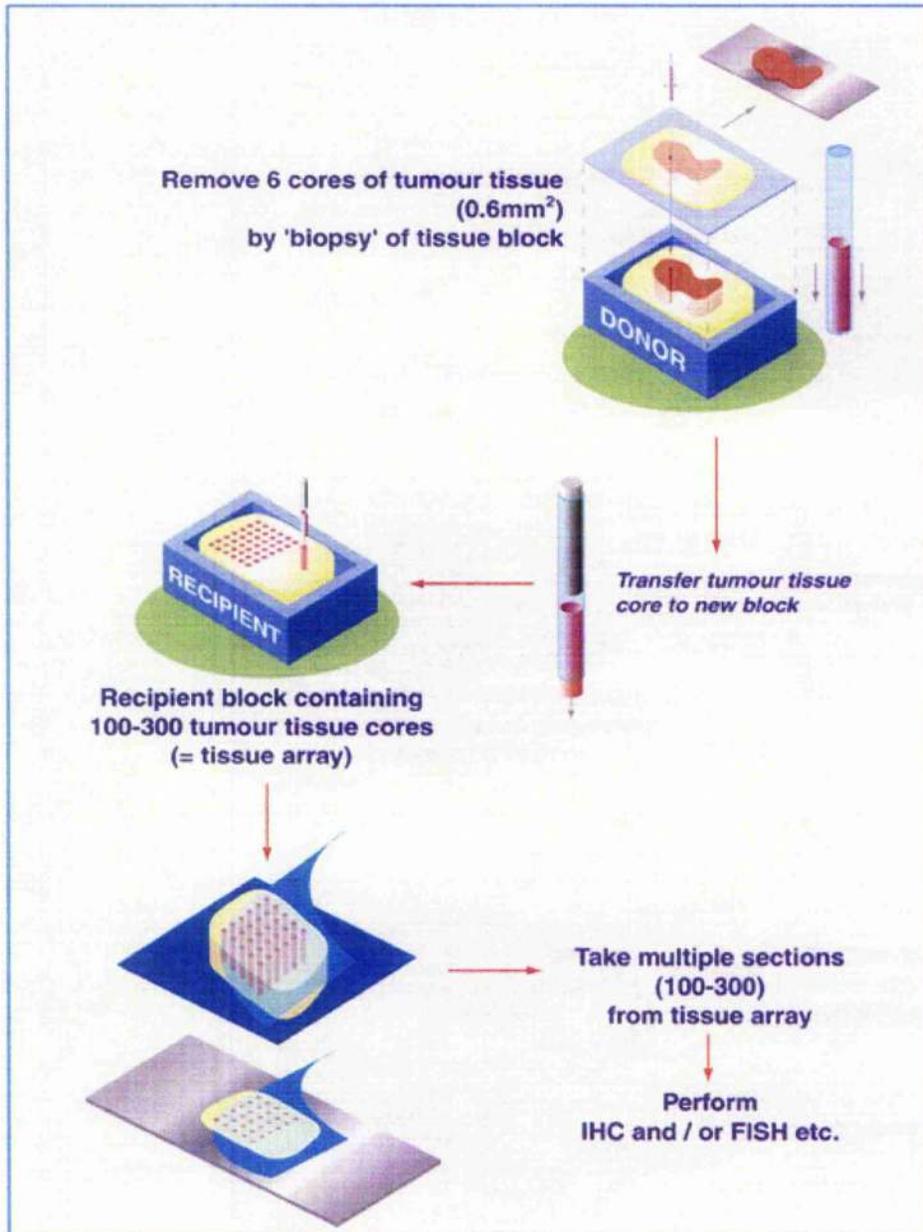
2.3.2 CONSTRUCTION OF ARRAY

Once the appropriate tumour areas were identified in each section (high and low grade where possible), one-millimetre tissue cores were removed from each paraffin block using a manual tissue arrayer (Beecher Instruments[®] Micro-Array Technology). Cores were then placed within a fresh paraffin block in order to construct an array (see **Figure 2**). Only blocks with sufficient tumour tissue arising from TURP chippings (i.e. contained areas substantially greater than 1mm in total) were used for TMA construction, allowing the return of sufficient tissue to the archives. A plan of each array was made to track the identity of each core by their co-ordinate position.

Five micrometer sections were then prepared as above for immunohistochemical staining (see **Section 2.2**).

Figure 2. Illustration of Tissue Microarray Construction

Fig.



2.4 IMMUNOHISTOCHEMISTRY

2.4.1 PRINCIPLES OF IMMUNOHISTOCHEMISTRY

Protein expression was determined using immunohistochemistry. This is a semi-quantitative staining technique which relies on the use of specific antibodies raised against epitopes of protein antigens to be analysed. In this particular study, a standard indirect immunoperoxidase technique was used to stain tissue sections. Slides containing 5µm sections of tissue, cut from paraffin embedded tissue-blocks, require dewaxing in xylene and progressive rehydration through graded alcohol solutions in order to remove the remnant paraffin. As some cells contain endogenous peroxidase enzymes, this can interfere with the use of peroxidase-labelled antibodies later on during the procedure. Therefore treatment with hydrogen peroxide (3%) solution is required to destroy pre-existing peroxidase within the tissue. Thereafter an antigen-retrieval process is required to counter the effects of formaldehyde fixatives, which react with basic amino-acids to form cross-links, which have the effect of masking antigen from an antibody. These cross-links may be removed by a variety of processes (including incubation slides in heated trypsinised solution or citrate buffer solution), according to the particular antigen under investigation, as different epitopes are affected by tissue fixation to various extents. Non-specific binding of antibody to protein can result in "background staining" which interferes with the presence of specific staining. These sites require "blocking" therefore with the addition of serum, usually derived from horse.

Addition of primary antibody follows. These may be polyclonal or monoclonal antibodies. Polyclonal antibodies, by definition, are raised from several different clones of plasma cells upon inoculation of protein. As a result, the antibodies produced will react to different epitopes on the same antigen, and can be used at low concentrations.

However, they may also contain non-specific antibodies, leading to background staining – this can be minimised upon titration to the most appropriate concentration. Monoclonal antibodies, produced from a single clone of B-cells, react with one specific epitope on the antigen, leading to less background staining. Primary antibodies are incubated to varying lengths of time, depending on the antibody used (e.g. from 1 hour at room temperature to overnight at 4°C). Following incubation with primary antibody, addition of a secondary antibody is required as part of an indirect method of immunostaining. This is a 2 stage process, and involves the use of a biotinylated secondary antibody, which is then linked to a peroxide enzyme-streptavidin conjugate. Streptavidin has a low potential for non-specific binding, resulting in more sensitive staining. Endogenous biotin may however result in non-specific binding of secondary antibody, which may be reduced by addition of a biotin block. This however was not required with the antibodies used in this study. Staining is achieved upon addition of the chemical 3,3-diaminobenzidine tetrahydrochloride, which polymerises, in the presence of peroxidase at the site of the antigen, to form a visible brown reaction product. Following this, nuclei are counterstained blue with haematoxylin and Scotts tapwater substitute. The sections are then dehydrated in graded alcohol solutions and cleared in xylene, before mounting in DPX resin-coated coverslips. The protein in the tissue sections, DPX and glass coverslips have a similar refractive index, producing optimal resolution when viewed under the microscope.

2.4.2. IMMUNOHISTOCHEMICAL PROTOCOL

2.4.2.1 Antibodies

The specificity of all antibodies used in this study was confirmed by western blotting. These procedures were conducted by a PhD student within the Laboratory at the time of the project (LMcG).

Raf-1

Tumour protein expression of Raf-1 was assessed using a mouse monoclonal antibody (IgG₁ raised against a peptide mapping at the carboxy terminus of Raf-1 p74 of human origin, Cat.No.sc-7267, Santa Cruz Biotechnology, USA) at a 1:10 concentration. The antibody was initially worked up in human tonsillar tissue to confirm staining (as per the product datasheet). The most suitable concentration was then titrated using prostatic controls.

Phosphorylated Raf-1 (serine 259)

Tumour phospho-Raf-1 expression was assessed using a rabbit polyclonal antibody (raised against residues surrounding phosphorylated serine 259 of human Raf-1, Cat.No.#9421, Cell Signaling Technology, USA) at a 1:25 concentration. The antibody was initially worked up in human placental tissue to confirm staining (as per the product datasheet). The most suitable concentration was then titrated using prostatic controls.

Phosphorylated Raf-1 (serine 338)

Tumour phospho-Raf-1 expression was assessed using a rat monoclonal antibody (IgG₁ raised against residues surrounding phosphorylated serine 334 - 347 of human Raf-1, Cat.No.#05-534, Upstate, USA) at a 1:250 concentration. The antibody was worked up

and titrated in human prostatic tissue, which is known to express phosphorylated Raf-1 (serine 338).

MAP Kinase (p42/44)

Tumour protein expression of MAP Kinase was assessed using a rabbit polyclonal antibody (derived from the sequence of rat p42 MAP Kinase, Cat.No.#9102, Cell Signaling Technology, USA) at a 1:25 concentration. The antibody was worked up and titrated in human prostate tissue, which is known to express MAP kinase.

Phosphorylated MAP Kinase (p42/44)

Tumour protein expression of phosphorylated MAP Kinase (pMAP Kinase) was assessed using rabbit polyclonal antibody (raised against residues surrounding phosphorylated Thr202/Tyr204 of human p44 Map Kinase, Cat.No.#9101, Cell Signaling Technology, USA). at a 1:50 concentration. The antibody was worked up and titrated in human prostate tissue which is known to express the phosphorylated protein (and as recommended by the product datasheet).

2.4.2.2. IHC Procedure

Tissue sections (5µm) were dewaxed in xylene and rehydrated through graded alcohols. Sections were then treated with 0.3% hydrogen peroxide solution, to remove endogenous peroxidases, for 20 minutes. Antigen retrieval was achieved by incubating sections in citrate buffer solution (DAKO Cytomation, 8ml of x10 concentrate in 80 ml distilled water) for 20 minutes at 97°C, allowing a further 20 minutes to cool down at room temperature. Sections were then blocked with horse serum solution (15µl per 1ml TBS-buffer) for 30 minutes at room temperature, before incubation with primary antibody at the appropriate concentration overnight at 4°C. Negative control sections were incubated

with an iso-type matched control antibody in which no staining was observed. Positive control specimens (consisting of prostatic cancer specimens not contained in the database) were also included in each assay. Bound antibody was visualised using a biotinylated secondary antibody, streptavidin-horseradish peroxidase complex (DAKO, UK) and 3,3'-diaminobenzidine (DAB) as chromogen (DAKO). Tissue sections were counter-stained with haematoxylin and dehydrated through graded alcohols and xylene.

2.5 HISTOSCORING AND DATA ANALYSIS

2.5.1 HISTOSCORING METHOD

All immunostained tumour sections were quantified blindly and independently by 2 observers, using a weighted histoscore method at high magnification (x 400). Specific staining in each tissue section (cytoplasmic and nuclear depending on the antigen studied) was allocated 0 (no staining), 1 (weak intensity of staining), 2 (moderate intensity), or 3 (strong intensity). The final score (maximum 300) is calculated from the sum of:

$$(1 \times \% \text{ weak staining}) + (2 \times \% \text{ moderate staining}) + (3 \times \% \text{ strong staining})$$

Histoscore analysis therefore provides a semi-quantitative classification of staining intensity (Fraser et al., 2003) (Edwards et al., 2003a).

2.5.2 STATISTICAL TESTS

Statistical analysis was performed using Microsoft Excel and the SPSS statistical package (version 9.0).

2.5.2.1 Inter-Observer Variation

Interclass correlation coefficients (ICCC) were used to assess inter-observer variation of histoscores (Cicchetti and Sparrow, 1981). In doing so, it compares and quantifies the variability between observers for each section to the overall variation in all scores. ICC values less than 0.4 is regarded as poor, 0.4-0.59 fair, 0.6-0.74 good, and 0.74 and above excellent.

Initially every slide was double scored by an independent observer. After initial calculation of ICC for Raf-1 expression, yielded scores greater than 0.9, which is classed as excellent, it was decided to reduce this to every tenth slide.

In addition, Pearson co-efficients, Scatter plots and Bland-Altman graphs were also calculated to confirm low inter-observer variation and the absence of a bias between scorers therefore further validating the results.

2.5.2.2 Protein Expression and Correlation

Protein expression data was not normally distributed and is shown as median and inter quartile ranges. Wilcoxon Signed Rank Tests were used to compare expression between ASPC and AIPC tumours. Significant changes (rising or falling) in protein expression between paired ASPC and AIPC tumours were defined as the mean (absolute) inter-observer variation plus 2 standard deviations of all sections scored.

Correlations (non-linear) between proteins were calculated using the Spearman Rank Test. All p values were 2-tailed and significance was defined as p values less than or equal to 0.05.

Time to relapse and survival analysis was conducted using the Kaplan-Meier method and curves were compared with the log-rank test.

RESULTS AND DISCUSSION

3.1 Patient Database

A patient cohort of 65 matched androgen sensitive and insensitive prostate tumours (130 tumours) was used in the study. The median age of the patient cohort was 70 years and median time of follow-up was 4.5 years. Median time to biochemical relapse was 2.5 years and median time to death from relapse was 1.4 years. Patients in the cohort were diagnosed with locally advanced (46 patients) or metastatic (19 patients) prostate cancer and subsequently received surgery and/or androgen deprivation therapy (27 orchidectomy, 59 GnRH analogue and/or anti-androgen therapy). Forty-six of the patients in the cohort died during the course of follow-up and 19 are still surviving at last follow-up. **Table 1** shows a summary of the clinical information available for this patient cohort. A more detailed representation of the clinical, pathological and biochemical data from all patients in the study (including PSA profiles) can be found in **Appendix 1**.

The presence of metastases at diagnosis was associated with a reduced time to biochemical relapse in this cohort ($p=0.037$) and a trend was seen with overall patient survival ($p=0.06$). High Gleason score was associated with reduced time to biochemical recurrence ($p=0.002$), death from biochemical recurrence ($p=0.02$) and overall survival ($p=0.001$). These results are consistent with our understanding of the clinical parameters associated with the progression of prostate cancer and therefore validate the use of the database in this study.

Table 1. Summary of clinical characteristics of patient cohort

| | Diagnosis | Relapse | Years | |
|---|------------------|----------------|--|---------------------|
| Median Age (inter-quartile range) | 70 (66-74) | 72 (67-78) | Median time to biochemical relapse (inter-quartile range) | 2.54 (1.51-4.62) |
| No. of patients alive | 65 | 19 | Time to death from relapse (inter-quartile range) | 1.37 (0.82-2.69) |
| No. of patients with metastasis | 19 | 40 | | |
| Median Gleason score | 8 (7-9) | 8 (8-9) | Overall survival (inter-quartile range) | 4.5 (3.00-7.01) |
| Patients undergone Surgical orchidectomy | 27 | - | | |
| Patients received GnRH analogue | 48 | - | | |
| Patients receiving Anti-androgen | 43 | - | | |
| Patients receiving GnRH and/or Anti-androgen | 59 | | | |

3.1.1 Definition of Androgen-Sensitive (ASPC) and Insensitive (AIPC) Prostate

Cancer

Androgen-sensitive tumour samples were taken from either TRUS biopsies or TURP specimens at diagnosis and prior to commencement of therapy. Thereafter, patients required a successful biochemical response, as demonstrated by a significant reduction in PSA levels (of at least 50%) in response to androgen deprivation therapy to be classified as ASPC in the study. Some patients at diagnosis were in the pre PSA era, however they had histological confirmation of disease (see **Appendix 1**). As such, patients were androgen or hormone-naïve (see **Materials and Methods**). A truer reflection of androgen-sensitive specimens may have been achieved from samples taken during therapy with a confirmed PSA at nadir. As a result an earlier immunohistochemical “snapshot” of what is occurring at a molecular level. One could speculate that different associations may have been seen, reflecting changes *en route* to androgen-escaped disease. The availability of such samples are generally rare in clinical practice, unless a TURP has been performed.

Androgen-insensitive or androgen-escaped prostate cancer can be defined as biochemical and/or clinical disease progression during androgen-deprivation therapy with castration levels of serum testosterone (Hernes et al., 2004). Retrieval of AIPC tissue was from the pathological archives (obtained following channel TURP for subsequent bladder outlet obstruction). Samples were thought to be indicative of AIPC if a sustained rise in PSA despite maximal treatment was noted, without a later decrease even with any change of therapy (see **Appendix 1**). Furthermore, palliative resections of the prostate are generally performed as a result of clinical disease progression.

The expansion of a matched patient database was a major advantage and indeed a unique feature of this study. The use of samples, taken sequentially from the same patient before androgen-deprivation therapy (ASPC) and after biochemically confirmed androgen-insensitive disease (AIPC), allowed tracking of the changes associated with its' development (see **Appendix 1**). As a result, the database can play a crucial role in unravelling the molecular mechanisms that underlie androgen-insensitive prostate cancer.

Investigations into the clinical role of signal transduction pathways in the progression to AIPC has been hindered in the past by the lack of clinical material, as these patients do not undergo routine biopsies (Craft et al., 1999). Discrepancies sometimes occur (for example in studies of HER2 expression) when radical prostatectomy specimens are used, as they generally contain localised disease which is not androgen-insensitive (Craft et al., 1999). The current study, however, was a dynamic, comparative analysis of the overall change in protein expression with the development of androgen-insensitive disease. In doing so, it was possible to identify subsets of patients who demonstrated rising, falling, or no change in expression of members of the Raf/MAP Kinase pathway.

It could be argued that as only patients who survived long enough to develop outflow symptoms, and were clinically suitable for surgical intervention, the cohort of patients maybe therefore skewed in favour of those with less aggressive disease. The development of a prospective database, which includes 3 sets of matched biopsies in future (at diagnosis, during treatment, and at point of relapse) is currently underway to address this. Furthermore, a variety of treatment modalities were used, from surgical castration, various GnRH analogues and androgens, sometimes in combination. An accurate

assessment of individual therapies alone on the Raf/MAP Kinase pathway would be of interest, but could only be achieved if significant numbers of each was achieved with further expansion of the patient cohort.

Variability in reports with respect to levels of protein expression have always been noted, for example with HER2 (Solit and Agus, 2001). Whilst these may reflect methodological differences with immunohistochemistry (as will be discussed later), they may be related to genetic differences in the patient populations assessed. Our cohort of patients were recruited from patients in central Scotland, and it may be of interest to compare results with similar databases in Europe or further a field. These may implicate a greater role for a variety of different pathways. In turn, this could influence drug development at a regional level and have therapeutic implications.

3.2 Inter-observer correlations/variation

Changes (fall or rise) in protein expression were determined according to mean (absolute) inter-observer differences in histoscores for each protein, plus 2 standard deviations (95% confidence interval), as previously described and further explained before (see **MATERIALS & METHODS**). **Table 2** reveals values for each protein studied including ICCCs and Pearson co-efficients. ICCCs were consistently higher than 0.7 and were therefore classed as excellent. In order to eliminate observer bias, scatter graph and Bland-Altman plots were drawn for each protein and are shown in **Appendix 2 (Figures 1-7)**.

Small changes in protein expression between paired ASPC and AIPC tumours could be due to random errors in the assessment of histoscores. To identify individual patients in whom there was strong evidence of a genuine rise or fall in protein expression, it was required that the change in expression exceed a threshold equal to mean plus two standard deviations of the inter-observer difference for that protein. This threshold was chosen because, if there was in reality no difference in protein expression between ASPC and AIPC tumours in a given patient, there would be only a 5% probability of an apparent difference being observed that exceeded the threshold due to random variation. This assumes that the mean random variation between two different observers assessing the same tumour is of a similar magnitude to the random variation that would affect a single observer assessing two different tumours with the same level of protein expression. Changes in protein expression in individual patients that exceeded this threshold were termed significant.

| | Mean inter-observer difference | Mean + 2 standard deviations | Inter class correlation co-efficient | Pearson co-efficient (r^2 from graph) |
|--------------------------------|--------------------------------|------------------------------|--------------------------------------|--|
| Raf-1 | 19.0 | 49.6 | 0.91 | 0.91 (0.83) |
| pRaf (ser259) | 25.7 | 66.8 | 0.86 | 0.84 (0.71) |
| pRaf (ser338) (Nuclear) | 21.6 | 65.9 | 0.91 | 0.91 (0.83) |
| pRaf (ser338) (Cytoplasmic) | 14.9 | 45.9 | 0.96 | 0.96 (0.92) |
| MAP Kinase (Nuclear) | 9.3 | 28.1 | 0.95 | 0.95 (0.91) |
| MAP Kinase (Cytoplasmic) | 10.7 | 32.5 | 0.92 | 0.92 (0.84) |
| pMAP Kinase (Nuclear) | 17.2 | 41.2 | 0.94 | 0.95 (0.90) |
| pMAP Kinase (Cytoplasmic) | 15.7 | 41.4 | 0.91 | 0.90 (0.80) |

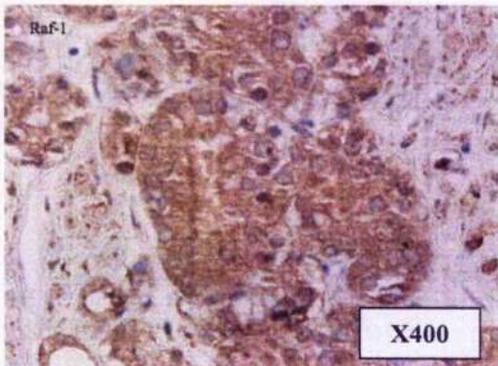
Table 2. Mean inter-observer difference, Mean + 2 standard deviation, inter-class correlation co-efficients and Pearson co- efficient for each protein studied.

3.3 Immunohistochemistry

3.3.1 Raf-1 Immunohistochemistry

Raf-1 staining was seen exclusively in the cytoplasm. Examples of immunohistochemical staining for Raf-1 can be seen in **Figures 1**.

Figure 1. Example of immunohistochemical staining for Raf-1.



When median Raf-1 protein expression in ASPC tumours was compared with AIPC tumours, no statistically significant change was observed ($p=0.74$) (**Table 3, Figure 2** below).

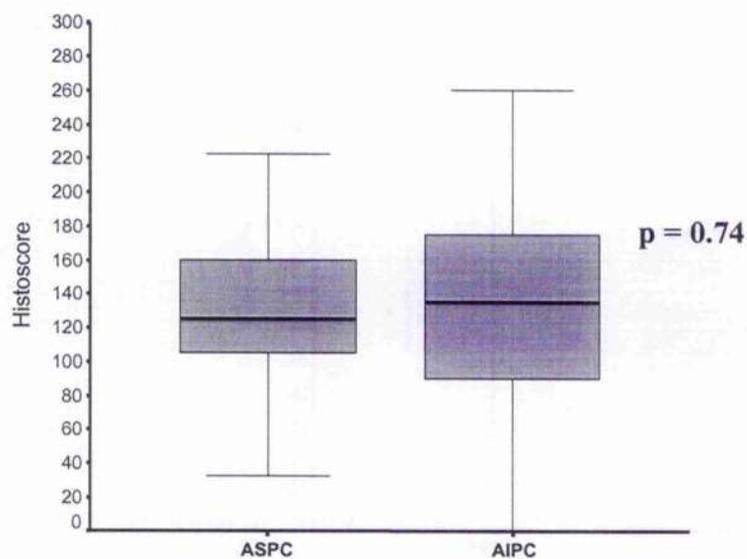


Figure 2. Median Raf-1 histoscores for ASPC and AIPC tumours, with p value as assessed by Wilcoxon Signed Ranks Test.

However a subgroup analysis revealed that 17% (11/65) of cases had a significant rise in Raf-1 expression with development of AIPC (as measured by a rise of greater than 49.6 histoscore units), while the remainder (54/65) demonstrated no significant rise or a fall in Raf-1 expression (see **Table 3** and **Figure 3** below).

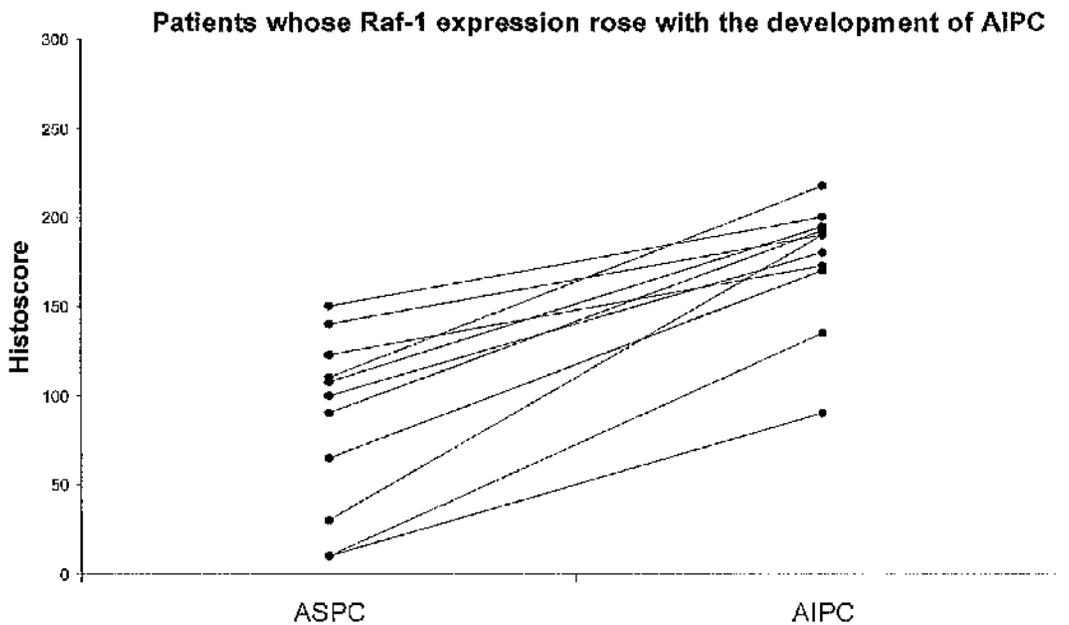


Figure 3. Changes in Raf-1 expression with the development of AIPC

In summary no significant increase in median Raf-1 expression was observed in the transition from ASPC to AIPC, however changes in expression were observed for individual patients (**Table 3**).

Table 3. Raf-1 histoscores in androgen-sensitive and androgen-insensitive tumours.

| | ASPC | AIPC | p value | % Fall | % Unchanged | % Rise |
|-------|----------------------|----------------------|------------|---------------|----------------|---------------|
| Raf-1 | 135 (102.5-182.5) | 137.5 (110-171.5) | 0.74 | 20 (13/65) | 63 (41/65) | 17 (11/65) |

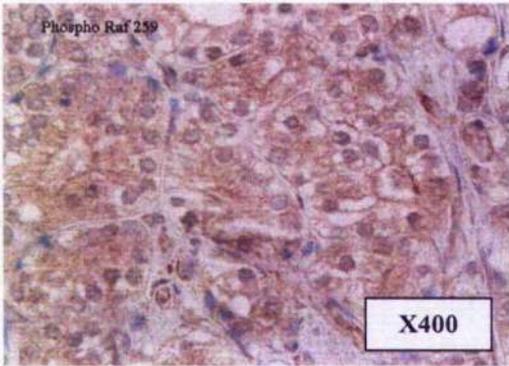
Table 3 shows median histoscores and interquartile ranges for Raf-1. P values, as assessed by the Wilcoxon Signed Ranks Test, were used to determine any significant change in histoscore between ASPC and AIPC. A further subanalysis was made to identify groups of patients, whose expression levels fell, rose or remained unchanged between ASPC and AIPC (depending on changes within or outside the 95% confidence interval for inter-observer variation).

ASPC, androgen-sensitive prostate cancer ; AIPC, androgen-insensitive prostate cancer.

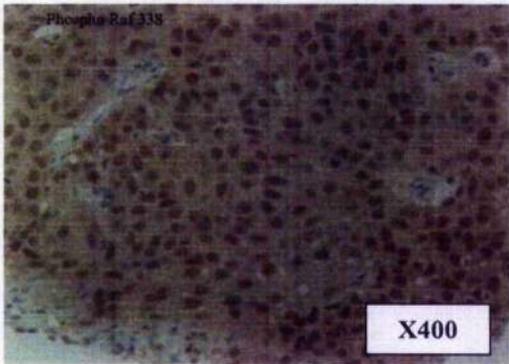
3.3.2 Phospho-Raf (serine 259 and serine 338) Immunohistochemistry

Inactive phosphorylated Raf, pRaf (ser259), staining was seen exclusively in the perimembranous region of the cell whilst activated phosphorylated Raf, pRaf (ser338), was seen in the cytoplasm and nucleus (which were given individual histoscores). Examples can be seen in **Figures 4** and **Figure 5** below.

Figures 4. Example of immunohistochemical staining for pRaf (serine 259).



Figures 5. Example of immunohistochemical staining for pRaf (serine 338).



The total number of paired specimens analysed were 65 for pRaf (ser259) and 50 for pRaf (ser338). There was no statistically significant increase in protein expression for pRaf (ser259) ($p=0.42$) or pRaf (ser338) ($p=0.22$ and 0.43 for nuclear and cytoplasmic expression respectively) associated with the development of AIPC (see **Table 4** and **Appendix 3 - Figure 13a,b,c**). However a subgroup analysis once again revealed that protein expression in individual tumour pairs either rose, fell, or remained unchanged after escape.

In 18% (12/65) of cases, there was a significant rise in pRaf (ser259) expression with development of AIPC (corresponding to a rise of greater than 66.8 histoscore units),

while the remainder demonstrated no significant change or a fall in expression (see **Table 4** and **Appendix 3 - Figure 14a**).

In the case of nuclear and cytoplasmic pRaf (ser338), a significant rise was noted in 30% (15/50) and 28% (14/50) of cases respectively (corresponding to a rise of greater than 65 and 45 histoscore units respectively). The remainder demonstrated no significant change or a fall in expression (see **Table 4** and **Appendix 3 - Figure 14b**).

Table 4. Protein histoscores for phosphorylated Raf in ASPC and AIPC tumours.

| | ASPC | AIPC | p value | % Fall | % Unchanged | % Rise |
|----------------------------------|--------------------|-------------------|----------------|---------------|--------------------|---------------|
| pRaf (ser259) | 127.5 (105-160) | 137.5 (89-175) | 0.42 | 14 (9/65) | 69 (45/65) | 17 (11/65) |
| pRaf (ser338) nuclear | 55 (0-125) | 50 (0-200) | 0.22 | 16 (8/50) | 54 (27/50) | 30 (15/50) |
| pRaf (ser338) cytoplasmic | 100 (20-114) | 92.5 (23 -103) | 0.43 | 32 (16/50) | 40 (20/50) | 28 (14/50) |

Table 4 shows median histoscores and interquartile ranges for activated and inactivated phosphorylated Raf expression. P values, as assessed by the Wilcoxon Signed Ranks Test, were used to assess any significant change in histoscore between ASPC and AIPC tumours. A further subanalysis was made to identify groups of patients, whose expression levels fell, rose

remained unchanged between ASPC and AIPC (depending changes within or outside the median plus 95% confidence interval for inter-observer variation).

pRaf (ser259), inactivated, phosphorylated Raf-1.

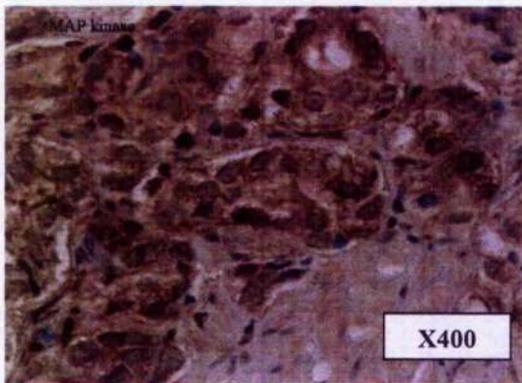
pRaf (ser338), activated, phosphorylated Raf-1.

ASPC, androgen-sensitive prostate cancer ; AIPC, androgen-insensitive prostate cancer.

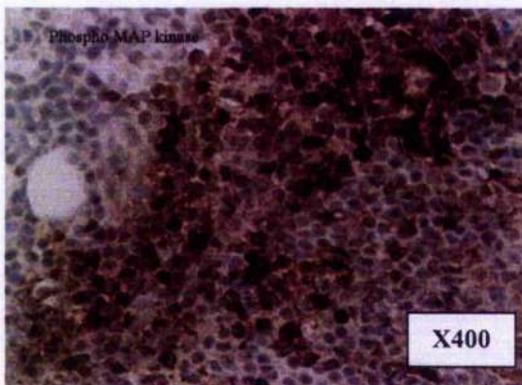
3.3.3 MAP Kinase / Phosphorylated MAP Kinase Immunohistochemistry

MAP Kinase expression (total and phosphorylated) was present in both cytoplasm and nucleus (and given individual histoscores) (see **Figure 6** and **7**).

Figures 6. Example of immunohistochemical staining for MAP Kinase.



Figures 7. Example of immunohistochemical staining for activated phosphorylated MAP Kinase (pMAP).



The total number of paired specimens analysed was 56. There was no statistically significant increase in nuclear or cytoplasmic expression of MAP Kinase nor its' active phosphorylated form (pMAP Kinase), with the development of AIPC (**Table 5** and **Appendix 3 - Figures 15** and **16**). Once again, sub-groups of tumours rose, fell, or remained unchanged with the development of AIPC. Nuclear and cytoplasmic expression of MAP kinase rose in 34% and 23% of patients respectively (19/56 and 13/56 patients) and in 27% and 26% of patients in the case of pMAP kinase (15/56 and 11/56 patients) (see **Table 5** and **Appendix 3 - Figures 17** and **18**).

Table 5. MAP Kinase protein histoscores in ASPC and AIPC tumours.

| | ASPC | AIPC | p value | % | % | % |
|--------------------|-------------|-------------|----------------|-------------|------------------|-------------|
| | | | | Fall | Unchanged | Rise |
| MAP Kinase | 60 | 80 | 0.27 | 27 | 39 | 34 |
| (Nuclear) | (20-100) | (30-100) | | (15/56) | (22/56) | (19/56) |
| MAP Kinase | 150 | 160 | 0.52 | 18 | 59 | 23 |
| (Cytoplasm) | (120-190) | (130-190) | | (10/56) | (33/56) | (13/56) |
| pMAP | 90 | 90 | 0.82 | 23 | 50 | 27 |
| Kinase | (32-144) | (52-119) | | (13/56) | (28/56) | (15/56) |
| (Nuclear) | | | | | | |
| pMAP | 120 | 120 | 0.93 | 20 | 60 | 20 |
| Kinase | (76-160) | (92-150) | | (11/56) | (34/56) | (11/56) |
| (Cytoplasm) | | | | | | |

Table 5 shows median histoscores with interquartile ranges for MAP Kinase expression and its' activated, phosphorylated form, pMAP Kinase. P values, as assessed by the Wilcoxon Signed Ranks Test, were used to assess any significant change in histoscore between ASPC and AIPC. A further subanalysis was made to identify groups of patients, whose expression levels fell, rose or remained unchanged between ASPC and AIPC

pMAP Kinase, activated, phosphorylated MAP Kinase ; ASPC, androgen-sensitive prostate cancer ; AIPC, androgen-insensitive prostate cancer.

3.4 Discussion of Raf-1 and MAP Kinase Protein Expression

3.4.1 Use of Immunohistochemistry in Assessing Protein Expression

Any demonstration of a functional pathway *in vivo* may be more accurate when based on actual protein expression, rather than gene amplification or mRNA expression. Gene amplification does not always result in increased protein expression and certainly does not provide any information on the activation status of the protein. In support of this, HER2 amplification is not associated with protein overexpression in prostate cancer (Bartlett et al., 2005). Similarly mRNA is subject to various external influences before final translation into a functional protein. Over expression of c-myc mRNA has been noted in prostate cancer tissue homogenates, but mRNA and protein was not overexpressed when using actual tissue sections (Karayi and Markham, 2004). To this effect, immunohistochemistry was selected as the primary mode of determining extent and pattern of protein expression.

However there are inherent difficulties associated with the technique of IHC. One of the main concerns regarding IHC as a technique relates to inconsistency of reports in the literature. Inconsistency of results may relate to various factors such as use of different reagents, differences in tissue-processing techniques (including fixation and antigen-retrieval), and the use of antibodies with different sensitivities and specificities (Solit and Agus, 2001). Studies of HER2 expression in prostate cancer, for example have revealed conflicting results due to varying sample size, and methodological variation, not just in terms of antibodies used but also the techniques used in determining degree of expression (Karayi and Markham, 2004).

It has also been suggested previously that IHC can also be limited in determining protein expression levels due to the semi-quantitative nature of scoring techniques (Linja et al., 2001). Furthermore, the use of grading systems in breast cancer may not be as useful in prostate cancer, which may explain the lack of predictive value as yet for HER2 expression in prostate cancer (Solit and Agus, 2001). With the inherent subjectivity associated with IHC, the use of a standardised grading scale for protein quantification is required if prognostic or therapeutic implications can be drawn from IHC based studies in prostate cancer (Solit and Agus, 2001).

Despite these known limitations, analysis of protein expression by IHC is central to current diagnostic pathology and future clinical therapies based on translational research (Bartlett et al., 2001). Its' use as a diagnostic and experimental tool is expanding, as demonstrated by the emergence of Herceptin[®] test in metastatic breast cancer (Bartlett et al., 2001). Diagnostic evaluation using IHC commonly involves the use of a histoscore technique. We employ a weighted histoscore, which translates the histoscore into a continuous variable (Kirkegaard et al., 2005). Although IHC can only ever be semi quantitative due to the nature of the technique, use of the weighted histoscore goes some way to address the problems associated with determining levels of protein expression as a continuous variable. The accuracy of this method is dependent on limiting subjective inter-observer variation. This can be achieved in a research setting (regardless of tumour type and sample size) if sections are dual scored by trained independent observers (Kirkegaard et al., 2005). Once a high standard of consistency and reproducibility is achieved, it has been demonstrated that dual scoring may be reduced to 10% of samples without resulting loss of accuracy (Bartlett et al., 2001) (Kirkegaard et al., 2005). Quality assurance and standards of inter-observer variation can be accurately quantified

statistically by calculation of inter-class correlation co-efficients (Cicchetti and Sparrow, 1981) (Kirkegaard et al., 2005). The ICCs in this study were consistently greater than 0.86, which is classified as "excellent" under current criteria. This validates our histoscoreing technique to the extent that any correlations can be deemed accurate. The study further demonstrates the potential reliability and validity of weighted histoscoreing when used in a specialised laboratory using the same blinded observers.

However, protein expression itself does not equate to protein activation (Gioeli et al., 1999) (Mandell, 2003). The use of phospho- and activation-state specific antibodies is well described and may provide a means by which the activation status of signal transduction pathways can be demonstrated at a histological level (Gioeli et al., 1999). Although other studies have demonstrated expression and activation of MAP Kinase in human tumours, these have used immune-complex assays on tissue homogenates which include non-cancerous cells (Ito et al., 1998) (Magi-Galluzzi et al., 1997). To date therefore, this is the largest study to demonstrate immunohistochemical activation of the Raf/MAP Kinase pathway in clinical prostate cancer specimens.

As one would expect, variability exists in the expression of phosphorylated proteins when compared to total protein expression (Bacus et al., 1996). In support of this, total MAP Kinase expression was not associated with its' phosphorylated form in one study in direct contrast to ours (Gioeli et al., 1999). In this study, significant associations were sometimes demonstrated between total expression of protein but not activated forms (such as between the activated forms of Raf-1 and MAP Kinase). This may be a result of the reduced time each protein exists in its' activated state due to rapid deactivation, particularly Raf-1. As a result, tissue sections stained with phosphorylated antibodies

portray a “snapshot” of activation state at the moment sampling of tissue took place. Lack of correlations may reflect inherent difficulties with the use of antibodies directed against phosphorylated proteins, and to this effect total expression of Raf-1 and MAP Kinase is still highly valuable and may sometimes be a more reliable indicator. Alternatively, cross-immunoreactivity for phosphorylated antibodies may account for some of the loss of correlations.

3.4.2 Localisation of Members of the MAP Kinase Pathway

All antibodies used in the current study had specificity confirmed by Western blotting. The immunohistochemical localisation of Raf-1 and MAP Kinase was consistent with our current understanding of the subcellular distribution of Raf and MAP Kinase following activation. Raf-1 is known to be expressed in both non-metastatic and metastatic prostate cancer cells (Weinstein-Oppenheimer et al., 2000) (Fu et al., 2003). The presence of the active and inactive forms of Raf-1, as demonstrated in this study, also confirms their presence in prostate cancer and provides an insight into their localisation in AIPC. Raf-1 expression in this study was seen diffusely in the cytoplasm and peri-membranous areas. Its' inactivated form was seen only at the peri-membranous region, where phosphorylation at the serine 259 occurs. Once activated (by phosphorylation at serine 338) at the membrane, it migrates into the cytoplasm where it targets MEK. In addition, activated Raf-1 is known to travel to the nucleus where it can function independently of the MAP Kinase pathway by targeting the retinoblastoma gene (Wang et al., 1998). However total Raf-1 expression was not consistently seen in the nucleus possibly due to the antibody being less sensitive.

Once phosphorylated in the cytoplasm MAP Kinase migrates to the nucleus (Gioeli et al., 1999). In the current study intense staining for total and activated MAP Kinase was present in the nuclei as well as the cytoplasm of neoplastic epithelium. Adjacent, non-cancerous epithelium revealed little or no staining for total and activated MAP Kinase, although staining was present in surrounding smooth muscle stroma and endothelium consistent with previous work by Gioeli et al. 1999 (Gioeli et al., 1999). However, unlike Gioeli et al. 1999, we also observed marked expression for phospho-MAP Kinase in lymphocytic aggregates.

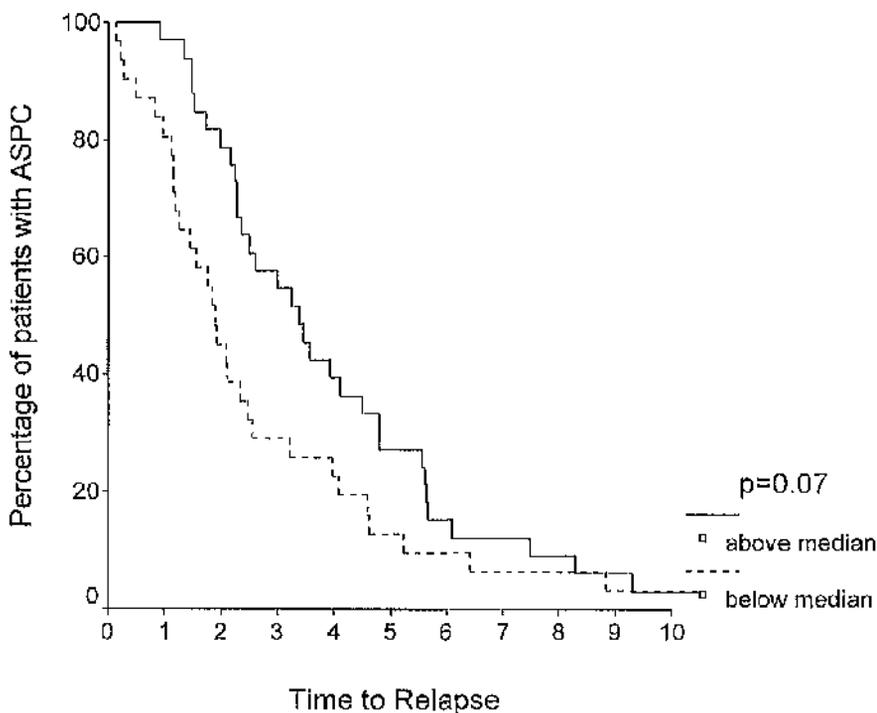
In conclusion, using immunohistochemistry we observed protein expression of total and phosphorylated Raf-1 and MAP Kinase in areas consistent with those reported in current literature. The specificity of antibodies used in this study were previously confirmed by Western blotting. This combined with the localisation of the protein expression suggests that staining observed was specific for Raf-1 and MAP Kinase.

3.5 The Role of the Raf/MAP Kinase Pathway in the Development of Androgen-Insensitive Prostate Cancer – Impact on Time to Relapse and Survival

3.5.1 Raf-1 and phosphorylated Raf (serine 259 and serine 338)

When Raf-1 histoscores were divided by median, no correlation was seen (Kaplan-Meier analysis) between high histoscores (above median) and time to relapse, although a trend was seen for shortened relapse time and Raf-1 histoscores below the median (1.9 years vs 3.4 years, $p=0.07$, **Figure 8a** and **Appendix 4 - Table 1a**).

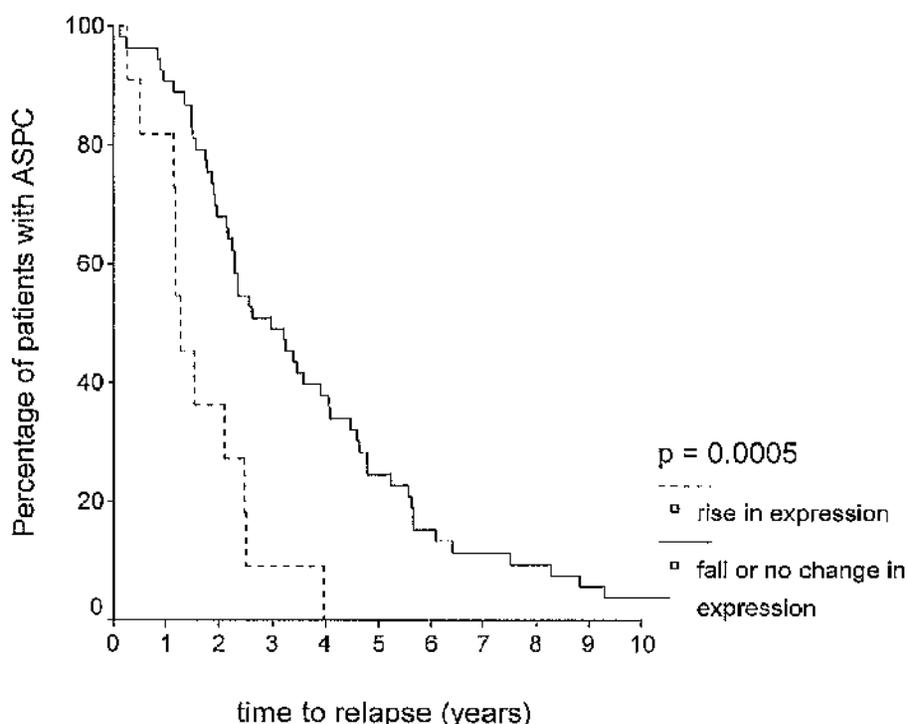
Figure 8a



Kaplan-Meier plot for patients with high Raf-1 expression (above the median histoscore) in ASPC tumours, compared to those with low expression. There was no significant difference in time to relapse in these patient subgroups ($p=0.07$), although a trend was seen.

Patients whose Raf-1 expression rose with the development of AIPC, however, had a significantly shorter time to relapse than those patients who had a fall or no change (p=0.0005, see **Figure 8b**). Time to relapse in those patients with a rise in Raf-1 expression was 1.16 years (1.12 years - 1.20 years) compared to 2.62 years (1.52 years - 3.70 years) for those who had a fall or no change in Raf-1 expression (see **Appendix 4 - Table 1b**).

Figure 8b.

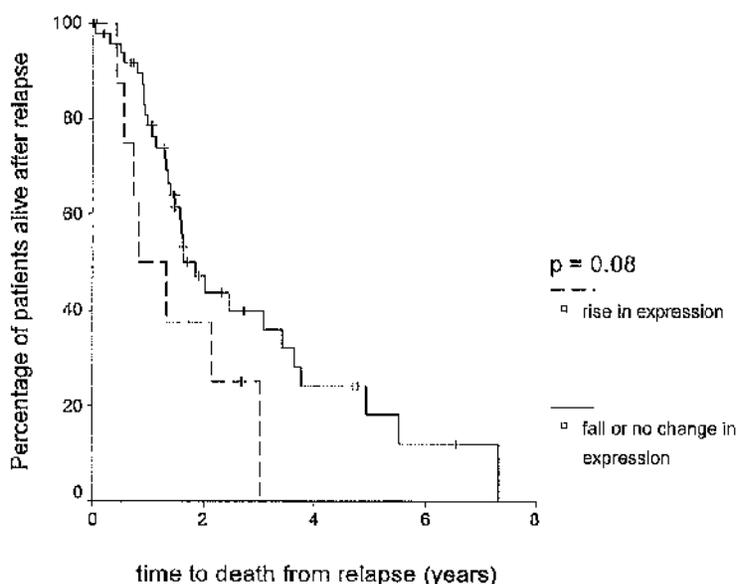


Kaplan-Meier plot for patients who demonstrated a rise in Raf-1 expression with the development of AIPC, compared to those that did not. There was a significant difference in time to relapse in these patient subgroups (p = 0.0005).

There was no association between Raf-1 expression and time to death from relapse, and overall survival (see **Appendix 4 – Tables 2 and 3**).

Whilst there was no correlation between pRaf (ser259) expression in the primary tumours and time to relapse, there was a trend seen between patients whose pRaf expression rose with the development of AIPC and a shorter time to death from relapse (0.83 years vs 1.87 years, $p=0.08$, see **Figure 9a** and **Appendix 4 - Table 2b**). pRaf (ser259) expression did not influence overall survival however (see **Appendix 4 – Table 3**).

Figure 9a.

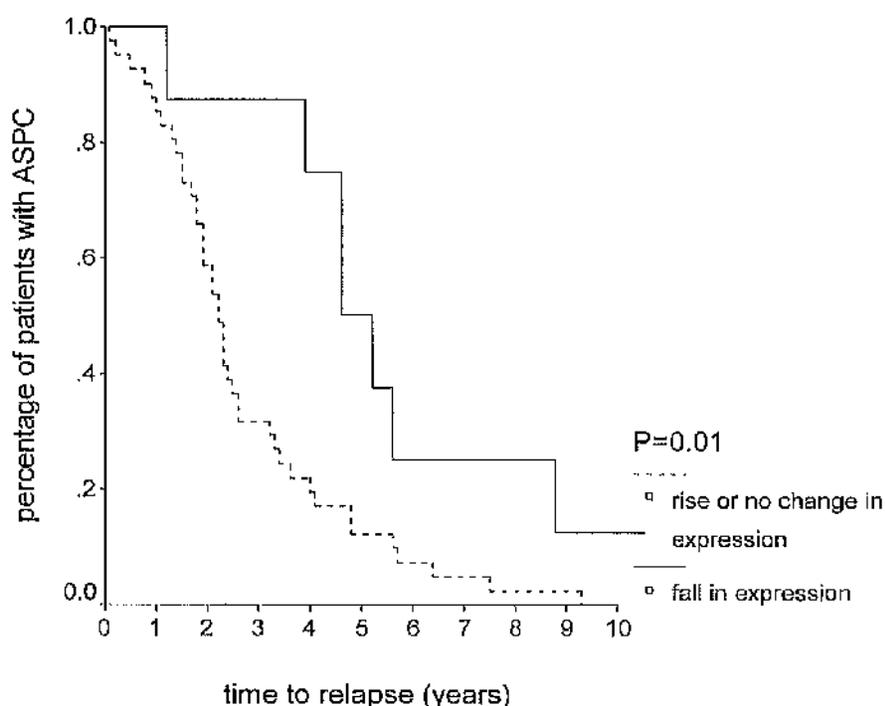


Kaplan-Meier plot for patients who demonstrated a rise in pRaf (ser259) expression with the development of AIPC, compared to those that did not. There was a trend for shortened time to death from relapse in these patient subgroups, although this was not significant ($p = 0.08$).

No correlations were seen between pRaf (ser338) expression when divided according to median and time to relapse, death from relapse and overall survival. Similarly, no

correlations were seen when subgroups of patients with rising expression with the development of AIPC (see **Appendix 4 – Tables 1, 2 and 3**). Interestingly, unlike any of the other proteins analysed in the study, a distinct association was seen between time to prolonged biochemical relapse when nuclear expression fell. The median time to relapse in patients with falling nuclear pRaf (ser338) expression with the development of AIPC was 4.6 years (3.4 years to 5.8 years) compared to 2.2 years (1.84 years to 2.56 years) for patients whose levels remained unchanged or rose ($p=0.01$, see **Figure 9b** and **Appendix 4 - Table 1b**).

Figure 9b



Kaplan-Meier plot for patients who demonstrated a significant fall in pRaf (ser338) expression with the development of AIPC, compared to those that did not. There was a significant difference in time to relapse in these patient subgroups ($p = 0.01$).

3.5.2 MAP Kinase and pMAP Kinase expression

When MAP Kinase and pMAP Kinase histoscores were divided by median, no correlation was seen (Kaplan-Meier analysis) between high histoscores (above median) and time to relapse. However a trend was seen for increased time with high nuclear (2.62 years vs 2.13 years, $p=0.06$) and high cytoplasmic pMAP Kinase expression (2.98 years vs 2.29 years, $p=0.08$). (see **Appendix 4 - Table 4a**).

When histoscores were divided into patients whose expression rose with development of AIPC and those that did not, once again there was no correlation seen, although a trend was seen for shorter time to relapse with a rising cytoplasmic expression of pMAP Kinase (2.25 years vs 2.62 years, $p=0.06$). (see **Appendix 4 - Table 4b**).

MAP Kinase expression and Survival Time

There was no correlation seen between time to death from relapse and overall survival with MAP Kinase and pMAP Kinase expression when divided according to median (ASPC and AIPC) (see **Appendix 4 - Tables 5a and 6a**). Patients whose nuclear MAP Kinase expression rose with the development of AIPC did however have a significantly shorter time to death from relapse compared to those that had a fall or no change in expression (1.40 years (1.20 years - 1.61 years) vs 3.00 years (1.43 years – 4.57 years), $p=0.0255$) (see **Figure 10a and Appendix 4 - Table 5b**). This translated into a shorter overall survival (3.37 years (1.58 years – 5.16 years) vs 6.89 years (5.70 years – 8.08 years), $p=0.0068$) (see **Figure 10b and Appendix 4 - Table 6b**).

Figure 10a.

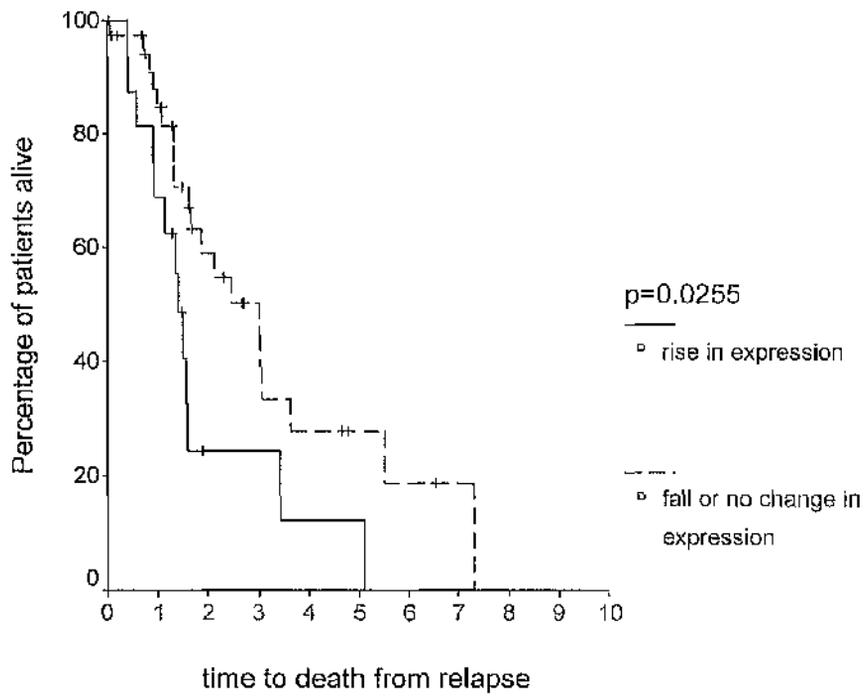
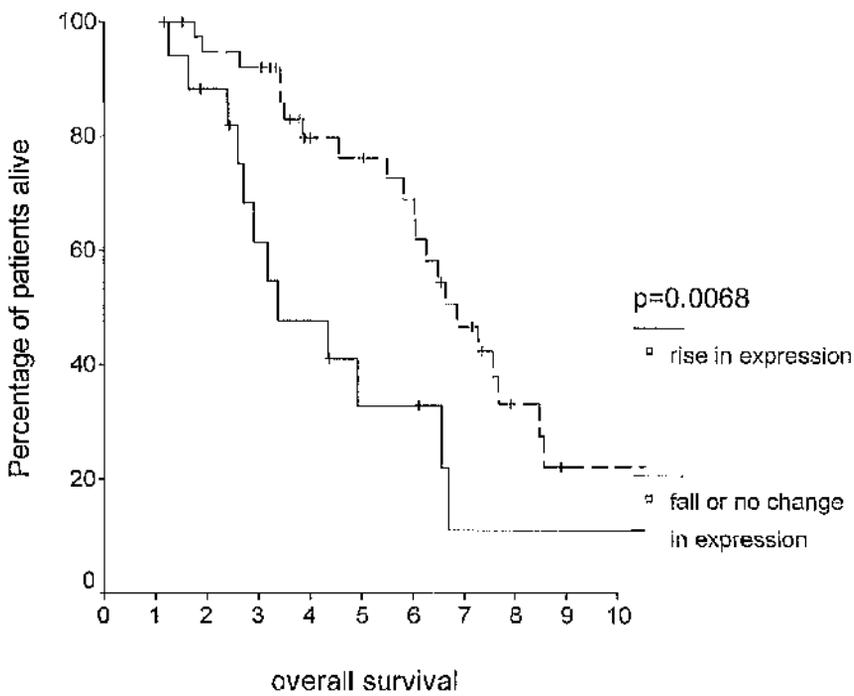


Figure 10b.



Kaplan-Meier plot for patients who demonstrated a rise in nuclear MAP Kinase expression with the development of AIPC, compared to those that did not. There was a significant difference in in time to death from relapse in these patient subgroups ($p = 0.0255$) and overall survival in these patient subgroups ($p = 0.0068$).

3.5.3 The Impact Raf/MAP Kinase Pathway on Time to Relapse and Survival – A Prognostic Indicator?

The data from this study revealed that expression levels in ASPC and AIPC were not individually significant. However changes in protein expression (rise or fall) were more clinically relevant as correlations affecting time to relapse and overall survival were demonstrated using Kaplan-Meier analysis. In the case of nuclear pRaf (ser338) falling expression versus non-fallers (rising expression and no change combined) also appeared to be significant and a separate analysis was therefore performed.

Raf-1

Eleven patients (17%) showed a significant increase in the mean Raf-1 histoscore in AIPC. Patients with elevated Raf-1 expression at relapse demonstrated a reduction of 1.46 years in time to biochemical relapse - a fall of greater than 50% ($p = 0.0005$). This data would suggest, as we hypothesised, that an increase in Raf-1 expression leads to a more aggressive and rapid disease progression to AIPC. In the current study this association did not, however, translate into altered overall survival. Since only 63% (24/65) of patients were dead at time of follow up, and 5 out of 11 of these were included in those with a rise in Raf-1 expression, this study maybe insufficiently powered to demonstrate a significant difference in overall survival.

It was also observed that those with low Raf-1 expression (above the median) also have reduced time to relapse ($p=0.07$). This may be due to patients with rising Raf-1 expression having low expression at the androgen-sensitive stage. It would appear that the increase in Raf-1 occurring with relapse (which is associated with low initial expression) leads to a more aggressive disease progression. High initial expression of Raf-1, conversely, is in fact associated with more stable disease. Its' ability to predict time to relapse, might enable it to act as a potential prognostic marker.

No correlations were seen with rising expression of activated Raf. As mentioned earlier however, when patients with rising expression of nuclear pRaf (ser338) were combined with those who demonstrated no significant change (non-fallers), there was a significantly lower time to biochemical relapse (by 2.4 years) when compared to patients who demonstrated a fall in expression. This would still be consistent with the findings of total Raf-1.

A trend was noted with shorter time to death from relapse in AIPC when levels of the inactive form of Raf-1 (ser259) rose ($p=0.08$). This may be explained by the negative feed back loop that exists to control Raf-1 activation via MAP Kinase i.e. activation of Raf-1 subsequent leads to inactivation and therefore an increase in levels of pRaf (ser259) (Rushworth et al., 2005). To support this we would predict rising MAP Kinase to also influence time to death from relapse and correlations to be seen between its' expression and pRaf (ser259). This proved to be the case (see below).

MAP Kinase

15 patients (27%) demonstrated a rise in nuclear expression of MAP Kinase with the development of AIPC, and subsequently developed a significantly shorter time to death

from relapse, by 1.6 years, compared to those that had a fall or no change in expression ($p=0.02$). This translated into a reduction in overall survival by 3.5 years ($p=0.0068$).

Interestingly, there was also an association seen between rising cytoplasmic pMAP Kinase expression and reduced time to biochemical relapse (by 0.38 years, $p=0.06$). There was also an association with low cytoplasmic and nuclear pMAP Kinase expression at diagnosis (ASPC) and reduced time to relapse (by 0.71 and 0.50 years, $p=0.08$ and $p=0.06$ respectively). Whilst these were not significant, their trend was consistent with our previous findings albeit with a more modest reduction in time. As with Raf-1, it would appear that increasing protein expression irrespective of the actual initial level at diagnosis or at the androgen-insensitive stage, is a more significant clinical parameter.

In summary, our results implicated elevated expression of members of the MAP Kinase pathway in a more aggressive and rapid disease process, with the development of AIPC. Whilst a rise in Raf-1 expression leads to a quicker biochemical relapse, rising expression of MAP Kinase impacts directly on time to death from relapse and overall patient survival thereafter.

3.6 The Role of the Raf/MAP Kinase Pathway in the Development of Androgen-Insensitive Prostate Cancer – Raf-1 and MAP Kinase Protein Correlations

3.6.1 Correlations between Raf-1 and its' phosphorylated forms

Whilst Raf-1 correlated weakly with its' inactivated form, pRaf (ser259), before relapse ($p=0.028$), a stronger correlation was seen after the development of AIPC ($p=0.008$) (see **Table 6a**). No correlations were seen between Raf-1 with the cytoplasmic expression of its' activated form, pRaf (ser338).

Table 6a. Correlation between Raf-1 and pRaf (serine 259 and 338) expression before and after AIPC.

| | pRaf (ser259) | pRaf (ser338) cytoplasmic |
|--------------|---------------------|---------------------------|
| Raf-1 (ASPC) | 0.028 (0.27) | 0.971 (0.05) |
| Raf-1 (AIPC) | 0.008 (0.32) | 0.445 (-0.10) |

Table 6a shows significant correlations (p values) between Raf-1 and its' phosphorylated forms in ASPC and AIPC tumours. Correlation coefficients are shown in brackets. P values were assessed by Spearman's Rank Correlation Test.

Protein expression of the inactive form of pRaf (ser259) and the active form of pRaf (ser338) in the cell cytoplasm did not correlate in ASPC or AIPC ($p=0.35$ and $p=0.96$ respectively). However, the nuclear and cytoplasmic forms of pRaf (ser338) strongly correlated with each other in ASPC ($p=0.01$) and AIPC tumours ($p<0.001$) (see **Table 6b**).

Table 6b. Correlation between pRaf (serine 259 and 338) expression before and after AIPC.

| | pRaf (ser338) cytoplasmic |
|------------------------------|---------------------------|
| pRaf (ser338) nuclear (ASPC) | 0.01 (0.34) |
| pRaf (ser338) nuclear (AIPC) | <0.001 (0.74) |

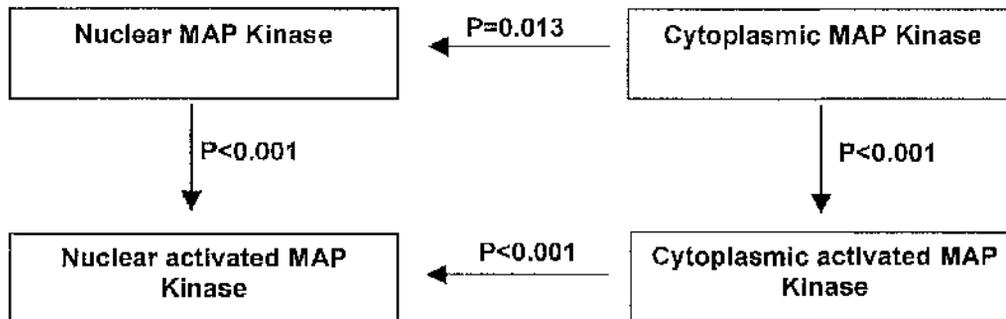
Table 6b shows significant correlations (p values) between the Cytoplasmic and nuclear forms of activated Raf-1 – pRaf (ser338) – in ASPC and AIPC tumours. Correlation coefficients are shown in brackets. P values were assessed by Spearman’s Rank Correlation Test.

3.6.2 Correlations between MAP Kinase and its’ phosphorylated form

Significant correlations were seen between the cytoplasmic and nuclear expression of MAP Kinase and activated, phosphorylated MAP Kinase (pMAP Kinase) in ASPC and AIPC tumours individually (Appendix 5 - Tables 7a and 7b).

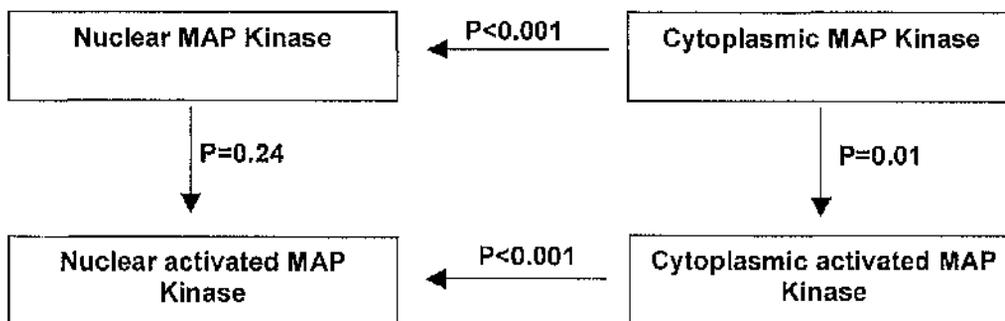
In ASPC tumours, cytoplasmic expression of MAP Kinase correlated with its’ nuclear expression (p=0.013) as well as the cytoplasmic expression of its’ activated form (p<0.001). Nuclear MAP Kinase expression in addition correlated very strongly with its’ activated form in the nucleus (p<0.001). Activated MAP Kinase in the cytoplasm was also very strongly associated with its’ activated expression in the nucleus (p<0.001). A summary can be seen in Figure 11a.

Figure 11a – Flow diagram summarising MAP Kinase correlations in ASPC tumours.



After the development of AIPC, cytoplasm expression of MAP Kinase became strongly associated with its' expression in the nucleus ($p < 0.001$) but no longer correlated anymore with its' activated form in the nucleus. Cytoplasmic expression of MAP Kinase still correlated with its' activated form in the cytoplasm ($p = 0.010$), which remained strongly associated with the activated form in the nucleus ($p < 0.001$). A summary can be seen in **Figure 11b**.

Figure 11b. Flow diagram summarising MAP Kinase correlations in AIPC tumours.



3.6.3 Correlations between Raf-1 and MAP Kinase

In ASPC tumours, Raf-1 and its' inactive form, pRaf, correlated with the cytoplasmic expression of MAP Kinase ($p = 0.002$ and $p = 0.003$ respectively), and activated MAP Kinase ($p = 0.002$ and $p = 0.049$ respectively). Raf-1 also correlated with nuclear activated

MAP Kinase ($p=0.003$). There were no correlations seen between pRaf (ser338) and MAP Kinase or pMAP Kinase (see Table 7a).

Table 7a. Correlations between Raf-1 and MAP Kinase in ASPC tumours

| | Raf-1 | pRaf (ser259) | pRaf (ser338)(cyto) |
|--------------------|---------------|----------------------|----------------------------|
| MAP Kinase | 0.319 | 0.441 | 0.280 |
| (nuclear) | (0.13) | (0.10) | (-0.16) |
| MAP Kinase | 0.002 | 0.003 | 0.826 |
| (cytoplasm) | (0.40) | (0.39) | (0.03) |
| pMAP Kinase | 0.003 | 0.431 | 0.221 |
| (nuclear) | (0.38) | (0.11) | (-0.17) |
| pMAP Kinase | 0.002 | 0.049 | 0.982 |
| (cytoplasm) | (0.40) | (0.26) | (0.00) |

Tables 7a shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, and the nuclear and cytoplasmic expression of MAP Kinase, and its' activated, phosphorylated form. Significant correlations ($p<0.05$) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

pRaf (ser259), inactive phosphorylated Raf ; pRaf (ser338) active phosphorylated Raf
 pMAP Kinase, activated phosphorylated MAP Kinase

In AIPC tumours, Raf-1 was still associated with cytoplasmic and now nuclear MAP Kinase expression ($p=0.003$ and 0.007). Cytoplasmic MAP Kinase also correlated with pRaf

(ser259) (p=0.008). There were still no correlations seen between pRaf (ser338) and MAP Kinase (See **Table 7b**).

Table 7b. Correlations between Raf-1 and MAP Kinase in AIPC tumours

| | Raf-1 | pRaf (ser259) | pRaf (ser338)(cyto) |
|--------------------|---------------|----------------------|----------------------------|
| MAP Kinase | 0.007 | 0.351 | 0.087 |
| (nuclear) | (0.34) | (0.12) | (-0.24) |
| MAP Kinase | 0.003 | 0.008 | 0.503 |
| (cytoplasm) | (0.37) | (0.33) | (-0.09) |
| pMAP Kinase | 0.355 | 0.204 | 0.949 |
| (nuclear) | (0.12) | (0.16) | (0.01) |
| pMAP Kinase | 0.612 | 0.102 | 0.293 |
| (cytoplasm) | (0.07) | (0.21) | (-0.14) |

Tables 7b shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, and the nuclear and cytoplasmic expression of MAP Kinase, and its' activated, phosphorylated form. Significant correlations (p<0.05) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

pRaf (ser259), inactive phosphorylated Raf ; pRaf (ser338) active phosphorylated Raf

pMAP Kinase, activated phosphorylated MAP Kinase

3.6.4 Raf-1 Activation – The Role of Serine 259 and 338 Phosphorylation

The serine 259 and 338 phosphorylation sites play a crucial role in determining the activity and biological outcome of Raf-1 (Dhillon et al., 2002a) (Dhillon et al., 2002b). Dephosphorylation of serine 259 in response to Ras stimulation is essential for peak activation of Raf-1. This facilitates Raf-1 membrane translocation, enhancing its' interaction with Ras, and stabilizing its' subsequent activation by phosphorylation of serine 338 (**Figure 12**) (Dhillon et al., 2002a) (Tzivion et al., 1998). Re-phosphorylation at serine 259, thereby deactivating Raf-1, provides a regulatory feedback mechanism to limit Raf-1 signalling and may occur via Ras cross-talk with PKA, Akt (serine kinases which are known to deactivate Raf-1 at serine 259), or an as yet unknown protein kinase (Dhillon et al., 2002a) (Abraham et al., 2000). Therefore, the activation state of Raf-1 is complex and dynamic, and exposed to rapid turnover of phosphorylated and dephosphorylated states.

In this study, total Raf-1 expression correlated with its' inactive form ($p=0.028$) in ASPC tumours. This correlation was strengthened in AIPC tumours ($p=0.008$). There were no correlations with its' active form, however this is consistent with the current understanding of Raf-1 dynamics. Once Raf-1 is activated at serine 338, it is marked for immediate dephosphorylation and re-phosphorylation at serine 259 resulting in its' return to the cytoplasm in an inactive and more stable state (Kubicek et al., 2002). Direct protein correlations with pRaf (ser338), as noted in this study, may reflect this and therefore maybe difficult to interpret.

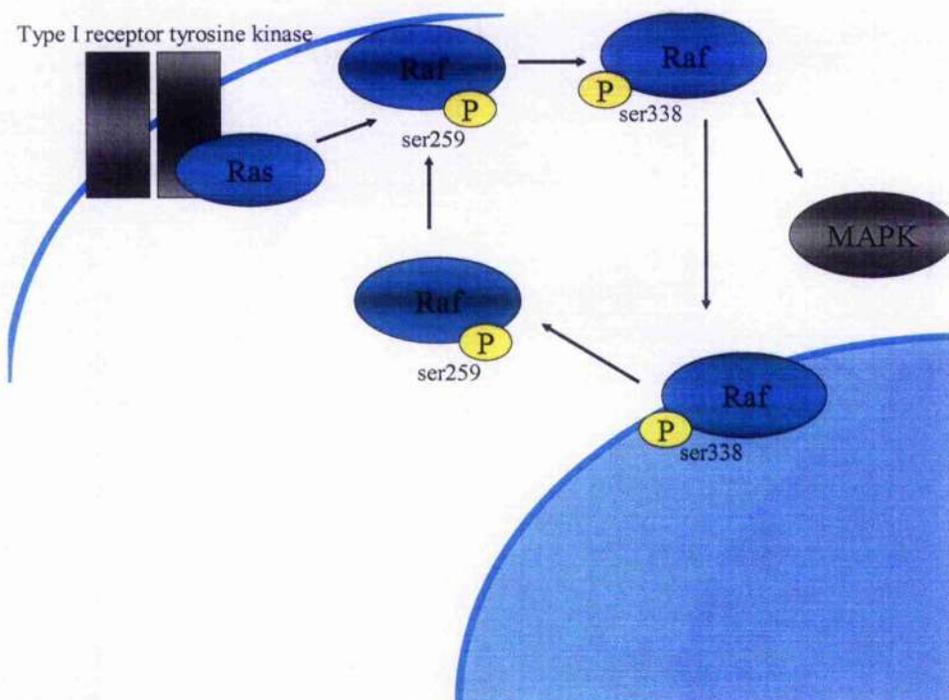


Figure 12. Raf-1 Activation – The Role of Serine 259 and 338 Phosphorylation

3.6.5 Raf-1 Activation of MAP Kinase

ASPC

Both Raf-1 and its' inactive form, pRaf (ser259) correlated with expression of total and activated MAP Kinase. Cytoplasmic MAP Kinase expression correlated with activated cytoplasmic MAP Kinase expression, which correlated with nuclear activated MAP Kinase expression ($p < 0.001$ and $p < 0.001$). This data suggests that Raf-1 activates MAP Kinase in the cytoplasm, which in turn translocates to the nucleus. No correlations were observed between activated Raf-1 and activated MAP Kinase, however this was not unexpected as Raf-1 becomes rapidly inactivated and phosphorylated at serine 259 as discussed above.

AIPC

With development of AIPC, Raf-1 was still associated with cytoplasmic and now nuclear MAP Kinase expression ($p = 0.003$ and 0.007). Correlations with activated MAP Kinase

interestingly were now lost. The inactive form of Raf-1 still correlated with cytoplasmic MAP Kinase ($p=0.008$), but not with its' activated form. Despite this, cytoplasmic MAP Kinase still strongly correlated with its' activated form in the cytoplasm ($p=0.01$) which correlated with the activated form in the nucleus, so a pathway from Raf-1 to activated nuclear MAP Kinase was still present.

Correlations between Raf protein expression, its' kinase activity and MEK activation is not always seen *in vitro*, implying that Raf/MAP Kinase coupling is complex, and carefully adjusted in cells (Dhillon et al., 2003). It is also possible that, whilst Raf-1 continues to activate nuclear MAP Kinase expression, the loss of some of the direct Raf/MAP Kinase correlations is related to an increasing degree of independent Raf-1 function, outwith the MAP Kinase cascade, with the development of clinical AIPC (Wang et al., 1998) (**Figure 13**). This may be supported by the fact that cytoplasmic and nuclear forms of activated Raf-1 strongly correlated with each other in androgen-sensitive ($p=0.01$) but even more strongly in androgen-insensitive tumours ($p<0.001$).

There is increasing evidence for such independent Raf-1 activity outwith the MAP Kinase cascade (Dhillon et al., 2003) (Dasgupta et al., 2004). Raf-1 can bind and inactivate the retinoblastoma tumour suppressor protein (Rb) directly resulting in cell cycle progression beyond the G1/S barrier (Wang et al., 1998). Raf-1 overexpression can thereby facilitate cellular proliferation and, as *in vitro* studies would suggest, vascular endothelial growth factor (VEGF)-induced angiogenesis, independently of MAP Kinase (Dasgupta et al., 2004). The inactivation of Rb through phosphorylation is therefore thought to play a significant role in carcinogenesis.

Once stimulated, significant translocation of Raf-1 occurs from the cytoplasm into the nucleus, where its' interaction with Rb, a nuclear protein, takes place (Wang et al., 1998) (Dasgupta et al., 2004). This is consistent with the immunohistochemical findings in this study, which demonstrated activated Raf-1 presence in the nucleus as well as cytoplasm. This was not seen with total Raf-1 and inactive Raf-1 presumably as Raf-1 only enters the nucleus when activated and its' subsequent inactivation only occurs in the cytoplasm secondary to Akt or PKA. The mechanism by which Raf-1 enters the nucleus, without interacting with the MAP Kinase pathway, is unknown but may involve the presence of as yet unidentified chaperone proteins (Wang et al., 1998). In doing so, Raf-1 may well directly affect other nuclear targets in addition to Rb.

Raf-driven inhibition of apoptosis has also been noted to be independent of MAP Kinase (Dhillon et al., 2003) (Cox and Der, 2003). In support of this, Raf-1 can physically interact with the pro-apoptotic bcl-2, shifting to the mitochondria whilst bound to it and inactivating the pro-apoptotic protein, BAD (Wang et al., 1998) (Cox and Der, 2003). Upregulation of bcl-2 has already been associated with the development of AIPC (Grossmann et al., 2001) (Chen et al., 2004).

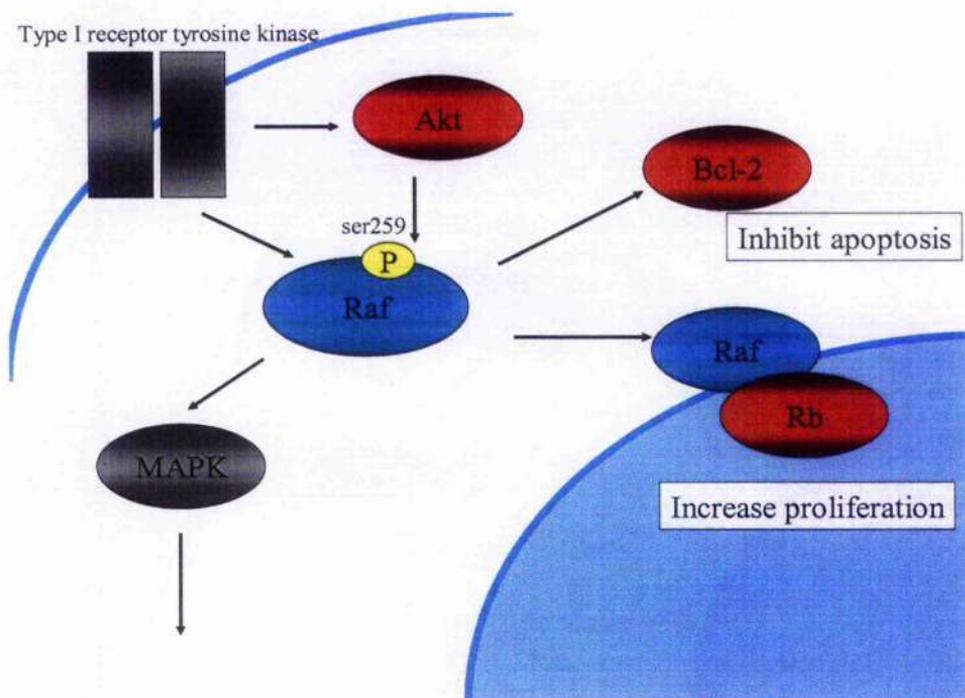


Figure 13. Raf-1 targets outwith the MAP Kinase pathway

Raf-1 independent activity may therefore explain the loss of some of the correlations in particular between Raf-1 and activated MAP Kinase with the development of AIPC. This may imply increased dysregulation of the Raf/MAP Kinase pathway as a result of cancer progression.

In line with these observations, high MAP Kinase expression has previously been observed in early stages of prostate cancer, correlating with cell proliferation (Uzgare et al., 2003). Intriguingly, the constitutively active form of MAP Kinase also induces Raf-1 activation in cell-line studies, forming the positive feedback loop required for chronic autocrine stimulation (Weinstein-Oppenheimer et al., 2000). This may explain the degree of correlations seen here. The data also supports the hypothesis that stimulation of the Raf/MAP Kinase pathway possibly via type 1 receptor tyrosine kinases, results in the elevated expression of activated members of the pathway (Gioeli et al., 1999).

3.7 Correlations with Other Proteins in the Signalling Pathway

3.7.1 Correlations with Other Proteins in ASPC Tumours

In androgen-sensitive tumours, expression of the growth factor receptor HER2 correlated with Raf-1 expression ($p=0.019$). HER2 also correlated with cytoplasmic MAP Kinase expression ($p=0.02$), and with the nuclear and cytoplasmic expression of pMAP Kinase ($p=0.03$ and $p<0.001$ respectively). There was a weak negative association with HER2 and activated pRaf (ser338) in the cytoplasm ($p=0.06$). Once activated, its phosphorylated form (pHER2) correlated strongly with pRaf (ser259) ($p=0.001$), but not the activated forms of Raf and MAP Kinase (see Table 8a).

Table 8a. Protein correlations between Raf/MAP Kinase and HER2 in ASPC tumours.

| | HER2 | pHER2 |
|--------------------------|-------------------------|---------------------|
| Raf-1 | 0.019 (0.34) | |
| pRaf (ser259) | 0.162 (0.21) | 0.001 (0.49) |
| pRaf (ser338) (nuclear) | 0.397 (-0.14) | 0.801 (0.04) |
| pRaf(ser338) (cytoplasm) | 0.06 (-0.30) | 0.824 (0.04) |
| MAP Kinase (nuclear) | 0.292 (0.16) | |
| MAP Kinase (cytoplasm) | 0.002 (0.45) | |
| pMAP Kinase (nuclear) | 0.003 (0.45) | 0.54 (-0.10) |
| pMAP Kinase (cytoplasm) | <0.001 (0.60) | 0.50 (0.11) |

Table 8a shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, nuclear and cytoplasmic expression of MAP Kinase and its activated, phosphorylated form, with HER2 in ASPC tumours. Significant correlations

($p < 0.05$) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

pHER2, phosphorylated HER2

pRaf (ser259), inactive phosphorylated Raf ; pRaf (ser338) activated phosphorylated Raf

pMAP Kinase, activated phosphorylated MAP Kinase

There were no correlations seen with EGFR in ASPC tumours, however its' activated, phosphorylated form weakly correlated with cytoplasmic pRaf (ser338) ($p = 0.037$). The mutant variant, EGFR VIII, did correlate strongly with cytoplasmic expression of MAP Kinase and pMAP Kinase ($p < 0.001$ and 0.014 respectively) and with Raf-1 to a weaker extent ($p = 0.034$). There was weak negative association with nuclear pRaf (ser338) ($p = 0.052$) (see Table 8b).

Table 8b. Protein correlations between Raf/MAP Kinase Protein Expression and EGFR in ASPC tumours.

| | EGFR | pEGFR | EGFR VIII |
|---------------------------|--------------------|--------------------|------------------------|
| Raf-1 | 0.050(0.25) | | 0.034(0.27) |
| pRaf (ser259) | 0.635(0.60) | 0.779(0.04) | 0.368(0.12) |
| pRaf (ser338) (nuclear) | 0.973(0.01) | 0.688(-0.06) | 0.052(-0.27) |
| pRaf (ser338) (cytoplasm) | 0.159(-0.20) | 0.037(0.28) | 0.628(-0.07) |
| MAP Kinase (nuclear) | 0.96(-0.07) | | 0.19(0.18) |
| MAP Kinase (cytoplasm) | 0.45(0.10) | | <0.001(0.46) |
| pMAP Kinase (nuclear) | 0.054(0.26) | 0.59(-0.07) | 0.053(0.27) |
| pMAP Kinase (cytoplasm) | 0.14(0.20) | 0.47(0.1) | 0.014(0.33) |

Table 8b shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, nuclear and cytoplasmic expression of MAP Kinase, and its' activated, phosphorylated form, with EGFR in ASPC tumours. Significant correlations (p<0.05) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

pEGFR, phosphorylated EGFR ; EGFR vIII, variant III EGFR

Negative associations were seen between AR and Raf-1 (p=0.016), and between AR and nuclear and cytoplasmic expression of pMAP Kinase (p=0.04 and p=0.009 respectively). Expression of the androgen-regulated gene, PSA, correlated with nuclear and cytoplasmic expression of MAP Kinase (p=0.04 and p=0.016 respectively). This correlation was strengthened with activation (pMAP Kinase) (p=0.001 and p<0.001 respectively) (See Table 8c).

Table 8c. Correlation Between Raf-1/MAP Kinase Protein Expression with AR and PSA in ASPC tumours.

| | AR | PSA |
|---------------------------|----------------------|-------------------------|
| Raf-1 | 0.016 (-0.30) | 0.09 (0.22) |
| pRaf (ser259) | 0.911 (-0.01) | 0.298 (0.13) |
| pRaf (ser338) (nuclear) | 0.244 (0.16) | 0.385 (0.12) |
| pRaf (ser338) (cytoplasm) | 0.473 (0.10) | 0.277 (0.15) |
| MAP Kinase (nuclear) | 0.331 (-0.13) | 0.04 (0.38) |
| MAP Kinase (cytoplasm) | 0.124 (-0.20) | 0.016 (0.32) |
| pMAP Kinase (nuclear) | 0.043 (-0.27) | 0.001 (0.44) |
| pMAP Kinase (cytoplasm) | 0.009 (-0.34) | <0.001 (0.45) |

Table 8c shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, nuclear and cytoplasmic expression of MAP Kinase and its' activated, phosphorylated form with AR and PSA in ASPC tumours. Significant correlations (p<0.05) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

AR, androgen receptor

pRaf (ser259), inactive phosphorylated Raf ; pRaf (ser338) active phosphorylated Raf

pMAP Kinase, activated phosphorylated MAP Kinase

Further downstream, activated pRaf (ser338) in the cytoplasm correlated with phosphorylated c-jun (p=0.014). There were no correlation seen between MAP Kinase and its' activated form with c-jun nor c-fos (See Table 8d).

Table 8d. Correlation Between Raf-1/MAP Kinase Protein Expression with components of AP-1 in ASPC tumours.

| | c-jun | Phospho c-jun | c-fos |
|---------------------------|--------------|--------------------|--------------|
| Raf-1 | 0.330(-0.15) | 0.411(-0.13) | 0.782(0.04) |
| pRaf (ser259) | 0.166(0.21) | 0.086(-0.27) | 0.364(-0.14) |
| pRaf (ser338) (nuclear) | 0.577(-0.93) | 0.134(0.26) | 0.528(0.11) |
| pRaf (ser338) (cytoplasm) | 0.577(0.93) | 0.014(0.41) | 0.437(-0.13) |
| MAP Kinase (nuclear) | 0.13(-0.24) | 0.08(-0.28) | 0.45(-0.12) |
| MAP Kinase (cytoplasm) | 0.78(0.04) | 0.95(-0.01) | 0.39(0.14) |
| pMAP Kinase (nuclear) | 0.176(-0.22) | 0.101(-0.27) | 0.336(0.15) |
| pMAP Kinase (cytoplasm) | 0.192(-0.21) | 0.097(-0.27) | 0.190(0.21) |

Table 8d shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, nuclear and cytoplasmic expression of MAP Kinase and its' activated, phosphorylated form with the components of AP-1. Significant correlations (p<0.05) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

phospho c-jun, phosphorylated c-jun

pRaf (ser259), inactive phosphorylated Raf ; pRaf (ser338) active phosphorylated Raf

pMAP Kinase, activated phosphorylated MAP Kinase

3.7.2 Correlation with Other Members of the Pathway – A Functional Signalling Cascade in ASPC?

Type 1 Receptor Tyrosine Kinase Activation in ASPC

Growth factors and their receptors have been consistently linked with the development of AIPC. In particular, increased expression of the EGF-receptor family of type 1 receptors tyrosine kinases, including the EGFR itself, HER-2 and EGFR vIII (Craft et al., 1999) (Shi et al., 2001) (Olapade-Olaopa et al., 2000). Membrane bound receptor tyrosine kinases activate several known cytoplasmic signal transduction cascades, of which the Raf/MAP Kinase is one of the best characterised (Karayi and Markham, 2004). Type 1 receptor tyrosine kinases have been shown to activate Raf/MAP Kinase pathway in prostate cancer cell line experiments (Bell et al., 2003) (Craft et al., 1999) (Yeh et al., 1999). HER2 and MAP Kinase have both been postulated to act as potential markers of disease progression and as therapeutic targets on the basis of *in vitro* work (Yeh et al., 1999). The role of type 1 receptor tyrosine kinases in activating the Raf/MAP Kinase pathway was further supported by our findings *in vivo*.

In this study, expression of the upstream type 1 tyrosine kinase receptor, HER2, was associated with Raf-1 ($p=0.019$), cytoplasmic MAP Kinase ($p=0.02$), and nuclear and cytoplasmic expression of activated MAP Kinase ($p=0.03$ and $p<0.001$ respectively). Although these correlations are generally weak (with the exception of HER2 and cytoplasmic activated MAP Kinase) the number of associations would indicate that in clinical ASPC, HER2 is involved in regulating the MAP Kinase cascade, as previously demonstrated in *in vitro* studies (Yeh et al., 1999) (Craft et al., 1999) (**Figure 14**). A weak negative association with HER2 and activated Raf-1 ($p=0.06$) was also observed, whilst HER2 activation correlated positively with inactive Raf-1 ($p=0.001$). This may imply that HER2 in fact inactivates Raf-1, possibly via activation of the PI3K/Akt cascade which can result in phosphorylation of Raf-1 at serine 259 (Zimmermann and Moelling, 1999) (**Figure 13**). This association may then drive the overall positive correlation between HER2 and total Raf-1. This however would not explain the correlations between HER2 and activated MAP Kinase. Furthermore, the negative relationship between HER2 and activated Raf-1 did not achieve significance. An alternative explanation for the strong correlation between HER2 and with pRaf (ser259) may be that HER2 drives the activation of MAP Kinase via Raf-1 resulting in its' rapid inactivation.

A positive correlation was also seen between activated EGFR and activated Raf-1 ($p=0.037$). In addition, EGFR vIII correlated with Raf-1 ($p=0.034$), and cytoplasmic expression of MAP Kinase and its' phosphorylated form ($p<0.001$ and $p=0.014$) respectively. This may imply that EGFR and its' variant, in conjunction with HER2, can also result in the subsequent downstream activation of the MAP Kinase pathway via Raf-1 (**Figure 14**). Furthermore, a positive correlation was seen between pRaf (ser338) and phosphorylated c-jun -- a component of the downstream transcription factor AP-1. It is

tempting to speculate that an activated EGFR/Raf-1/c-jun pathway is present in ASPC as correlations between all 3 members are now present.

Interaction with the AR

Interestingly, *in vitro* studies have speculated a role for HER2 in promoting ARA-AR interaction, via MAP Kinase (Yeh et al., 1999). In support of this, MAP Kinase inhibitors have also been demonstrated to inhibit HER2-induced transcriptional activity of AR (Yeh et al., 1999). However, in our study, AR expression inversely correlated with Raf-1 expression ($p=0.016$) and with the nuclear and cytoplasmic expression of activated MAP Kinase ($p=0.04$ and $p=0.009$ respectively). As the AR signalling pathway is active in ASPC, a possible explanation for this could be that the AR axis is the preferred mode of cell growth regulation in ASPC cells, independent of HER2/MAP Kinase (**Figure 14**).

During androgen deprivation therapy, prostate cancer cells undergo growth arrest and apoptosis. Upregulation of the Raf/MAP Kinase pathway may enable AIPC cells to utilise the type 1 receptor tyrosine kinase/Raf-1/AP-1 cascade to promote cell growth independently of the AR, and compensate for androgen ablation-induced apoptosis. Activation of the pathway therefore occurs and eventually drives progression to death. In a separate study using the same dataset, rising HER2 expression with the development of AIPC was shown to correlate with reduced survival (Bartlett et al., 2005). This together with the survival data on rising MAP Kinase expression from this study would appear to support this hypothesis. Furthermore, correlations between all 3 levels of this pathway, which begin to appear in ASPC, develop further in AIPC tumours (see next section) (**Figure 15**).

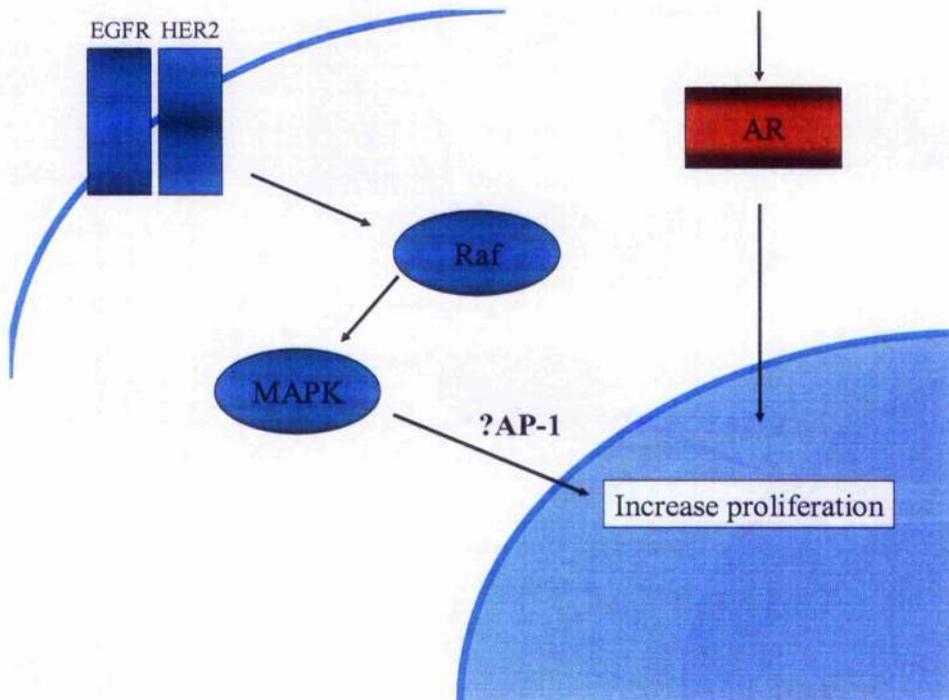


Figure 14 – Possible interpretation of protein correlations seen in patients with ASPC

Interestingly, expression of the androgen-regulated gene, PSA, correlated with nuclear and cytoplasmic expression of MAP Kinase ($p=0.04$ and $p=0.016$ respectively). This correlation was strengthened with activated MAP Kinase ($p=0.001$ and $p<0.001$ respectively), which may imply that MAP Kinase activation can drive the transcription of androgen-dependent genes in androgen-sensitive disease, independently of the AR (as AR correlated inversely with Raf-1 and MAP Kinase). However the ability of PSA protein expression to act as a marker of AR activation and AR-regulated gene expression has been questioned as, unlike serum levels, correlations between PSA and AR protein expression are often absent. It may be that MAP Kinase expression, like PSA, acts as a general marker for prostate cancer, and their association is merely casual rather than causal. Interestingly, these correlations were lost in AIPC, however by this stage levels of PSA expression do not reflect the extent of disease progression and are less useful.

3.7.3 Correlations with Other Proteins in AIPC Tumours

After the development of AIPC, HER2 once again correlated with Raf-1 ($p=0.04$). Its' weak negative association, seen in ASPC tumours, with cytoplasmic activated pRaf (ser338) was now significant ($p=0.013$) as was a negative correlation with the nuclear form ($p=0.027$). In addition, its' activated form (pHER2) once again correlated weakly with Raf-1 and strongly with pRaf (ser259) ($p=0.046$ and <0.001 respectively) as well as with cytoplasmic MAP Kinase expression ($p=0.014$) (see **Table 9a**).

Table 9a. Protein correlations between Raf/MAP Kinase and HER2 in AIPC tumours.

| | HER2 | pHER2 |
|---------------------------------|----------------------|-------------------------|
| Raf-1 | 0.040 (0.31) | |
| pRaf (ser259) | 0.456 (0.11) | <0.001 (0.60) |
| pRaf (ser338) (nuclear) | 0.027 (-0.35) | 0.176 (0.23) |
| pRaf(ser338) (cytoplasm) | 0.013 (-0.38) | 0.311 (0.17) |
| MAP Kinase (nuclear) | 0.588 (0.08) | |
| MAP Kinase (cytoplasm) | 0.579 (0.09) | |
| pMAP Kinase (nuclear) | 0.593 (-0.08) | 0.285 (0.18) |
| pMAP Kinase (cytoplasm) | 0.394 (0.13) | 0.363 (0.15) |

Table 9a shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, nuclear and cytoplasmic expression of MAP Kinase and its' activated, phosphorylated form, with HER2 in AIPC tumours. Significant correlations ($p<0.05$) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

pHER2, phosphorylated HER2

The association between EGFR vIII and Raf-1 ($p=0.024$) and cytoplasmic MAP Kinase ($p=0.004$) was still present in addition to an association with pRaf (ser259) ($p=0.014$) and nuclear expression of MAP Kinase ($p=0.012$). Furthermore, the weak negative association, seen in ASPC tumours, between with EGFR vIII and cytoplasmic activated pRaf (ser338) was now significant ($p=0.049$) but a much stronger negative correlation was seen with the nuclear form ($p=0.008$) (see Table 9b).

Table 9b. Protein correlations between Raf/MAP Kinase Protein Expression and EGFR in AIPC tumours.

| | EGFR | pEGFR | EGFR vIII |
|-------------------------------------|---------------|---------------|----------------------|
| Raf-1 | 0.548 (0.07) | | 0.024 (0.28) |
| pRaf (ser259) | 0.560 (-0.07) | 0.111 (0.20) | 0.014 (0.30) |
| pRaf (ser338) (nuclear) | 0.963 (0.01) | 0.672 (-0.06) | 0.008 (-0.34) |
| pRaf (ser338) (cytoplasm) | 0.423 (0.11) | 0.483 (0.10) | 0.049 (-0.26) |
| MAP Kinase (nuclear) | 0.860 (-0.02) | | 0.012 (0.32) |
| MAP Kinase (cytoplasm) | 0.850 (0.02) | | 0.004 (0.37) |
| pMAP Kinase (nuclear) | 0.054 (0.26) | 0.590 (-0.07) | 0.053 (0.27) |
| pMAP Kinase (cytoplasm) | 0.250 (0.15) | 0.127 (0.20) | 0.580 (0.07) |

Table 9b shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, nuclear and cytoplasmic expression of MAP Kinase and its' activated, phosphorylated form, with EGFR in AIPC tumours. Significant correlations

($p < 0.05$) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

pEGFR, phosphorylated EGFR ; EGFR vIII, variant III EGFR

A weak, positive correlation was seen between cytoplasmic pRaf (ser259) and pMAP Kinase with the AR ($p=0.04$ and 0.023 respectively). There was no correlation (positive or negative) now between Raf-1 expression and AR. There were no correlations noted with PSA expression (see Table 9c).

Table 9c. Correlation Between Raf-1/MAP Kinase Protein Expression with AR and PSA in AIPC tumours.

| | AR | PSA |
|---------------------------|---------------------|---------------|
| Raf-1 | 0.750 (-0.04) | 0.960 (-0.07) |
| pRaf (ser259) | 0.023 (0.28) | 0.987 (0.00) |
| pRaf (ser338) (nuclear) | 0.720 (-0.05) | 0.332 (0.13) |
| pRaf (ser338) (cytoplasm) | 0.748 (-0.04) | 0.627 (0.07) |
| MAP Kinase (nuclear) | 0.099 (-0.22) | 0.780 (-0.04) |
| MAP Kinase (cytoplasm) | 0.462 (-0.1) | 0.806 (0.03) |
| pMAP Kinase (nuclear) | 0.159 (0.18) | 0.76 (0.04) |
| pMAP Kinase (cytoplasm) | 0.040 (0.26) | 0.95 (0.01) |

Table 9c shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, nuclear and cytoplasmic expression of MAP Kinase and its' activated, phosphorylated form with AR and PSA in AIPC tumours. Significant

correlations ($p < 0.05$) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

AR, androgen receptor

pRaf (ser259), inactive phosphorylated Raf ; pRaf (ser338) active phosphorylated Raf

pMAP Kinase, activated phosphorylated MAP Kinase

More correlations were now seen with components of the downstream transcription factor AP-1. A strong correlation was still seen between cytoplasmic pRaf (ser338) and phosphorylated c-jun ($p = 0.008$), but now there was also one with pRaf (ser259) ($p = 0.005$). There was also a correlation seen between Raf-1 and c-fos ($p = 0.006$), and between the nuclear expression of pMAP Kinase and c-jun ($p = 0.025$) (see Table 9d).

Table 9d. Correlation Between Raf-1/MAP Kinase Protein Expression with components of AP-1 in AIPC tumours.

| | c-jun | Phospho c-jun | c-fos |
|---------------------------|---------------------|---------------------|---------------------|
| Raf-1 | 0.460 (0.12) | 0.878 (0.02) | 0.006 (0.44) |
| pRaf (ser259) | 0.385 (0.14) | 0.005 (0.42) | 0.286 (0.17) |
| pRaf (ser338) (nuclear) | 0.335 (0.16) | 0.123 (0.26) | 0.568 (-0.10) |
| pRaf (ser338) (cytoplasm) | 0.330 (0.17) | 0.008 (0.43) | 0.061 (-0.32) |
| MAP Kinase (nuclear) | 0.706 (0.06) | 0.115 (-0.26) | 0.532 (0.11) |
| MAP Kinase (cytoplasm) | 0.710 (0.06) | 0.820 (0.04) | 0.898 (0.02) |
| pMAP Kinase (nuclear) | 0.025 (0.35) | 0.245 (0.19) | 0.823 (0.04) |
| pMAP Kinase (cytoplasm) | 0.092 (0.27) | 0.732 (0.05) | 0.780 (0.05) |

Table 9d shows correlations (p values) between Raf-1, inactive and active phosphorylated Raf, nuclear and cytoplasmic expression of MAP Kinase and its'

activated, phosphorylated form with the components of AP-1. Significant correlations ($p < 0.05$) are highlighted. P values were assessed by Spearman's Rank Correlation Test. Correlation co-efficients in brackets.

phospho c-jun, phosphorylated c-jun

pRaf (ser259), inactive phosphorylated Raf ; pRaf (ser338) active phosphorylated Raf

pMAP Kinase, activated phosphorylated MAP Kinase

3.7.4 Correlation with Other Members of the Pathway – A Functional Signalling Cascade in AIPC?

Having demonstrated a role for Raf-1 and MAP Kinase expression in the development of AIPC, it was important to correlate this expression with other proteins, upstream and downstream, involved in the MAP Kinase signal cascade. Immunohistochemical determination of these proteins had already been carried out in the laboratory, using the same matched cohort of tumours (JE, NSK and KP). By using these results we were able to demonstrate that Raf-1 and MAP Kinase protein expression correlated with other proteins known to interact with the MAP Kinase signal transduction cascade (**Figure 15**).

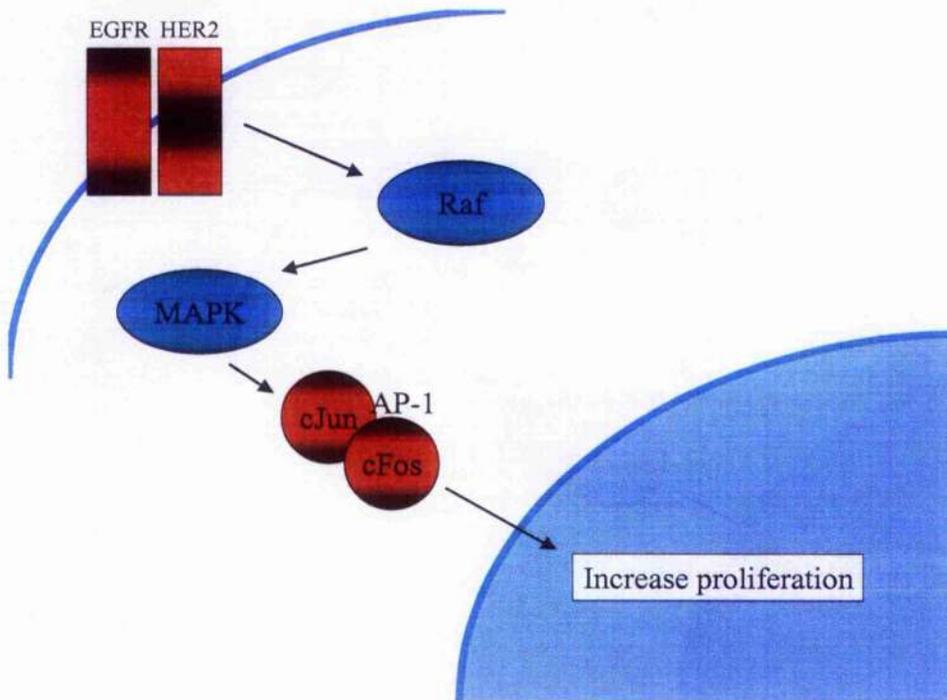


Figure 15 – The Raf/MAP Kinase Pathway – a functioning signalling cascade in AIPC development?

Type 1 Receptor Tyrosine Kinase Activation in AIPC

Correlations with the type 1 receptor tyrosine kinases continued with the development of AIPC. However the correlations between HER2 and its' activated form with Raf-1 were weaker ($p=0.040$ and $p=0.046$ respectively). Furthermore, activated HER2 correlated even more strongly with the inactive form of Raf-1 ($p<0.001$). Its' weak negative association, seen in ASPC tumours, with cytoplasmic activated Raf-1 was now significant ($p=0.013$) as was a negative correlation with the nuclear form of activated Raf-1 ($p=0.027$). This may imply that HER2 inactivates the Raf-1/MAP Kinase pathway – a relationship that may have been developing in ASPC. In support of this, the association between HER2 and MAP Kinase was also weakened with the development of AIPC, with only a single correlation with cytoplasmic MAP Kinase expression present ($p=0.014$).

In the case of EGFR vIII, the pattern of correlations with Raf-1 was similar to HER2. The association between EGFR vIII and Raf-1 ($p=0.024$) was still present perhaps driven by a strong association with inactive Raf-1 ($p=0.014$). In support of this, the weak negative association seen in ASPC tumours, between with EGFR vIII and cytoplasmic, activated Raf-1 (ser338), was now significant ($p=0.049$) along with a much stronger negative correlation with the nuclear form ($p=0.008$). Taken together this data implies that EGFR vIII also inactivates Raf-1. Despite this, strong positive correlations between EGFR vIII were still present with the nuclear and cytoplasmic expression of MAP Kinase ($p=0.012$ and $p=0.004$ respectively), although the relationship with its' activated form was now lost. No correlations were seen between EGFR and its' activated form with Raf-1 or MAP Kinase.

Whilst there is evidence linking type 1 receptor tyrosine kinases with AIPC via the MAP Kinase pathway in cell-line studies (Yeh et al., 1999) (Craft et al., 1999), this study may suggest that this axis, whilst present in ASPC, is lost in AIPC *in vivo*. HER2 and EGFR may be acting via other signal pathways, such as PI3K/Akt which inhibits Raf-1 by direct phosphorylation of serine 259 as mentioned earlier (**Figure 13**) (Zimmermann and Moelling, 1999). Both HER2 and EGFR are both known to activate the PI3K/Akt pathway, promoting androgen-independent tumourigenesis via the AR in the absence of androgen (Zhou et al., 2000) (Wen et al., 2000). In support of this, it has been shown that inhibition of MAP Kinase does not completely block HER2 activation of the AR, further implicating the role of other signal cascades in the promotion of androgen-insensitive growth. Furthermore, MAP Kinase expression has been noted to decrease with prostate cancer progression in a study using a transgenic mouse model, whilst remaining high in the early stages of cancer and correlating with cell proliferation (Uzgare et al., 2003).

Whilst this study speculated a role for MAP Kinase in initiating prostate carcinogenesis, inactivation was thought to be associated with the development of androgen-independence. It is important, however, to use caution when comparing these results with those using clinical prostate cancer specimens as any differences noted may be a reflection of the difficulties in creating accurate model systems for studying prostate cancer.

The survival data yielded from this study however would indicate that the Raf/MAP Kinase pathway still plays a crucial role in the development of AIPC. It may be the case that in AIPC alternative receptor tyrosine kinases can activate Raf-1. One may speculate a greater role for other members of the type 1 receptor tyrosine kinase family, such as HER3 or HER4 in the activation of the Raf/MAP Kinase cascade in AIPC. Other growth factor pathways may also be involved, for example the IL-6R cascade, which is known to interact and stimulate the MAP Kinase cascade directly (Ueda et al., 2002b). Interestingly, HER2 is also thought to be a central component of IL-6 signalling via MAP Kinase in prostate cancer with co-activation of HER3 (Qiu et al., 1998). It appears that the control and activation of the cascade, in general, becomes increasingly dysregulated with the development of androgen-insensitive disease, as one would expect. The AIPC samples in this study were retrieved once biochemical relapse was well established, and it may be that earlier sampling, (perhaps closer to the moment of actual moment of AIPC development) would still have detected ongoing strong correlations with HER2/EGFR and the Raf/MAP Kinase pathway.

However, as discussed earlier in ASPC tumours, an alternative explanation for the negative and positive correlations seen with active and inactive Raf-1 respectively may yet

be that HER2 or EGFR vIII drives the activation of MAP Kinase via Raf-1 resulting in its' rapid inactivation. Raf-1 activation status is also regulated by cross-talk with other signal transduction cascades, for example its' negative regulation by the PI3K/Akt pathway (Zimmermann and Moelling, 1999) (**Figure 16**). Alternatively, Raf-1 may be activated by PKC (Edwards et al., 2004). As mentioned earlier, these pathways may also be co-activated by growth factor receptors including the type 1 receptor tyrosine kinases with the development of AIPC (Wen et al., 2000) (Lee et al., 2002). PKA is also known to directly phosphorylate serine 259 and activate MAP Kinase (Dhillon et al., 2002b) (Ueda et al., 2002b) (Lee et al., 2002). In doing so, androgen-independent growth may be furthered, whilst Raf-1 is re-cycled to the membrane for further activation.

Interaction with the AR

Interestingly, the negative correlations previously noted between the Raf/MAP Kinase and the AR were no longer present. Instead, weak positive correlations were now seen between cytoplasmic pMAP Kinase and pRaf (ser259) with the AR ($p=0.04$ and 0.023 respectively). MAP Kinase activation of the AR by phosphorylation via phosphorylation at serine 515 *in vitro* is well documented. MAP Kinase can increase transcription of androgen-dependent genes, independently of androgens, via AR phosphorylation (Abreu-Martin et al., 1999) (Bakin et al., 2003) (Ueda et al., 2002b) (Franco et al., 2003) (Rochette-Egly, 2003). The AR therefore may now be acting as a target for MAP Kinase in AIPC (**Figure 16**). However, there is currently no evidence to demonstrate that this occurs in AIPC *in vivo* or even if phosphorylation by MAP Kinase has any clinical relevance. Correlations between activated MAP Kinase and phosphorylated AR (serine 515) would support a role for MAP Kinase phosphorylation of the AR however there is no commercially available antibody, as yet, to determine this.

Raf/MAP Kinase Interaction with AP-1

The MAP Kinase cascade has also been demonstrated *in vitro* to stimulate prostate cancer cell growth independently of the AR (Magi-Galluzzi et al., 1997). This study demonstrated activation of the transcription factor AP-1 as an alternative target for the Raf/MAP Kinase pathway, resulting in cell growth and survival. Raf-1 and MAP Kinase now correlated with c-fos and c-jun, components of the downstream transcription factor AP-1 - a known target of the pathway (Abreu-Martin et al., 1999) (Weinstein-Oppenheimer et al., 2000). Strong correlations were seen between Raf-1 and c-fos ($p=0.006$), activated and inactivated Raf-1 and phosphorylated c-jun ($p=0.008$ and 0.005 respectively), and the nuclear expression of pMAP Kinase with c-jun ($p=0.025$). Hence, activation of the Raf-1/MAP Kinase pathway may influence AP-1 formation, leading to increased levels of its' components, in clinical AIPC patient specimens. *In vitro* studies have already demonstrated that Raf-1 regulates c-fos expression and, once activated, Raf-1 can phosphorylate and hence activate c-jun, via MAP kinase (Weinstein-Oppenheimer et al., 2000). Once again, the rapid inactivation of Raf-1 once AP-1 has formed may explain why pRaf (ser259) correlated with phosphorylated c-jun.

Activated MAP Kinase may therefore stimulate prostate cancer cell growth independently of the AR via activation of AP-1 (**Figure 16**) (Weinstein-Oppenheimer et al., 2000). The AP-1 transcription factor is involved in the control of cell growth and differentiation as well as apoptosis, and is thought to be the convergence point of several signalling pathways leading to carcinogenesis (Church et al., 2005). As such, it has already been linked with AIPC progression (Sato et al., 1997) (Bubulya et al., 2001) (Church et al., 2005). Its' formation and activation requires phosphorylated c-jun forming homodimers or heterodimers with c-fos. Phosphorylation of c-jun may be stimulated by MAP Kinase

in cell-line studies (Sato et al., 1997) (Wyke et al., 1996). The concentration of c-jun and c-fos has been noted to be several times greater in androgen-insensitive compared to sensitive cell-lines (Sato et al., 1997). Furthermore, the MAP Kinase pathway is known to stimulate AP-1 transcriptional activity in AR-positive and negative cells (Abreu-Martin et al., 1999). MAP Kinase may also effect transcription of c-fos resulting in its' upregulation (Wyke et al., 1996). Upregulation of RKIP (Raf-1 inhibitory protein), which is known to disrupt Raf-1 and MEK interaction, causes inhibition of the cascade and subsequent AP-1 dependent transcription (Yeung et al., 2000).

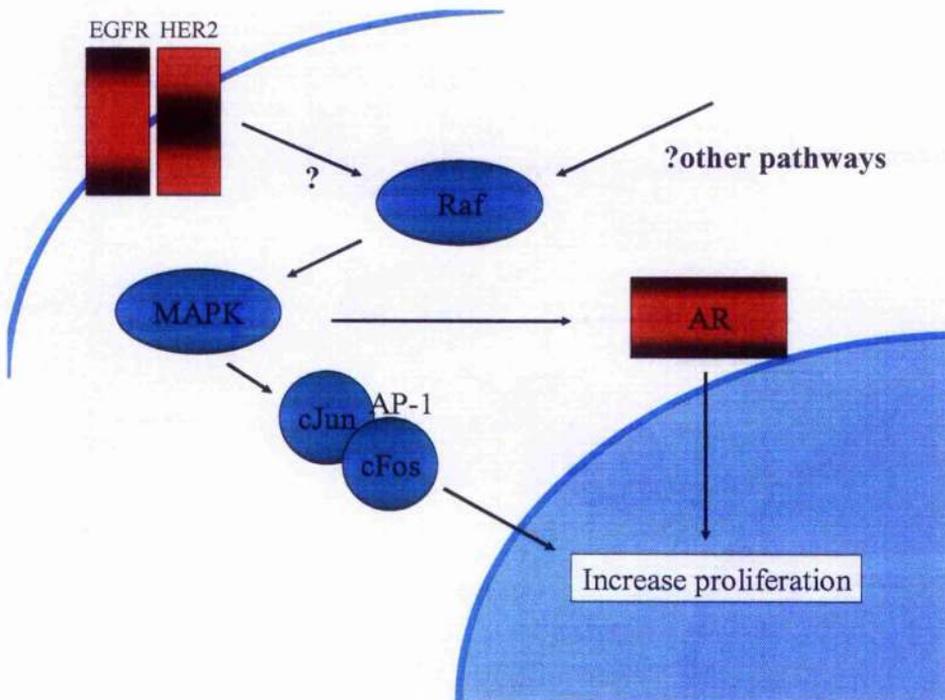


Figure 16 – Possible interpretation of protein correlations seen in patients with AIPC

AP-1 and its' components are known to cross-talk with the AR (Church et al., 2005). AP-1 can inhibit AR gene transcription by direct binding with the receptor (Sato et al., 1997) (Edwards et al., 2004). In turn, AP-1, acting as an independent transcription factor, may activate androgen-regulated genes, stimulating prostate cancer cell growth independently

of the AR (Sato et al., 1997). The PSA promoter site, for example, contains a specific binding site for AP-1 possibly allowing for AR-independent transcription via the MAP Kinase pathway. It has been postulated that a relative increase in the cellular AP-1/AR ratio would result in a "switch" to AP-1 regulation of (androgen-dependent) gene expression (Edwards et al., 2004). Interestingly, activation of the AR in a recent study using the LNCaP cell-line, can also result in growth arrest, with co-stimulation of AP-1 mediated gene transcription (Church et al., 2005). The composition of AP-1 in this study was also altered, as a result, in favour of c-jun. In addition, c-jun, in its unphosphorylated form, acts as an AR co-activator, promoting AR homodimerisation and increasing its' transactivation ability in the absence of ligand (1516) (Bubulya et al., 2001). In support of this, overexpression of c-jun in androgen-insensitive cell lines, can increase AR activation (Tillman et al., 1998). C-jun can also prevent the inhibitory effects of the AR co-repressor, p53 (Lee and Chang, 2003). Upregulation of c-jun may therefore be another mechanism by which MAP kinase can activate the AR to effect androgen-independent growth (Zhu and Liu, 1997).

The role of AP-1 and its' components is therefore complex. Work in our own laboratory has already linked increased phosphorylated c-jun expression with androgen-resistance (Edwards et al., 2004). Using the same database of paired patient samples, this study demonstrated high phosphorylated c-jun expression in over 60% of patients with AIPC. Interestingly, this resulted in a reduction in time to death from relapse (from 5.5 years to 1.87 years) in patients with high expression of phosphorylated c-jun (Edwards et al., 2004). Concomitant progression of disease should translate into potential cancer specific survival which may explain the association between rising MAP Kinase expression and reduced overall survival.

The mechanism of AIPC development in this patient cohort may well be increased AP-1 expression, driven by the phosphorylation of c-jun by MAP Kinase. This in turn would lead to AR suppression and AP-1 mediated "bypass", as speculated earlier. In support of this, data in the current study revealed positive correlations between Raf-1, c-fos and phosphorylated c-jun with the development of AIPC and ASPC. However the correlation between MAP Kinase with unphosphorylated c-jun may indicate that the pathway can also act via c-jun alone, albeit to a lesser extent.

However it may be argued that such protein correlations may be casual rather than causal. Rising Raf-1 and MAP Kinase expression in prostate cancer may be a result of, rather than the effect of, tumour progression. However the sheer extent and number of protein correlations with known activators and targets of the pathway, and, more importantly, the impact of Raf-1 and MAP Kinase on relapse and survival would imply a more direct role for the pathway in the development of AIPC. To further test the clinical relevance of the Raf/MAP Kinase pathway *in vitro*, assays simulating the physiological state of androgen-deprivation with activation by growth factors are required (Yeh et al., 1999).

3.8 Tissue Micro Arrays

3.8.1 Correlations with Tissue Micro Arrays

Interclass correlation coefficients were then calculated to assess the accuracy of each core in predicting histoscore from the full section (see **Table 10**). In total 55 sections had tissue suitable for obtaining cores. As correlations were generally poor (ICCC<0.50), TMAs were not stained for pRaf (ser338) nor pMAP Kinase.

Table 10

| | Raf-1 | pRaf (ser259) | MAP Kinase (nuclear) | MAP Kinase (cytoplasm) |
|---|-------|------------------|----------------------------|------------------------------|
| ICCC (Full section vs correspondingTMA) | 0.24 | 0.50 | 0.17 | 0.06 |

Table 10 shows interclass correlation coefficients between median histoscores for TMA cores compared with median histoscores for the corresponding full section.

ICCC, inter-class correlation co-efficient

pRaf (ser259), inactive phosphorylated Raf

pMAP Kinase, activated phosphorylated MAP Kinase

3.8.2 Use of TMAs in prostate cancer

High density TMAs are widely used for the rapid screening of multiple tumour sections for protein expression, allowing large-scale retrospective studies of archival tissue (Camp et al., 2000) (Kononen et al., 1998) (Kirkegaard et al., 2005). By constructing a single tissue block containing cores from several patients, TMAs have the advantage of conserving tissue and standardising experimental conditions (Perrone et al., 2000). The sampling and coring of small specific tumour areas should reduce tissue staining heterogeneity due to non-cancerous tissue which can be observed from analysis of full tissue sections (Kirkegaard et al., 2005). Despite the small size of cores, inter-observer variability has been shown to be just as low in TMAs, compared with full sections (Kirkegaard et al., 2005).

TMAs have already been used for high throughput analysis of prostate cancer specimens (Perrone et al., 2000). In order to analyse the effectiveness of TMAs in prostate cancer in this study, cores were taken from tissue blocks (if suitable) and transferred into an array block. Fifty five sections were cored in total. In breast TMAs, 0.6 mm cores are routinely used, however prostate cancer is a more heterogeneous cancer both in terms of tumour histology and differentiation. As a result the grades from the most prevalent regions are combined to form the Gleason score. Larger areas of tissue (containing as much of the cancerous regions as possible) are therefore required to provide a true representation as different tumour regions may have varying levels of protein expression (Camp et al., 2000) (Perrone et al., 2000). We therefore increased the core size to 1 mm and included 2 cores from high and low Gleason areas to combat this.

However, we still observed poor correlations between TMA cores and full sections. This is in direct contrast with previous results in breast cancer, where use of 2 cores alone were representative of the entire tumour (Camp et al., 2000). It may be possible to compensate for this by extracting multiple cores (up to 6) from tissue blocks to truly reflect the tumour specimen, as has been carried out previously in tissue derived from radical prostatectomies (Perrone et al., 2000) (Fergenbaum et al., 2004). However this is often not technically feasible using tissue blocks containing TURP chippings (and certainly not for TRUS biopsies) which are not large enough in terms of depth and surface area for multiple coring to take place. As such, this study supports the use of full tissue sections only when conducting immunohistochemical analyses of protein expression in samples of prostate cancer only.

**GENERAL DISCUSSION AND
CONCLUSIONS**

4.1 Introduction

Androgen-insensitive prostate cancer is forecast to be an increasing problem in the immediate future (Beemsterboer et al., 1999). Whilst the AR signalling pathway plays a central role in prostate carcinogenesis as well as its' treatment, it is now understood that the progression to AIPC may involve several possible changes, which may not be confined to the AR itself. The unravelling of the various molecular profiles or pathways which result in AIPC is crucial therefore for the development of novel therapeutic strategies.

Previous research in our laboratory has implicated the MAP Kinase pathway in the development of AIPC using comparative gene array analysis (Edwards et al., 2003b). Based on the genetic changes found in this study, we hypothesised that the MAP Kinase pathway plays a crucial role in promoting the development of androgen-insensitive disease. To test this hypothesis we assessed the protein expression and activation status of Raf-1 and MAP Kinase, critical members of the pathway, using immunohistochemistry and determined if this had any impact on clinical parameters. We then correlated their expression with the expression of known activators of the MAP Kinase cascade (the type 1 receptor tyrosine kinases EGFR and HER2), a known downstream target (AP-1), as well as expression of the androgen receptor itself. This would provide evidence to suggest a functional pathway was in fact present with the development of AIPC. In order to carry out the study, a unique database consisting of matched, paired ASPC and AIPC specimens from the same patients was developed.

4.2 Raf/MAP Kinase Pathway As A Novel Therapeutic Target

The Raf/MAP Kinase pathway is thought to play an important role in promoting cell proliferation and preventing apoptosis. If modest statistical benefits in terms of survival can be shown, then these may well represent significant clinical benefits in individual, often elderly patients (Klotz, 2001). Therefore it could be argued that the molecular selection of patients may be valuable in identifying groups of individuals or subset of patients who would benefit from Raf/MAP Kinase pathway inhibition. The ability of the MAP Kinase pathway to inhibit apoptosis depends on its' environmental context, for example in response to chemotherapeutic or radiation-induced stress (Abreu-Martin et al., 1999) (Hagan et al., 2000). This may render targeting of its' activation status a useful therapeutic strategy. Interestingly, activation of Raf-1 and MAP Kinase has been shown to be inhibited by the 5-alpha-reductase inhibitor, finasteride (Huynh, 2002), which may result its' anti-proliferative and pro-apoptotic effects. Specific inhibitors to Ras and Raf function have been developed e.g farnesyl tranferase (FT) inhibitors and anti-sense oligodeoxyribonucleotides. Many of these compounds are showing promising results in clinical trials (Weinstein-Oppenheimer et al., 2000). Interference with the pathway by specific drugs in such a manner may represent a novel means of improving anti-cancer therapy, perhaps as an adjunct to current chemotherapeutic approaches (**Figure 1**) (Karayi and Markham, 2004).

Recent studies have yielded promising results in the form of combination therapy, between standard chemotherapeutic agents or radiotherapy and novel drugs aimed at molecular targets. Radiotherapy is known to activate various signalling pathways and irradiation of DU-145 cells has been shown to cause EGFR-dependent activation of the MAP Kinase pathway, resulting in cell proliferation and survival (Hagan et al., 2000).

Inhibition of the MAP Kinase pathway by the known pharmacological inhibitors UO126 and PD98059 (which target different sites on MEK) however results in increased radiation-induced G₂/M phase growth arrest and apoptosis. As such, EGFR/MAP Kinase inhibition may be used to increase radiation-induced cell kill as an adjunct to radical or palliative radiotherapy. Another recent study demonstrated a 20% increase in docetaxel-induced growth suppression of androgen-independent LNCaP cells when MAP Kinase inhibition (by PD98059) was included (Zelivianski et al., 2003). In addition, enhanced apoptosis secondary to further bcl-2 inactivation and bax upregulation was also seen. Its' combination with docetaxel may result in reduced clinical cytotoxicity as a result of reduced dosage. Together, this implies a further role for MAP Kinase inhibition in AIPC multidrug chemotherapy, especially in cases where activation of the pathway occurs (**Figure 1**) (Zelivianski et al., 2003).

New specific strategies aimed at reversing the molecular changes that underlie prostate tumourigenesis are being developed. It is perhaps not surprising however that no single oncogene product has been linked exclusively or conclusively with the progression of prostate cancer to androgen-insensitive disease (Karayi and Markham, 2004) and instead a variety of biological markers and signal transduction pathways have been linked with AIPC. It is becoming increasingly apparent that several of these, when altered or overexpressed, will play a role in the disease at least in a proportion of patients. As multiple mechanisms or pathways are responsible for escaping androgen control, a multimodal management of AIPC is likely in the future (Feldman BJ and Feldman D, 2001) (Culine and Droz, 2000).

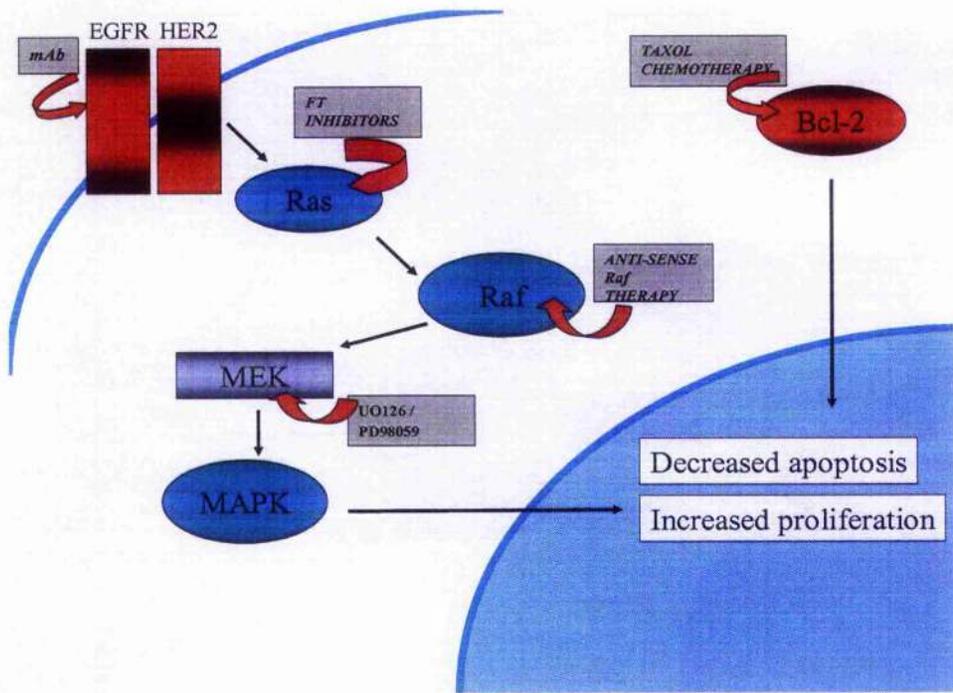


Figure 1. Raf/MAP Kinase Pathway with therapeutic sites of action.

mAb – monoclonal antibodies e.g. herceptin

FT inhibitors – farnesyl transferase (FT) inhibitors (aimed at Ras)

Anti-sense Raf therapy - anti-sense oligodeoxyribonucleotides (aimed at Raf)

UO126 / PD98059 – specific MEK inhibitors

4.3 CONCLUSIONS

In summary, the data compiled in this thesis provides the first clinical evidence to support the hypothesis that the Raf/MAP Kinase pathway is utilised in AIPC. This pathway plays a role in promoting its' development and progression as their expression significantly impacts on time to biochemical relapse and overall survival. In addition, correlations with type 1 tyrosine kinase receptors and components of AP-1 (c-fos and c-jun) in AIPC tumours, may indicate a functional pathway in the development of AIPC.

Further analysis of this pathway, and its' role in AIPC using clinical material, are clearly warranted. These findings lead us to hypothesise that expression of Raf-1 and MAP Kinase would yield useful predictive information for patients at risk of AIPC. Targeting of the type 1-tyrosine kinase receptor/Raf-MAP Kinase /AP-1 axis in selected patients may yield a novel and exciting therapeutic avenue, consistent with early trials that are already underway.

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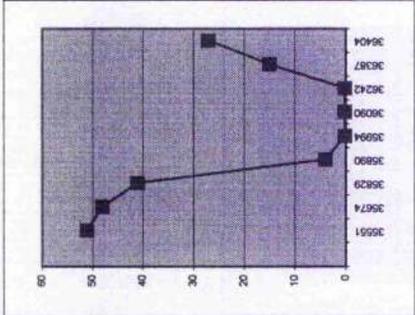
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APPENDICES

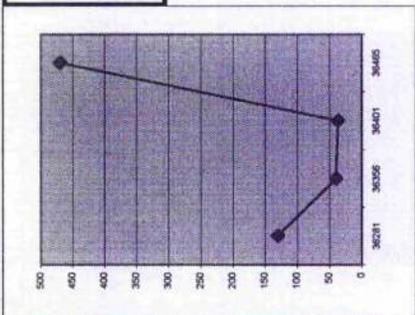
APPENDIX 1

Full clinical, pathological and biochemical data of all patients in the study.

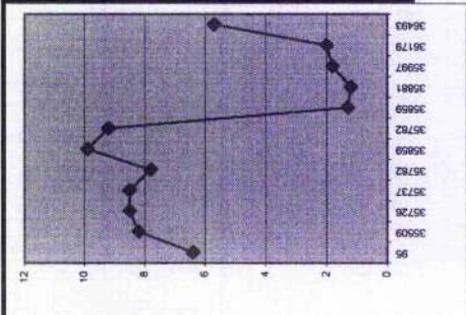
| Study No | Date of Birth | Age at Diagnosis | Date Diagnosis | Diagnosis-BX / Turp | PSA at diagnosis | Gleason score | Prevalence of mets | Op site (Zrelaps) | Gleason sum | Time to PSA relapsa(Yrs) | Mets at relapsa | Chemotherapy | GnRH analogues | anti-androgen | Alive/Dead | Follow-up(years) | Followup from relapsa | Cause of death |
|----------|---------------|------------------|----------------|---------------------|------------------|---------------|--------------------|-------------------|-------------|--------------------------|-----------------|--------------|----------------|---------------|------------|------------------|-----------------------|---------------------|
| AR132 | 12/08/1955 | 72 | 01/05/1997 | TURP | 51 | 8 | No metastases | 01/21/1999 | 8 | 2.25 | No metastases | | May-99 | | Alive | 3.36 | 1.07 | NA |
| AR334 | 12/08/1950 | 78 | 01/05/1999 | Biopsy | 130 | 8 | No metastases | 01/11/1999 | 8 | 0.50 | No metastases | | May-99 | | Alive | 0.50 | 0.00 | NA |
| AR334 | 12/08/1950 | 69 | 01/01/1982 | Biopsy | 54 | 5 | No metastases | 11/01/1988 | 8 | 3.68 | No metastases | | Aug-88 | | DEAD | 6.53 | 2.56 | NOT KNOWN |
| AR188 | 02/11/1910 | 72 | 26/03/1983 | TURP | 6.4 | 6 | No metastases | 02/21/1988 | 9 | 5.05 | No metastases | | May-88 | | DEAD | 7.34 | 1.99 | NA |
| AR188 | 14/07/1917 | 74 | 16/04/1982 | TURP | 8.2 | 4 | No metastases | 00/00/00 | 10 | 5.52 | No metastases | 26/03/1983 | May-88 | Feb-99 | DEAD | 7.27 | 1.65 | DISEASE PROGRESSION |
| AR254 | 06/09/1923 | 71 | 04/02/1995 | TURP | 152 | 4 | No metastases | 01/01/1997 | 10 | 4.03 | No metastases | 10/09/1982 | Mar-95 | Oct-98 | DEAD | 5.51 | 0.92 | DISEASE PROGRESSION |
| AR334 | 06/04/1923 | 68 | 04/09/1995 | TURP | 308 | 10 | No metastases | 01/02/1995 | 10 | 2.38 | No metastases | 31/06/1985 | Jul-95 | Jul-98 | DEAD | 3.94 | 0.58 | DISEASE PROGRESSION |
| AR334 | 07/03/1915 | 83 | 12/01/1986 | TURP | 4.2 | 10 | No metastases | 01/06/1992 | 8 | 1.87 | No metastases | | Apr-97 | Jul-98 | DEAD | 3.92 | 0.53 | NOT KNOWN |
| AR184 | 14/01/1922 | 76 | 01/04/1998 | TURP | 1300 | 8 | No metastases | 01/06/1992 | 8 | 1.80 | No metastases | | Apr-98 | Jan-00 | DEAD | 2.42 | 0.56 | DISEASE PROGRESSION |
| AR334 | 09/04/1928 | 69 | 10/12/1995 | TURP | 19.3 | 9 | No metastases | 03/05/1999 | 9 | 2.28 | No metastases | | Jan-97 | | DEAD | 4.31 | 0.96 | NOT KNOWN |
| AR334 | 09/17/1914 | 71 | 30/10/1996 | TURP | 3 | 7 | No metastases | 12/11/1990 | 9 | 3.85 | No metastases | 30/10/1986 | | | DEAD | 0.00 | 0.00 | DISEASE PROGRESSION |
| AR334 | 03/04/1916 | 73 | 06/05/1989 | TURP | 208 | 9 | No metastases | 06/05/1992 | 9 | 4.50 | No metastases | 19/09/1986 | | Feb-89 | DEAD | 5.82 | 5.08 | DISEASE PROGRESSION |
| AR334 | 06/05/1922 | 63 | 18/06/1986 | TURP | 23 | 7 | No metastases | 08/21/1986 | 9 | 3.28 | No metastases | 19/09/1986 | Feb-89 | Feb-87 | DEAD | 4.57 | 1.33 | NOT KNOWN |
| AR334 | 03/03/1905 | 81 | 11/02/1987 | TURP | 61 | 7 | No metastases | 06/02/1992 | 6 | 2.62 | No metastases | 29/10/1987 | | | DEAD | 6.26 | 3.64 | DISEASE PROGRESSION |
| AR334 | 21/11/1910 | 77 | 01/03/1988 | TURP | 1 | 9 | No metastases | 06/02/1991 | 7 | 1.36 | No metastases | 24/09/1988 | | | DEAD | 3.51 | 2.15 | DISEASE PROGRESSION |
| AR334 | 03/09/1912 | 75 | 26/04/1988 | TURP | 165 | 9 | No metastases | 14/03/1984 | 9 | 3.98 | No metastases | 04/05/1988 | | | DEAD | 1.91 | 1.01 | NOT KNOWN |
| AR334 | 06/01/1909 | 67 | 04/01/1989 | TURP | 29 | 9 | No metastases | 06/01/1989 | 9 | 2.89 | No metastases | 02/11/1980 | | | DEAD | 6.07 | 3.08 | NOT KNOWN |
| AR334 | 14/01/1922 | 67 | 04/01/1989 | TURP | 8.6 | 6 | No metastases | 06/03/1983 | 9 | 2.89 | No metastases | 02/11/1980 | | | DEAD | 6.07 | 3.08 | NOT KNOWN |
| AR334 | 19/11/1918 | 65 | 02/05/1990 | TURP | 78 | 7 | No metastases | 02/12/1997 | 9 | 5.57 | No metastases | 07/05/1980 | | | DEAD | 3.00 | 3.00 | DISEASE PROGRESSION |
| AR334 | 19/11/1918 | 65 | 02/05/1990 | TURP | 141 | 7 | No metastases | 02/12/1997 | 9 | 2.17 | No metastases | 09/03/1981 | | Jul-90 | DEAD | 7.68 | 5.51 | NOT KNOWN |
| AR334 | 09/12/1920 | 68 | 11/09/1989 | TURP | 105 | 9 | No metastases | 14/02/1984 | 9 | 1.90 | No metastases | 09/03/1981 | | | DEAD | 4.92 | 3.42 | DISEASE PROGRESSION |
| AR334 | 05/03/1915 | 75 | 26/02/1991 | TURP | 165 | 4 | No metastases | 12/07/1992 | 8 | 1.58 | No metastases | 27/09/1989 | | | DEAD | 4.15 | 2.87 | NOT KNOWN |
| AR334 | 06/01/1922 | 70 | 04/21/1982 | TURP | 29 | 9 | No metastases | 08/12/1982 | 8 | 4.78 | No metastases | 02/05/1991 | Apr-92 | | DEAD | 6.65 | 1.87 | DISEASE PROGRESSION |
| AR334 | 19/12/1925 | 68 | 16/11/1984 | TURP | 19 | 6 | No metastases | 08/12/1982 | 5 | 1.14 | No metastases | 14/11/1984 | | Jul-94 | DEAD | 2.63 | 1.49 | NOT KNOWN |
| AR334 | 19/11/1918 | 67 | 09/01/1984 | TURP | 2 | 7 | No metastases | 26/11/1985 | 5 | 4.03 | No metastases | | | Apr-95 | DEAD | 6.83 | 2.60 | NOT KNOWN |
| AR334 | 11/09/1930 | 63 | 09/09/1984 | TURP | 70.9 | 6 | No metastases | 27/10/1988 | 8 | 4.03 | No metastases | 18/09/1991 | Nov-90 | | DEAD | 6.83 | 2.60 | NOT KNOWN |
| AR334 | 16/07/1915 | 74 | 07/11/1993 | TURP | 23 | 7 | No metastases | 01/01/1995 | 8 | 0.77 | No metastases | 18/09/1991 | Mar-97 | | DEAD | 1.64 | 1.37 | NOT KNOWN |
| AR1028 | 11/02/1919 | 63 | 08/01/1988 | TURP | 2360 | 10 | No metastases | 15/03/1990 | 8 | 1.15 | No metastases | 15/03/1990 | Jan-98 | | ALIVE | 1.18 | 0.03 | NA |
| AR1048 | 23/07/1913 | 70 | 16/05/1984 | TURP | 25 | 8 | No metastases | 23/04/1983 | 10 | 9.32 | No metastases | 19/02/1980 | | Oct-92 | DEAD | 10.89 | 1.58 | NOT KNOWN |
| AR1078 | 09/11/1926 | 68 | 10/05/1985 | TURP | 127 | 9 | No metastases | 10/02/1988 | 9 | 1.77 | No metastases | 19/02/1980 | May-95 | May-95 | DEAD | 2.91 | 1.14 | NOT KNOWN |
| AR1095 | 11/02/1922 | 73 | 06/02/1986 | TURP | 41 | 9 | No metastases | 11/09/1988 | 10 | 2.85 | No metastases | 26/03/1983 | Feb-96 | | DEAD | 21.36 | 9.71 | NOT KNOWN |
| AR1181 | 17/02/1913 | 67 | 26/09/1979 | TURP | 178 | 6 | No metastases | 11/10/1983 | 8 | 0.13 | No metastases | 26/03/1983 | | Nov-94 | DEAD | 1.75 | 1.62 | DISEASE PROGRESSION |
| AR1181 | 29/10/1911 | 65 | 26/09/1980 | TURP | 178 | 6 | No metastases | 11/10/1983 | 8 | 0.13 | No metastases | 26/03/1983 | | Nov-94 | DEAD | 1.75 | 1.62 | DISEASE PROGRESSION |
| AR1181 | 29/10/1911 | 65 | 11/11/1984 | TURP | 70 | 7 | No metastases | 25/03/1988 | 10 | 2.47 | No metastases | 23/01/1986 | | Nov-94 | DEAD | 4.92 | 2.45 | NOT KNOWN |
| AR1181 | 21/11/1987 | 59 | 11/09/1997 | TURP | 44 | 9 | No metastases | 24/09/1988 | 9 | 1.15 | No metastases | | Jun-95 | Jun-95 | DEAD | 4.92 | 2.45 | NOT KNOWN |
| AR120 | 29/07/1936 | 59 | 21/12/1995 | TURP | 24 | 10 | No metastases | 24/09/1988 | 10 | 0.84 | No metastases | | Jul-97 | Jul-97 | DEAD | 2.58 | 1.38 | DISEASE PROGRESSION |
| AR126 | 27/10/1920 | 72 | 12/07/1983 | TURP | 84 | 7 | No metastases | 11/05/1985 | 9 | 6.09 | No metastases | 04/05/1988 | Mar-96 | Mar-96 | DEAD | 1.27 | 0.43 | DISEASE PROGRESSION |
| AR134 | 03/01/1925 | 65 | 05/01/1985 | Biopsy | 82 | 5 | No metastases | 05/01/1985 | 9 | 8.41 | No metastases | 04/05/1988 | Jul-93 | Mar-97 | DEAD | 3.37 | 1.40 | DISEASE PROGRESSION |
| AR134 | 19/04/1919 | 60 | 05/09/1985 | TURP | 8.2 | 6 | No metastases | 26/02/2000 | 7 | 8.41 | No metastases | 26/02/2000 | Dec-95 | Feb-93 | ALIVE | 7.18 | 0.75 | NA |
| AR134 | 13/02/1928 | 67 | 06/01/1986 | TURP | 64.7 | 8 | No metastases | 27/01/1986 | 9 | 1.92 | No metastases | | Apr-96 | Dec-97 | DEAD | 2.65 | 0.74 | DISEASE PROGRESSION |
| AR136 | 14/09/1920 | 72 | 03/07/1992 | TURP | 125 | 8 | No metastases | 01/01/2000 | 6 | 7.50 | No metastases | | Apr-96 | Dec-97 | DEAD | 7.55 | 0.05 | DISEASE PROGRESSION |
| AR136 | 15/10/1919 | 71 | 16/07/1991 | Biopsy | 15 | 8 | No metastases | 01/07/1997 | 6 | 3.47 | No metastases | | Nov-98 | Nov-98 | DEAD | 6.50 | 3.04 | DISEASE PROGRESSION |
| AR140 | 14/12/1912 | 80 | 13/12/1983 | TURP | 98 | 9 | No metastases | 01/01/1985 | 9 | 3.22 | No metastases | 01/01/1985 | Feb-94 | Nov-98 | DEAD | 6.50 | 3.04 | DISEASE PROGRESSION |
| AR144 | 20/09/1922 | 67 | 15/03/1980 | TURP | 278 | 10 | No metastases | 01/10/1986 | 8 | 4.90 | No metastases | | Mar-95 | Mar-95 | DEAD | 6.98 | 3.76 | DISEASE PROGRESSION |
| AR144 | 05/11/1925 | 71 | 27/08/1987 | TURP | 40.7 | 8 | No metastases | 01/05/2000 | 10 | 2.10 | No metastases | | Apr-96 | Oct-97 | DEAD | 9.72 | 4.92 | DISEASE PROGRESSION |
| AR150 | 19/09/1919 | 77 | 26/05/1987 | Biopsy | 284 | 6 | No metastases | 01/05/2001 | 7 | 3.83 | No metastases | | Apr-96 | Oct-97 | DEAD | 4.38 | 1.34 | DISEASE PROGRESSION |
| AR152 | 12/09/1923 | 76 | 13/01/2000 | TURP | 254 | 9 | No metastases | 01/12/2001 | 7 | 2.13 | No metastases | | Jan-98 | Jan-98 | DEAD | 4.38 | 1.34 | DISEASE PROGRESSION |
| AR152 | 12/09/1923 | 67 | 01/05/1989 | Biopsy | 41 | 10 | No metastases | 01/05/1989 | 8 | 2.50 | No metastases | | Mar-95 | Mar-95 | DEAD | 3.10 | 0.58 | DISEASE PROGRESSION |
| AR158 | 23/02/1928 | 67 | 01/05/1989 | Biopsy | 41 | 10 | No metastases | 01/05/1989 | 8 | 2.50 | No metastases | | Mar-95 | Mar-95 | DEAD | 3.10 | 0.58 | DISEASE PROGRESSION |
| AR158 | 13/01/1915 | 83 | 18/02/1988 | Biopsy | 149 | 5 | No metastases | 01/05/1989 | 8 | 1.53 | No metastases | | Mar-98 | Jan-00 | DEAD | 2.45 | 0.52 | DISEASE PROGRESSION |
| AR164 | 30/05/1924 | 73 | 03/10/1987 | TURP | 7.7 | 9 | No metastases | 01/05/1989 | 8 | 1.74 | No metastases | | Nov-97 | Nov-97 | DEAD | 3.77 | 2.02 | DISEASE PROGRESSION |
| AR168 | 19/10/1928 | 67 | 07/02/2000 | TURP | 47 | 8 | No metastases | 11/01/2002 | 8 | 8.84 | No metastases | | Mar-01 | Mar-01 | DEAD | 9.07 | 0.23 | NOT KNOWN |
| AR168 | 23/01/1928 | 74 | 07/02/2000 | Biopsy | 358 | 8 | No metastases | 12/04/2002 | 8 | 1.49 | No metastases | | Jan-98 | Jan-98 | DEAD | 6.71 | 5.08 | DISEASE PROGRESSION |
| AR172 | 17/04/1933 | 62 | 18/12/1985 | Biopsy | 358 | 8 | No metastases | 01/02/2003 | 8 | 1.02 | No metastases | | Jan-98 | Jan-98 | DEAD | 6.71 | 5.08 | DISEASE PROGRESSION |



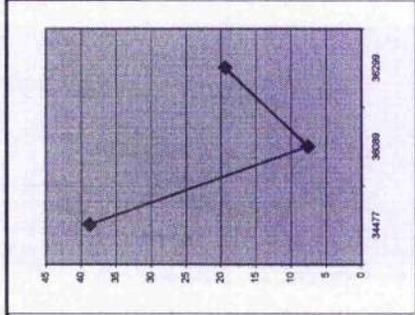
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| 0.2 | 22/10/1998 |
| 0.2 | 23/03/1999 |
| 15 | 15/08/1999 |
| 27 | 01/09/1999 |



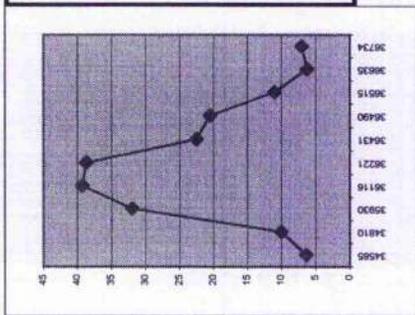
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| 470 | 01/11/1999 |



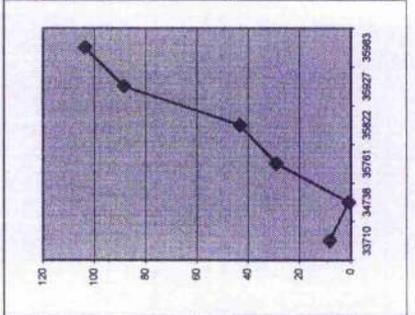
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| 8.5 | 03/11/1997 |
| 7.8 | 18/12/1997 |
| 9.9 | 05/03/1998 |
| 9.2 | 18/12/1997 |
| 1.3 | 05/03/1998 |
| 1.2 | 27/03/1998 |
| 1.8 | 21/07/1998 |
| 2 | 19/01/1999 |
| 5.7 | 29/11/1999 |



| AR14 | AR15 |
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| 38.8 | 23/05/1994 |
| 7.6 | 21/10/1998 |
| 19.4 | 19/05/1999 |

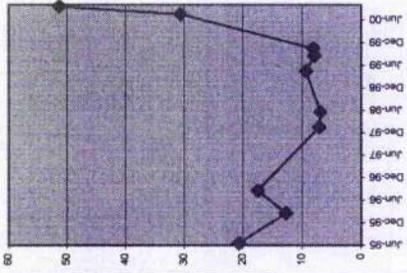


| AR16 | AR17 |
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| 6.4 | 08/09/1994 |
| 10 | 21/04/1995 |
| 32 | 15/05/1998 |
| 39.3 | 17/11/1998 |
| 38.7 | 02/03/1999 |
| 22.5 | 28/09/1999 |
| 20.5 | 26/11/1999 |
| 11 | 21/12/1999 |
| 6.2 | 19/04/2000 |
| 7 | 27/07/2000 |

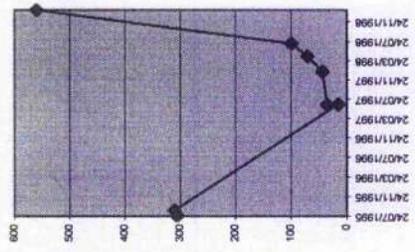


| AR18 | AR19 |
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| 8.2 | 16/04/1992 |
| 0.9 | 08/02/1995 |
| 29 | 27/11/1997 |
| 43.1 | 27/01/1998 |
| 88.5 | 12/05/1998 |
| 103.6 | 07/07/1998 |

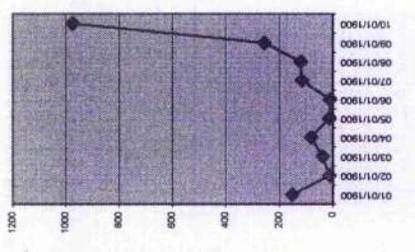
| AR33 | AR34 |
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| 20.7 | 27/06/1995 |
| 12.7 | 06/02/1996 |
| 17.5 | 27/08/1996 |
| 7.1 | 16/01/1998 |
| 6.9 | 14/05/1998 |
| 9.3 | 20/04/1999 |
| 7.9 | 31/08/1999 |
| 8.1 | 29/10/1999 |
| 30.6 | 10/07/2000 |
| 51.3 | 28/09/2000 |



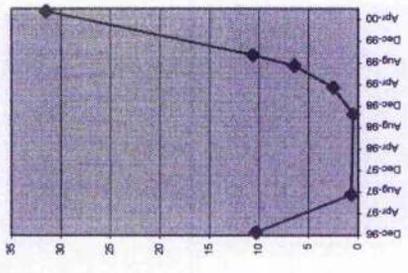
| AR31 | AR32 |
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| 306 | 24/07/1995 |
| 309.8 | 28/08/1995 |
| 15.67 | 19/06/1997 |
| 35.7 | 19/06/1997 |
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| 70.7 | 20/04/1998 |
| 99.7 | 10/07/1998 |
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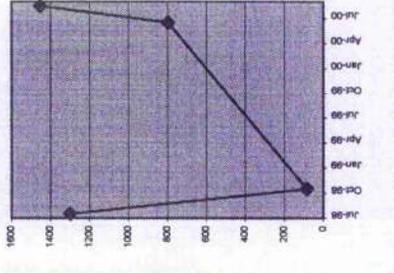
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| 152 | 06/12/1994 |
| 13.4 | 10/06/1997 |
| 38 | 10/10/1997 |
| 82 | 14/07/1998 |
| 12.3 | 21/12/1998 |
| 10 | 13/04/1999 |
| 116.1 | 13/09/1999 |
| 120.7 | 19/10/1999 |
| 256 | 14/03/2000 |
| 975 | 23/05/2000 |



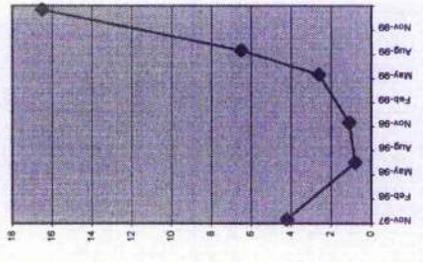
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| 0.7 | 31/07/1997 |
| 0.5 | 14/10/1998 |
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| 6.4 | 14/07/1999 |
| 10.6 | 15/09/1999 |
| 31.5 | 22/05/2000 |

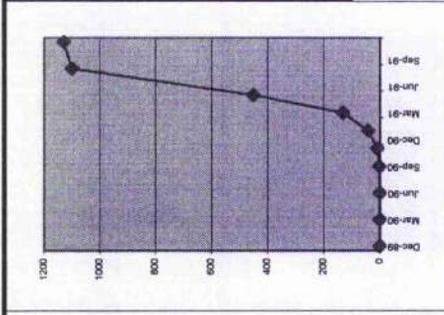


| AR41 | AR42 |
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| 85.2 | 27/10/1998 |
| 796.4 | 15/06/2000 |
| 1452.8 | 10/08/2000 |



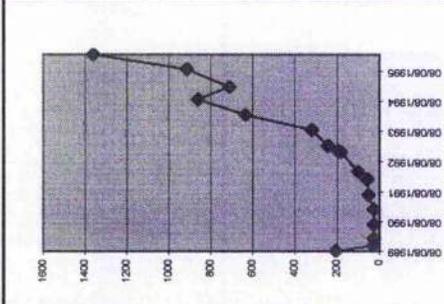
| AR35 | AR36 |
|------|------------|
| 4.2 | 10/11/1997 |
| 0.8 | 15/06/1998 |
| 1.1 | 03/11/1998 |
| 2.6 | 04/05/1999 |
| 6.5 | 31/08/1999 |
| 16.5 | 18/01/2000 |





| AR53 | AR54 |
|------|------------|
| 0.7 | 18/12/1989 |
| 0.6 | 06/03/1990 |
| 1 | 04/06/1990 |
| 2.3 | 03/09/1990 |
| 10.4 | 12/11/1990 |
| 42 | 07/01/1991 |
| 131 | 11/03/1991 |
| 450 | 27/05/1991 |
| 1100 | 26/08/1991 |
| 1130 | 25/11/1991 |

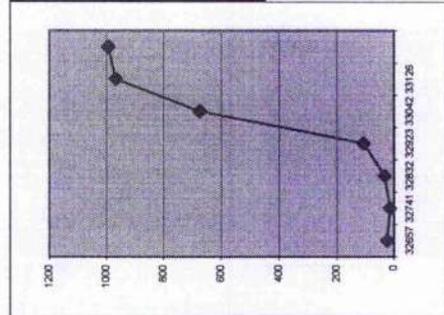
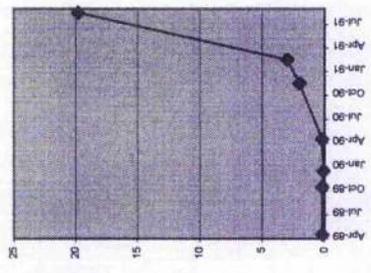
Pre PSA era at diagnosis



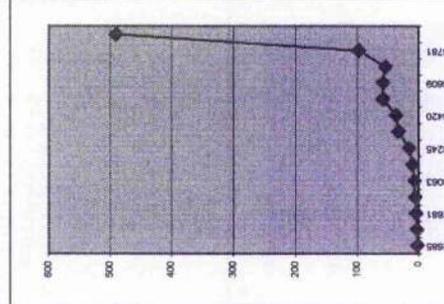
| AR55 | AR56 |
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| 208 | 08/08/1989 |
| 30 | 16/10/1989 |
| 27 | 15/01/1990 |
| 26 | 02/07/1990 |
| 26.9 | 07/01/1991 |
| 52 | 01/07/1991 |
| 58 | 06/01/1992 |
| 101 | 06/04/1992 |
| 182 | 30/11/1992 |
| 195 | 14/12/1992 |
| 245 | 15/02/1993 |
| 320 | 23/08/1993 |
| 634 | 21/02/1994 |
| 860 | 29/08/1994 |
| 710 | 30/01/1995 |
| 914 | 01/09/1995 |
| 1362 | 01/03/1996 |

Pre PSA era at diagnosis

| AR57 | AR58 |
|------|------------|
| 0.2 | 24/04/1989 |
| 0.2 | 16/10/1989 |
| 0.1 | 18/12/1989 |
| 0.2 | 23/04/1990 |
| 2 | 05/11/1990 |
| 3 | 04/02/1991 |
| 19.8 | 26/08/1991 |



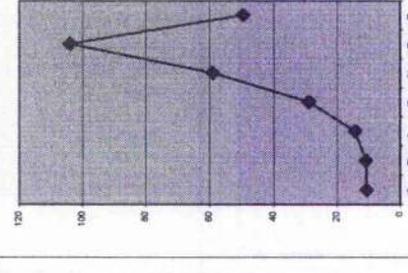
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| 23.7 | 29/05/1989 |
| 14.5 | 21/08/1989 |
| 32.8 | 20/11/1989 |
| 106 | 19/02/1990 |
| 674 | 18/06/1990 |
| 967 | 10/09/1990 |
| 994.0 | 19/11/1990 |



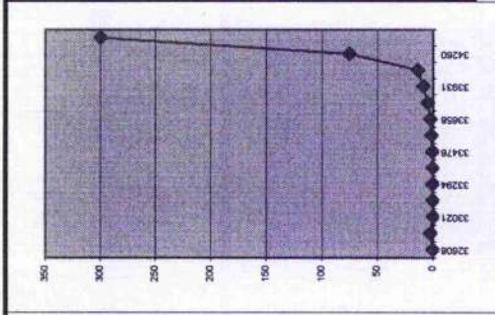
| AR61 | AR62 |
|------|------------|
| 2 | 26/06/1989 |
| 2.7 | 25/09/1989 |
| 4.1 | 08/01/1990 |
| 5.6 | 09/04/1990 |
| 6.5 | 09/07/1990 |
| 9.9 | 08/10/1990 |
| 15.5 | 07/01/1991 |
| 32.7 | 14/04/1991 |
| 36.8 | 01/07/1991 |
| 58 | 30/09/1991 |
| 58 | 06/01/1992 |
| 53.9 | 30/03/1992 |
| 97.7 | 26/06/1992 |
| 490 | 23/11/1992 |

Pre PSA era at diagnosis

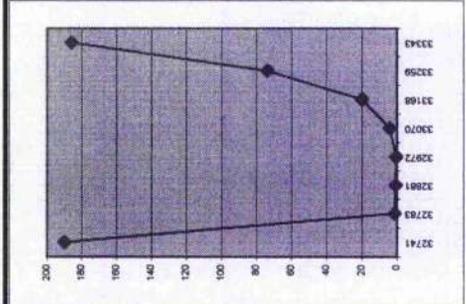
| AR63 | AR64 |
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| 10.7 | 10/04/1989 |
| 10.9 | 10/07/1989 |
| 14.3 | 16/10/1989 |
| 29 | 15/01/1990 |
| 59 | 09/04/1990 |
| 104 | 03/12/1990 |
| 49.5 | 22/04/1991 |



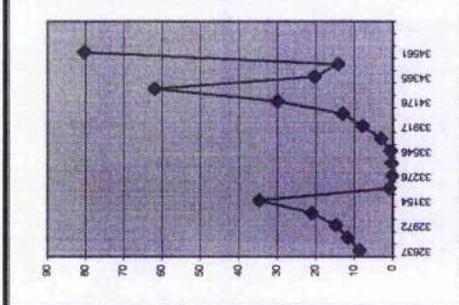
Pre PSA era at diagnosis



| AR65 | AR66 |
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| 2.4 | 15/01/1990 |
| 0.1 | 28/05/1990 |
| 0.2 | 26/11/1990 |
| 0.2 | 25/02/1991 |
| 0.3 | 27/05/1991 |
| 0.6 | 26/08/1991 |
| 1.3 | 25/11/1991 |
| 2.5 | 24/02/1992 |
| 4.8 | 25/05/1992 |
| 8.6 | 23/11/1992 |
| 13.7 | 22/02/1993 |
| 75.5 | 18/10/1993 |
| 300 | 18/04/1994 |

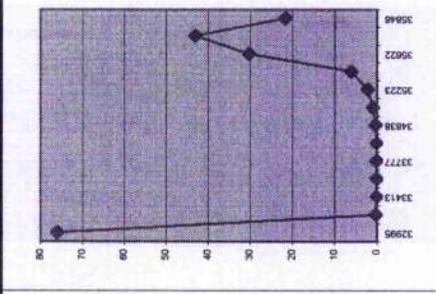


| AR67 | AR68 |
|------|------------|
| 190 | 21/08/1989 |
| 1.4 | 02/10/1989 |
| 0.7 | 08/01/1990 |
| 0.6 | 09/04/1990 |
| 4.1 | 16/07/1990 |
| 20 | 22/10/1990 |
| 73.8 | 21/01/1991 |
| 186 | 15/04/1991 |

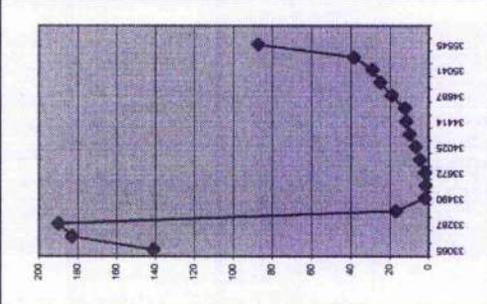


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| 8.6 | 09/05/1989 |
| 11.7 | 08/01/1990 |
| 14.8 | 09/04/1990 |
| 21 | 09/07/1990 |
| 34.7 | 08/10/1990 |
| 0.9 | 03/12/1990 |
| 0.2 | 07/02/1991 |
| 0.3 | 12/08/1991 |
| 0.6 | 04/11/1991 |
| 3.1 | 04/05/1992 |
| 7.9 | 09/11/1992 |
| 13 | 08/02/1993 |
| 30 | 26/07/1993 |
| 61.9 | 25/10/1993 |
| 20.3 | 31/01/1994 |
| 14.2 | 18/04/1994 |
| 80.3 | 15/08/1994 |

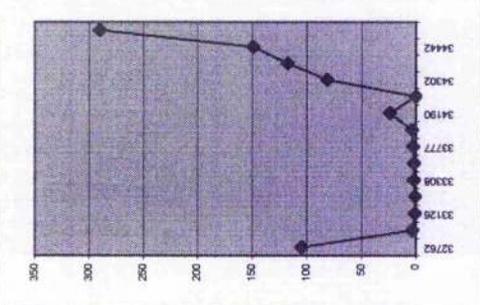
Pre PSA era at diagnosis



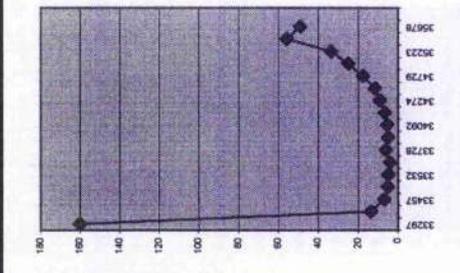
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| 0.1 | 24/06/1991 |
| 0.1 | 23/12/1991 |
| 0.1 | 22/06/1992 |
| 0.1 | 21/11/1994 |
| 0.3 | 19/05/1995 |
| 1 | 24/11/1995 |
| 2.2 | 07/06/1996 |
| 6.1 | 10/01/1997 |
| 30.3 | 11/07/1997 |
| 43 | 10/10/1997 |
| 21.7 | 20/02/1998 |



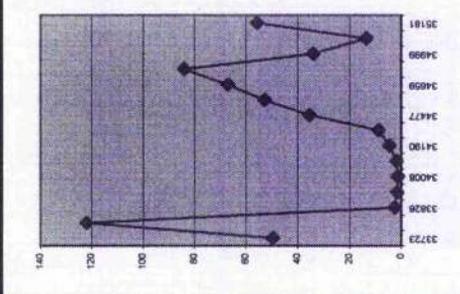
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| 183 | 19/11/1990 |
| 190 | 18/02/1991 |
| 17 | 25/03/1991 |
| 2 | 09/09/1991 |
| 1.6 | 02/12/1991 |
| 2 | 09/03/1992 |
| 4.5 | 10/09/1992 |
| 6.7 | 25/02/1993 |
| 9.7 | 27/09/1993 |
| 11.6 | 21/03/1994 |
| 12.4 | 27/06/1994 |
| 19.2 | 19/12/1994 |
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| 29.1 | 08/12/1995 |
| 38.5 | 24/01/1997 |
| 87.3 | 25/04/1997 |



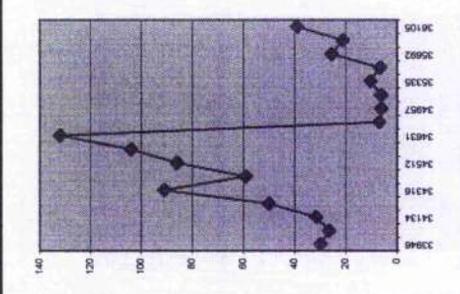
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| 0.9 | 10/12/1990 |
| 1.7 | 11/03/1991 |
| 1.6 | 23/09/1991 |
| 2.6 | 22/06/1992 |
| 3.8 | 08/02/1993 |
| 24.1 | 09/08/1993 |
| 42.4 | 04/10/1993 |
| 81.3 | 29/11/1993 |
| 117.5 | 31/01/1994 |
| 149 | 18/04/1994 |
| 290.5 | 18/07/1994 |



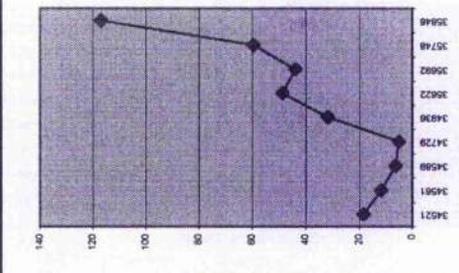
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| 5.2 | 29/07/1991 |
| 4.9 | 21/10/1991 |
| 4.1 | 02/03/1992 |
| 6.1 | 04/05/1992 |
| 5 | 02/11/1992 |
| 5.5 | 03/05/1993 |
| 6.7 | 02/08/1993 |
| 9.1 | 01/11/1993 |
| 11.6 | 02/05/1994 |
| 17.6 | 30/01/1995 |
| 24.9 | 01/09/1995 |
| 33.6 | 07/06/1996 |
| 55.7 | 24/01/1997 |
| 49 | 05/09/1997 |



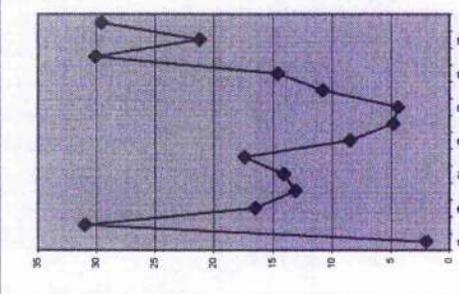
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| 122 | 29/06/1992 |
| 2.6 | 10/08/1992 |
| 1.6 | 09/11/1992 |
| 1.3 | 08/02/1993 |
| 1.8 | 10/05/1993 |
| 4.6 | 09/08/1993 |
| 8.7 | 08/11/1993 |
| 35.5 | 23/05/1994 |
| 52.7 | 22/08/1994 |
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| 84 | 27/02/1995 |
| 34 | 27/10/1995 |
| 13.6 | 26/01/1996 |
| 55.7 | 26/04/1996 |



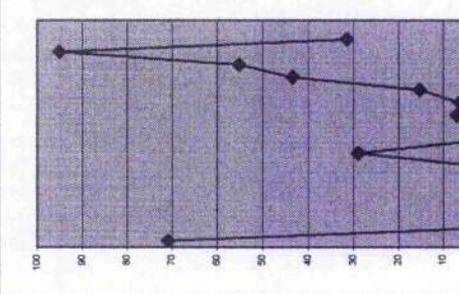
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| 31.7 | 14/06/1993 |
| 50 | 13/09/1993 |
| 91 | 13/12/1993 |
| 59 | 28/03/1994 |
| 86 | 27/06/1994 |
| 104 | 03/10/1994 |
| 132 | 24/10/1994 |
| 7 | 23/01/1995 |
| 6.2 | 15/09/1995 |
| 6.1 | 22/03/1996 |
| 10.4 | 27/09/1996 |
| 6.6 | 14/03/1997 |
| 25.5 | 19/09/1997 |
| 21.1 | 09/01/1998 |
| 39 | 06/11/1998 |



| AR86 | AR87 |
|------|------------|
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| 59.6 | 14/11/1997 |
| 117 | 20/02/1998 |

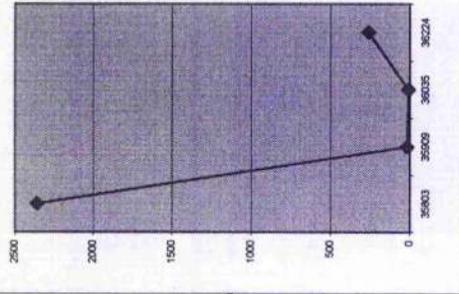


| AR88 | AR89 |
|------|------------|
| 2 | 17/10/1994 |
| 31 | 10/04/1995 |
| 16.5 | 09/06/1995 |
| 13.1 | 08/09/1995 |
| 14.1 | 08/03/1996 |
| 17.4 | 20/09/1996 |
| 8.5 | 26/09/1997 |
| 4.8 | 30/01/1998 |
| 4.4 | 15/05/1998 |
| 10.8 | 28/05/1999 |
| 14.6 | 15/10/1999 |
| 30.1 | 18/02/2000 |
| 21.2 | 18/08/2000 |
| 29.6 | 10/11/2000 |

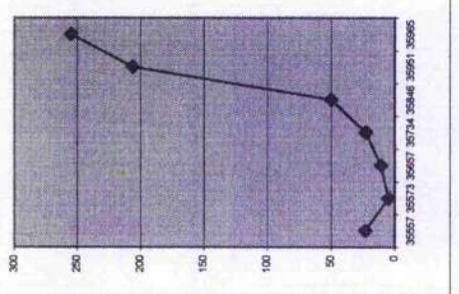


| AR90 | AR91 |
|-------|------------|
| 70.9 | 08/08/1994 |
| 1.6 | 28/11/1994 |
| 0.6 | 03/04/1995 |
| 0.9 | 15/09/1995 |
| 1.3 | 29/03/1996 |
| 1.7 | 20/09/1996 |
| 2.5 | 04/04/1997 |
| 28.97 | 11/04/1997 |
| 3.1 | 13/06/1997 |
| 1 | 12/09/1997 |
| 7 | 01/07/1998 |
| 6.3 | 04/12/1998 |
| 15.3 | 24/03/1999 |
| 43.4 | 13/08/1999 |
| 55.2 | 19/11/1999 |
| 95.11 | 10/03/2000 |
| 31.49 | 09/06/2000 |

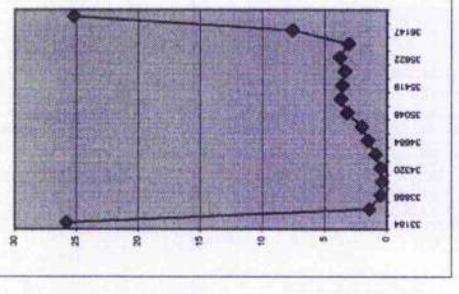
| | |
|--------------|--------------|
| AR102 | AR103 |
| 2360 | 08/01/1998 |
| 16.1 | 24/04/1998 |
| 7.6 | 28/08/1998 |
| 261 | 05/03/1999 |



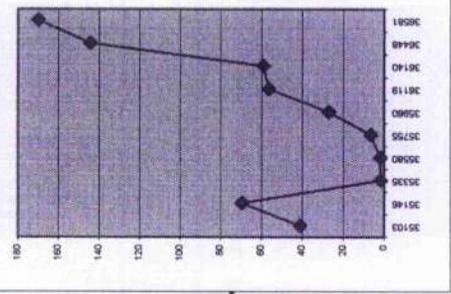
| | |
|--------------|--------------|
| AR100 | AR101 |
| 23.2 | 07/05/1997 |
| 4.9 | 23/05/1997 |
| 10.7 | 15/08/1997 |
| 22.7 | 31/10/1997 |
| 49.8 | 20/02/1998 |
| 206 | 05/06/1998 |
| 255 | 19/06/1998 |



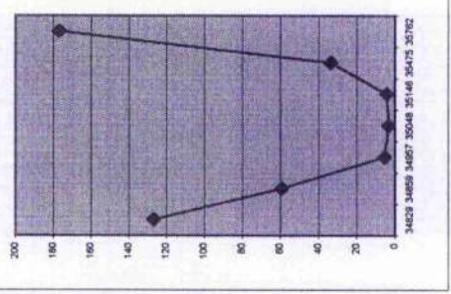
| | |
|-------------|-------------|
| AR96 | AR97 |
| 25.8 | 07/11/1990 |
| 1.4 | 04/10/1991 |
| 0.5 | 09/10/1992 |
| 0.4 | 23/04/1993 |
| 0.5 | 17/12/1993 |
| 0.9 | 17/06/1994 |
| 1.5 | 16/12/1994 |
| 2 | 16/06/1995 |
| 3.2 | 15/12/1995 |
| 3.7 | 21/06/1996 |
| 3.6 | 20/12/1996 |
| 3.4 | 04/04/1997 |
| 3.8 | 11/07/1997 |
| 3.1 | 12/06/1998 |
| 7.6 | 18/12/1998 |
| 25.2 | 18/06/1999 |



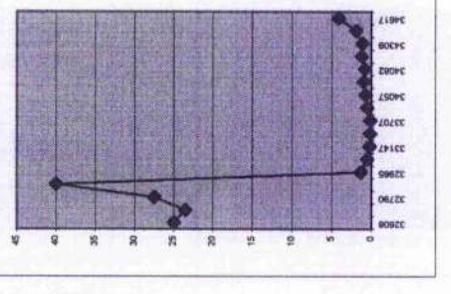
| | |
|--------------|--------------|
| AR109 | AR110 |
| 41 | 08/02/1996 |
| 69.5 | 22/03/1996 |
| 1.7 | 27/09/1996 |
| 1.8 | 30/05/1997 |
| 6.4 | 21/11/1997 |
| 27.1 | 14/06/1998 |
| 56.2 | 20/11/1998 |
| 59 | 11/12/1998 |
| 144.3 | 15/10/1999 |
| 169.9 | 25/02/2000 |

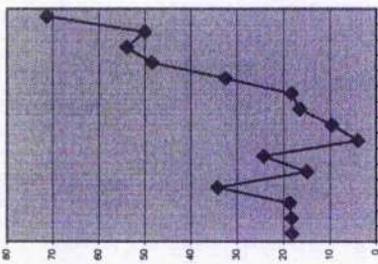


| | |
|--------------|--------------|
| AR107 | AR108 |
| 127 | 10/05/1995 |
| 59.2 | 09/06/1995 |
| 5.4 | 15/09/1995 |
| 3.3 | 15/12/1995 |
| 4.3 | 22/03/1996 |
| 33.6 | 14/02/1997 |
| 177 | 28/11/1997 |

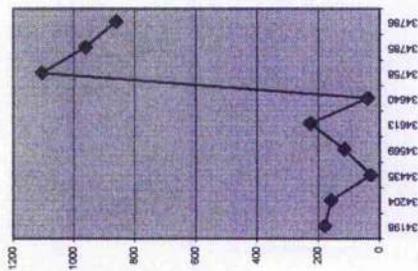


| | |
|--------------|--------------|
| AR104 | AR106 |
| 25 | 10/04/1989 |
| 23.5 | 10/07/1989 |
| 27.5 | 09/10/1989 |
| 40 | 08/01/1990 |
| 1.3 | 02/04/1990 |
| 0.5 | 02/07/1990 |
| 0.2 | 01/10/1990 |
| 0.1 | 21/10/1991 |
| 0.1 | 13/04/1992 |
| 0.5 | 12/10/1992 |
| 0.7 | 29/03/1993 |
| 0.9 | 07/06/1993 |
| 0.8 | 23/04/1993 |
| 1.2 | 06/09/1993 |
| 1.1 | 06/12/1993 |
| 1.9 | 06/06/1994 |
| 4.1 | 10/10/1994 |



AR111**AR113**

| | |
|------------|------|
| 08/05/1989 | 18.2 |
| 06/11/1989 | 18.3 |
| 07/05/1990 | 18.7 |
| 26/09/1990 | 34.3 |
| 19/11/1990 | 15 |
| 27/02/1991 | 24.3 |
| 22/04/1991 | 4 |
| 21/10/1991 | 9.6 |
| 02/03/1992 | 16.5 |
| 31/08/1992 | 18.3 |
| 30/08/1993 | 32.5 |
| 25/04/1994 | 48.4 |
| 24/10/1994 | 53.9 |
| 10/04/1995 | 50 |
| 06/10/1995 | 71.3 |

**AR114**

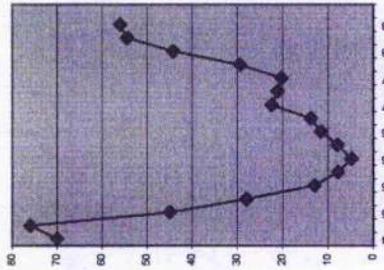
| | |
|------------|-------|
| 17/08/1993 | 178.6 |
| 23/08/1993 | 158 |
| 11/04/1994 | 29 |
| 23/08/1994 | 114 |
| 06/10/1994 | 226 |
| 02/11/1994 | 38.8 |
| 28/02/1995 | 1105 |
| 27/03/1995 | 961 |
| 28/03/1995 | 860 |

AR115

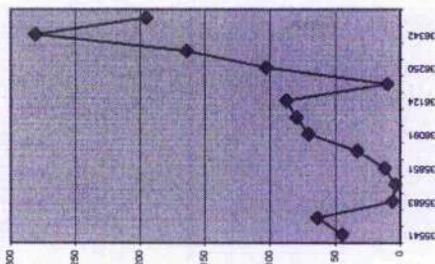
| | |
|------------|-------|
| 17/08/1993 | 178.6 |
| 23/08/1993 | 158 |
| 11/04/1994 | 29 |
| 23/08/1994 | 114 |
| 06/10/1994 | 226 |
| 02/11/1994 | 38.8 |
| 28/02/1995 | 1105 |
| 27/03/1995 | 961 |
| 28/03/1995 | 860 |

AR116

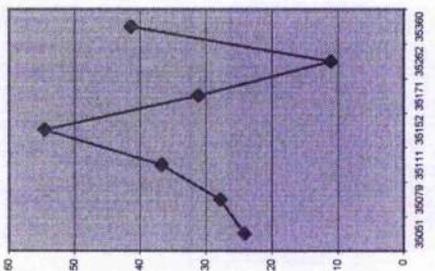
| | |
|------------|------|
| 30/09/1994 | 70 |
| 28/11/1994 | 76 |
| 08/03/1995 | 45 |
| 07/06/1995 | 28 |
| 02/08/1995 | 13 |
| 01/11/1995 | 7.8 |
| 60/03/96 | 4.8 |
| 19/06/1996 | 7.9 |
| 23/10/1996 | 11.6 |
| 26/02/1997 | 13.8 |
| 07/05/1997 | 22.4 |
| 01/10/1997 | 21.1 |
| 30/10/1997 | 20.3 |
| 19/02/1998 | 29.4 |
| 14/05/1998 | 44.2 |
| 10/07/1998 | 54.4 |
| 15/07/1998 | 56 |

**AR118**

| | |
|------------|------|
| 21/04/1997 | 44.9 |
| 28/05/1997 | 63.8 |
| 10/09/1997 | 6.2 |
| 17/12/1997 | 3.9 |
| 25/02/1998 | 12 |
| 10/08/1998 | 33.4 |
| 23/10/1998 | 70.6 |
| 26/10/1998 | 79.8 |
| 25/11/1998 | 87.6 |
| 01/12/1998 | 10 |
| 31/03/1999 | 103 |
| 02/06/1999 | 164 |
| 01/07/1999 | 281 |
| 17/11/1999 | 195 |

**AR120**

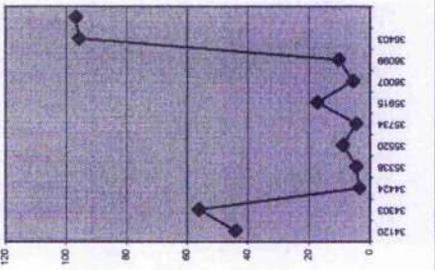
| | |
|------------|------|
| 18/12/1995 | 24.1 |
| 15/01/1996 | 27.8 |
| 16/02/1996 | 36.7 |
| 28/03/1996 | 54.6 |
| 16/04/1996 | 31.1 |
| 16/07/1996 | 11.1 |
| 22/10/1996 | 41.4 |

**AR121**

| | |
|------------|------|
| 18/12/1995 | 24.1 |
| 15/01/1996 | 27.8 |
| 16/02/1996 | 36.7 |
| 28/03/1996 | 54.6 |
| 16/04/1996 | 31.1 |
| 16/07/1996 | 11.1 |
| 22/10/1996 | 41.4 |

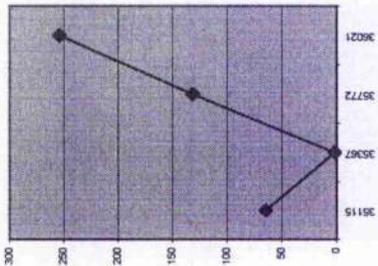
AR126

| | |
|--------|------|
| May-93 | 44 |
| Nov-93 | 56 |
| Mar-94 | 3.5 |
| Sep-96 | 4.5 |
| Mar-97 | 8.8 |
| Oct-97 | 4.5 |
| Apr-98 | 17.2 |
| Jul-98 | 5.5 |
| Oct-98 | 10.3 |
| Aug-99 | 95.8 |
| Apr-00 | 96.8 |

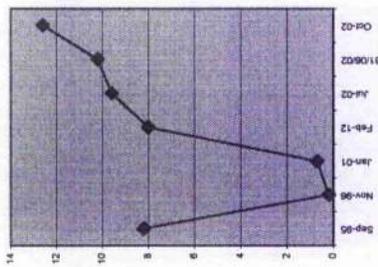
**AR127**

| | |
|--------|------|
| May-93 | 44 |
| Nov-93 | 56 |
| Mar-94 | 3.5 |
| Sep-96 | 4.5 |
| Mar-97 | 8.8 |
| Oct-97 | 4.5 |
| Apr-98 | 17.2 |
| Jul-98 | 5.5 |
| Oct-98 | 10.3 |
| Aug-99 | 95.8 |
| Apr-00 | 96.8 |

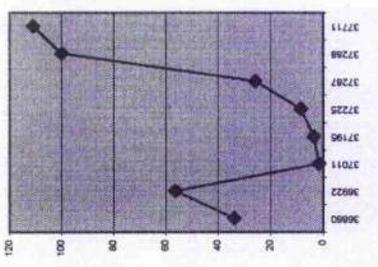
| | |
|--------------|--------------|
| AR134 | AR135 |
| 64.7 | 20/02/1996 |
| 1.8 | 29/10/1996 |
| 131.4 | 08/12/1997 |
| 254.1 | 14/08/1998 |



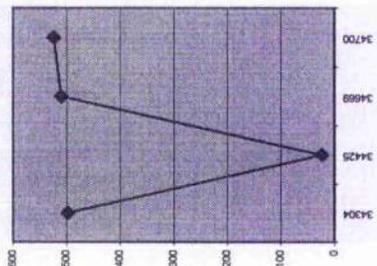
| | |
|--------------|--------------|
| AR130 | AR131 |
| 8.2 | Sep-95 |
| 0.2 | Nov-96 |
| 0.7 | Jan-01 |
| 8 | Feb-12 |
| 9.6 | Jul-02 |
| 10.2 | 31/06/02 |
| 12.6 | Oct-02 |



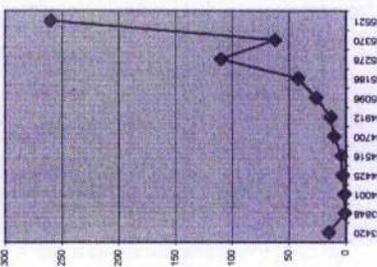
| | |
|--------------|--------------|
| AR128 | AR129 |
| 33.9 | Nov-00 |
| 56.2 | Jan-01 |
| 1.6 | Apr-01 |
| 3.6 | Oct-01 |
| 8.5 | Nov-01 |
| 25.9 | Jan-02 |
| 100 | Feb-02 |
| 111 | Mar-03 |



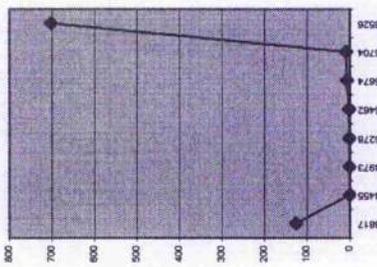
| | |
|--------------|--------------|
| AR140 | AR141 |
| 498 | Dec-93 |
| 23.4 | Apr-94 |
| 510 | Dec-94 |
| 524 | Jan-95 |

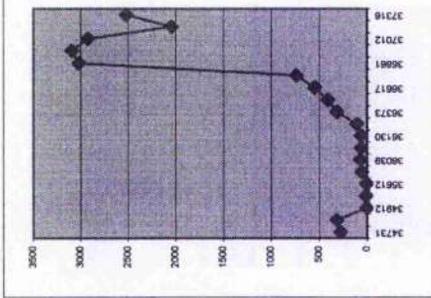


| | |
|--------------|--------------|
| AR138 | AR139 |
| 15 | Jul-91 |
| 1.1 | Sep-92 |
| 1.5 | Feb-93 |
| 3 | Apr-94 |
| 4.1 | Jul-94 |
| 10.3 | Jan-95 |
| 13.3 | Aug-95 |
| 26 | Feb-96 |
| 42 | May-96 |
| 110 | Aug-96 |
| 62 | Nov-96 |
| 260 | Apr-97 |



| | |
|--------------|--------------|
| AR136 | AR137 |
| 125.9 | Aug-92 |
| 0.2 | May-94 |
| 0.7 | Oct-95 |
| 1.4 | Aug-96 |
| 2.4 | Feb-97 |
| 7.2 | Sep-97 |
| 8.8 | Oct-97 |
| 702 | Jan-00 |



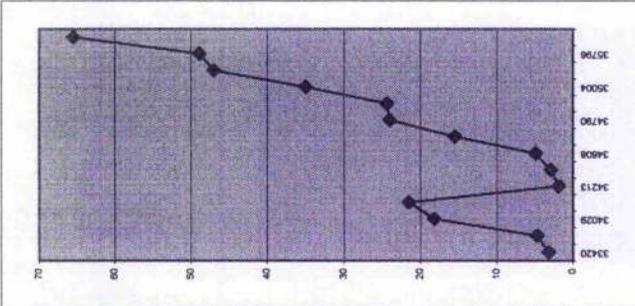


AR142

278
318
2.4
14
5.6
55.2
72.6
61.9
64.7
108.5
313.2
407
546
739
3019
3091
2926
2041
2520

AR143

Feb-95
Mar-95
Aug-95
Nov-95
Jul-97
Jul-98
Sep-98
Nov-98
Dec-98
Apr-99
Aug-99
Jan-00
Apr-00
Jul-00
Dec-00
01-Feb
01-May
01-Aug
01-Mar

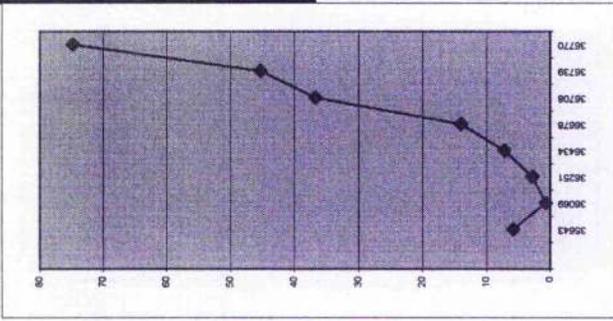


AR144

3.2
4.7
18.2
21.5
1.9
2.9
4.9
15.5
24
24.4
35
47
48.9
65.5

AR145

Jul-91
Dec-91
Mar-93
Apr-93
Sep-93
May-94
Oct-94
Jan-95
Apr-95
Jul-95
Nov-95
Oct-96
Jan-98
Apr-98

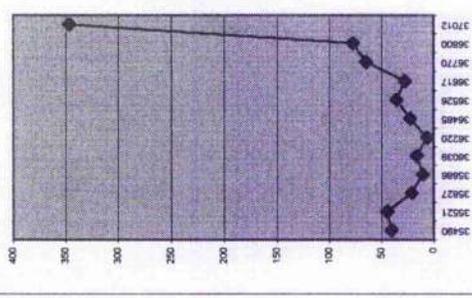


AR146

5.8
0.7
2.8
7.2
14
36.7
45.3
74.8

AR147

Aug-97
Oct-98
Apr-99
Oct-99
Jun-00
Jul-00
Aug-00
Sep-00

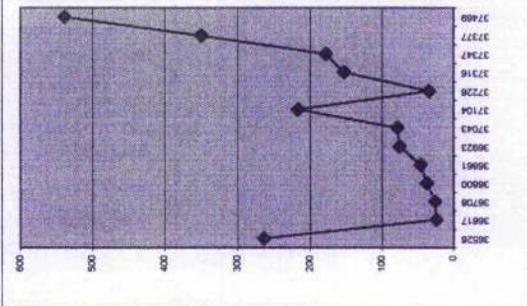


AR150

40.7
44.5
21.2
10.3
16.3
6.2
22.6
35.7
27.5
64.8
77.3
346.2

AR151

Mar-97
Apr-97
Feb-98
Apr-98
Sep-98
Mar-99
Nov-99
Jan-00
Apr-00
Sep-00
Oct-00
May-01

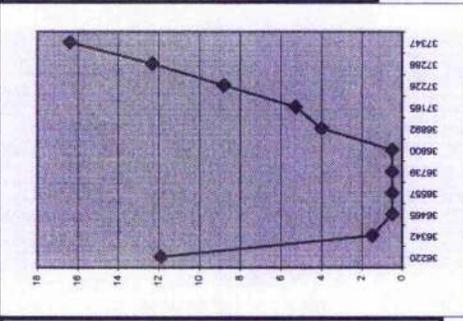


AR152

264
24
26
37
46
75
78
217
34
153
178
350
539

AR153

Jan-00
Apr-00
Jul-00
Oct-00
Dec-00
Feb-01
Jun-01
Aug-01
Dec-01
Mar-02
Apr-02
May-02
Aug-02



AR154

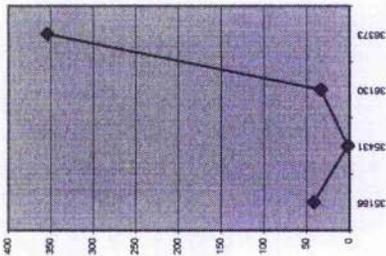
11.9
1.5
0.5
0.5
0.5
0.5
4
5.3
8.8
12.3
16.4

AR155

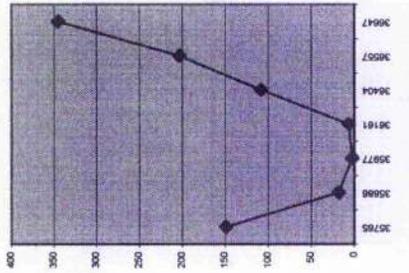
Mar-99
Jul-99
Nov-99
Feb-00
Aug-00
Oct-00
Jan-01
Oct-01
Dec-01
Feb-02
Apr-02

AR156

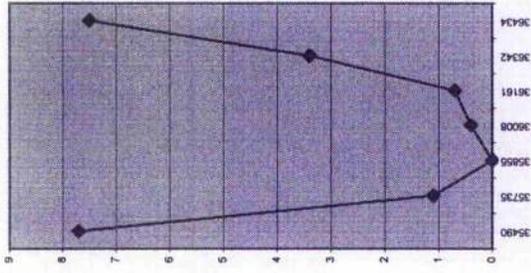
| | |
|------|--------------|
| 41.6 | AR157 |
| 2.3 | May-96 |
| 33.5 | Jan-97 |
| 354 | Dec-98 |
| | Aug-99 |

**AR158**

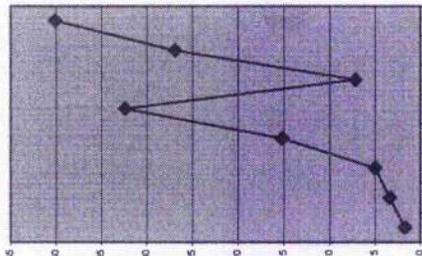
| | |
|-----|--------------|
| 149 | AR159 |
| 18 | Dec-97 |
| 2.5 | Apr-98 |
| 6.6 | Jul-98 |
| 108 | Jan-99 |
| 203 | Sep-99 |
| 345 | Feb-00 |
| | May-00 |

**AR164**

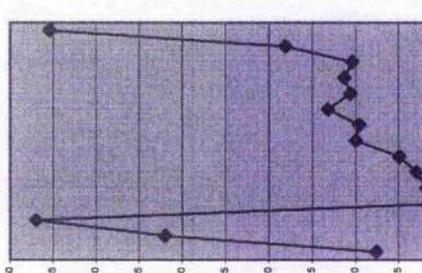
| | |
|------|--------------|
| 7.7 | AR165 |
| 1.1 | Mar-97 |
| <0.2 | Nov-97 |
| 0.4 | Mar-98 |
| 0.7 | Aug-98 |
| 3.4 | Jan-99 |
| 7.5 | Jul-99 |
| | Oct-99 |

**AR166**

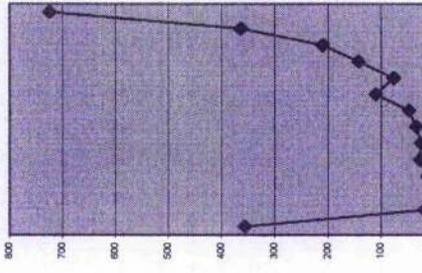
| | |
|------|--------------|
| 1.7 | AR167 |
| 3.4 | May-95 |
| 5 | Aug-98 |
| 15.2 | Mar-99 |
| 32.4 | Apr-00 |
| 7.2 | Oct-00 |
| 26.9 | Jul-01 |
| 40.1 | Oct-02 |
| | Nov-02 |

**AR168**

| | |
|------|--------------|
| 7.6 | AR169 |
| 32 | Nov-98 |
| 47 | Jul-00 |
| 1.6 | Sep-00 |
| 1.7 | Jan-01 |
| 2.9 | Apr-01 |
| 4.9 | Jul-01 |
| 10 | Sep-01 |
| 9.6 | Jan-02 |
| 13.2 | Feb-02 |
| 10.6 | Mar-02 |
| 11.3 | Mar-02 |
| 10.4 | Apr-02 |
| 18.1 | Apr-02 |
| 45.4 | Jun-02 |
| | Jun-02 |

**AR172**

| | |
|------|--------------|
| 356 | AR173 |
| 17 | Nov-95 |
| 1.5 | Feb-96 |
| 10.9 | Oct-96 |
| 25.8 | Aug-97 |
| 23.5 | Feb-98 |
| 33 | Nov-98 |
| 47 | Aug-99 |
| 110 | Feb-00 |
| 76 | Aug-01 |
| 143 | Oct-01 |
| 210 | Nov-01 |
| 364 | Dec-01 |
| 724 | Feb-02 |
| | Jun-02 |



APPENDIX 2

Scatter graph and Bland-Altman plots demonstrating inter-observer variation for each protein.

Figure 1a – Scatter-graph plot demonstrating inter-observer variation for Raf-1

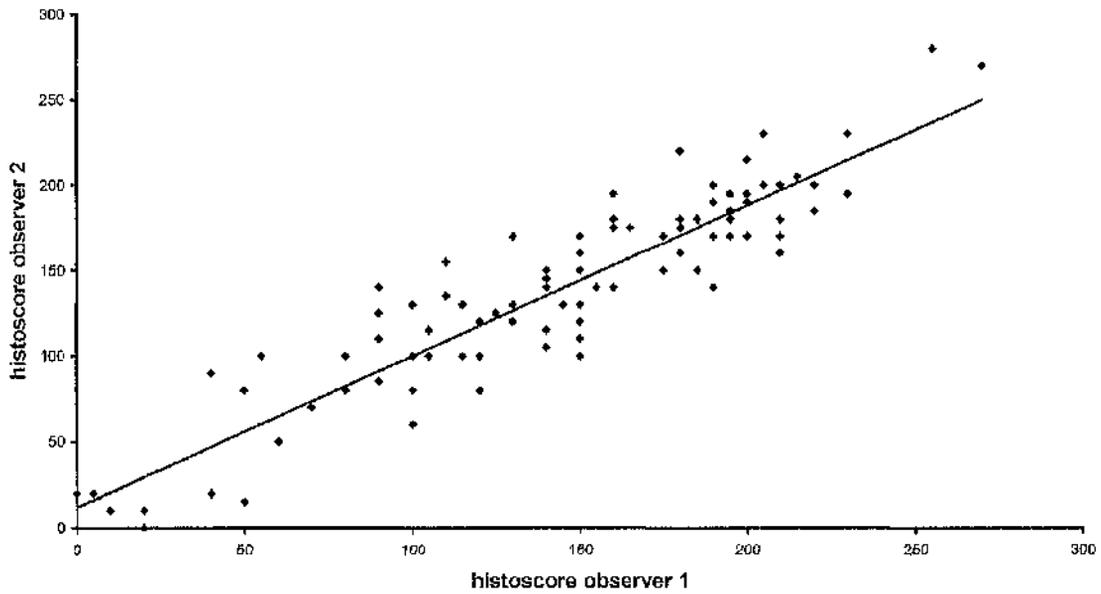


Figure 1b – Bland-Altman plot demonstrating inter-observer variation for Raf-1

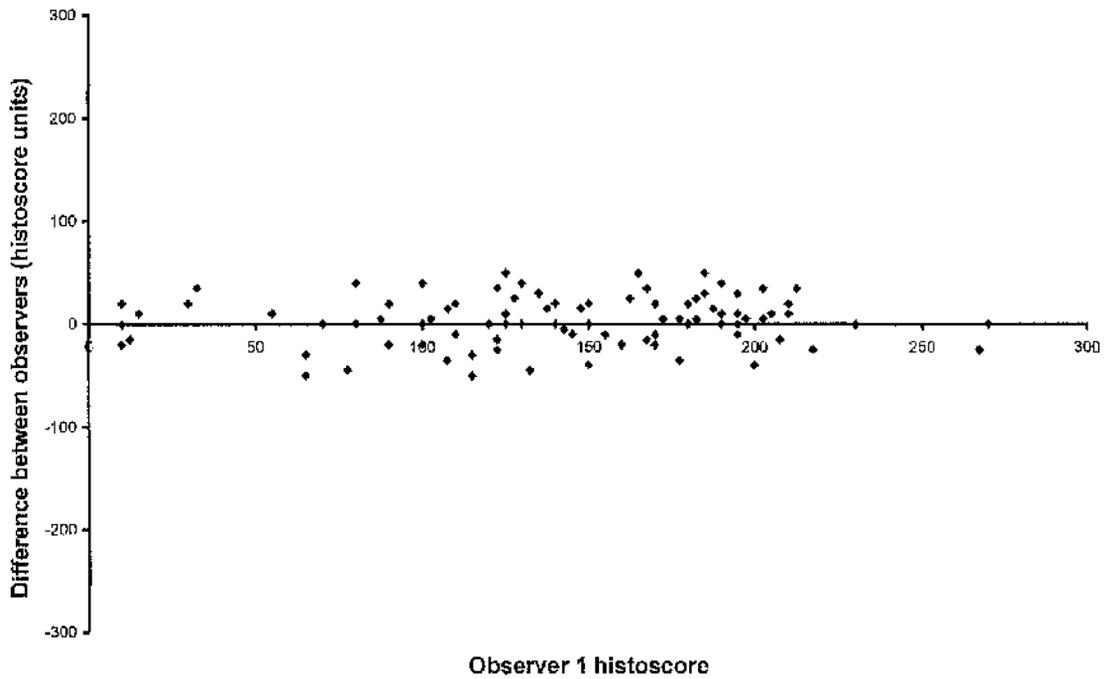


Figure 2a – Scatter-graph plot demonstrating inter-observer variation for pRaf (ser259)

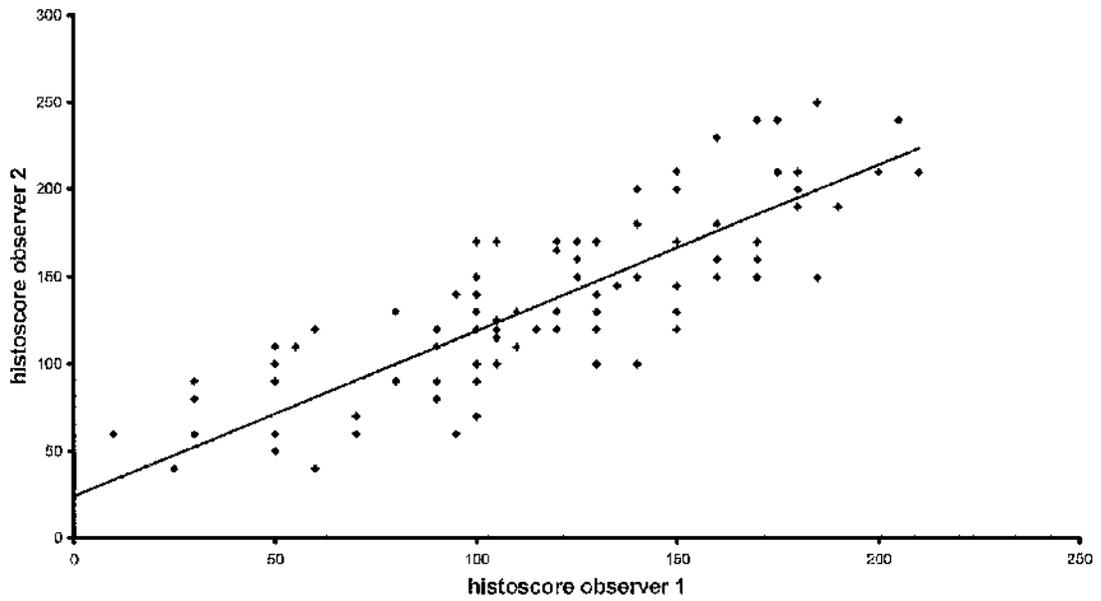


Figure 2b – Bland-Altman plot demonstrating inter-observer variation for pRaf (ser259)

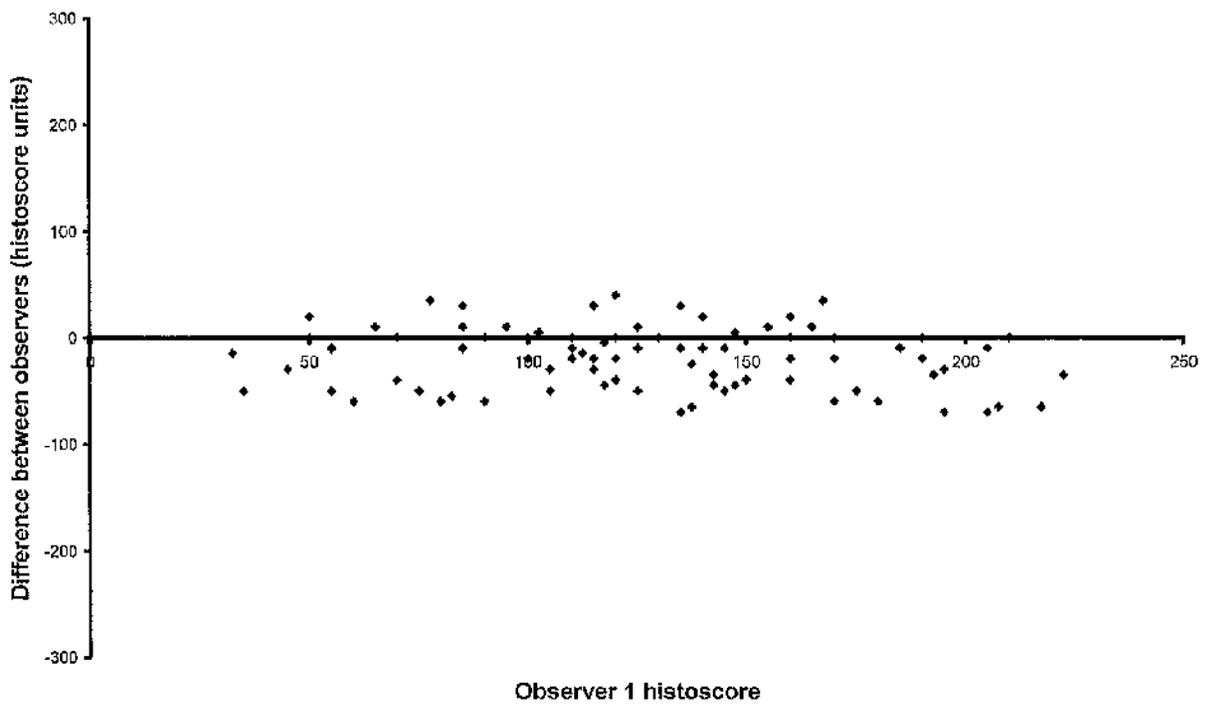


Figure 3a – Scatter-graph plot demonstrating inter-observer variation for nuclear pRaf (ser338)

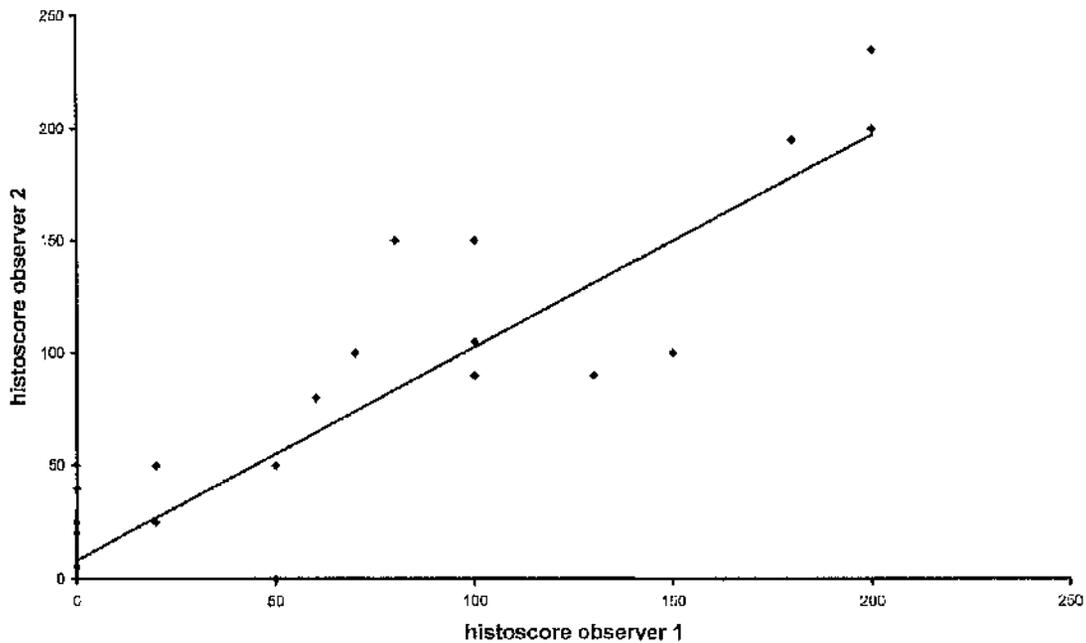


Figure 3b – Bland-Altman plot demonstrating inter-observer variation for nuclear pRaf (ser338)

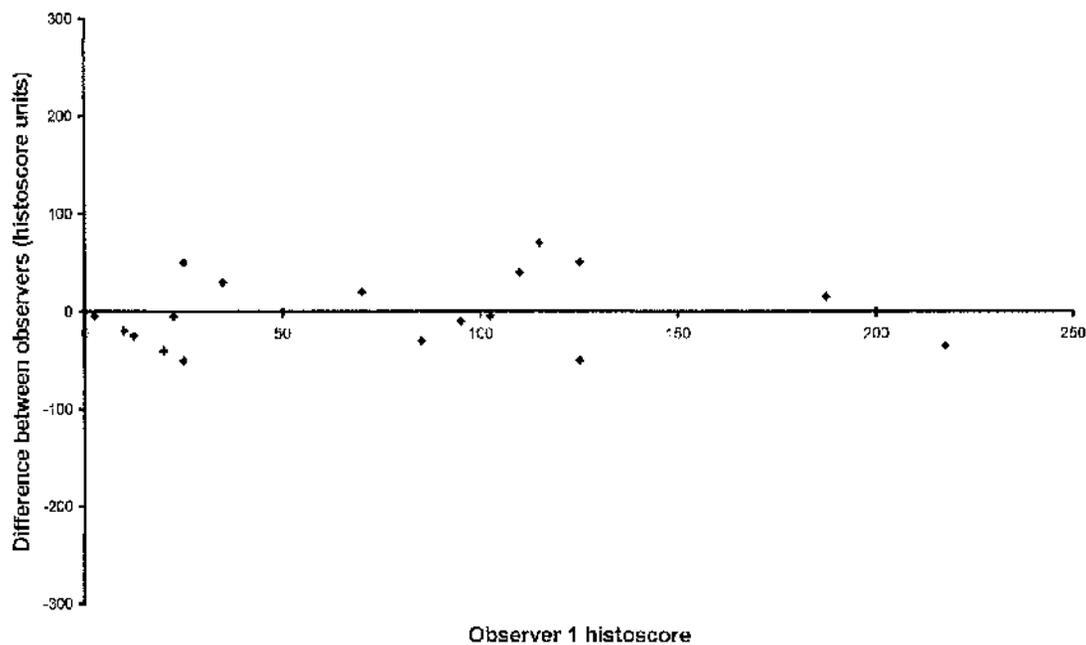


Figure 3c – Scatter-graph plot demonstrating inter-observer variation for cytoplasmic pRaf (ser338)

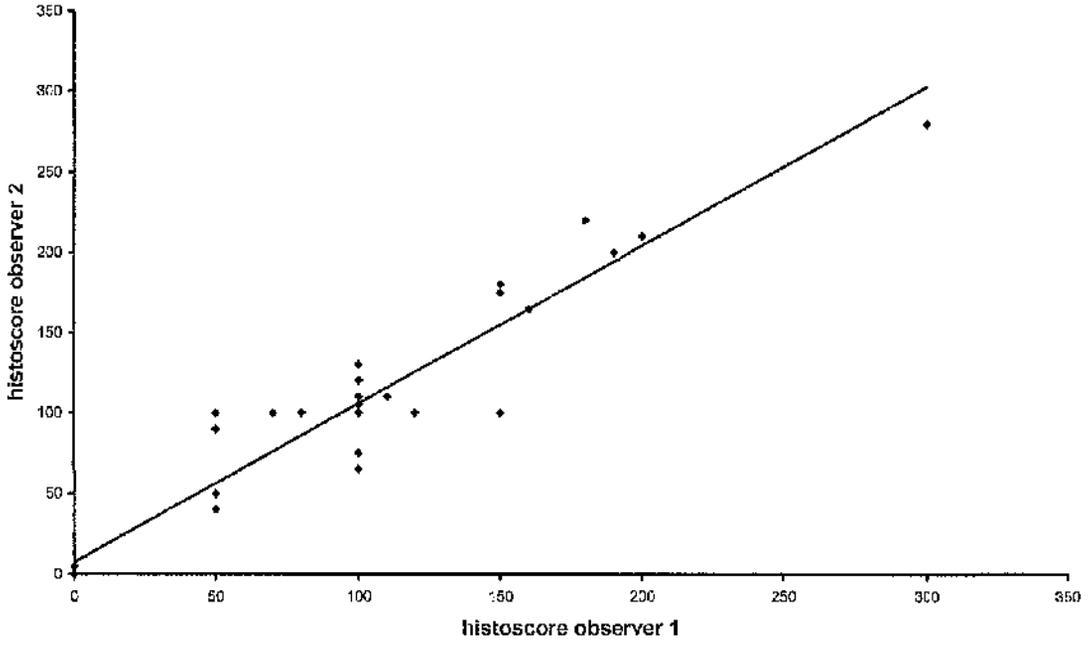


Figure 3d – Bland-Altman plot demonstrating inter-observer variation for cytoplasmic pRaf (ser338)

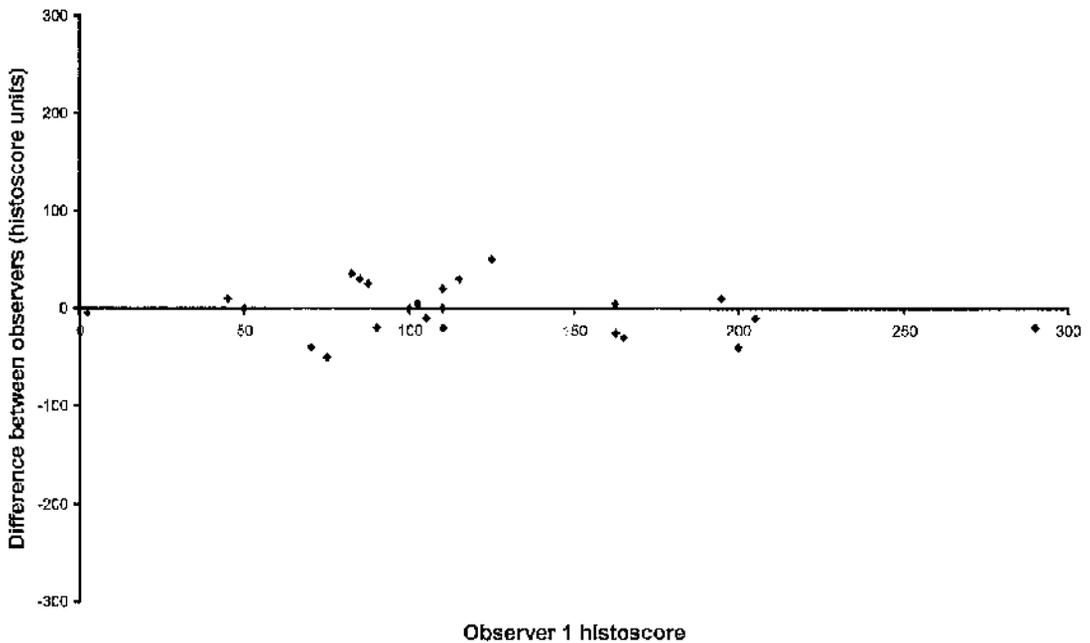


Figure 4a – Scatter-graph plot demonstrating inter-observer variation for cytoplasmic MAP Kinase expression

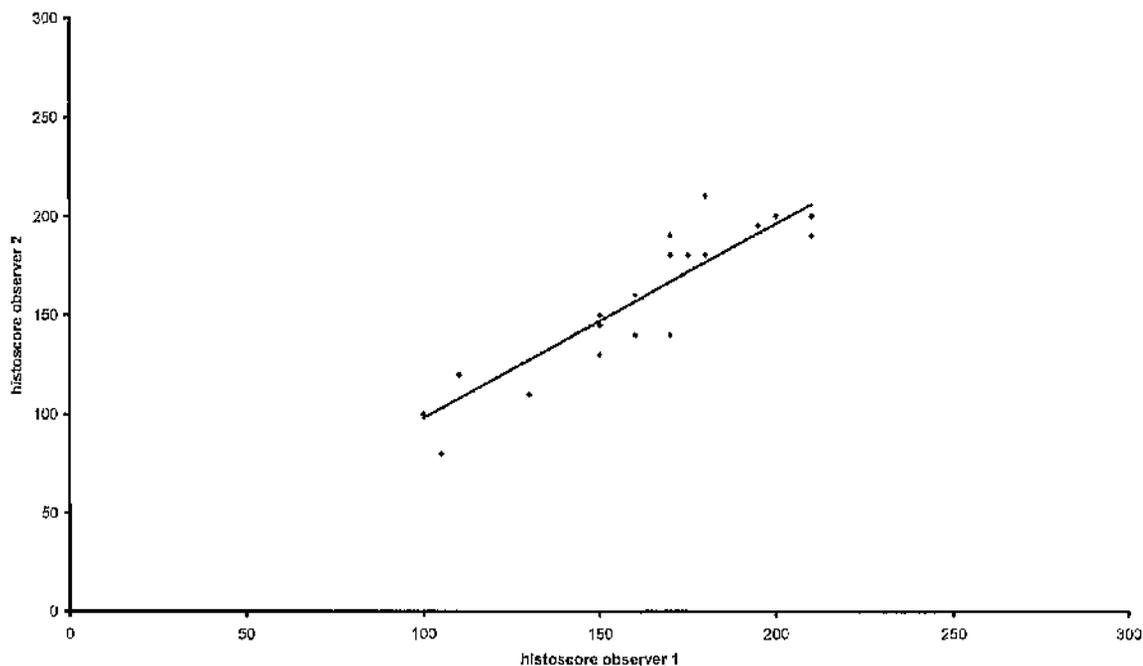


Figure 4b – Bland-Altman plot demonstrating inter-observer variation for cytoplasmic MAP Kinase expression

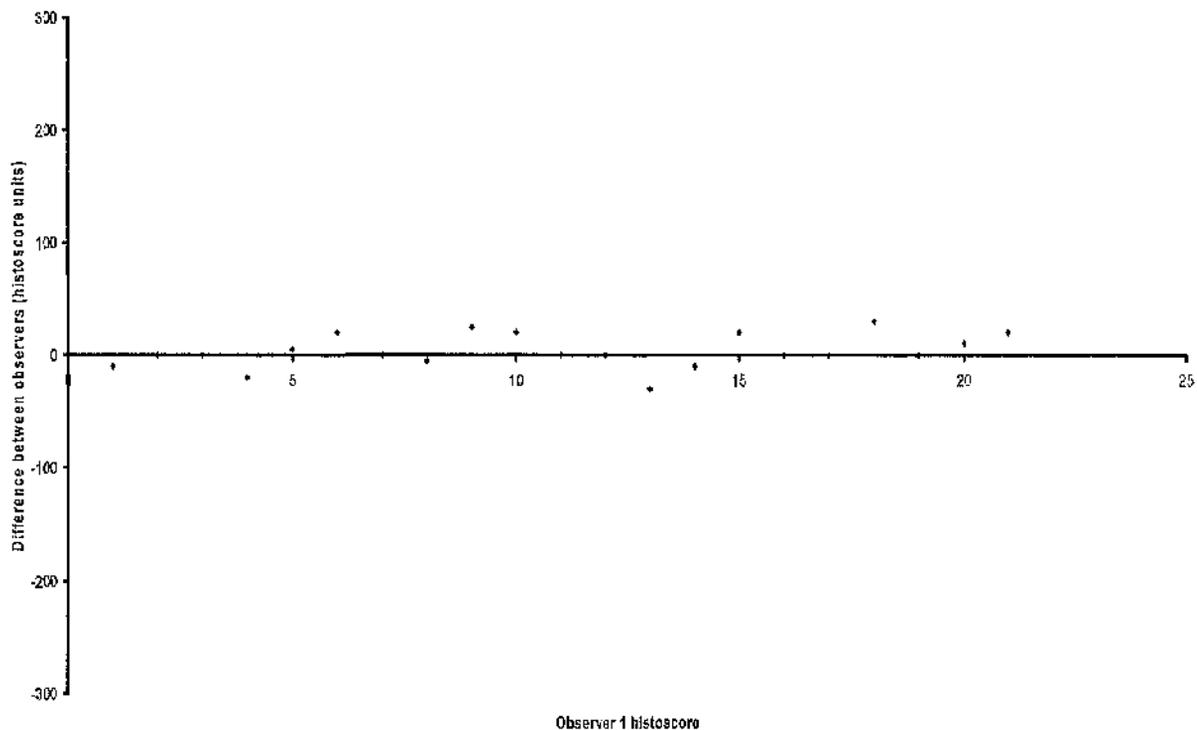


Figure 5a – Scatter-graph plot demonstrating inter-observer variation for nuclear MAP Kinase expression

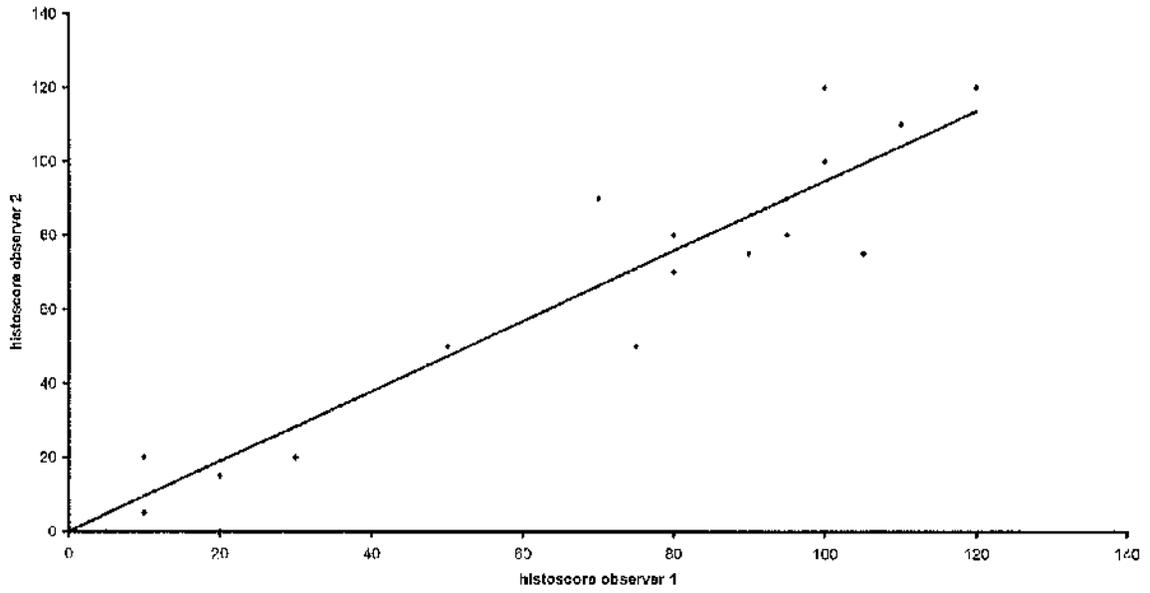


Figure 5b – Bland-Altman plot demonstrating inter-observer variation for nuclear MAP Kinase expression

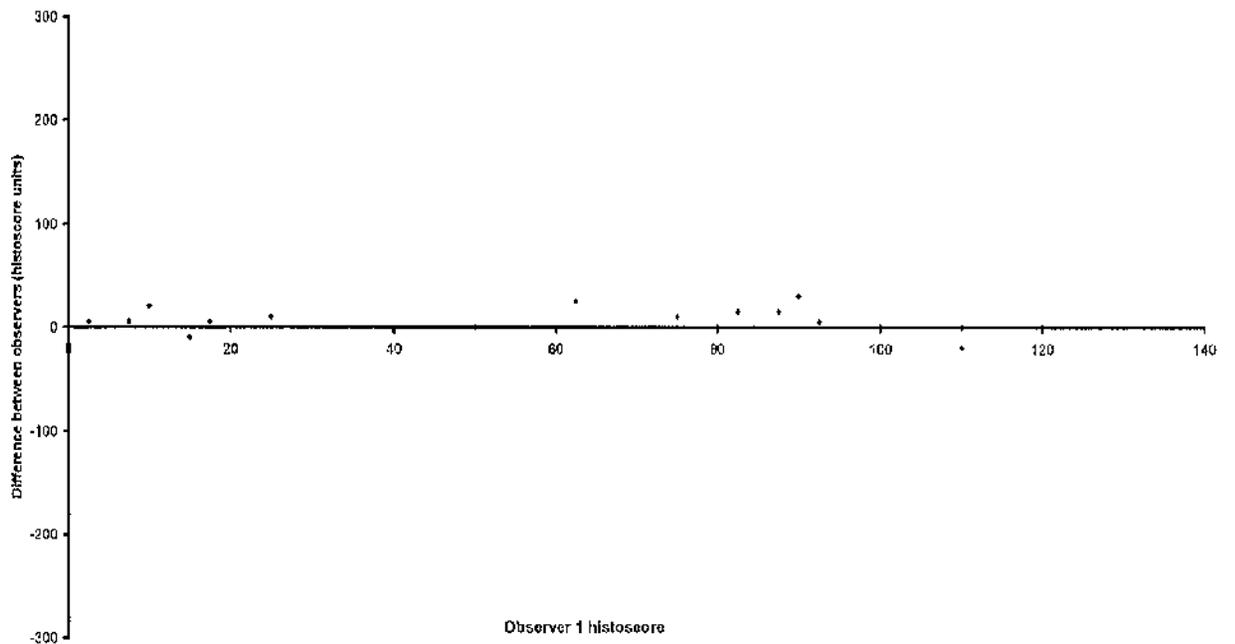


Figure 6a – Scatter-graph plot demonstrating inter-observer variation for cytoplasmic pMAP Kinase expression

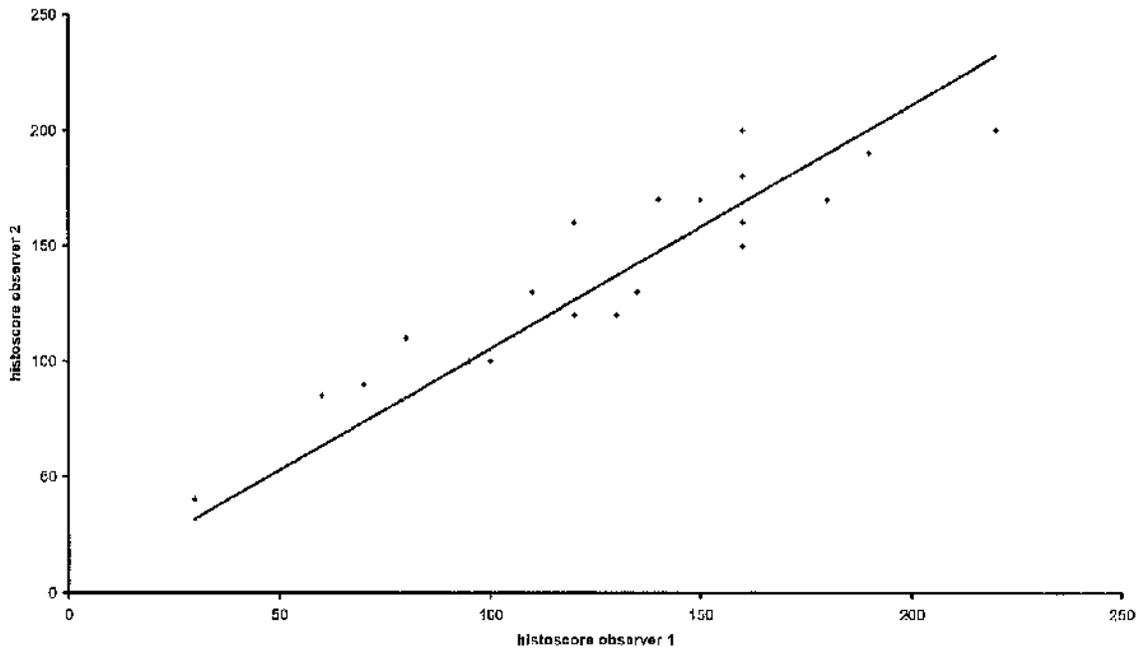


Figure 6b – Bland-Altman plot demonstrating inter-observer variation for cytoplasmic pMAP Kinase expression

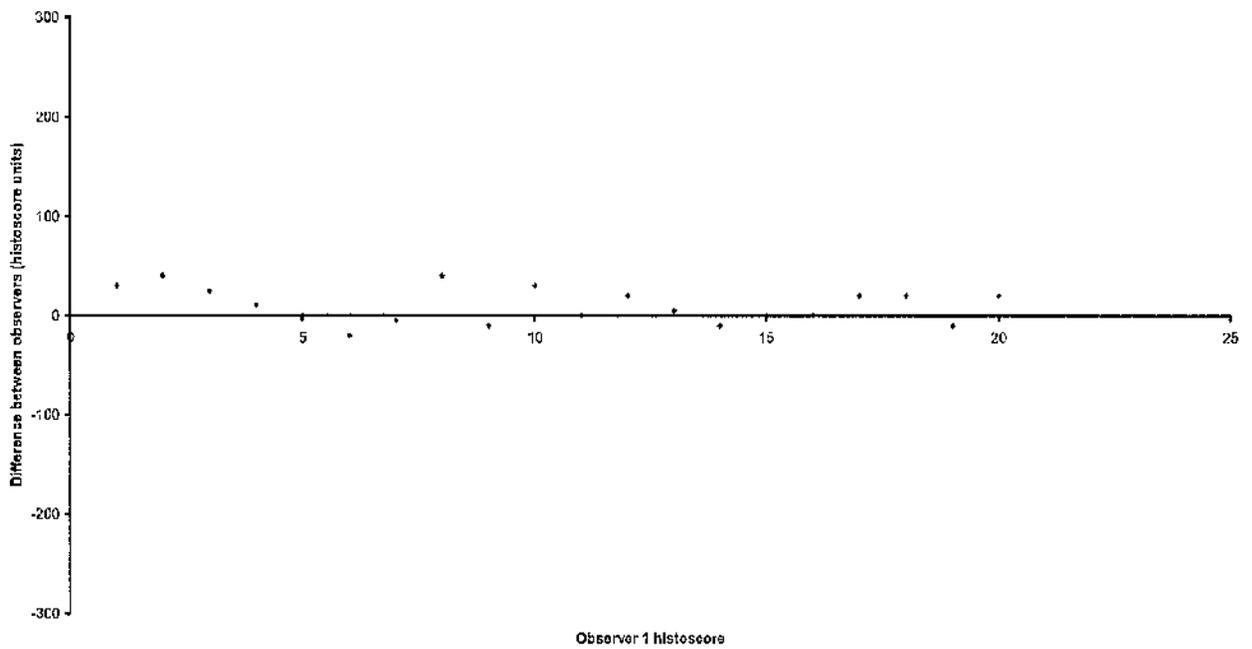


Figure 7a – Scatter-graph plot demonstrating inter-observer variation for nuclear pMAP Kinase expression

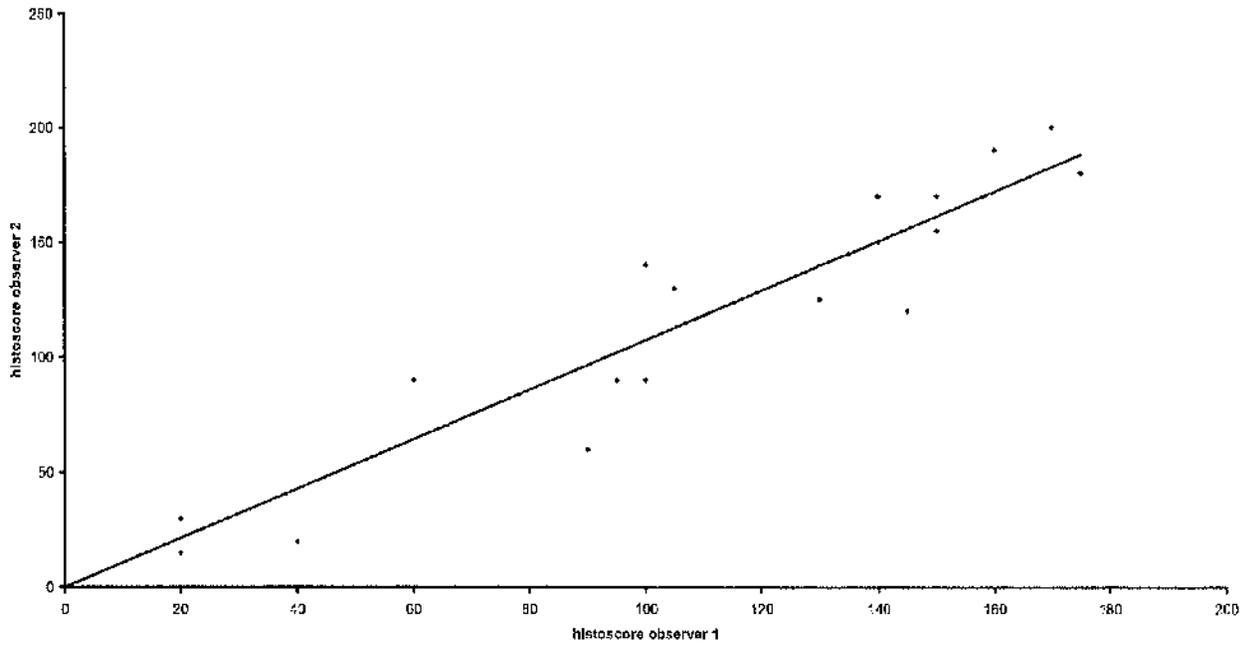
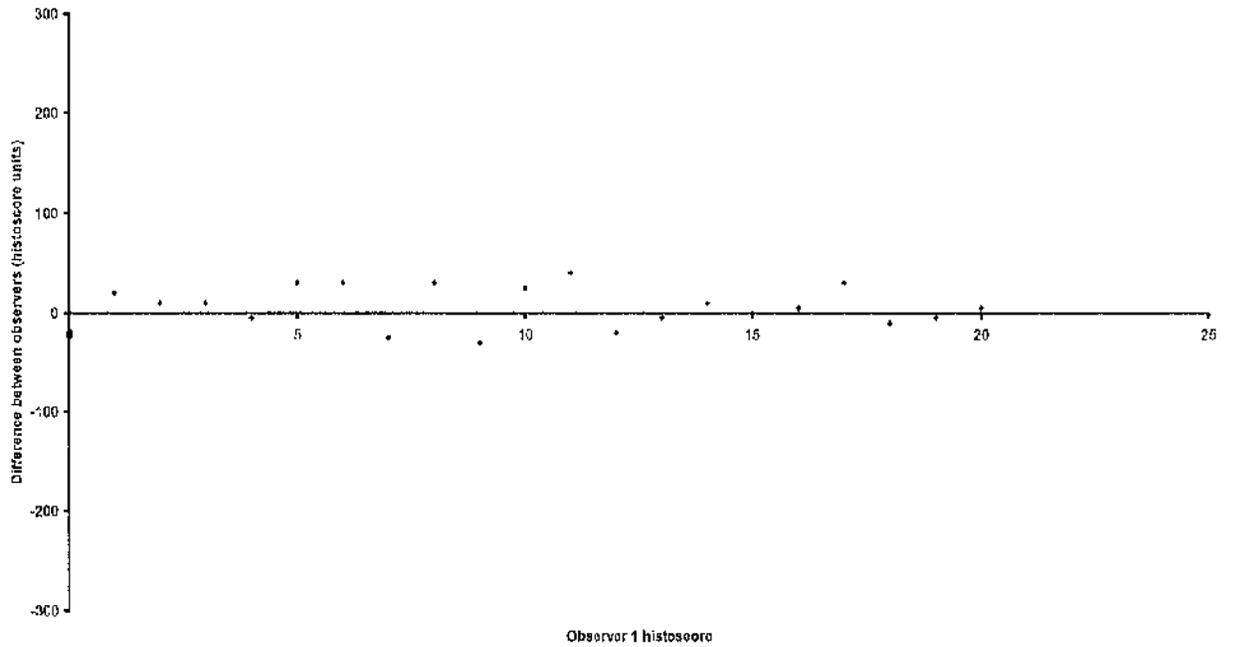


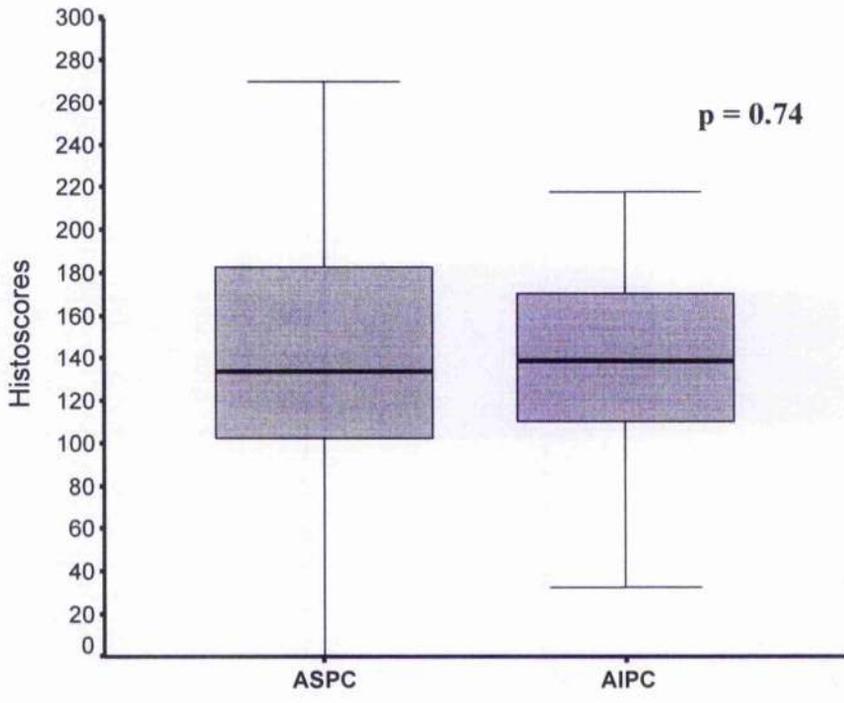
Figure 7b – Bland-Altman plot demonstrating inter-observer variation for nuclear pMAP Kinase expression



APPENDIX 3

Box-plots and Line Graphs highlighting changes in protein histoscore for each protein.

Figure 9.



Median Raf-1 histoscores for ASPC and AIPC tumours, with p value as assessed by Wilcoxon Signed Ranks Test.

Figure 10. Changes in Raf-1 expression with the development of AIPC

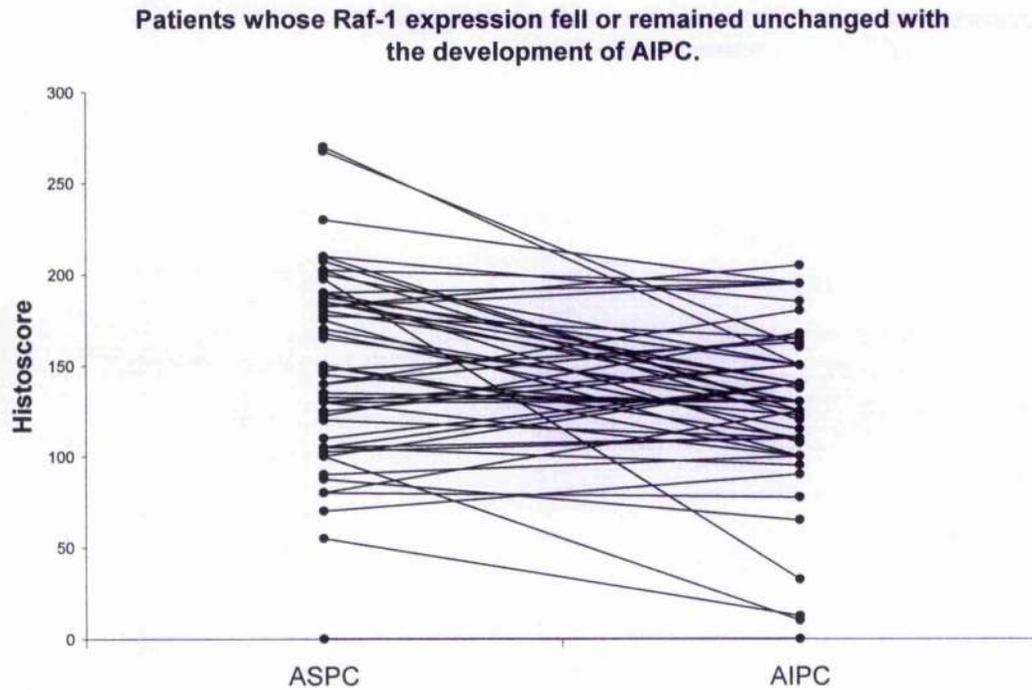
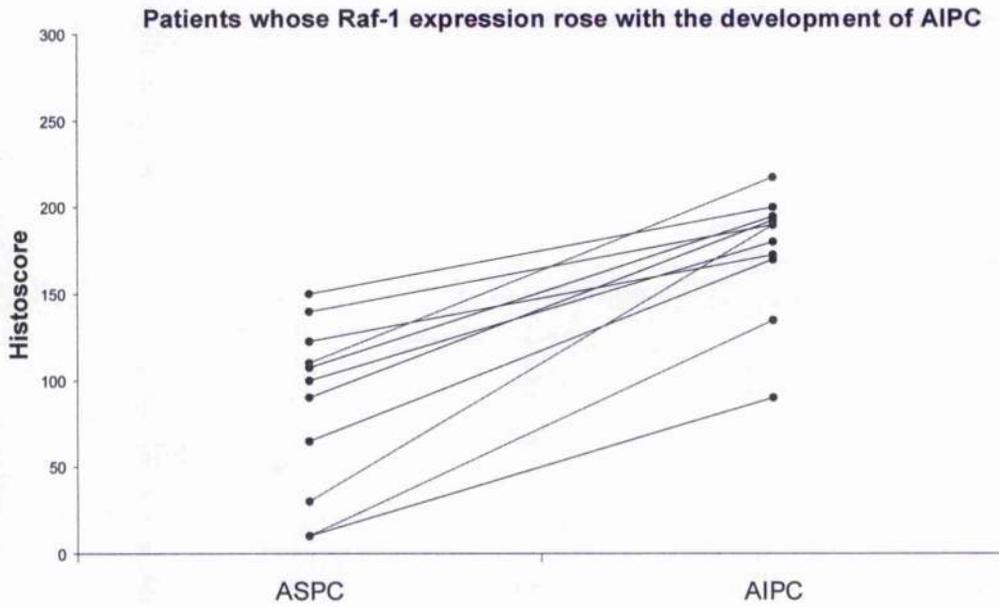


Figure 13a. Median pRaf (ser259) histoscores for ASPC and AIPC tumours, with p value as assessed by Wilcoxon Signed Ranks Test.

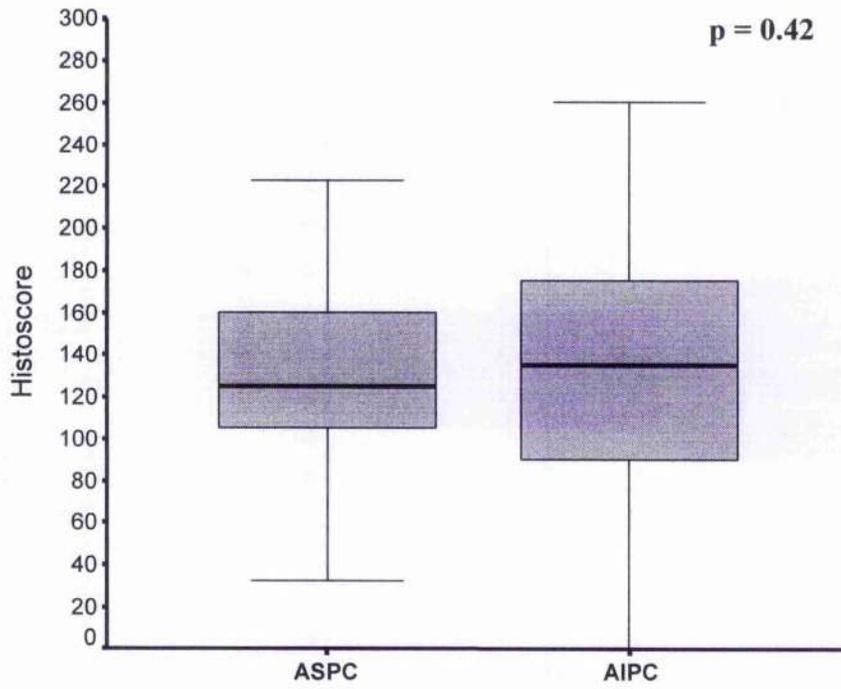


Figure 13b. Median pRaf (ser338) nuclear and cytoplasmic histoscores for ASPC and AIPC tumours, with p value as assessed by Wilcoxon Signed Ranks Test.

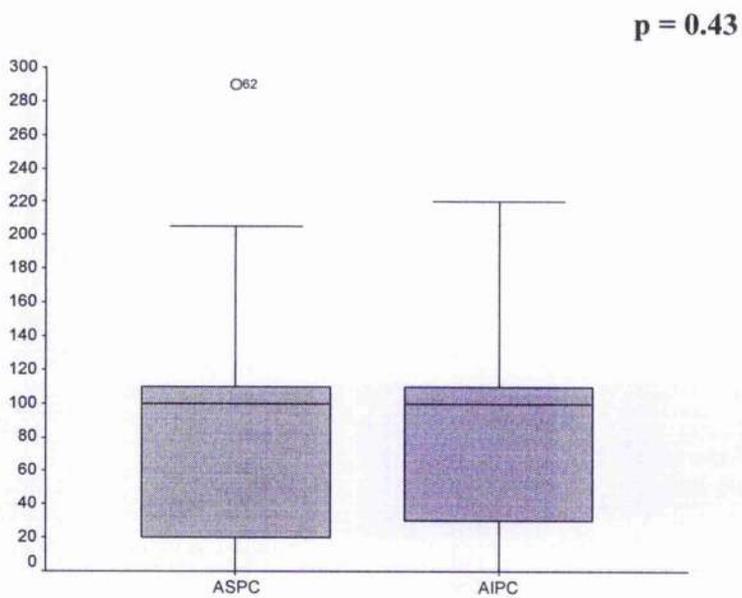
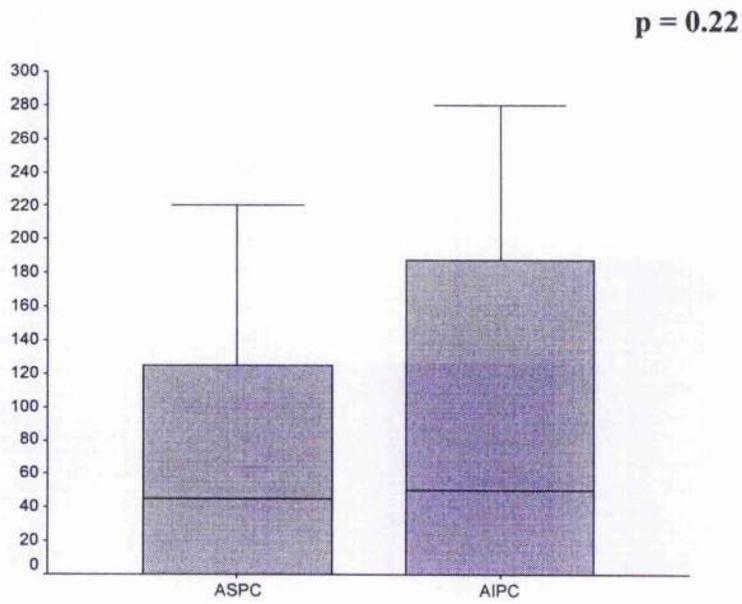


Figure 14a.

Changes in nuclear pRaf (ser259) expression with the development of AIPC

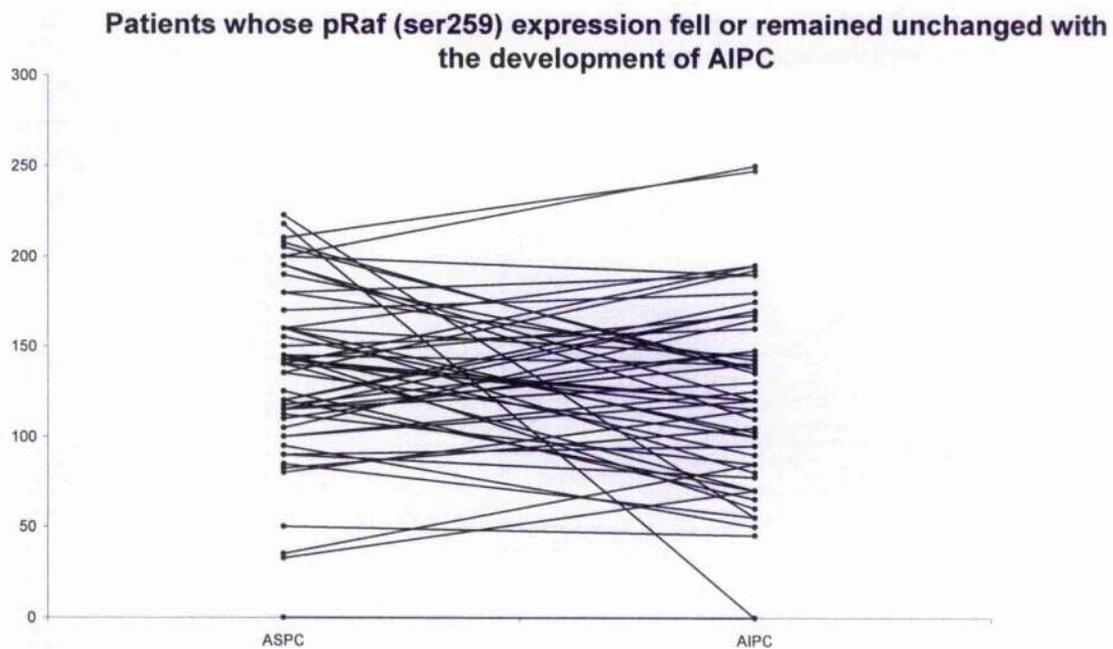
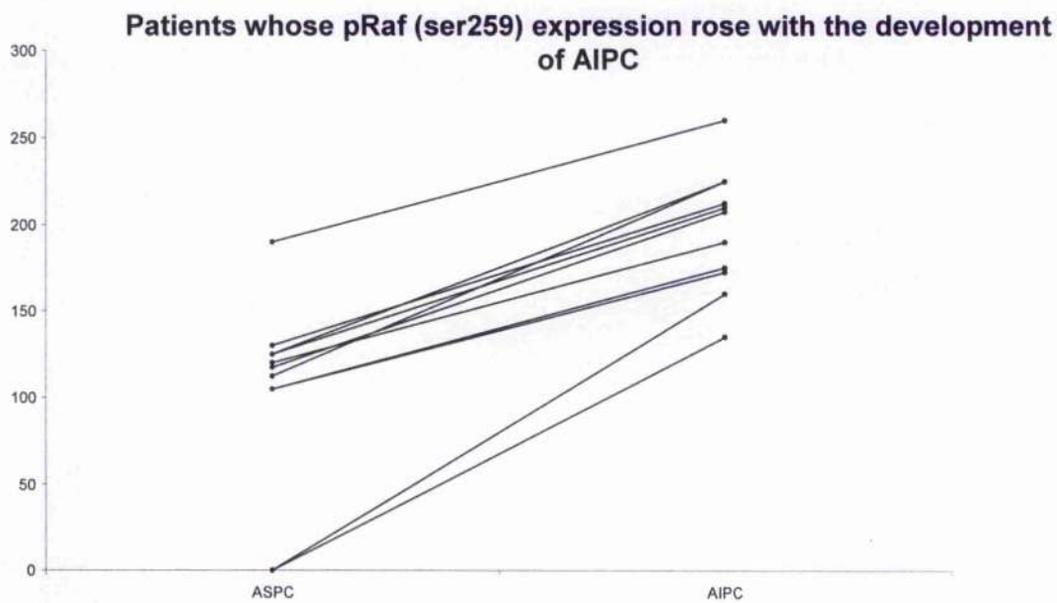


Figure 14b.

Changes in nuclear pRaf (ser338) expression with the development of AIPC

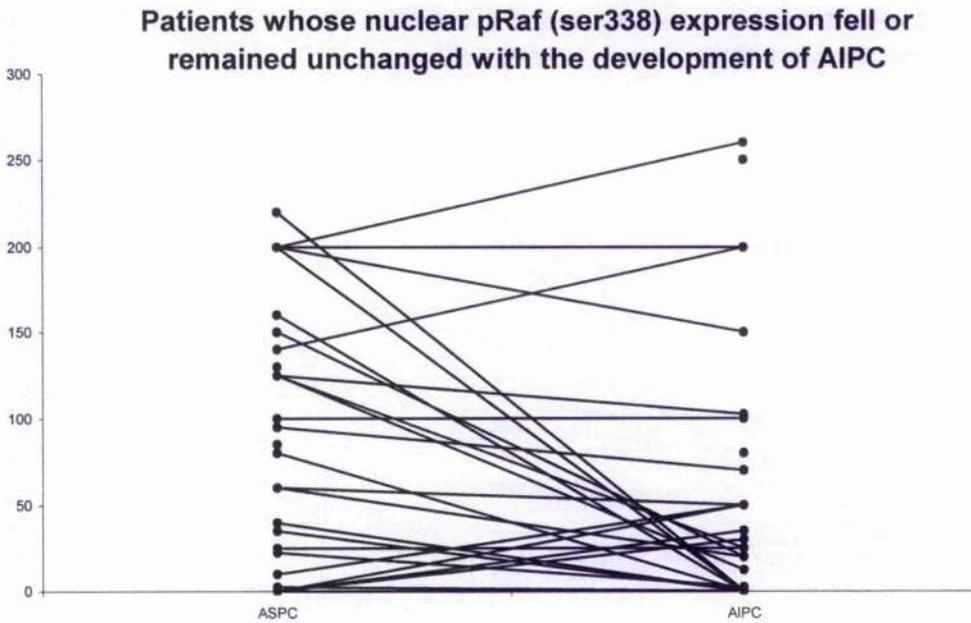
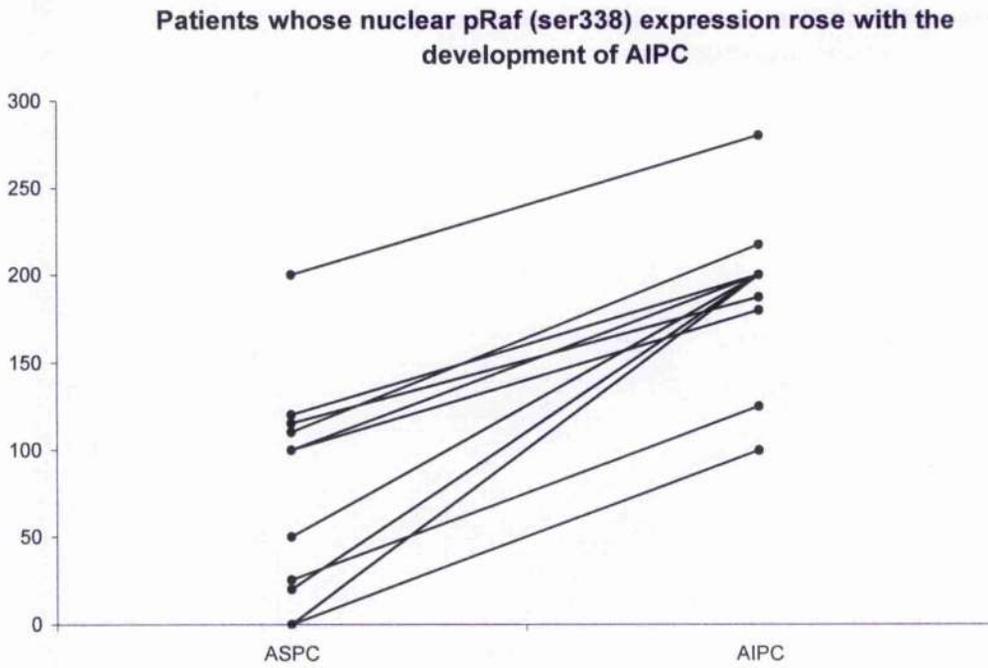


Figure 14c.

Changes in cytoplasmic pRaf (ser338) expression with the development of AIPC

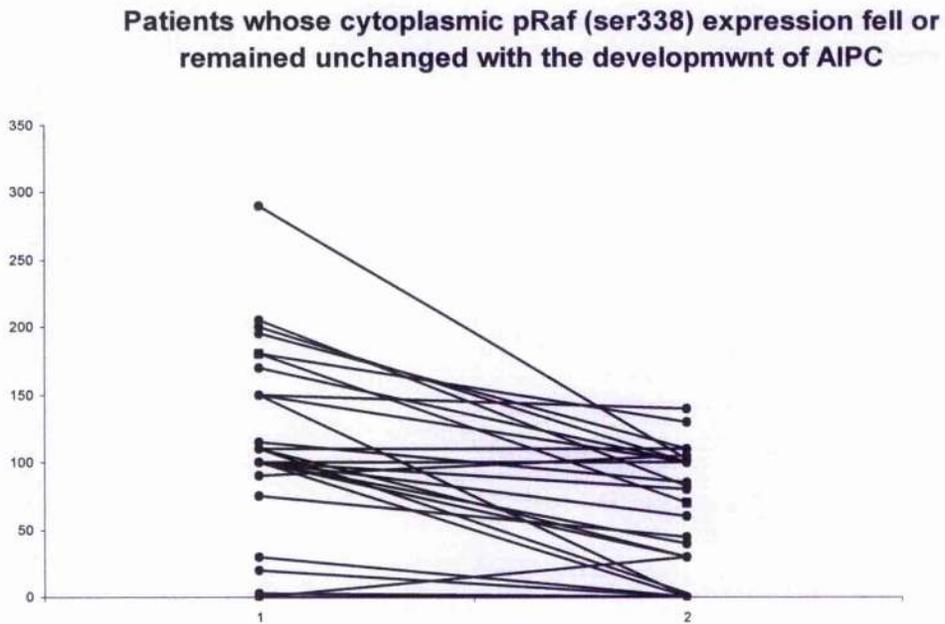
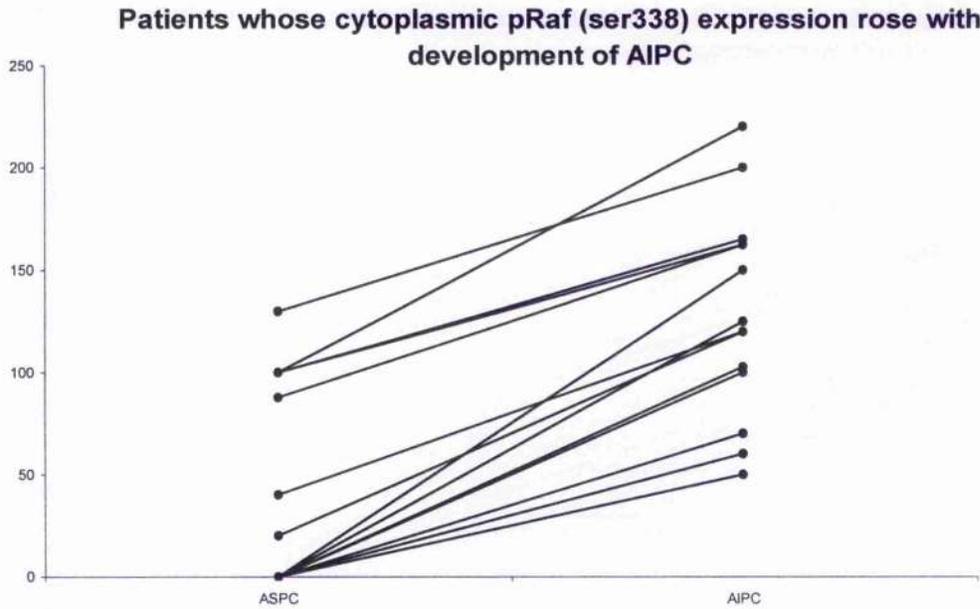


Figure 15.

Median MAP Kinase nuclear and cytoplasmic histoscores for ASPC and AIPC tumours, with p value as assessed by Wilcoxon Signed Ranks Test

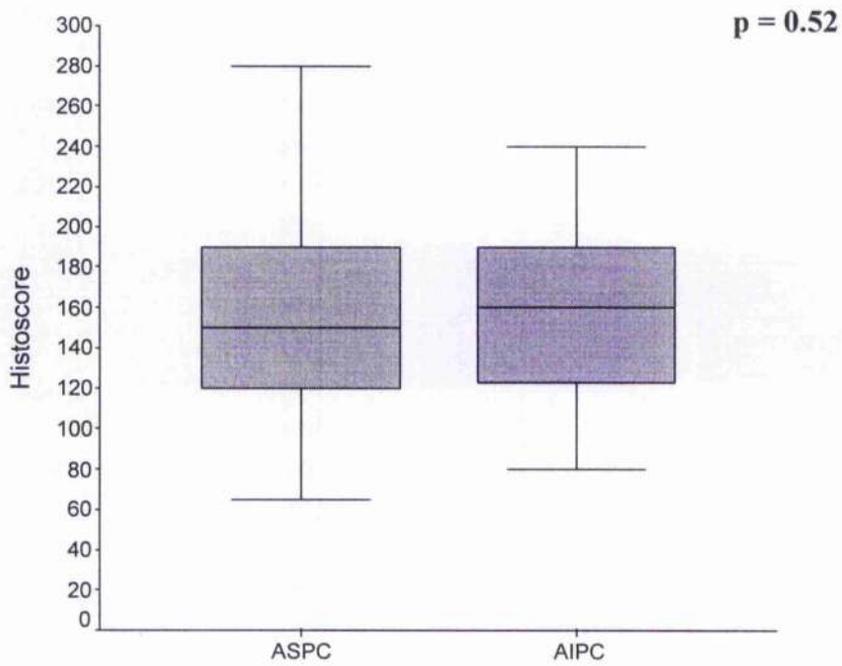
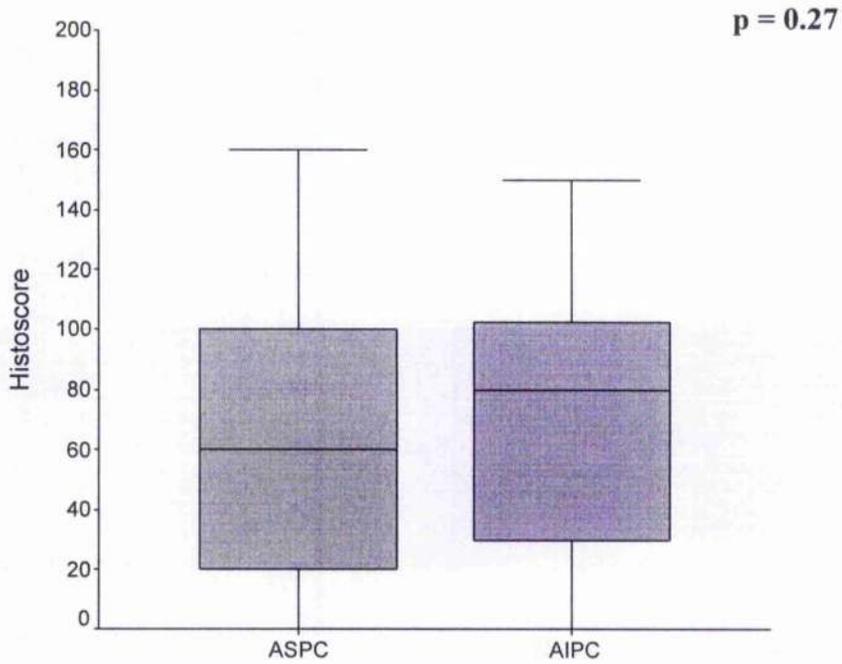


Figure 16.

Median pMAP Kinase nuclear and cytoplasmic histoscores for ASPC and AIPC tumours, with p value as assessed by Wilcoxon Signed Ranks Test

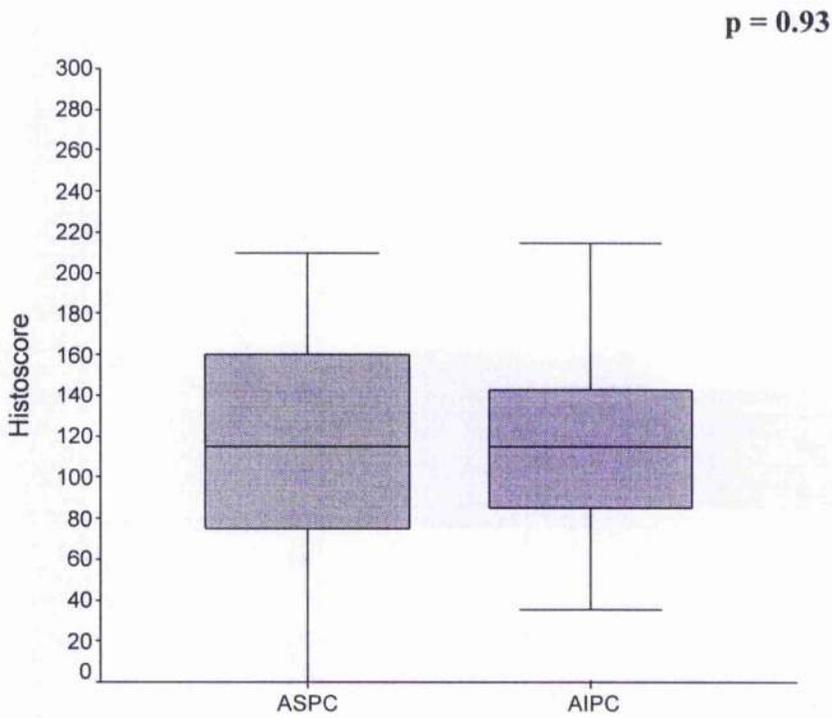
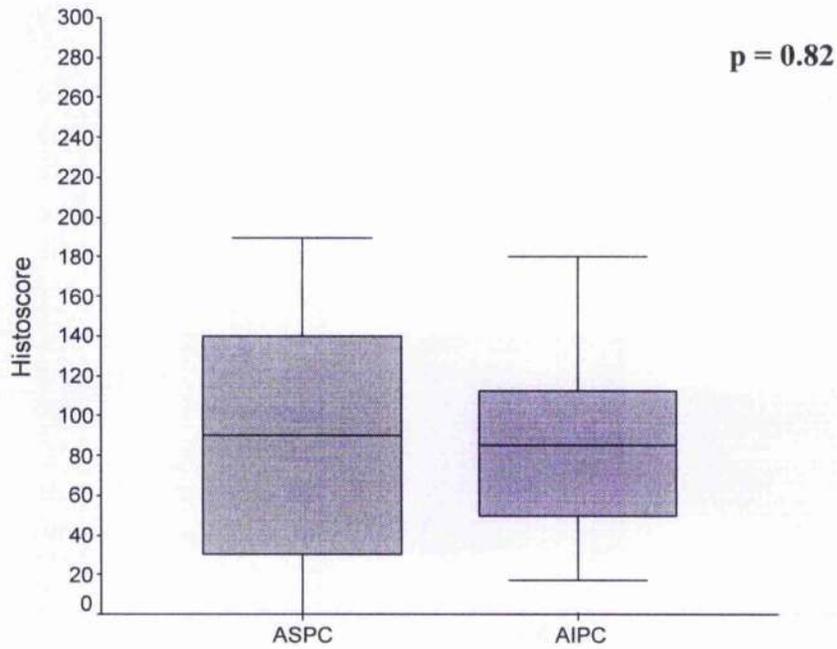


Figure 17b.

Changes in cytoplasmic MAP Kinase expression with the development of AIPC.

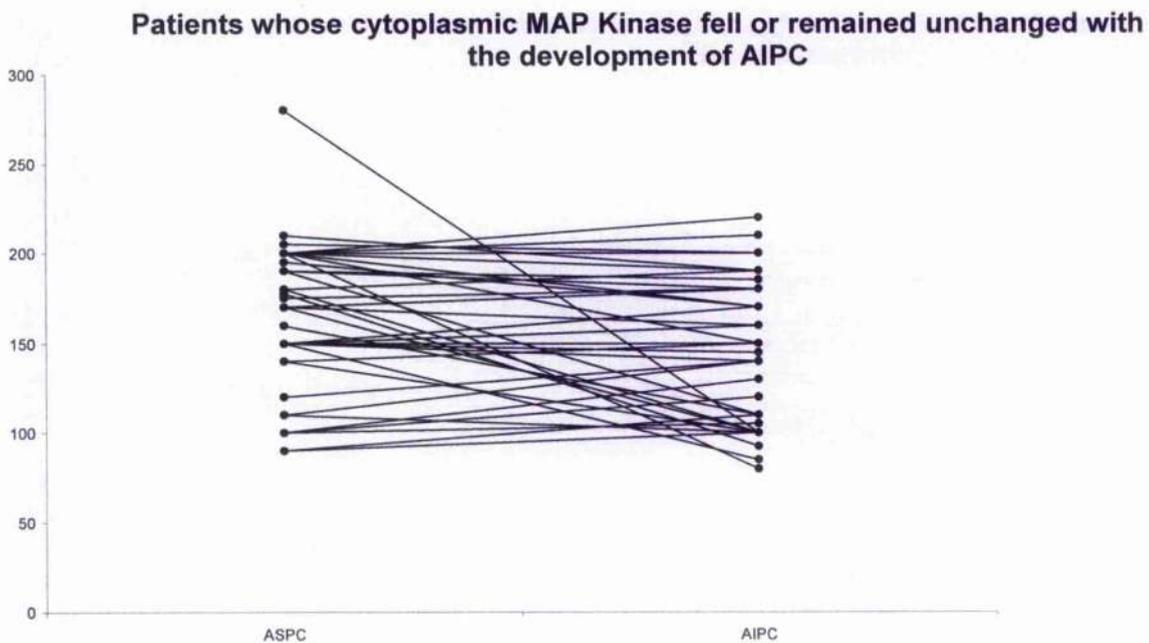
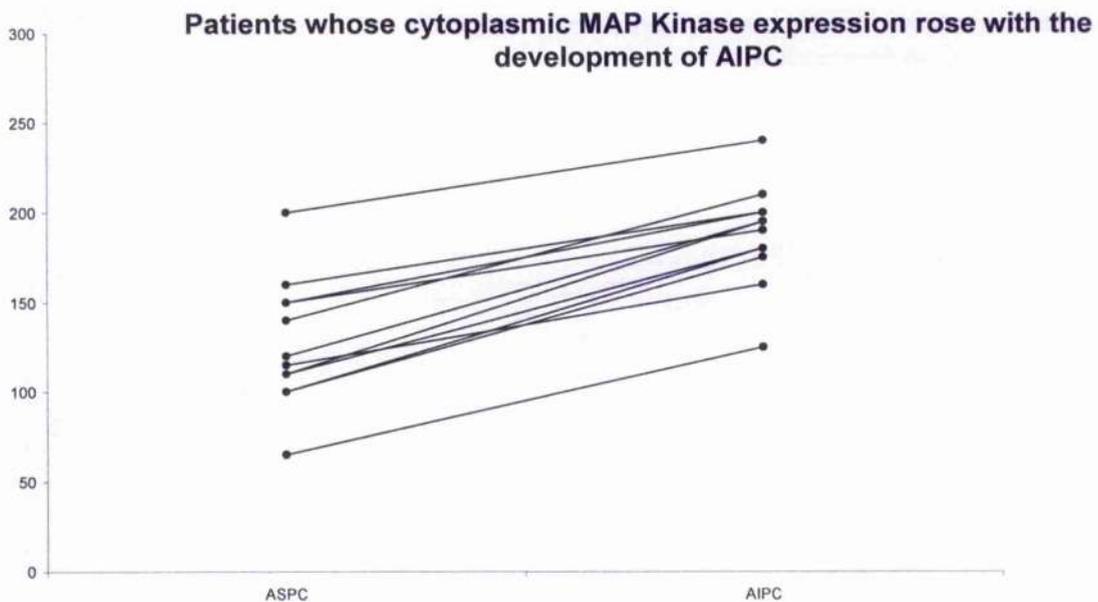


Figure 18a.

Changes in nuclear pMAP Kinase expression with the development of AIPC.

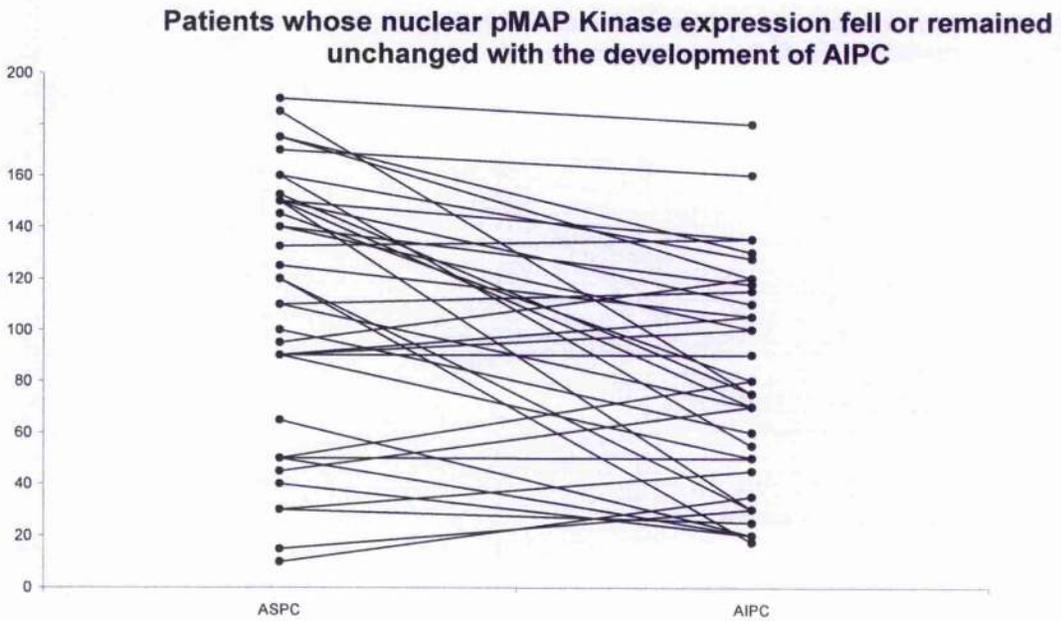
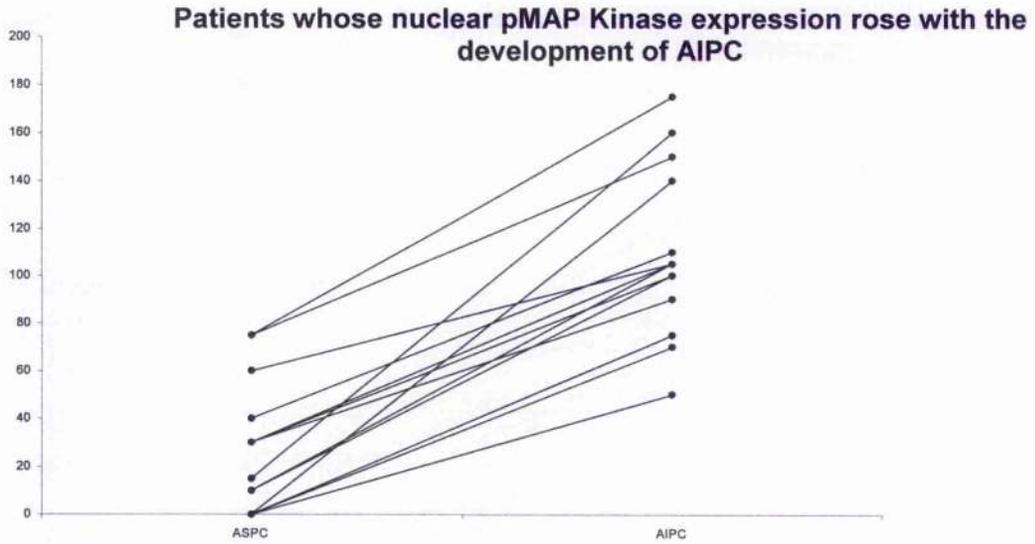
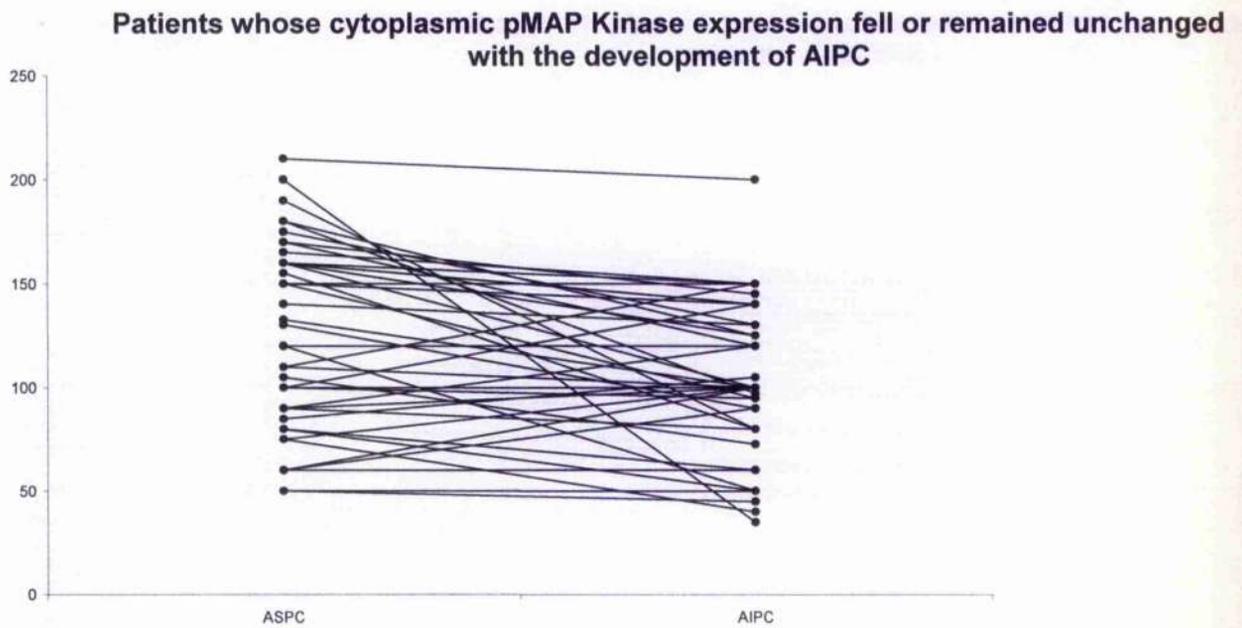
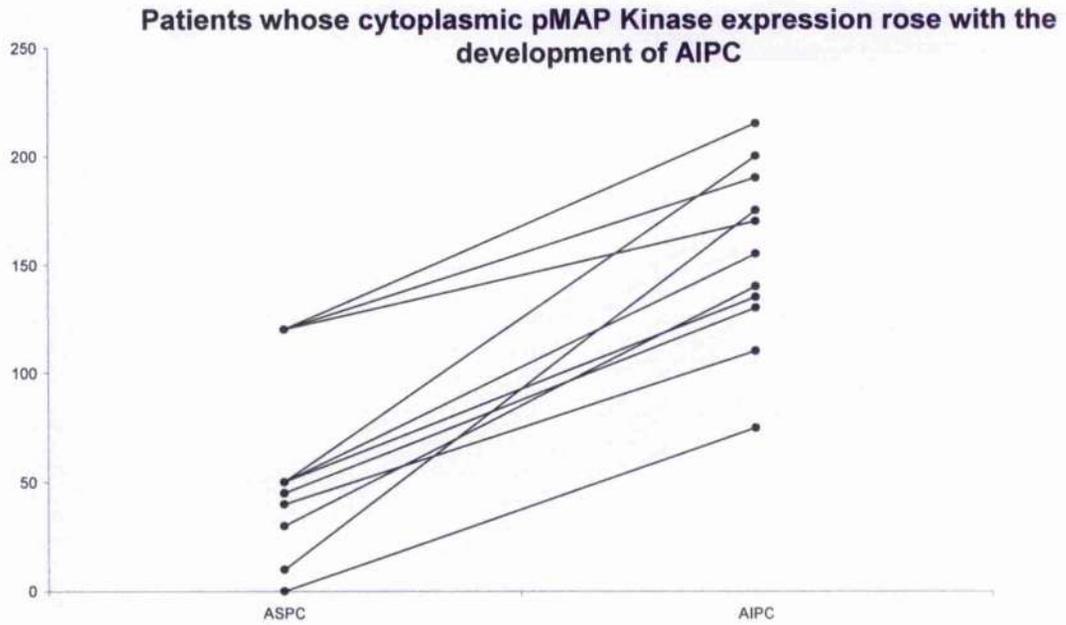


Figure 18b.

Changes in cytoplasmic pMAP Kinase expression with the development of AIPC.



APPENDIX 4

Tables highlighting Time to Relapse, Time to Death from relapse, and Overall Survival for each protein.

Table 1a. Time to Relapse (TTR) and Raf-1 / pRaf expression divided by median.

| | | TTR (years) | P value |
|--------------------------------|--------------|--------------------|----------------|
| Raf-1 | Below Median | 1.90 (1.45 – 2.35) | |
| | Above Median | 3.40 (2.27- 4.53) | 0.07 |
| pRaf (ser259) | Below Median | 2.28 (1.88-2.69) | |
| | Above Median | 2.58 (1.31-3.83) | 0.79 |
| pRaf (ser338) Nuclear | Below Median | 2.35 (1.92-2.77) | |
| | Above Median | 2.98 (1.46-4.50) | 0.93 |
| pRaf (ser338) Cytoplasm | Below Median | 2.36 (2.08-2.64) | |
| | Above Median | 2.98 (0.64-5.32) | 0.16 |

Raf-I and pRaf expression, split into 2 subgroups - above and below median - is shown related to median time to relapse in days (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 1b. Time to Relapse (TTR) and Raf-1 / pRaf expression divided according to rising histoscore with development of AIPC.

| | | TTR (years) | P value |
|--------------------------------|-------------|---------------------------|----------------|
| Raf-1 | Non-Risers | 2.62 (1.52 - 3.70) | |
| | Risers | 1.16 (1.12 - 1.20) | 0.0005 |
| pRaf (ser259) | Non-Risers | 2.36 (1.98-2.73) | |
| | Risers | 2.57 (0-5.41) | 0.68 |
| pRaf (ser338) Nuclear | Non-Risers | 2.30 (1.72-2.88) | |
| | Risers | 2.30 (1.75-2.85) | 0.75 |
| pRaf (ser338) Cytoplasm | Non-Risers | 2.30 (1.86-2.74) | |
| | Risers | 2.60 (2.14-3.06) | 0.79 |
| pRaf (ser338) Nuclear | Fallers | 4.60 (3.40-5.80) | |
| | Non-Fallers | 2.20 (1.84-2.56) | 0.01 |
| pRaf (ser338) Cytoplasm | Fallers | 2.40 (0-4.95) | |
| | Non-fallers | 2.30 (1.93-2.67) | 0.56 |

Raf-1 and pRaf expression, split into 2 subgroups - risers and non-risers with the development of AIPC - is shown related to median time to relapse in years (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 2a. Time to Death from Relapse (TTDR) and Raf-1/ pRaf expression divided according to median.

| | TTDR (years) | P value |
|---|--------------------------------|---------|
| Raf-1 AIPC | Below Median 1.6 (1.47 – 1.73) | |
| | Above Median 2.1 (0.43 - 3.77) | 0.25 |
| pRaf (ser259) AIPC | Below Median 2.47 (0.76-4.18) | |
| | Above Median 1.40 (1.20-1.60) | 0.11 |
| pRaf (ser338) nuclear AIPC | Below Median 1.34 (1.16-1.52) | |
| | Above Median 2.47 (1.14-3.79) | 0.15 |
| pRaf (ser338) cytoplasm AIPC | Below Median 1.37 (1.15-1.59) | |
| | Above Median 2.15 (1.10-3.20) | 0.36 |

Raf-1 and pRaf expression (ASPC and AIPC), split into 2 subgroups - above and below median - is shown related to median time to death from relapse in years (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 2b. Time to Death from Relapse (TTDR) and Raf-1 / pRaf expression divided according to rising histoscore with development of AIPC.

| | | TTDR (years) | P value |
|-------------------------------------|------------|---------------------|----------------|
| Raf-1 AIPC | Non-Risers | 1.62 (1.19 – 2.05) | |
| | Risers | 7.30 (-) | 0.13 |
| pRaf (ser259) AIPC | Non-Risers | 1.87 (1.23 – 2.41) | |
| | Risers | 0.83 (0 – 1.66) | 0.08 |
| pRaf (ser338) nuclear AIPC | Non-Risers | 1.62 (1.15-2.10) | |
| | Risers | 2.47 (0.93-4.00) | 0.94 |
| pRaf (ser338) cytoplasm AIPC | Non-Risers | 1.65 (1.23-2.07) | |
| | Risers | 1.49 (0.65-2.33) | 0.30 |

Raf-1 and pRaf expression, split into 2 subgroups - risers and non-risers with the development of AIPC - is shown related to median time to relapse in years (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 3a. Overall Survival (OS) and Raf-1/ pRaf expression divided according to median.

| | | OS (years) | P value |
|---|--------------|-------------------|----------------|
| Raf-1 ASPC | Below Median | 4.39 (1.58-7.2) | |
| | Above Median | 6.50 (5.83-7.17) | 0.60 |
| Raf-1 AIPC | Below Median | 6.04 (2.77-9.31) | |
| | Above Median | 6.26 (4.40-8.12) | 0.55 |
| pRaf (ser259) ASPC | Below Median | 6.26 (3.51-9.01) | |
| | Above Median | 6.07 (3.86-8.28) | 0.68 |
| pRaf (ser259) AIPC | Below Median | 6.07 (5.03-7.11) | |
| | Above Median | 4.39 (0.77-8.01) | 0.32 |
| pRaf (ser338) nuclear ASPC | Below Median | 6.26 (3.61-8.91) | |
| | Above Median | 6.65 (5.37-7.93) | 0.29 |
| pRaf (ser338) cytoplasm ASPC | Below Median | 6.57 (4.02-9.12) | |
| | Above Median | 6.04 (5.23-6.85) | 0.23 |
| pRaf (ser338) nuclear AIPC | Below Median | 5.51 (3.24-7.78) | |
| | Above Median | 6.65 (5.54-7.76) | 0.84 |
| pRaf (ser338) cytoplasm AIPC | Below Median | 6.07 (3.48-8.65) | |
| | Above Median | 6.65 (3.51-9.79) | 0.55 |

Raf-1 and pRaf expression (ASPC and AIPC), split into 2 subgroups - above and below median - is shown related to median overall survival in years (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 3b. Overall Survival (OS) and Raf-1 / pRaf expression divided according to rising histoscore with development of AIPC.

| | | OS (years) | P value |
|--------------------------------|------------|--------------------|---------|
| Raf-1 | Non-Risers | 6.07 (4.85 – 7.29) | |
| | Risers | 8.48 (-) | 0.88 |
| pRaf (ser259) | Non-Risers | 6.07 (4.91-7.23) | |
| | Risers | 4.36 (1.88 – 6.84) | 0.88 |
| pRaf (ser338) nuclear | Non-Risers | 5.51 (3.07-7.95) | |
| | Risers | 7.55 (3.49-11.61) | 0.84 |
| pRaf (ser338) cytoplasm | Non-Risers | 6.65 (3.28-10.02) | |
| | Risers | 6.04 (3.78-8.30) | 0.31 |

Raf-1 and pRaf expression, split into 2 subgroups - risers and non-risers with the development of AIPC - is shown related to median overall survival in years (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 4a. Time to Relapse (TTR) and MAP Kinase / pMAP Kinase expression divided by median.

| | | TTR (years) | P value |
|------------------------------|--------------|--------------------|----------------|
| MAP Kinase (Nuclear) | Below Median | 2.47 (0.62-4.32) | |
| | Above Median | 2.36 (1.90-2.82) | 0.65 |
| MAP Kinase (Cyto) | Below Median | 2.36 (1.73-2.99) | |
| | Above Median | 2.57 (1.71-3.43) | 0.82 |
| pMAP Kinase (Nuclear) | Below Median | 2.13 (1.39-2.87) | |
| | Above Median | 2.62 (1.09-4.16) | 0.06 |
| pMAP Kinase (Cyto) | Below Median | 2.29 (1.87-2.72) | |
| | Above Median | 2.98 (1.49-4.46) | 0.08 |

Nuclear and cytoplasmic MAP Kinase and pMAP Kinase expression, split into 2 subgroups - above and below median - is shown related to median time to relapse in days (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 4b. Time to Relapse and MAP Kinase / pMAP Kinase expression divided according to rising histoscore with development of AIPC.

| | | TTR (years) | P value |
|------------------------------|------------|------------------|---------|
| MAP Kinase (Nuclear) | Non-Risers | 3.26 (1.94-4.58) | |
| | Risers | 1.77 (1.39-2.15) | 0.10 |
| MAP Kinase (Cyto) | Non-Risers | 2.35 (1.42-3.28) | |
| | Risers | 2.36 (1.71-3.01) | 0.94 |
| pMAP Kinase (Nuclear) | Non-Risers | 2.57 (1.39-3.76) | |
| | Risers | 2.47 (1.20-3.75) | 0.25 |
| pMAP Kinase (Cyto) | Non-Risers | 2.62 (1.60-3.65) | |
| | Risers | 2.25 (1.42-3.07) | 0.06 |

Nuclear and cytoplasmic MAP Kinase expression, divided into 2 subgroups – risers and non-risers with the development of AIPC - is shown related to median time to relapse in days (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 5a. Time to Death from Relapse (TTDR) and MAP Kinase / pMAP

Kinase expression divided according to median.

| | | TTDR (days) | P value |
|---------------------------------------|--------------|------------------|---------|
| MAP Kinase (Nuclear) AIPC | Below Median | 1.87 (1.11-2.63) | |
| | Above Median | 1.60 (0.83-2.37) | 0.73 |
| MAP Kinase (Cyto) AIPC | Below Median | 2.47 (0.03-4.90) | |
| | Above Median | 1.65 (1.12-2.18) | 0.40 |
| pMAP Kinase (Nuclear) AIPC | Below Median | 1.65 (0.81-2.48) | |
| | Above Median | 1.87 (1.03-2.71) | 0.42 |
| pMAP Kinase (Cyto) AIPC | Below Median | 2.15 (1.44-2.86) | |
| | Above Median | 1.60 (1.25-1.96) | 0.86 |

Nuclear and cytoplasmic MAP Kinase expression (ASPC and AIPC), split into 2 subgroups - above and below median - is shown related to median time to death from relapse in days (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 5b. Time to death from relapse (TTDR) and MAP Kinase / pMAP Kinase expression divided according to rising histoscore with development of AIPC.

| | | TTDR (years) | P value |
|------------------------------|-------------------|-------------------------|---------------|
| MAP Kinase (Nuclear) | Non-Risers | 3.00 (1.43-4.57) | |
| | Risers | 1.40 (1.20-1.61) | 0.0255 |
| MAP Kinase (Cyto) | Non-Risers | 2.15 (1.14-3.16) | |
| | Risers | 1.58 (0.70-2.46) | 0.61 |
| pMAP Kinase (Nuclear) | Non-Risers | 1.62 (1.17-2.08) | |
| | Risers | 3.08 (1.92-4.24) | 0.58 |
| pMAP Kinase (Cyto) | Non-Risers | 1.65 (1.09-2.21) | |
| | Risers | 2.47 (1.05-3.88) | 0.36 |

Nuclear and cytoplasmic MAP Kinase expression (ASPC and AIPC), divided into 2 subgroups – risers and non-risers with the development of AIPC - is shown related to median time to death from relapse in days (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis. Significant correlations are highlighted.

Table 6a. Overall Survival (OS) and MAP Kinase expression divided according to median.

| | | OS (years) | P value |
|---------------------------------------|--------------|-------------------|----------------|
| MAP Kinase (Nuclear) ASPC | Below Median | 6.07 (5.06-7.08) | |
| | Above Median | 6.65 (5.76-7.54) | 0.60 |
| MAP Kinase (Nuclear) AIPC | Below Median | 6.04 (3.93-8.15) | |
| | Above Median | 6.26 (3.98-8.54) | 0.45 |
| MAP Kinase (Cyto) ASPC | Below Median | 7.68 (4.71-10.65) | |
| | Above Median | 6.50 (5.50-7.50) | 0.62 |
| MAP Kinase (Cyto) AIPC | Below Median | 6.04 (4.93-7.15) | |
| | Above Median | 6.50 (3.48-9.52) | 0.92 |
| pMAP Kinase (Nuclear) ASPC | Below Median | 6.07 (3.65-8.49) | |
| | Above Median | 6.50 (5.26-7.74) | 0.39 |
| pMAP Kinase (Nuclear) AIPC | Below Median | 4.92 (2.36-7.48) | |
| | Above Median | 6.44 (5.19-7.69) | 0.69 |
| pMAP Kinase (Cyto) ASPC | Below Median | 6.07 (5.14-7.00) | |
| | Above Median | 6.50 (4.55-8.45) | 0.56 |
| pMAP Kinase (Cyto) AIPC | Below Median | 6.26 (3.94-8.58) | |
| | Above Median | 5.82 (3.54-8.10) | 0.83 |

Nuclear and cytoplasmic MAP Kinase expression (ASPC and AIPC), split into 2 subgroups - above and below median - is shown related to median overall survival in years (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis.

Table 6b. Overall survival (OS) and MAP Kinase expression divided according to rising histoscore with development of AIPC.

| | | OS (years) | P value |
|------------------------------|-------------------|-------------------------|---------------|
| MAP Kinase (Nuclear) | Non-Risers | 6.89 (5.70-8.08) | |
| | Risers | 3.37 (1.58-5.16) | 0.0068 |
| MAP Kinase (Cyto) | Non-Risers | 6.26 (5.47-7.05) | |
| | Risers | 6.89 (0-14.53) | 0.98 |
| pMAP Kinase (Nuclear) | Non-Risers | 6.57 (5.15-7.99) | |
| | Risers | 6.07 (5.52-6.62) | 0.98 |
| pMAP Kinase (Cyto) | Non-Risers | 6.57 (5.49-7.65) | |
| | Risers | 6.04 (3.21-8.87) | 0.60 |

Nuclear and cytoplasmic MAP Kinase expression (ASPC and AIPC), divided into 2 subgroups – risers and non-risers with the development of AIPC - is shown related to median overall survival in years (with inter-quartile ranges in brackets). Significance is shown by p values, assessed by Kaplan-Meier analysis. Significant correlations are highlighted.

APPENDIX 5

Significant Correlations Between the Cytoplasmic and Nuclear Expression of MAP Kinase and its activated, phosphorylated form in ASPC and AIPC tumours.

Table 7a. Correlations between MAP Kinase protein histoscores in ASPC tumours.

| | MAP Kinase (Nuclear) | MAP Kinase (Cytoplasm) | pMAP Kinase (Nuclear) | pMAP Kinase (Cytoplasm) |
|-------------------------------|-----------------------------|-------------------------------|------------------------------|--------------------------------|
| MAP Kinase (Nuclear) | | 0.013 (0.321) | <0.001 (0.570) | 0.014 (0.334) |
| MAP Kinase (Cytoplasm) | | | 0.083 (0.238) | <0.001 (0.523) |
| pMAP Kinase (Nuclear) | | | | <0.001 (0.703) |

Table 7a shows correlations (p values) between the cytoplasmic and nuclear expression of MAP Kinase and its' activated, phosphorylated form, in ASPC tumours. Significant correlations (p<0.05) are highlighted. P values were assessed by Spearman's Rank Correlation Test, and correlation co-efficients are shown in brackets.

pMAP Kinase, activated phosphorylated MAP Kinase

Table 7b. Correlations between MAP Kinase protein histoscores in AIPC tumours.

(Correlation co-efficients in brackets)

| | MAP Kinase (Nuclear) | MAP Kinase (Cytoplasm) | pMAP Kinase (Nuclear) | pMAP Kinase (Cytoplasm) |
|------------------------|----------------------|------------------------|-----------------------|-------------------------|
| MAP Kinase (Nuclear) | | <0.001 (0.613) | 0.24 (0.156) | 0.39 (0.113) |
| MAP Kinase (Cytoplasm) | | | 0.037 (0.273) | 0.01 (0.335) |
| pMAP Kinase (Nuclear) | | | | <0.001 (0.697) |

Table 7b shows correlations (p values) between the cytoplasmic and nuclear expression of MAP Kinase and its' activated, phosphorylated form, in AIPC tumours. Significant correlations ($p < 0.05$) are highlighted. P values were assessed by Spearman's Rank Correlation Test, and correlation co-efficients are shown in brackets.

pMAP Kinase, activated phosphorylated MAP Kinase.

APPENDIX 7

Full List of Presentations

Mukherjee R, Krishna N S, Underwood M, Bartlett J M S, Edwards J.
The Raf/MAP Kinase Pathway Influences Survival in Androgen-Insensitive Prostate Cancer
American Urological Association Annual Meeting-Podium Presentation (San Antonio, May 2005).

Mukherjee R, Krishna N S, Edwards J, Underwood M, Bartlett J M S.
Type 1 Receptor Tyrosine-Kinase Activation of Raf-1 in Androgen-Escaped Prostate Cancer.
A Novel Target for Future Therapy?
American Urological Association Annual Meeting (San Francisco, May 2004).

Mukherjee R, Edwards J, Krishna N S, Underwood M, Bartlett J M S.
The MAP Kinase Pathway in Prostate Cancer – A Role in Hormone-Escape?
European Association of Urology Annual Congress-Podium Presentation (Vienna, March 2004).

Mukherjee R, Edwards J, Underwood M, Bartlett J M S.
Raf-1 Expression is Related to Early Hormone-Relapse in Prostate Cancer.
5th World Congress of Urological Research (London, September 2003).

Mukherjee R, Edwards J, Underwood M, Bartlett J M S.
COX-2 Expression and Angiogenesis in Prostate Cancer.
British Prostate Group Annual Meeting (Edinburgh, April 2003).

Mukherjee R, Edwards J, Underwood M, Bartlett J M S.
COX-2 Expression in Prostate Cancer.
Urological Research Society Annual Meeting (London, January 2003).

Mukherjee R, Edwards J, Wells A C, Almushatat A, Bartlett J M S.
Expression of HER2 and COX-2 in Prostate Cancer.
PROSTATE 2002 (Liverpool, September 2002).

Mukherjee R, Edwards J, Underwood M, Bartlett J M S.
Angiogenic Pathways in Hormone-Relapsed Prostate Cancer – A Target For Future Therapy?
Scottish Urological Society (Dunfermline, March 2002).

Mukherjee R, Edwards J, Krishna N S, Underwood M, Bartlett J M S.
The MAP Kinase Pathway in Prostate Cancer – A Role in Hormone-Escape?
West of Scotland Urology Group (Stobhill Hospital, March 2004).

