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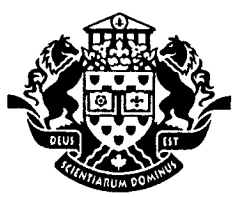

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Prediction of Recurrence in Prostate Cancer Following Radiotherapy: Value of Biomarkers Microvessel Density, MIB-1, P-53, BCL2, and Bax

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Prediction of recurrence in prostate cancer following radiotherapy: Value of biomarkers microvessel density, MIB-1, P-53, BCL2, and Bax

Submitted by: Simone Dahrouge

Thesis supervisor: George Wells, PhD

Co-supervisors: Nick Birkett, MD, Shawn Malone, MD

Abstract

Background: Standard traditional parameters relied on for estimating the risk of disease recurrence after curative radiotherapy in prostate cancer are stage, Gleason score and PSA.

Objectives: To elucidate the prognostic role of biomarkers: P-53, MIB-1, MVD, Bax and BCL2 in prostate cancer.

Method: Cox proportional hazard model was used to estimate the risk of disease progression associated with these biomarkers and develop models based on traditional parameters only or incorporating biomarkers. Models were compared for their predictive potential using Akaike information criteria and concordance index.

Results: Statistically significant associations were found between all biomarkers and risk of progression. MVD, Bax and Bax/BCL2 were independent predictors of outcome. Models incorporating biomarkers were superior to the traditional one in identifying patients at risk of local progression.

Conclusions: Biomarkers may be useful in forecasting the risk of local recurrence following radiotherapy in prostate cancer patients. The results require validation in a separate cohort.

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List of Acronyms

-2LL	-2 Log likelihood
-ve	Negative
+ve	Positive
3D CRT	Three dimensional conformal radiotherapy
ADT	Androgen deprivation therapy
BCL2	B-cell leukemia/lymphoma 2
CI	Confidence intervals
EBRT	External beam radiotherapy
EORTC	European Organisation for Research and Treatment of Cancer
Gy	Grays
HIER	Heat Induced Epitope Retrieval
LL	Log likelihood
LR	Likelihood ratio
MO	Months
MVD	Microvessel density
ORCC	Ottawa Regional Cancer Centre
PAP	Peroxidase antiperoxidase
PFS	Progression free survival
PH	Proportional hazard
PSA	Prostatic specific antigen
ROC	Receiver operating characteristic
RTOG	Radiation Therapy Oncology Group
TRUS	Trans Urethral US
XRT	Radiotherapy
Yr	Year

1. INTRODUCTION

Prostate cancer makes up 26% of all newly diagnosed cancers amongst Canadian men. In 2002, 18,200 new cases will be diagnosed in Canada. The risk of prostate cancer increases dramatically with age(1,2). As a result, the number of new cases of prostate cancer is expected to triple in the twenty-five year period during which the "baby boomer" population becomes seniors(3).

Prostate cancer that has not spread to adjacent nodes or outside the pelvic area is potentially curable by external beam radiotherapy (EBRT). The five year progression free survival of patients treated with EBRT ranges between 50-82%, depending on the extent of disease burden at the time of diagnosis and pathological features of the tumor(4,5). Under the current standard treatment approach, patients receive 66-78 Grays (Gy) to the small field in the pelvic area that contains the prostate using three dimensional conformal radiotherapy techniques(6,7). Patients considered at higher risk of recurrence may be eligible for high doses of radiotherapy and may be given adjuvant androgen deprivation therapy for a period of 3 months or more, depending on their estimated risk level(8,9). Because these approaches can increase the risk of morbidity associated with treatment, they should be reserved for those patients who can be reliably identified to have a significantly elevated risk of recurrence.

Clinicians currently rely on the patient's stage, tumor grade (Gleason score: a measure of the tumor's differentiation level; or having the appearance of its tissue of origin) and pre-treatment prostatic specific antigen (PSA) scores (a measure related to overall tumor burden) to establish risk levels. In Canada, clinicians use a nomogram that is based on these factors to categorize patients into low, intermediate or high risk levels(10). Other more

sophisticated models have been developed, but despite their complexity they still have limited predictive ability(11,12). Another limitation of currently available models is that they do not distinguish between the local and distant recurrences(12-20). Patients treated with EBRT may recur within the irradiated field (local recurrence) or may develop metastatic disease (distant recurrence). If a patient is known to have an elevated risk of recurrence within the irradiated field, the local management approach selected for that patient may be more aggressive. For example, higher doses of radiotherapy may be given, or radiotherapy may be administered with a radiosensitizer to enhance its tumoricidal effect. In contrast, this more aggressive approach would likely have no impact on the risk of developing metastatic disease and would produce unnecessary morbidity. Therefore, if predictive models are to be used to plan therapy, the site of recurrence is considered by the author an essential component that must be incorporated in the model development.

Investigators have sought new factors that could improve the prediction of recurrence. In the past decade, an increasing volume of information has become available suggesting that biomarkers may play a role in determining the recurrence risk level in cancer patients. The National Institute of Health defines a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Potentially valuable biomarkers in the prediction of outcome in prostate cancer include microvessel density (MVD), Ki-56 (detected by MIB-1), P-53, Bax and B-cell leukemia/lymphoma 2 (BCL2).

Microvessel density is a measure of tumor tissue vascularization level. All normal tissue is vascularized to support its nutritional and oxygen requirements. Growing tissue, like neoplasms, must stimulate the production of new blood vessels (angiogenesis) to

survive(21). The density of that new vasculature has been found to be associated with a more aggressive phenotype in various cancers(22-25), including prostate cancer(26).

Normal tissue homeostasis is achieved by a well-orchestrated balance of cell proliferation and apoptosis (programmed cell death). Cancer develops from an uncontrolled cell proliferation. Biological factors that have a role in cell cycle regulation may therefore influence the risk of cancer growth or re-growth following treatment. Examples of these cell cycle regulators include P-53, a tumor suppressor gene, and BCL2 and Bax, apoptosis regulators.

P-53 is important in the regulation of cell growth because it controls cell cycle duration, DNA repair, and apoptosis(27). P-53 mutations are frequently found in cancer tissue(28-33). Overexpression of mutated P-53 has been associated with radioresistance in some(34,35) but not all(36,37) prostate cancer studies.

BCL2 is an oncoprotein that inhibits apoptosis, and Bax is an apoptosis inducer. Overexpression of BCL2 promotes oncogenesis by inhibiting cell death and extending cell life. Overexpression of BCL2 was associated with reduced disease control in two studies following EBERT(34,38), but an improved outcome in another prostate cancer study(39). Bax has been poorly studied in prostate cancer.

Ki-67 is nuclear antigen associated with cell division detected. Because ki-67 is elevated during cell division, it is postulated to reflect biological aggressiveness(40). Ki-67 expression levels are determined by MIB-1 antibody staining. A larger proportion of cell staining positive for MIB-1, reflecting the presence of higher ki-67 levels, was found associated with a higher risk of local recurrence in prostate cancer(41). Specimens from

locally recurrent tissue were also observed to have a ki-67 measure of proliferation rate two times higher than their pre-treated counterparts(42).

In sum, there is evidence that the biological markers MVD, P-53, Bax, BCL2 and MIB-1 may be useful in helping to estimate the risk of disease recurrence following EBRT in prostate cancer. The present study aims to determine whether these biomarkers add to the predictive value of the traditional factors relied upon (stage, PSA and Gleason score) in estimating recurrence risk level in prostate cancer patients treated curatively with EBRT. This was studied in a cohort of patients having received EBRT at the Ottawa Regional Cancer Center (ORCC). Because of the reasons presented above, the value of the biomarkers in predicting local progression and distant progression were assessed separately. To allow a comparison to be made with published models, the predictive value of these markers in quantifying the risk of biochemical progression was also studied. Biochemical progression is detected based solely on PSA levels without regard to the site of progression. The objectives of the present study were to determine whether any association between the biomarkers and outcome was present, and to evaluate whether the information provided by the biomarkers supplemented that already available from the traditional factors of stage, Gleason score and pre-treatment PSA levels; all of which are currently standard assessments performed at diagnosis.

To do so, models for the prediction of each outcome that is based on the traditional factors only were developed. The analysis strived to optimize the predictive ability of each variable by closely studying its relationship with outcome and selecting the best traditional factor variable transformation to include in the model. Independently predictive biomarkers were then added to the traditional factor model, and the larger biomarker model was

compared to the nested traditional factor model to determine whether its predictive value was superior.

2. BACKGROUND

2.1 Epidemiology

Prostate cancer is the most commonly diagnosed malignancy in North American men. In Canada, prostate cancer makes up 26% of all newly diagnosed cancers in men. In comparison, lung and colorectal cancer represent 18% and 14% of all cancers, respectively. Based on Health Canada estimates, 18,200 new cases were diagnosed in 2002, and the current lifetime probability of developing prostate cancer is 12%(1).

An individual's genetic predisposition plays a significant role in the risk of developing prostate cancer. Studies have reported that Americans of African descent are two times more likely to develop prostate cancer than white North American men, and are 50 times more likely to be diagnosed with prostate cancer than Chinese men(2,43,44). Environmental factors are also implicated in risk determination, but these are not yet well understood. Diet, exercise and smoking are some of the environmental factors cited as influencing risk(45,46). Supporting this notion, migration studies have reported that, while Asian men are generally at a lower risk of developing prostate cancer, the risk for Asian immigrants' progeny living in North America and having adapted local culture eventually reaches that of the American nation(45).

Within North America, by far, the largest determinant of risk is age(1,2). In Canada, the probability of being diagnosed with prostate cancer is 60 times higher amongst men between the ages of 70 and 79 years (6.1%) compared to men between the ages 40 and 49 years (0.1%). In contrast, the comparable magnitude of risk increase for lung or colorectal cancer is 15 to 20 times(1). The crude incidence of prostate cancer is dramatically increasing, in

part owing to the increase in the prevalence of PSA testing and the incidental detection of carcinoma through TURP and in part due to the aging Canadian cohort (47,48). The incidence amongst Canadian men is projected to triple between the years 1990 and 2016 as the population continues to age, thereby considerably increasing the burden to our health care system associated with that disease(3).

2.2 Treatment and outcome

Prostate cancer clinically confined to the prostate is curable. Standard treatments for localized prostate cancer include prostatectomy and EBRT, with both approaches providing comparable disease control(49,50). The five year progression free survival (PFS) following standard EBRT ranges between 50-82%, depending on the extent of disease burden at the time of diagnosis and on the pathological features of the tumor(4,5). Three dimensional conformal radiotherapy (3D CRT) is a precise form of EBRT that enables irradiation of tumorous tissue with reduced normal tissue exposure and, consequently, morbidity(6,7). The advent of 3D CRT in the past decade has permitted higher doses to be delivered to the prostate, and has produced significant improvement in PFS and overall survival(4,51,52). Studies suggest that the 5 year PFS amongst patients with a predicted 50% risk could potentially be improved to 75%-85% if higher doses of radiation are delivered(4,53-55).

Following the discovery in the early 1980s that prostate cancer was sensitive to testosterone levels (in a similar way that breast cancer is dependent on estrogen for its growth), androgen deprivation therapy (ADT) was introduced in the management of disseminated prostate cancer. Prostate cancer cells deprived of testosterone through ADT undergo apoptosis. Historically, androgen deprivation was realized through surgical castration. Today, ADT is achieved by medical castration consisting of the combined use of

an oral steroidal or non-steroidal anti-androgen agent combined with parenteral injections of a luteinizing hormone-releasing hormone agonist. ADT is currently the standard first line treatment of patients with metastatic disease and achieves one-year progression free control of 50% in that patient population(56). In the 1990s, ADT was studied as an adjunct therapy to curative EBRT. Prospective randomized Radiation Therapy Oncology Group (RTOG) and European Organization for Research and Treatment of Cancer (EORTC) trials have recently demonstrated that the combination is superior to EBRT alone(8,9). The most impressive risk reduction gained was in patients with intermediate or high risks of recurrence(57).

Today, innovative approaches such as anti-angiogenic therapy and gene therapy are being investigated for their roles in controlling the growth and spread of prostate cancer and are showing promising results(58,59). Because of the morbidity generally associated with more aggressive therapy like higher doses of EBRT or prolonged adjunct ADT, these approaches should be reserved for patients with a significantly elevated risk of recurrence. Improved tools are required to more reliably identify these patients.

2.3 Traditional factors

The current standard nomograms for establishing individual patients' risk levels are based primarily on three tumor-related assessments available at the time of diagnosis: tumor Gleason score, clinical tumor stage, and baseline PSA levels.

The Gleason score is a tumor grading system constructed by Donald Gleason to measure the extent of cell differentiation, a measure of the proliferative potential of the tumor(60). More differentiated cells (i.e. having the appearance of their tissue of origin) have less

proliferative potential than cells with a less differentiated morphology (appearing more like stem cells). Prostate cancer tissue is heterogeneous, and within a tumorous tissue section, more than one level of differentiation may be observed. For that reason, the Gleason score is a summated measure of the two most prominent architectural patterns that reflect the differentiation levels, each scored on a scale of 1 to 5. The Gleason score is therefore reported on a scale of 2 to 10, with 10 representing the least differentiated, most malignant form. The Gleason score measured from prostatectomy specimens is especially valuable in identifying patient risk level. However, for patients receiving EBRT, Gleason score must be determined from the small tissue block samples derived from biopsy specimens. These estimates are less reliable and are reported to underestimate the true grade(61,62). Despite this limitation, the Gleason score has been shown to be a useful independent predictor of outcome following treatment with EBRT in most studies (63-66), but not in all(67,68).

Clinical tumor stage is a measure of the extent of tumor spread(69). Like all solid tumors, prostate cancer is staged according to three factors: the extent of organ involvement at the primary site (T1-T4); the spread to regional lymph nodes (N0-2); and the presence of distant metastases (M0-1). In the absence of resection, as is the case for patients treated with EBRT, clinicians cannot rely on a pathological evaluation of the tumor and excised nodes to determine stage. Instead T and N stages are determined clinically, a measure predictably less reliable than a pathological determination(70). The extent of spread to the regional lymph node (N status) can be inferred only by imaging studies. The absence of abnormalities within the regional lymph node on CT scan suggests a lack of nodal involvement. Since this has not been confirmed pathologically, the staging nomenclature is Nx (instead of N0 which is traditionally reserved for the pathologically confirmed absence

of regional lymph node involvement). Because of the localized nature of radiotherapy, it has traditionally been reserved for patients with disease localized to the prostate and immediately adjacent tissue. However, enlarged treatment fields that encompass regional lymph nodes have recently been introduced in an attempt to curatively treat patients with suspected nodal involvement (N1-2). Therefore, until recently, patients undergoing EBRT had to be deemed Nx and M0.

The presence of distant metastases is only investigated in patients at high risk of having metastatic seeds as indicated by PSA levels greater than 10 ng/ml. Since bone is the principal site of distant spread for prostate cancer, the main imaging evaluation required to confirm the absence of metastatic disease (M0) is a bone scan. Patients with evidence of metastatic disease are treated palliatively with ADT. Patients are required to be M0 to receive curative EBRT.

Therefore, stage assignment for patients undergoing EBRT is only based on the extent of organ involvement by the primary tumor (T staging) as determined by clinical studies. The clinical T stage classification according to the 1992 edition of the TNM prostate cancer staging system is as follows(69):

T1 – Clinically inapparent tumor; not palpable or visible. These are limited to tumors identified on biopsy only which was indicated by elevated PSA levels at screening.

T2 – Tumor confined within the prostate. The extent of prostatic involvement determines the subcategory of T2:

T2a – Involving $< 50\%$ of one lobe

T2b – Involving $\geq 50\%$ of one lobe

T2c – Involving both lobes.

T3 – Tumor extending through the capsule or invading the seminal vesicles.

T4 – Tumor fixed to or invading adjacent structures other than seminal vesicles.

Clinically determined T stage is directly associated with the risk of progression following EBRT(57,66,71). More recently, the distinction between T2a and b has been abolished because of the apparently poor reliability in assessing the extent of lobar involvement. However, at the time this project was initiated, this distinction was still being used and, as the results will demonstrate, remains relevant.

PSA is a glycoprotein produced at low levels by normal prostatic tissue. Because it is abnormally elevated in individuals with prostate cancer, it is being studied in screening programs of prostate cancer. However, PSA screening has mediocre specificity, and its use in that setting remains controversial(72). The uncontroverted utility of PSA level remains in predicting the risk of recurrence with curative therapy, and in detecting the presence of that recurrence following curative therapy. PSA levels measured at the time of diagnosis (pre-treatment PSA levels) have been well documented to be highly predictive of the progression free and disease specific survivals following curative EBRT(20,68,73). During and after EBRT, PSA levels will drop as cancerous prostate cells are abolished. A consistent rise in PSA indicates that the disease bulk is increasing, and reflects disease progression (see section 3.4.1)(74).

While treatment itself is not considered a prognostic factor, it must be considered in studies where it was heterogeneous. The standard approach to EBRT at the ORCC during the period studied in this report was modified in keeping with changes in standards. Until the early 1990s, patients were treated with daily fractions of 1.8 Gy for 36 days, and received a total dose of 64.8 Gy. Subsequently, the daily fraction dose was increased to 2.0 Gy and the treatment was delivered for 33 days over 6.6 weeks, for a total dose of 66 Gy. Despite the apparent similarity in total dose level, the tumoricidal potential of the lower

daily fraction size (1.8 Gy) is smaller. Radiobiologically, a total dose 64.8 Gy delivered at 1.8 Gy daily fractions is equipotent to 60 Gy delivered at 2.0 Gy fractions(75). More recently, escalated doses of 68-78 Gy (2.0 Gy daily) have been used because they have been demonstrated to provide better control(4,51,52), but few patients received that treatment during the period covered by the present analysis.

Although age has only rarely been demonstrated a significant predictor of recurrence in prostate cancer(15,18), because it is important in estimating the risk of death associated with prostate cancer, studies continue to evaluate its risk in predicting progression. Therefore, while age is not expected to be a significant factor in predicting recurrence following EBRT, age will be included in the analysis to ensure that all patient information readily available prior to EBRT is considered in the traditional factor model.

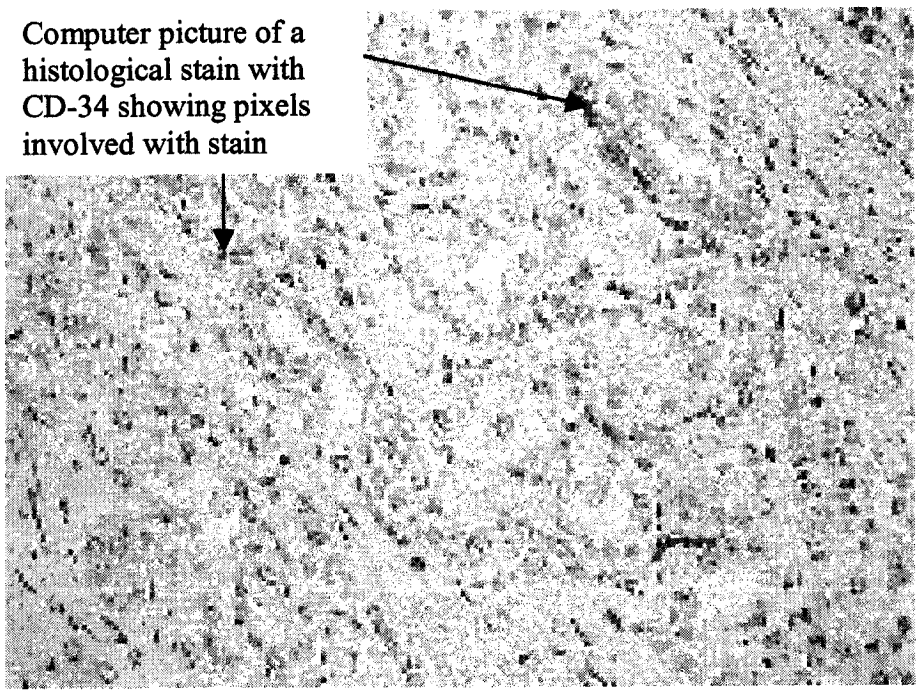
2.4 Biomarkers

Biomarkers have been implicated in the risk of developing cancer as well as the risk of recurrence following curative treatment in various cancer types. There is a wealth of studies reporting the value of biomarkers in predicting failure following prostatectomy(76), but fewer considered patients treated by radiotherapy. Understanding the prognostic value of biomarkers in patients treated with EBRT could lead to improved prediction of disease recurrence and could potentially be valuable in developing innovative treatment approaches. Potentially promising biomarkers include MVD, MIB-1, P-53, Bax and BCL2. A brief review of these biomarkers follows.

All normal tissue is vascularized to support its nutritional and oxygen requirements. When new tissue is formed, as in tumor growth, signals are released that stimulate the

development of new vasculature (angiogenesis). In fact, induction of neovascularization is required for the transition from benign growth to neoplasia(21). Angiogenesis has also been shown to be a pre-requisite for prostate cancer growth and development of metastases(77). The most common technique used to assess the tumor's neo-vascularization levels is the immunohistochemical evaluation of endothelial cells presence level. Endothelial cells make the vasculature wall. One measure of the vascular density is derived from the extent of staining by the monoclonal antibody to vascular endothelial cells CD-34. In the present study, the microvessel density was detected by an automated process that estimated the proportion of pixels on a computer screen with CD-34 stain. An example of this is shown in Figure 1. Higher levels of MVD have been found to be significantly correlated with disease progression in several prostatectomy studies (22,78-83) and one EBRT study (26).

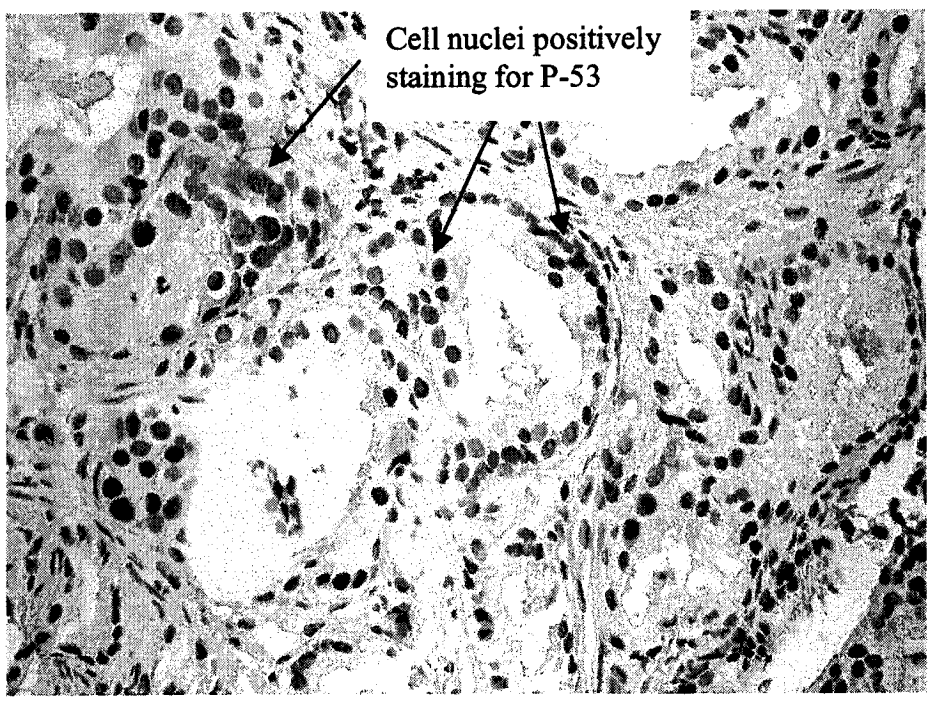
Figure 1: Microvessel density in prostate cancer tissue section.



Histochemical section of prostate cancer tissue stained with endothelial antibody CD-34 showing tumor vasculature measured by an automated electronic process (x40).

P-53 is a tumor suppressor gene that plays a role in cell cycle control, DNA repair, and apoptosis(27). The P-53 protein is important in the regulation of cell growth. P-53 mutations are a common genetic alteration found in several human cancers(84). Mutated P-53 is theorized to have lost its ability to control cell growth, potentially resulting in or promoting neoplasm development. In keeping with this hypothesis, studies have shown that mutated P-53 levels correlate with stage as well as tumor differentiation in prostate cancer (28-33,85). The mutated, but not normal, form of P-53 protein is detectable immunohistochemically. An example of P-53 stain is found in Figure 2. P-53 overexpression was found to be significantly associated with radioresistance in some prostate cancer studies (34),(35) but not all (36,37).

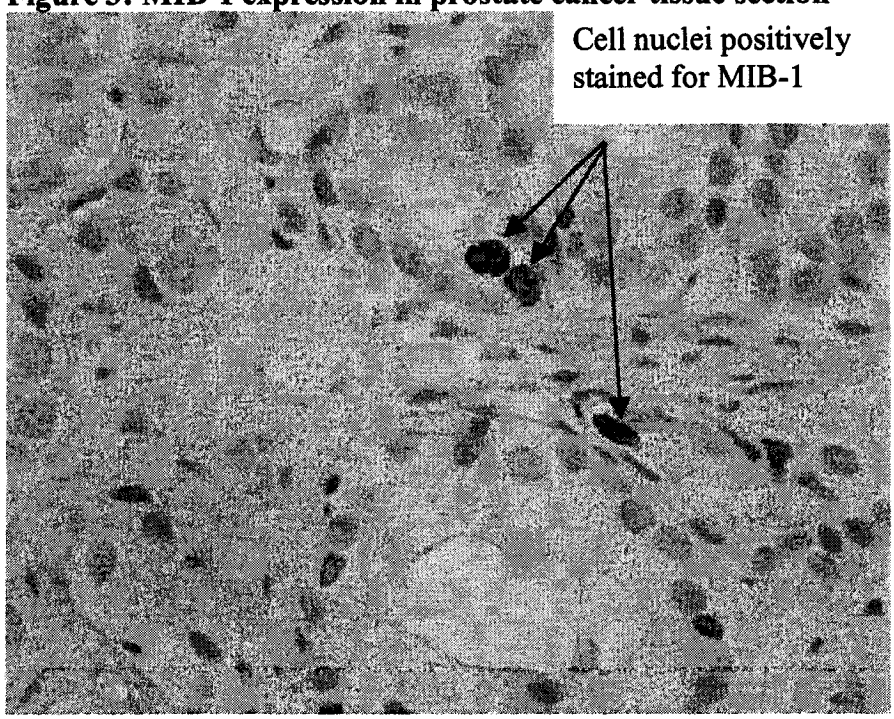
Figure 2: P-53 expression in prostate cancer tissue section



Histochemical section of prostate cancer tissue stained with Novacastra monoclonal antibody DO7 showing presence of mutated P-53 protein in a small proportion of cell nuclei (x40).

Tumor cells divide at different rates. Ki-67 is a nuclear antigen elevated during cell division. Ki-67 is measured using the MIB-1 antibody. Reports of this test in the literature use the two names interchangeably. Examples of MIB-1 staining are found in Figure 3. MIB-1 is an indicator of the proliferation rate, and therefore, is expected to reflect biological aggressiveness(40). Clinical evaluations of MIB-1 in prostate patients receiving EBRT suggest that the MIB-1 as a measure of proliferation rate is a predictor of disease recurrence. One study reported that patients with recurrences following EBRT were more likely to have elevated MIB-1 activity in their pre-treatment specimens (78%) compared to patients without recurrence (32%)(41). Another study found that specimens from locally recurrent tissue have a proliferation rate two times higher than their pre-treated counterparts(42).

Figure 3: MIB-1 expression in prostate cancer tissue section

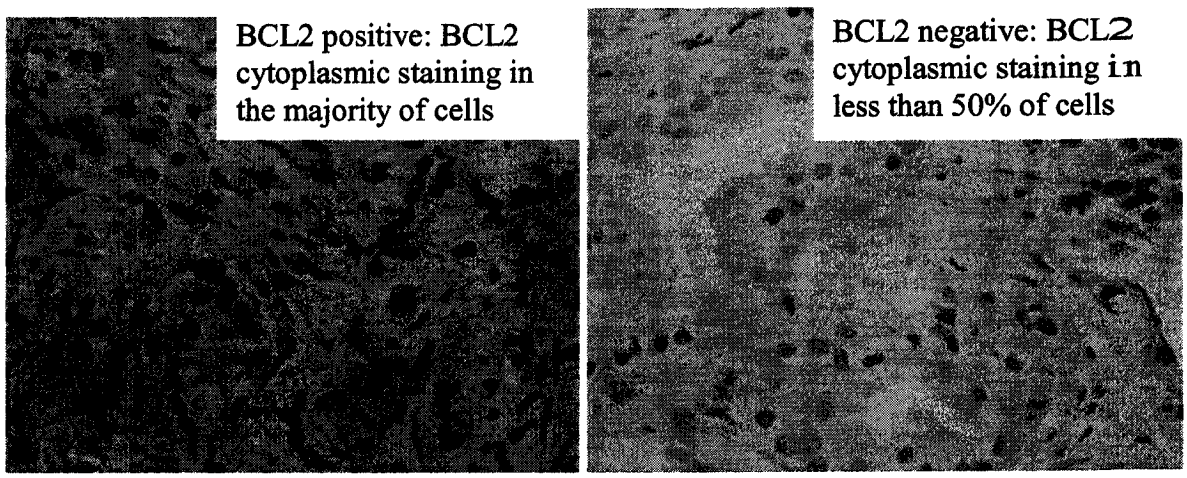


Histochemical section of prostate cancer tissue stained with monoclonal antibody to MIB-1 demonstrating presence of MIB-1 in 3 of positive cell nuclei (x40)

Normal tissue homeostasis is maintained by a balance between cell proliferation and apoptosis. BCL2 is an oncoprotein that inhibits apoptosis. Therefore, overexpression of

BCL2 promotes oncogenesis by inhibiting cell death and extending cell life. Overexpression can also lead to increased cell cycle duration by prolonging of the G1 phase of the cycle. BCL2 immunoreactivity (see Figure 4) was found to be significantly associated with outcome following EBRT in several studies, although reports of its predictive effect are conflicting. One study described BCL2 immunoreactivity to be associated with a better disease control(39) while others report it associated with a higher risk disease progression in EBRT(34,38). BCL2 is also believed to play a role in response to ADT. One study found that cancer growth inhibition following castration of nude mice was significantly higher in BCL2 negative tumors compared to BCL2 overexpressing tumors(86).

Figure 4: BCL2 expression in prostate cancer tissue section

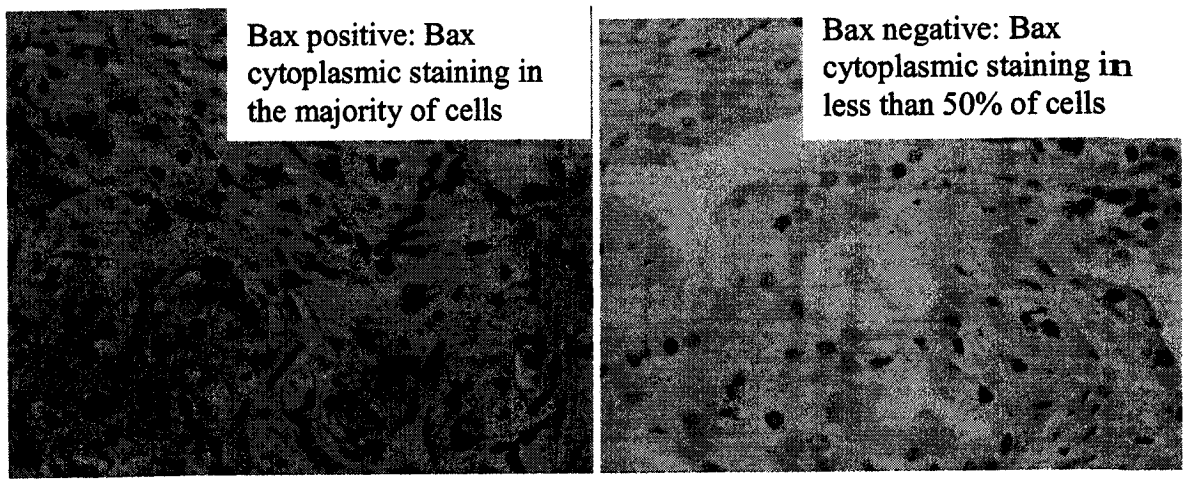


Histochemical sections of BCL2 immunoreactive prostate cancer tissue stained with monoclonal antibody for BCL2 showing BCL2 positive and negative sections (x40)

Bax protein regulates apoptosis in cellular pathways involving both BCL2 and P-53. While BCL2 is an apoptosis suppressor, Bax is an apoptosis inducer. Bax has not been extensively studied in prostate cancer treated with EBRT. One study found that Bax

immunureactivity (Figure 5) may be marginally predictive of good disease control, and suggested an interaction between the two apoptosis regulators BCL2 and Bax in predicting outcome(38).

Figure 5: Bax expression in prostate cancer tissue section



Histochemical section of Bax immunoreactive prostate cancer tissue stained with Coulter monoclonal antibody to Bax

2.4 Prediction models

Prediction models are nomograms or equations that rely on prognostic factors to help distinguish those patients in whom the treatment planned is more likely to be successful from those that could potentially benefit from more aggressive treatment. Currently available models have limited clinical usefulness in predicting outcome for prostate cancer patients treated with EBRT for two reasons. The first is that the current prognostic factors can only explain some of the variability in outcome, and the second is that the choice of outcome measure studied.

All models currently available to predict outcome following EBRT were developed to estimate the risk of biochemical progression. While a rise in PSA is indeed usually the first indication of treatment failure, it does not identify the site of recurrence. Patients treated with curative radiotherapy may suffer a local relapse (within the site of radiotherapy) or develop distant metastases, both of which are accompanied by a rising PSA. The biological basis for tumor regrowth at the site of original disease (local failure) and for dissemination of disease (distant progression) are different. Therefore, combining these two outcomes into a single category (biochemical or PSA failure) would confound the effect of a factor on one outcome by its effect (or lack of) on the other outcome. Also, if a model does not distinguish between treatment failure within the irradiated field and disease recurrence outside that area, it cannot be effectively used to determine what change in the management of the patient would potentially improve control. For example, patients at a higher risk of local failure may benefit from more aggressive local treatment like higher doses of EBRT or use of a radiosensitizer in combination with EBRT, while patients at higher risk of developing distant metastatic disease may have better disease control if a systematic form of therapy is used adjunctively to EBRT.

I propose that models should be developed separately for the prediction of local and distant progression. I postulate that biomarkers associated with a generally more aggressive phenotype would contribute to the overall risk of disease recurrence, regardless of site, whereas those conferring radioresistance would identify patients at risk of local but not necessarily distant recurrences, and those facilitating dissemination and survival of metastatic seeds would be associated with a higher risk of distant progression.

All algorithms/models currently available for the prediction of treatment failure following EBRT consider PSA levels, Gleason scores and clinical T stage(12,13,13-18,87,88) and, in some cases, age(15,18). All these measures are routinely available at the time of diagnosis and, because they have been demonstrated very useful, they have become part of standard patient care assessments. In a subset of studies, the model developed also relies on information that becomes available only after the start of therapy to make a determination about the probability of disease control. For example, the model developed by Ben-Josef and colleagues considered the nadir PSA level following EBRT as a covariate in the model. This measure is understandably highly predictive of the long term risk of recurrence since PSA nadir is itself a surrogate measure of outcome (87% sensitivity and 80% specificity)(15). However, since PSA nadir information only becomes available after the treatment selection has been made and treatment implemented, it cannot be used to make a prospective determination of the optimal treatment. Only models based on pre-treatment information can be used to select optimal management. In 1999, Vollmer studied several major models developed for predicting treatment failure following EBRT. He attempted to validate a subset of the best models in a cohort of 240 men treated with curative EBRT. Using a logistic regression analysis, he reported that the best model could only explain approximately 18% of the variance, and concluded that the models evaluated have limited usefulness. He suggested that models could be improved by adding new variables, such as biomarkers(11). More recently, Kattan and colleagues performed a similar evaluation of available models. They measured the validity and accuracy of various pre-treatment EBRT models, including one model developed by their group, in an independent patient cohort of 912 patients. Some of the models included in their analysis had been developed in very a

large cohort of patients and were expected to perform rather well. A measure of concordance was used to estimate the models' ability to discriminate between risk levels. That analysis compared models for their ability to select between two patients the one likely to have a better outcome. They reported on the concordance measure Somer's D, a value from which a more easily interpreted concordance measure, the c index, could be derived. A model without any predictive ability would randomly correctly identify the patient with the better outcome 50% of the time, and would produce a c index of 50%. Kattan reported Somer's D values for the models studied ranging between 30 and 52, indicating that the models could correctly identify the patient with the better outcome (c index) 65% to 76% of the time(12). His results demonstrate that currently available models have limited predictive ability, and other measures of disease aggressiveness are required to improve the predictive ability of models.

Since biomarkers have been shown to be associated with the risk of risk of biochemical recurrence, information derived from biomarker evaluation may improve the accuracy of a model that is based on traditional factors only. The majority of studies evaluating biomarkers in prostate cancer were conducted in patients undergoing prostatectomy(80,89-103). Fewer studies have been performed in patients receiving external beam radiotherapy(12-14,17,18,87,88).

To date, only two studies have incorporated biomarker information in model development. Both were performed in patients undergoing radical prostatectomy(97,104). Liebovich found P-53 to be an independent predictor of biochemical recurrence and reported a 5% improvement in the model's ability to discriminate risk level (c index) compared to the model excluding P-53 and based on traditional factors only(97). Bauer found P-53 and

BCL2 to be significant independent predictors of treatment failure, but did not quantify the improvement in predictive value by adding these markers to a model based on traditional factors only(104).

The present study is the only report of prediction model containing biomarkers performed in a cohort of patients receiving curative radiotherapy. Since each marker has a biologically distinct role in regulating cell growth and survival, it follows that their effects may be dependent on the type of therapy given, and therefore, interpretations must be limited to the context of that treatment type. More studies are required to elucidate the association between biomarkers and the risk of disease progression amongst patients receiving EBRT. These studies must also distinguish between the risks associated with local progression from those associated with distant progression so that therapy may be tailored appropriately.

For patients undergoing EBRT, the current supplemental therapeutic options include use of a short or prolonged course of neoadjuvant ADT and dose-escalated radiotherapy. Other adjunctive approaches are a prospect of the future. Scientists believe that targeted gene therapy is a plausible future therapeutic approach for prostate cancer(105), and current experimental studies support this notion. For example, if P-53 is demonstrated to be a risk factor in patients receiving EBRT, then patients expressing mutated P-53 proteins may be candidates for P-53 gene therapy. Pre-clinical evaluation of P-53 suggests that transfecting wild type P-53 into cells with the mutated gene reinstates the suppressive properties of its protein and resulted in significant cell growth inhibition and apoptosis(106,107), reduced risk of metastatic formations(106), and improved sensitivity to radiation in culture(108) and in *vivo*(109).

2.6 Objectives

The primary objective is to assess the value of biomarkers over that of traditional factors in predicting the risk of treatment failure following radical radiotherapy for prostate cancer.

The secondary objectives were 1) to elucidate the prognostic value of biomarkers in prostate cancer patients undergoing definitive radiotherapy (univariate analyses) and 2) to describe the relationship between traditional factors and biomarkers.

3. METHODOLOGY

3.1 Study design

This study is based on a retrospective cohort from a single large cancer treatment and research institution, the Ottawa Regional Cancer Center (ORCC). The ORCC is the only cancer treatment facility in the Capital region and serves a population of approximately one million in Eastern Ontario.

3.2 Patient population

In order to maintain a relatively uniform patient population, eligibility was limited to individuals meeting the following criteria:

1. Pathologically confirmed prostate carcinoma treated with curative intent at the ORCC.
2. Clinical stage T1-4, Nx (assumed N0) and M0.
3. Having received radical radiotherapy treatment (EBRT) at the ORCC between June 1987 and June 1997 (to ensure that adequate follow-up was available).
4. Availability of sufficient archival biopsy tissue for biomarker analysis (at least one marker).

Patients were required to meet all inclusion criteria. They were excluded if they had undergone radical prostatectomy or had suspected nodal disease. Patients having received adjuvant hormonal therapy were permitted in the study. In total, 199 patients were identified.

3.3 Data extraction

Data extraction was performed without the knowledge of biomarker expression level (and usually prior to that assessment). Approximately 75% of the data required for this study had been previously collected by a research assistant as part of an ongoing effort to capture patient clinical information for future research questions. For the purpose of the current study, information was extracted from this databank and tested for internal consistency. Missing information and unusual data were checked against original source documents (patient cancer clinic electronic and paper charts, and pathology reports of biomarker). Details of therapy and outcome were all independently reviewed and updated specifically for this analysis. The information collected is listed below.

Traditional characteristics:

1. Age: Date of diagnosis – Date of birth
2. Clinical stage: Recorded by the physician at the time of diagnosis
3. Gleason score: Available in the diagnostic pathology report derived from biopsy specimens
4. Pre-treatment PSA score: Last PSA evaluation prior to any form of therapy (hormonal or radiation)

Biomarkers (provided blinded by Dr. S Robertson (pathologist) using immunhistochemical studies of diagnostic biopsy specimens)

1. MVD: % pixel area involved by endothelial cells
2. MIB-1: Percent of cells stained positive
3. P-53: Percent of cells stained positive
4. Bax : Positive or negative

5. BCL2: Positive or negative

Treatment:

1. Adjuvant androgen deprivation therapy: Use and duration of ADT
2. Radiotherapy: Daily fraction size and total number of treatment fractions

Outcomes:

1. Biopsy status: Date of post therapy trans urethral ultrasound (TRUS) guided biopsy and pathological results throughout follow-up (coded as follows)
   a. Viable cells with little or no radiation effect (damage) – unequivocal positive biopsy
   b. Viable cells with moderate or severe radiation effect – Equivocal results, assumed negative, to re-evaluate
   c. No viable cells – Negative biopsy
2. Post therapy PSA levels – Date and serum level of total PSA throughout follow-up period
3. PSA progression status - Date and PSA progression status (occurred/did not occur), see outcome definition section 3.4.1
4. Local progression status – Date and local progression status (occurred/did not occur), see outcome definition section 3.4.2
5. Distant progression status – Date and distant progression status (occurred/did not occur/censored), see outcome definition section 3.4.3

3.4 Patient follow-up and outcome definition

Patients were followed with TRUS guided biopsies at two years following the end of radiotherapy, and then as clinically indicated by a rise in PSA or symptom development. PSA assessments were performed twice yearly for the first year, then yearly unless indicated

by symptoms. When a rise in PSA was documented, the interval between PSA assessments was usually reduced to 3-6 months so that the presence of biochemical progression (which requires repeated evaluations) can be reliably assessed. Imaging tests (primarily bone scan) to evaluate the presence of distant metastases were carried out only when indicated by a rise in PSA levels or symptom development. Patient outcome was divided into 3 clinically relevant categories: biochemical (PSA) failure; local failure; and distant failure.

3.4.1 Biochemical failure

In the majority of patients, EBRT produces a considerable drop in the PSA levels present before treatment start. In many patients, EBRT is successful in reducing PSA levels to within normal range. Because PSA is a measure of disease bulk, a rise is the first indication of disease progression(74,110). PSA assessments were performed every six months for one year, then yearly subsequently. However, the presence of symptoms prompted an immediate PSA evaluation. If that evaluation showed a rise in PSA levels, subsequent evaluations were performed at 3-6 months intervals to document (or exclude) progression. PSA failure was determined according to the American Society of Therapeutic Radiology and Oncology (ASTRO) consensus definition(74,110). According to that definition, 3 consecutive rises in PSA levels must be documented to establish biochemical failure. The time to biochemical failure was the interval from the end of radiotherapy until the midpoint between the last non-elevated PSA value and the first rise as exemplified in Figure 6.

Figure 6: Example of time to biochemical progression estimate

Time to biochemical progression: 63.2 months

06/19/90 → 09/22/95

PSA*	15.3	16.3	12.1	2.1	0.04	...	0.03	1.2	2.2	5.2
Date	05/01/90	05/05/90	06/19/90	12/12/90	05/31/91	...	06/21/95	12/22/95	05/15/96	09/31/96
Event	Diagnosis	XRT start	XRT end	6 mo post	1 yr post	...	5 yrs post	1st rise	2nd rise	Failure†

Patients having not had a confirmed biochemical failure were coded as having "no biochemical recurrence" for the interval between treatment end and last follow-up.

3.4.2 Local failure

The definition of local failure (or local recurrence) required a confirmed biochemical failure (as defined above) AND an unequivocal positive biopsy (see section 3.3 "Biopsy status" for definition). A single scheduled biopsy was performed at two years. Subsequently, biopsies were only performed if the patient reported pelvic symptoms suggesting a local recurrence or in the presence a biochemical progression to document the site of disease progression. Time to local failure was the interval between the end of radiotherapy and the date of biochemical failure or positive biopsy; whichever came later. Patients with no documented local failure were coded as having "no local recurrence" for the interval between treatment end and last follow-up.

* PSA levels in mg/ml
† Confirmed treatment failure

3.4.3 Distant failure

Distant failure is the presence of metastatic disease. The definition required confirmed biochemical failure (as defined above) AND the presence of radiologically documented tumor outside the pelvic area. There were no scheduled radiological evaluations after the start of treatment. These evaluations were only ordered if the patient reported symptoms that could have been associated with the development of a metastasis or in the presence of biochemical progression so that the site of disease progression could be established. Time to distant failure was the interval between end of radiotherapy and date of biochemical failure or radiological evidence of metastasis; whichever came last. Patients with no evidence of distant failure were coded as having "no distant recurrence" for the interval between treatment end and last follow-up.

3.4.4 Outcome assignment

Patients undergoing EBRT are at risk of recurring locally and/or of developing metastatic disease. The risk of one event is likely not independent of the other. Specifically, patients having recurred locally may be at a higher risk of developing metastatic disease(111-115). Also, patients with local progression usually received systemic treatment consisting of androgen ablation therapy shortly following progression to control local symptoms. Since the two events are not independent, and treatment intervention usually follows recurrence, patients having had a documented progression at one site could not continue to be followed for the recurrence at the other site without compromising the risk estimate. For that reason, individuals with a biochemical progression were coded as having had local OR distant progression, whichever came first, unless both occurred within 3

months. That is, unless an individual had documented local and distant progression occurring within 3 months, patients were always assigned the failure site documented first. In this cohort, when the two events were observed in the same patient, local recurrence always preceded distant progression. For these patients, the follow-up for distant progression was censored at the time of local recurrence. Patients who had simultaneously documented local and distant failures were considered in both analyses because the temporal relationship between the two events precluded an impact of one event on the risk of the other.

3.5 Sample

Based on the eligibility criteria listed above, 199 patients were included in this study. All biomarkers could not be evaluated in the entire cohort because tissue availability was limited to the core biopsy tissue extracted at diagnosis. Tumor vascularity (MVD), MIB-1 levels, P-53 status, Bax status, BCL2 status were measured on a subset of 166, 156, 149, 150, and 150 archival pretreatment biopsies specimens, respectively. A group of 126 patients had results for all biomarkers.

3.6 Immunohistochemistry measures of biomarkers

Biomarkers expression was determined from the diagnostic biopsy specimens. MVD, P-53, MIB-1 and BCL2 were visualized using the Avidin Biotin immunohistochemical method. Bax was visualized with the peroxidase antiperosidase (PAP) method. Tissue evaluated for P-53, MIB-1 and BCL2 (but not MVD or Bax) were primed using the heat induced epitope retrieval (HIER) with decloaker biocure at pH 6.0, then incubated with their respective antibody. Only BCL2 required overnight incubation. For MVD and Bax, HIER

was not required. Antibodies and their dilutions were as follows: for MVD - Vector NCI-end diluted at 1/75; for P-53 - undiluted NCICP53-D07 (vector laboratories), for MIB-1 - DAKO M722240 diluted at 1/150, for BCL2 - DAKO M887 diluted at 1/10, and for Bax - DAKO A3533 diluted at 1/50.

The measure of vascular surface area was determined from areas of highest vascularity, the so-called "hot spots". Hot spots were identified by scanning the biopsy sections under low power fields. MVD was estimated using an automated image analyzer and is expressed as a percentage pixel area on the computer screen containing CD34 stain. This was referred to as the microvessel density or MVD. MIB-1 and P-53 are expressed as a percentage of nuclei containing positive stain. BCL2 and Bax are expressed as positive or negative based on the cytoplasmic staining of the majority of tumour cells.

3.7 Statistical analysis

3.7.1 Data description

Patient demographic, treatment, biomarker, and outcome information are reported in frequency tables and/or diagrams. To demonstrate the relationship between biomarkers and traditional factors, cross tabulations and correlative graphs were produced. The purpose of evaluating the relationship between biomarkers and traditional factors was to visually evaluate whether an association was present that could help explain the loss of biomarker correlation with outcome measures in the multivariate setting. For that reason, no statistical analysis was carried out to evaluate the statistical significance of any association.

3.7.2 Imputing the site of progression

Since the palliative therapy prescribed to patients having progressed after EBRT is not dependent on the site of that progression (local versus distant), clinicians often do not see the need to carry out extensive investigations to identify that site, but rather begin palliative treatment based principally on the extent of disease measured by PSA levels. For this reason, there was a subset of patients (24%) with documented biochemical progression but in whom investigations were insufficient in determining the site of progression.

Since this study aims to establish risk factors associated with local recurrence and distant progression separately, identifying the site of progression was essential. Therefore, imputation of the site of progression was done where it was not known. PSA doubling time at the time of biochemical progression was used as an indicator of the recurrence site. PSA doubling time had previously been demonstrated to be considerably shorter in patients with distant metastases compared to those with local recurrences(116-118). The PSA doubling time could therefore be used to ascribe the site of recurrence. To establish the optimal PSA doubling time cut off mark for imputing the site of recurrence, a receiver operating characteristic (ROC) curve was developed that identifies patients with a local recurrence. By elimination, patients having not met the criteria for local recurrence were assigned a distant progression site. Only patients with a biochemical recurrence and clearly documented site of recurrence were used to produce the ROC curve.

Sensitivity of the cut off marks evaluated is the proportion of cases with local progression that would have been correctly assigned that site of progression using PSA doubling time. It was calculated as follows:

$$\frac{\text{Correctly predicted local progression}}{\text{Total observed local progression}}$$

Specificity of the cut off marks evaluated is the proportion of cases with absence of local progression (i.e. documented distant progression) that were also correctly assigned to have had no local progression when using PSA doubling time. It was calculated as follows:

$$\frac{\text{Correctly predicted non-local progression}}{\text{Total observed non-local progression}}$$

3.7.3 Univariate survival analyses of time to progression

3.7.3.1 Survival and hazard functions background

The survivorship function is the probability of observing a survival time (T) greater than or equal to some stated value t, and is denoted as:

$S(t) = \text{Probability } (T>t)$

Under the proportional hazards assumption, the survivorship function for a subject with a covariate "x" is denoted as:

$S(t,x,\beta) = [S_0(t)]^{\exp(x\beta)}$, where $[S_0(t)]$ is the baseline survival function.

The Cox model is a semi-parametric method of estimating a survival function since it does not explicitly describe the baseline function (ho (t)). The hazard function for a subject with covariate equal to x is denoted:

$h(t,x,\beta) = e^{-(\beta o + \beta 1 x)} = h_0(t)\, e^{x\beta}$, where $[h_0(t)]$ is the baseline hazard function.

Since the proportional hazards model does not explicitly specify the baseline function (and the error component of the model), it is not possible to use the log-likelihood function used in other regression models to evaluate the best fitting model. Instead, the Cox model uses a partial likelihood function. Without knowledge of the baseline survival function, only

ratios of the hazard for one group with a set of variables (vector) compared to another group can be estimated(119).

The hazard ratio (HR) for a subject with covariate equal to x_1 compared to one with covariate equal to the reference (x_0) is therefore denoted:

$$HR(t,x_1,x_0) = e^{\beta(x_1 - x0)}$$

Three statistics exist to evaluate the contribution of a variable to a model. These are the score test, the partial likelihood ratio test and the Wald test. These tests are founded on different statistical properties and assumptions but provide similar results.

The score test is useful in evaluating the potential significance of individual variables prior to entering the equation. The score test, denoted Z^*, follows a standard normal distribution with degrees of freedom equal to the number of categories in that variable. Z^* is a derivative of the log partial likelihood.

The log likelihood ratio (LR) test provides the significance level of adding an individual variable or a set of variables to the equation. That is, it compares the – 2 log likelihood (-2LL) values of the two nested models. The log likelihood ratio test, denoted by G, follows a chi-square distribution with degrees of freedom equal to the number of new variables in the more complex model. The likelihood ratio test is the preferred choice for evaluating the contribution of (a) variable(s).

G = (-2LL of larger model) - (-2LL of nested model)

The Wald statistic provides the significance level of variables (and their individual strata) contained in the equation. The Wald statistic, denoted Z, follows a standard normal distribution with degrees of freedom equal to the number of categories for that variable.

$Z = \beta/\text{standard error of } (\beta)$

3.7.3.2 Methodology

Kaplan Meier(120) and Cox regression(119) univariate analyses were used to depict and quantify the prognostic value of the variables on the risk of local, distant and biochemical progression separately. Univariate Cox regression analysis was also used to evaluate the linearity of the relationship between continuous variables and outcome as well as identify potential categorization of continuous and ordinal variables.

Linearity

The following procedure was applied to evaluate linearity:

1. The variable was coded into small groups. The size of these groups varied based on the number of events available in that each analysis (local, distant or biochemical). When events were clustered, the size of groups were adjusted to ensure that at least one event was present in each group. The coded variable was regressed (Cox model) against the outcome and the resulting coefficient value (β) associated with each stratum was plotted against the median value of the stratum. To produce a smoother curve, plotted coefficient values were weighted for the number of patients in the strata and running averages of three neighboring strata were plotted.
2. Linearity was visually assessed. Variable having a linear relationship with outcome were left untransformed. Variables found to be non-linear, were categorized into groups with apparently homogenous risk levels. Categorization was the simplest approach to correcting linearity in this preliminary phase of the evaluation. The best transformation for these variables was carefully evaluated in the multivariate setting.

Univariate risk

The risk associated with each variable was established using a Cox regression analysis and depicted with a Kaplan Meier plot. P values associated with Wald statistics for the variable in the Cox equation are reported.

Biomarker hormone interaction

ADT could potentially modify the effect of biomarkers in predicting disease control. That is, the prognostic value of a biomarker may be different amongst patients having received ADT then those ADT naïve. To evaluate the effect modifying properties of ADT on biomarker related prognosis, a Cox regression analysis stratified according to ADT was performed, and the resulting hazard ratio in the two strata reported. The statistical significance of that interaction was investigated by adding an interaction term between biomarker and ADT to a Cox regression containing that biomarker and ADT (see section 3.7.4.3). A p value < 0.05 was required to establish the presence of interaction.

3.7.4 Model building strategies

Each model was built in three steps. The initial step was used to select the variables that should be included. The second step consisted of re-evaluating the continuous variable linearity in the multivariate setting, and adjusting their form, where appropriate. And the final step consisted of investigating the presence of interaction, and adding interaction terms to the model, if required. The same strategy was used to build the traditional factor and biomarker models. Model development was guided by the procedures described by Harrell and colleagues for data reduction, model verification and model validation(121). To enforce parsimony and avoid overfitting, only variables with a significance level < 0.10 were

included. For local and biochemical models, the ratio of events to number of variable in the equation was not to exceed 10. Because of the small number of distant events, this criterion could not be adhered to in the distant progression model.

3.7.4.1 Step 1: Variable selection

Variable selection for inclusion into the multivariate model was carried out using a forward stepping model-building strategy. The process was as follows:

1. The best form of each variable, as determined in univariate analysis, was considered.
2. All variables were entered sequentially into the equation based on the score test significance level.
3. At each step, the value added by the new variable was measured using the LR test.
4. Because of the small sample size, variable selection p-value cut-off was established at $p < 0.10$. This permitted inclusion of variables that were meaningful contributors to the equation but did not reach significance because of the small cohort sample. Exceptionally, because there is strong evidence that pre-treatment PSA levels, Gleason score and clinical stage are significantly associated with outcome in prostate cancer, and because current clinical nomograms rely upon these parameters, these three variables would be forced into the equation if any did not meet the p value requirement. ‡

During model building, close attention was paid to the size of the standard error. Unusually large standard errors (as well as unusually large coefficient estimates) can be

‡ Changes in the coefficient estimates of variables already in the equation are considered a measure of the confounding effect of the new variable added and is sufficient basis for including that variable in some model building strategies. However, since the goal of this analysis was not to evaluate a main effect, changes in coefficient estimates induced by the entry of a variable were not considered.

indicators of numerical problems such as monotone likelihood (absence of events in a vector) or extreme collinearity between two variables, which require correction. Duration of ADT was not incorporated into the variable selection list, but was studied in the interaction analysis.

The biomarker models were extensions of the final traditional factor models (including variable transformation and interaction terms). For these models, the variables included in the final traditional factor model were forced into the model, and biomarkers individually stepped into the equation using the process described above. The traditional factor model is therefore always nested within the larger model containing biomarkers. Since results for all markers are only available on a subset of patients (63%), including all biomarker variables in a single analysis would considerably diminish the power of the analysis due to case-wise deletions. For that reason, biomarkers were manually forward stepped in separate equations individually. The LR significance level obtained from incorporating each biomarker in its equation was used to determine the order in which the biomarkers were entered in the model. After the first biomarker was incorporated into the model, the analysis was repeated, entering each of the remaining biomarkers in separate analyses to determine their significance levels, until no more biomarkers were found significant.

3.7.4.2 Step 2: Linearity verification and variable transformation

The best transformation for continuous variables was evaluated in the multivariate setting. Two approaches were used:

1. Visual evaluation: The graphical method of representing the relationship between the various levels of a continuous variable and the outcome as described in section 3.7.3.2

(Linearity) was repeated in the multivariate setting, and a visual evaluation of linearity was performed.

2. Fractional polynomials: This analytical process detects deviations from linearity and allows the presence of other forms of relationships to be evaluated. The process was as follows:
   a. Continuous variables were transformed to the following individual powers: p = {-2, -1, -0.5, 0, 0.5, 1, 2, 3}(122), where p = 0 denotes the log of the variable and the other p values represent the variable raised to that power. Negative powers represent 1/transformed variable.
   b. Multivariate regression analyses were performed in which each transformed version of the variable was added to the equation separately. The LR was evaluated to determine the significance level of each transformation and identify those most appropriate.
   c. When more than one transformation appeared adequate, each were visually evaluated for linearity using the visual approach described above, and the most appropriate form selected for inclusion in the model.

3.7.4.3 Step 3: Interactions

The following procedure was used to investigate the effect-modifying property of one variable on another (interaction):

1. An interaction term between the two variables of interest was added to the model and the significance of the resulting LR measured.
2. The interaction term was maintained in the model if the p value of the Wald statistics for the LR test was ≤ 0.05.

3. If the interaction term was included, the corresponding main effects were also maintained in the model regardless of whether a reduction in their significance level occurred.

When an interaction variable was found significant and included in the model, the risk associated with the groups formed by that interaction were calculated as shown in Table 1.

Table 1: Dichotomous variable interaction computation

	Coefficient of variable 1	Coefficient of variable 2	Coefficient of interaction	Sum of Coefficients
X 1 reference * X 2 reference	0	0	0	0
X 1 other strata * X 2 reference	β_1	0	0	β_1
X 1 reference * X 2 other strata	0	β_2	0	β_2
X 1 other strata * X 2 other strata	β_1	β_2	β_{1*2}	$\beta_1 + \beta_2 + \beta_{1*2}$

3.7.5 Verifying the proportional hazard (PH) assumption

In a proportional hazard model, the hazard function for a subject with a covariate set (vector) equal to x is denoted:

$h(t,x,\beta) = e^{-(\beta 0 + \beta 1 x)}$, therefore,

$\ln [h(t,x,\beta)] = \ln [h_0(t)] + x\beta$ (log of the baseline function + linear predictor)

The hazard ratio is a comparative measure of survival experience over the entire time period. Therefore, the hazard ratio must not depend on time, and the Cox model is contingent on the PH of the variables it contains. The PH assumption was evaluated visually using the –ln (-ln(S)) approach and mathematically through a time-dependent interaction term.

3.7.5.1 Using the univariate graphical approach: -ln (-ln (S))

The –ln (-ln(S)) plot was used to evaluate the proportional hazard of categorized predictors. The ln (-ln(S)) is the natural log of the negative natural log of the cumulative survival at any time (t). It can be mathematically equated to the cumulative hazard function. The negative of this second log may be produced to graphically depict mostly positive results. When visually evaluating –ln (-ln(S)) plots, the cumulative hazard of an individual strata is compared to the strata forming the reference group. To obtain –ln (-ln(S)) plots, a stratified Kaplan Meier analysis was done within levels of the predictor, and the –ln (-ln(S)) of each stratum was computed and plotted against time. The assumption of PH is not met if the curves intersect or are not parallel in some other way. It is generally recommended that a conservative approach be used so that only those curves that clearly diverge from parallelism be said to have not met the PH(122).

3.7.5.2 Using a time-dependent interaction variable

An interaction term between the variable being evaluated and time (a time-dependent variable) was introduced in the model to evaluate whether hazard rate was proportional over time. If that interaction variable was statistically significant ($p<0.05$), then the assumption of proportionality of hazard was rejected. For variables with non proportional hazard, an extended Cox model was developed in which a time-dependent interaction variable was included.

3.7.6 Depicting models

Mathematical models provide numerical estimates of risk ratios but, in the absence of graphical representations, these estimates are difficult to interpret. Most published models

use Kaplan Meier curves to illustrate the relationship between the risk level measured by a model and the corresponding patient outcome(13,13,14,17,18,87,88). Risk estimates obtained from the models are usually categorized into 3-6 strata, and the corresponding Kaplan Meier PFS curves for each stratum is plotted. In keeping with this practice, the PFS of the models developed in this study were also depicted graphically. The hazard ratio scores derived from model equation were divided into four strata of equal size (cut offs were established between 0-25% (strata #1), >25%-50% (strata #2), >50%-75% (strata #3) and >75% (strata #4) percentiles for the hazard ratios) and Kaplan Meier plots of the observed PFS in each strata was generated.

3.7.7 Comparing models

To quantify the impact of adding a biomarker to the model based on traditional factors only, each biomarker model was compared to its nested traditional factor model to determine the extent to which the additional variable(s) improved to the model's predictive value. Models were evaluated for their predictive accuracy and discrimination using two approaches: Information Criteria and c index.

3.7.7.1 Information criteria

In the Cox regression model, the –2LL is the measure of explanatory power of a model. However, the LR increases monotonically with number of independent variables in the model. Therefore, basing the comparison of models on the LR value alone is contrary to the parsimony principal. Alternative forms of evaluations that incorporate a penalty for larger models include the Akaike (AIC)(123) and Bayesian or Schwartz (BIC) information criteria(124). The AIC and BIC are useful for nested and non-nested models and have been

used in the prostate predictive model literature(12,15,125) . The premise of these measures is that the –2 LL of one model can be compared to the –2LL of another model if an adequate penalty is added for the larger number variables. The concept is that, to be selected, the larger model must have more explanatory power than the effect of adding more variables. The AIC and BIC represent trade-offs between underfitting and overfitting the sample data, which is the fundamental principle of parsimony. In these tests, the smaller AIC and BIC values reflect the better model. The computations are as follows:

Akaike information criterion:

AIC = -2 Log likelihood + 2 K, where K = number of parameters estimated in the model

Schwarz information criterion:

Schwarz adapted the AIC by adjusting the penalty component to include a measure of the sample size in which the model was developed.

BIC = -2 Log likelihood + log (N) K, where N = sample size of the model

Corrected Akaike information criterion:

When the ratio of the sample size to the number of parameters in the model (N/K) is smaller than 40, a bias adjustment to the AIC is recommended. The adjusted (or corrected) AIC has the following form:

$$AICc = \frac{-2 \text{ Log likelihood} + 2K + 2K(K+1)}{N-K-1}$$

The corrected Akaike information criterion was determined to be the least biased estimate of the goodness of fit in the present study because the ratio of sample size to number of parameters in the model was smaller than 40 in all analyses. All three criteria

will be reported so that changes in other information criterion may be available for comparisons with other reports in the literature.

3.7.7.2 Concordance index (c index)

The c index is a widely accepted measure of predictive discrimination that is related to the ROC curve(126). It reflects the ability of the model to discriminate between those patients less likely from those more likely to relapse. In the survival analysis, the risk level measure derived from the model is the hazard ratio. The c index is derived from comparing the hazard ratios for pairs of patients to the real outcome to determine whether the two are concordant. In survival analyses, the only evaluable pairs are those in whom the event has occurred in both patients, or has occurred in one patient and that survival duration was surpassed by the other. For pairs where the PFS of the second patient did not attain a follow-up equal to or greater than that in the patient who had progressed, or where both patients are progression free, the accuracy of the model cannot be assessed (non-evaluable). For all others, if the PFS duration is consistent with the hazard ratio (e.g. a longer PFS is observed in the patient for whom the PFS was predicted to be larger (smaller hazard ratio)), the pair is said to be concordant and is assigned a score of "1". Discordant pairs are assigned "0", and pairs in whom the PFS or hazard ratio is the same are assigned "0.5". The c index is generated by summing the scores obtained for all evaluable pairs and dividing it by the total number of evaluable pairs. A c index of 50% reflects the absence of predictive value, and would be expected from randomly assigned risk levels, whereas a c index of 100% would reflect 100% predictive accuracy. The c index has been used to measure the predictive value in some prostate models(15,97), and can be converted to a related measure

of concordance called Somer's D rank which has been used in others(12) by simply subtracting 0.5 from the measure and multiplying the result by two.

3.7.8 Reporting the results

Tables and figures have been used throughout the text to help display results. Tabular and graphical representations of results that are germane to the principal objectives of this study are included in the result section. Those determined to be ancillary to the main objectives of the study but relevant, were relegated to appendices. The appendices are grouped into 4 main sections (A, B, C and D) supporting analyses in section 4.1-4.3, 4.4, 4.5, and 4.6, respectively. When a table or figure contained in an appendix is referred to in the text, the appendix section label precedes its number. For example, Figure 1 refers to the first figure in the text whereas Figure A-1 refers to the first figure in appendix A.

4. RESULTS

4.1 Data verification and summary

4.1.1 Eligibility

Patients were only eligible for the present analysis if at least one biomarker evaluation had been performed. Patients had been identified for biomarker assessment from a cohort of patients identified for research and for whom clinical information had been partially captured in a clinical databank. At the time the clinical databank was created, the lead investigator carried out her clinical activities at the General division of the ORCC, and only patients treated at that division were included in the databank. In the mid 1990s, patients treated at both divisions, the Civic and General, were prospectively included in the databank. The only criterion for inclusion in the database was a diagnosis of organ confined prostate cancer treated with curative intent with radiotherapy. For the period specified for inclusion in this study, clinical information on 496 patients had been extracted and stored in the clinical information bank. On a subset of these subjects, biomarker evaluation had also been performed. Biomarker evaluation required that diagnostic biopsy tissue be available. Since special permission is required to obtain samples collected outside the Ottawa Hospital, only patients having had their biopsy at the Ottawa Hospital were selected initially. Amongst the 496 patients identified, biopsy tissue for 212 patients was archived at the Ottawa Hospital. Biomarker evaluation had been requested for all but 9 patients. These patients had apparently been overlooked. Amongst the 284 remaining patients, 152 had their diagnostic biopsy performed at the Queensview Carleton Hospital, the Riverside Hospital or outside the province. No attempt was made to collect these samples. Diagnostic

biopsies were performed at the Montfort Hospital for 132 patients, 20 of which were obtained by special request to increase the number of biomarker evaluations performed. In total, 223 patients were identified for biomarkers evaluation. Twenty-four (24) could not be analyzed because we were unable to obtain the tissue from the hospital at which the diagnosis was made. Of the remaining 199 samples, only a partial analysis of the biomarkers could be performed on 73 samples either because the small volume of tissue available precluded complete analysis or because only a partial analysis was requested to save tissue for future studies.

4.1.2 Patient characteristics

One hundred and ninety nine (199) patients were available for analysis. Patient characteristics for traditional prognostic factors are listed in Table 2. The median age at diagnosis was 68 years. The majority of patients (74%) had a Gleason score of 6 or 7. Since only 5 patients had Gleason scores of 4 or 9, these categories were combined with their neighboring group (i.e. 4 + 5 and 9 + 8) for most analyses. Clinically assessed t-stage varied from stage 1 (16%) through stages 3 or 4 (26%). Since only four patients had stage 4, stages 3 and 4 were combined into a single group for most analyses. All patients were clinically determined to have no lymph node involvement (assumed N0) and absence of metastatic disease (M0). Median pre-treatment PSA level was 10.7 ng/ml (range 0.90 ng/ml to 218 ng/ml with first and third quartile value of 6.8 ng/ml and 22.4 ng/ml, respectively).

Table 2: Frequency distribution (%) of traditional prognostic factors

Number (%) of patients												
Age at diagnosis (years)			**Gleason score**			**Clinical T-stage**			**Pre-treatment PSA (ng/ml)**		
<60	26	(13)	**4**	5	(3)	**1**	32	(16)	**0-<5**	21	(11)
60-64	32	(16)	**5**	19	(10)	**2a**	47	(24)	**5-<10**	67	(35)
65-69	52	(26)	**6**	86	(43)	**2b**	60	(30)	**10-<15**	30	(16)
70-75	53	(27)	**7**	62	(31)	**2c**	9	(5)	**15-<20**	13	(7)
>75	36	(18)	**8**	22	(11)	**3 or 4**	51	(26)	**20-<30**	24	(13)
Total	199		**9**	5	(3)	**Total**	199		**30-<40**	9	(5)
			Total	199					**40-<50**	8	(4)
									50+	17	(9)
									Total	189	
Mean	69								**Mean**	28.1	
Median	68		**Median**	6		**Median**	2b		**Median**	10.7	
Range[1]	50-83		**Range**[1]	4-9		**Range**[1]	1-4		**Range**[1]	0.9-218	

[1] *Range = minimum and maximum values of the variable*

Five biomarkers were evaluated in this cohort of 199 patients. A summary of biomarker results is listed in Table 3. The distribution of continuous variables (MVD, MIB-1 and P-53) is also depicted graphically in Figures A-1 through A-3. Bax and BCL2 were evaluated in 150 patients each and found to be positive in 33% and 25% of samples, respectively. The combination of negative Bax and positive BLC2 was present in 15% of tissues. The percent pixel area reactive to CD-34 stain, reflecting tumor microvessel density (MVD) was evaluated in 166 patients. The median MVD was 1.88% (range 0.01% -12.09%, with first and third quartile value of 0.96% and 3.40%, respectively). MIB-1 values were assessed in 156 tissues and found to be immunoreactive in the majority (97%). The median percentage of nuclei staining for MIB-1 was 5% (range 0%-45%, with first and third quartile values of 2% and 8%, respectively). The presence of P-53 mutations was evaluated in 149 samples and found absent in 53%. Amongst immunoreactive samples, the proportion of positively staining nuclei for P-53 ranged from 1% to 98% (median, first and third quartile values of 7%, 5% and 18%, respectively).

Table 3: Frequency distribution (%) of biomarker status

Number (%) of patients												
Bax and BCL2			**MVD (%)**			**MIB-1 (%)**			**P-53 (%)**			
Bax												
Negative	101	(68)	**< 0.5**	29	(17)	**0**	5	(3)	**0**	79	(53)	
Positive	49	(33)	**0.5-<1.0**	20	(12)	**1**	21	(13)	**1-5**	27	(18)	
Total	150		**1.0-<2.0**	35	(21)	**2**	15	(10)	**6-10**	14	(9)	
BCL2			**2.0-<3.0**	26	(16)	**3**	18	(12)	**11-20**	14	(9)	
Negative	113	(75)	**3.0-<4.0**	20	(12)	**4**	14	(9)	**21-50**	6	(4)	
Positive	37	(25)	**4.0-<5.0**	7	(4)	**5**	11	(7)	**≥50**	9	(6)	
Total	150		**5.0-<6.0**	8	(5)	**6**	13	(8)	**Total**	149		
Bax-ve & BCL2+ve			**6.0-<7.0**	9	(5)	**7**	11	(7)				
Not true	128	(85)	**7.0-<8.0**	4	(2)	**8**	10	(6)				
True	22	(15)	**> 8.0**	8	(5)	**9**	5	(3)				
Total	150		**Total**	166		**10**	6	(4)				
						11-15	19	(12)				
						16-20	3	(2)				
						>20	5	(3)				
						Total	156					
			Mean	2.71		**Mean**	6.6		**Mean**	7.9		
			Median	1.88		**Median**	5		**Median**	0		
			Range[1]	0.01-12.09		**Range**[1]	0-45		**Range**[1]	0-98		

[1]*Range = minimum and maximum values of the variable*

4.1.3 Treatment

Details of EBRT and ADT given in this cohort of patients are shown in Table 4. All patients received curative intent EBRT. The majority (90%) were prescribed a daily fraction size of 2.0 Gy. The intended total doses ranged from 66 Gy to 70 Gy. All received a dose within accepted standard for that period (between 60 Gy and 70 Gy). Nineteen patients (10%) treated before the daily 2.0 Gy protocol was implemented received a 1.8 Gy daily fraction. These patients were treated with 36 fractions for a total dose of 64.8 Gy. Because of the smaller daily fraction size, the tumoricidal potential for that dose was determined radiobiologically to be equipotent to 60 Gy delivered at 2.0 Gy fractions(75).

One third of patients were prescribed ADT. Approximately half of these patients received ADT for a period ranging between 3 and 6 months, and 15% were treated for up to one year. Four patients continued to receive hormonal ablation therapy following EBRT for a period ranging between 2 and 3 years. The prolonged use of ADT was introduced in more recent times as a means of improving outcome amongst patients at high risk of recurrence. Because of the known impact of prolonged ADT on the risk of recurrence, these 4 patients had an artificially reduced risk of recurrence and will not be considered in the model development. In 30% of the patients prescribed ADT, the documented duration of the treatment was less than the standard 3 months usually prescribed to patients with an intermediate risk level of recurring. Details of ADT are not systematically documented in clinical charts, and that information was derived from prescriptions and progress notes only. For that reason, duration of ADT is likely inaccurate; specifically, the end date may be underestimated. However, information about whether ADT had ever been used is likely valid. With that exception, the treatment was within expected range based on the standards for that period.

Table 4: Frequency distribution (%) of treatment types

Number (%) of patients								
External Beam Radiotherapy (daily dose x no. fractions)			**Androgen Deprivation Therapy**			**Duration of Androgen Deprivation Therapy**		
2.0 Gy, x 30-32	5	(3)	Neoadjuvant given	67	(34)	< 3 months	21	(30)
2.0 Gy, x 33	170	(85)	None given	128	(64)	3-6 months	34	(49)
2.0 Gy, x 34-35	5	(3)	Peri-EBRT given	4	(2)	6-12 months	10	(15)
1.8 Gy, x 36	19	(10)	Total	199		2-3 years	4	(6)
Total	199					Unknown	2	
						Total	71	

4.2 Outcome

At the time of analysis, the median follow-up from the end of radiotherapy was 4.5 years (range 1-12 years). One hundred and four (104) patients (52%) had documented biochemical recurrence. The site of recurrence (local or distant) could be ascertained in 79 patients (76%). In the remaining 25 patients, either insufficient investigations were carried out to determine the site of recurrence or these evaluations failed to reveal that site (Table 5). Sixty eight (68) and 22 patients had documented local recurrence and distant metastases, respectively. For patients with documented progression at both sites, local recurrence always preceded distant progression, by an average of 31 months (range 12.7-67.4 months).

Table 5: Frequency distribution (%) of patient outcome status and site of recurrence associated with biochemical progression

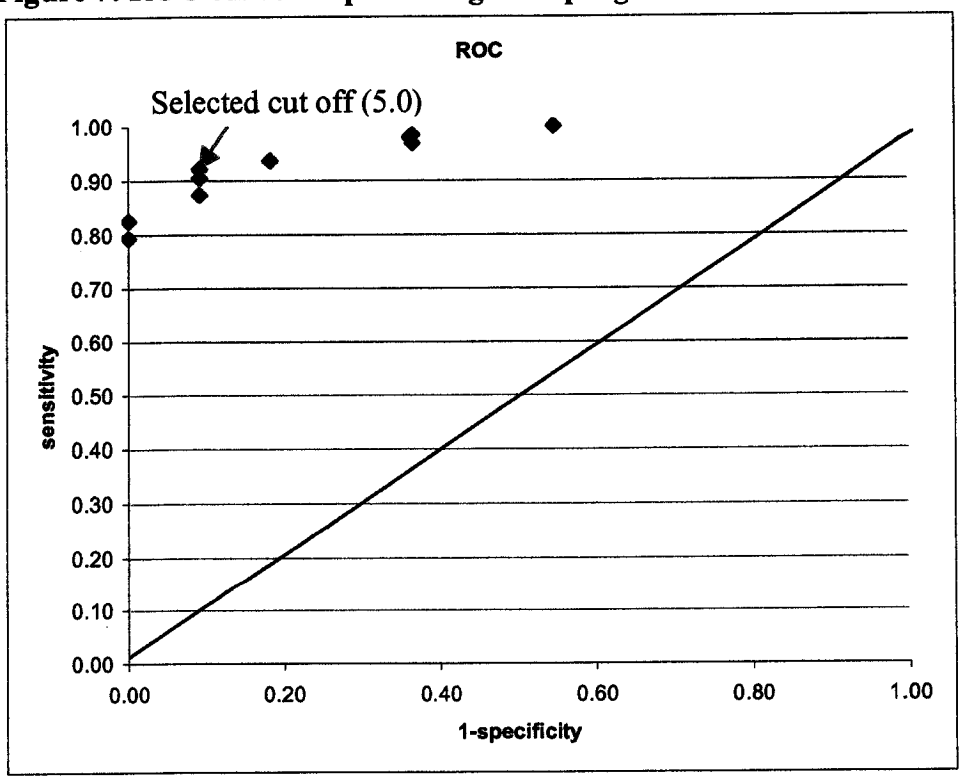
Recurrence status	**Number (%) of patients**
No recurrence	95 (48)
Documented biochemical recurrence with:	104 (52)
no documented site of recurrence	*25 (24)*
documented local recurrence	*57 (55)*
documented distant recurrence	*11 (11)*
documented local and distant recurrence (≤ 3 months)	*3 (3)*
documented local then distant recurrence (> 3 months)	*8 (8)*
Total	**199**

4.2.1 Imputing site of failure

To establish the optimal PSA doubling time cut off for imputing the site of failure, a receiver operating characteristic (ROC) curve was developed. The data from 74 patients with biochemical recurrence and documented site of recurrence were used to develop the ROC curve. Thirty (30) patients were excluded for the following reasons: 25 had an

unknown site of recurrence, 2 had local progression but had incomplete PSA information, and 3 had simultaneous local and distant progression. For patients with documented local then distant recurrences (8 patients), the PSA doubling time at the time of their first recurrence (always local) was used as an indicator of that progression only. Amongst the 74 patients used, 63 had local and 11 had distant progression. The ROC curve aimed at determining the best cut off mark for PSA doubling time that identified local recurrence. The ROC curve represents the relationship between the false positive rate (1-specifity) and the true positive rate (sensitivity). PSA doubling time cut off < 2.5 months produced 100% sensitivity but 45% specificity, and PSA doubling time cut off ≥ 6.5 produced 100% specificity but 83% sensitivity. The results of cut off points between 2.5 and 6.5 months are depicted in Figure 7.

Figure 7: ROC curve for predicting local progression



Receiver operating characteristic curve of PSA doubling time (months) cut off points for predicting local progression

The cut off doubling time producing the best combination of sensitivity (92%) and specificity (91%) was 5.0 months. This velocity was selected to classify 25 patients with biochemical recurrence but no site for recurrence. Of these subjects, 18 were imputed as having had a local recurrence, and 7 a distant recurrence (Table A-1). The final distribution of recurrence sites is shown in Table 6.

Table 6: Patient outcome, including imputed sites of recurrence

Recurrence status	Number (%) of patients
No recurrence	95 (48)
Documented biochemical	104 (52)
Local	*83 (80)*
Distant	*18 (17)*
Local and distant	*3 (3)*
Total	199

Patient outcome (%) based on documented and imputed sites of first recurrence.

4.3 Relationship between traditional factors and biomarkers

The relationship between traditional variables and biomarkers was explored. The purpose of this analysis was to reveal associations between the two groups of variables that could help explain biomarker significance level in the multivariate setting. A biomarker significant in predicting an outcome when investigated alone but which loses significance when investigated into the multivariate setting may be related to a traditional factor contained in the model.

Biomarker expression levels were found associated with clinical stage and Gleason score, but not PSA levels (Table 7). The relationship observed was always positive; that is, higher biomarker values (or positive markers) were associated with worse status of traditional prognostic factors. Associations were apparent between MVD and stage; MIB-1 and stage; Bax and stage; BCL2 and stage; MVD and Gleason score; P-53 and Gleason score.

Table 7: Relationships between traditional factors and biomarkers.

Factor	*n*	Mean %MVD	*n*	Mean %MIB-1	*n*	Mean %P-53	*n*	% Bax +ve	*n*	% BCL2 +ve
Gleason										
4-5	*16*	1.99	*23*	6.70	*22*	3.00	*21*	10	*22*	27
6	*72*	2.39	*65*	7.08	*61*	6.39	*62*	29	*61*	12
7	*53*	3.18	*46*	6.24	*43*	10.93	*44*	46	*44*	36
8	*18*	3.22	*14*	6.07	*15*	11.00	*15*	27	*15*	20
9	*3*	2.85	*4*	4.25	*4*	15.00	*4*	50	*4*	75
T stage										
1-2a	*63*	2.28	*63*	5.44	*60*	4.65	*59*	22	*60*	20
2b	*48*	2.65	*47*	6.45	*43*	10.42	*45*	29	*44*	21
2c	*7*	3.07	*8*	7.25	*7*	4.43	*7*	57	*7*	29
3 or 4	*44*	3.34	*34*	8.79	*35*	11.23	*35*	46	*35*	34
PSA (ng/ml)										
0-<5	*16*	1.80	*19*	5.95	*17*	2.29	*17*	35	*16*	6
5-<10	*52*	2.51	*54*	5.39	*51*	5.00	*50*	26	*51*	23
10-<15	*26*	2.09	*22*	6.32	*23*	12.78	*22*	32	*22*	27
15-<20	*11*	2.61	*9*	4.78	*10*	8.80	*11*	18	*11*	0
20-<30	*21*	4.11	*15*	7.67	*14*	17.07	*15*	47	*15*	40
30-<40	*7*	1.98	*6*	11.67	*5*	6.60	*5*	0	*5*	20
40-<50	*5*	1.79	*4*	4.25	*2*	4.00	*3*	0	*3*	33
50+	*15*	3.62	*14*	8.36	*14*	12.21	*14*	64	*14*	43

Mean % MVD, % MIB-1 and % P-53 levels, and % positive Bax and BCL2 specimens are reported for each strata of traditional factor. The number of patients contained in each analysis is shown in the adjacent column

Specimens extracted from the tissue of more advanced cancer were, on average, more vascularized, contained a larger proportion of proliferating cells (MIB-1), and were apparently more likely to express Bax or BCL2 in the majority of their cells. An analysis of the association between stage and median biomarker immunnoreactivity level was consistent with these results for MIB-1 (Figure A-4) but suggested that the higher MVD averages observed with higher stages were likely due to more frequent samples with dense vascularity rather than generally elevated density of all tissue (Figure A-5). Specimens with higher tumor grade levels were more vascularized (Figure A-6) and were unequivocally expressing higher average levels of mutant P-53 expression. The mean proportion of P-53 immunorreactive cells was 3% in specimens with Gleason scores 4-5 and increased

gradually to 15% in specimens of Gleason score 9. A cross tabulation between Gleason score and P-53 groups demonstrates that the increase in mean percent P-53 immunoreactivity with higher Gleason score is not due to a larger number of P-53 positive nuclei in individual specimens but rather to the larger number of samples with high mutated P-53 expression (P-53 $\geq$ 20%) in tissue with higher Gleason score (Table A-2). Surprisingly, Gleason score was not associated with MIB-1 immunoexpression.

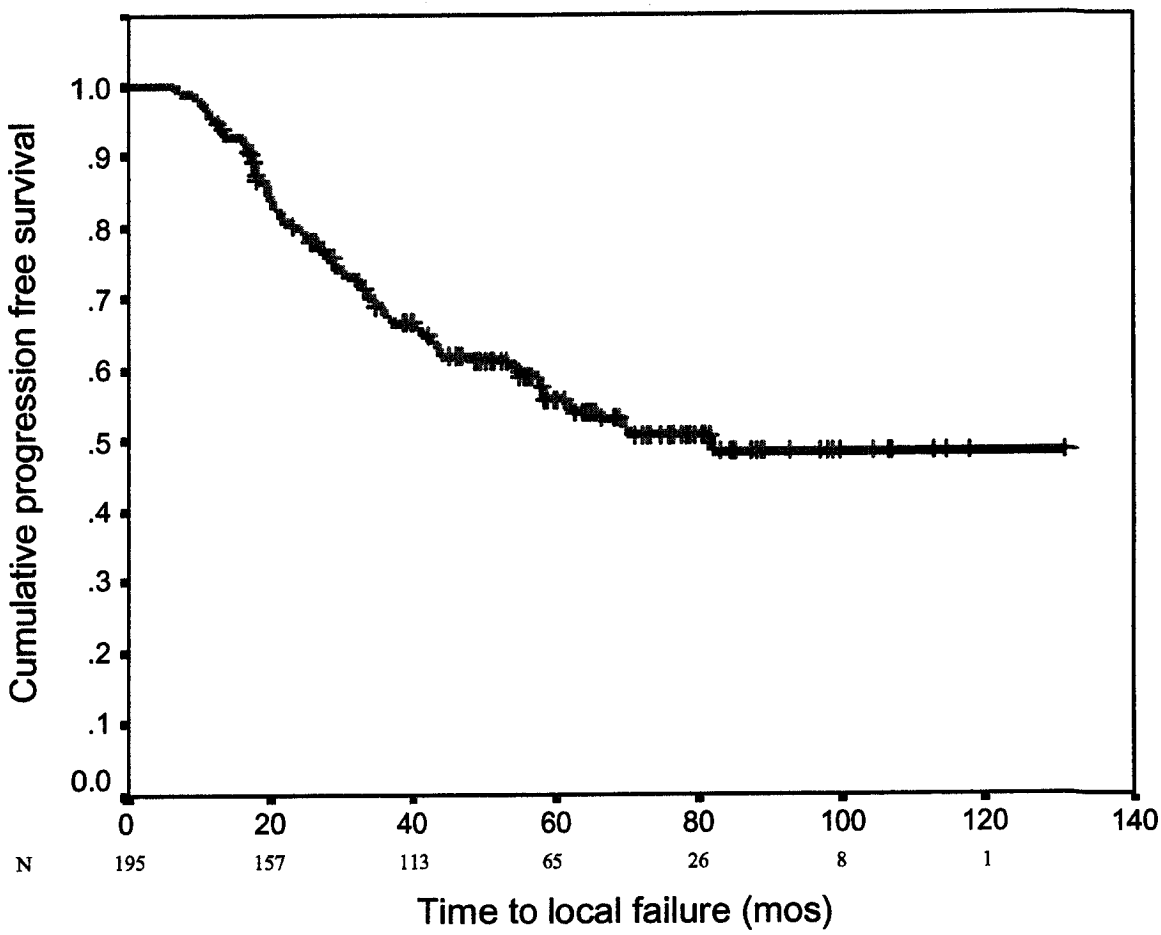
4.4 Local progression analysis

The purpose of this analysis was to produce the best traditional factor model predicting for local recurrence against which an extended model containing biomarkers could be compared. This approach could allow us to determine whether one or more biomarker was useful in supplementing, not supplanting, information contained in the traditional prognostic variables. To that end, the prognostic value of traditional factors, treatment and biomarkers, as well as their interaction with ADT was studied in the univariate setting (section 4.4.1), optimized traditional factors were incorporated into a multivariate model (section 4.4.2.1), linearity of continuous variables verified (section 4.4.2.2), and presence of interactions studied (section 4.4.2.3) to produce a preliminary model based on traditional factors only. To that model, independently significant biomarkers were incorporated (section 4.4.3.1), and linearity (section 4.4.3.2) and ADT interaction (section 4.4.3.3) of biomarkers re-evaluated to produce a preliminary biomarker model. The proportional hazard assumption for variables contained in the models were assessed and correction introduced, if required, (section 4.4.4) to produce final traditional factor and biomarker models (section 4.4.5) that were compared for their predictive discrimination value (section 4.4.6).

4.4.1 Univariate analysis of local progression free survival

At the time of analysis, 84 of the 195 patients eligible for analysis (43%) had progressed within the prostate (local progression). Median local PFS was 82 months, and the 2, 5 and 8 year PFS were 80%, 56%, and 48%, respectively (Figure 8).

Figure 8: Kaplan Meier estimate of local progression free survival



Number of patients remaining at risk is indicated below each time interval

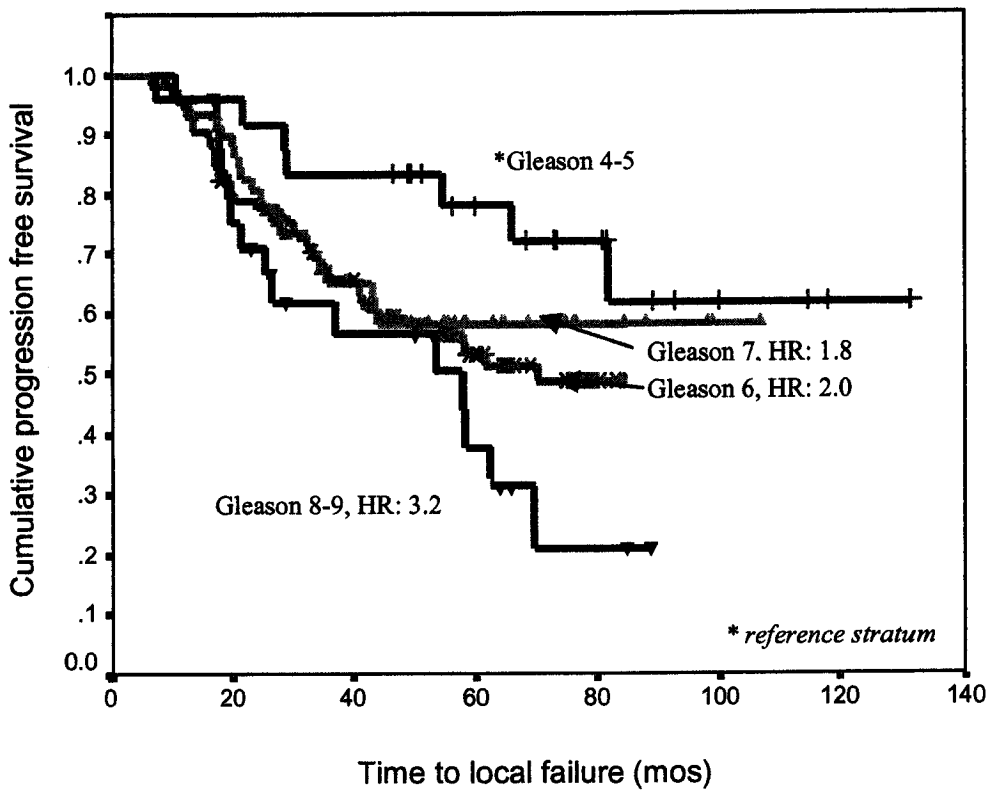
Each traditional variable and biomarker was analyzed for its value in predicting local progression. The local PFS associated with each stratum of categorized variables are represented by Kaplan Meier curves. The risk (Hazard ratio) associated with each stratum was estimating using a Cox regression analysis and is indicated on the graph. When neighboring strata of a variable were determined to have similar risk levels, these were collapsed to form new groups for subsequent analyses (model building). For these variables, the original strata are represented in the Kaplan Meier curves to demonstrate the risk

similarity (i.e. similar curves and hazard ratios) between the collapsed strata.

4.4.1.1 Traditional factors

The Kaplan Meier estimate of local PFS by Gleason score is shown in Figure 9. Gleason scores of 6 and 7 were associated with similar risks of local progression (HR 2.0 and 1.8, respectively) and were pooled for subsequent analyses. Using the 4-5 group as a reference, increasing Gleason scores 6-7 and 8-9 were significantly associated with a higher risk of recurrence (HR: 1.9, 95% CI: 0.9-4.3; and HR: 3.2, 95% CI: 1.3-7.8, respectively).

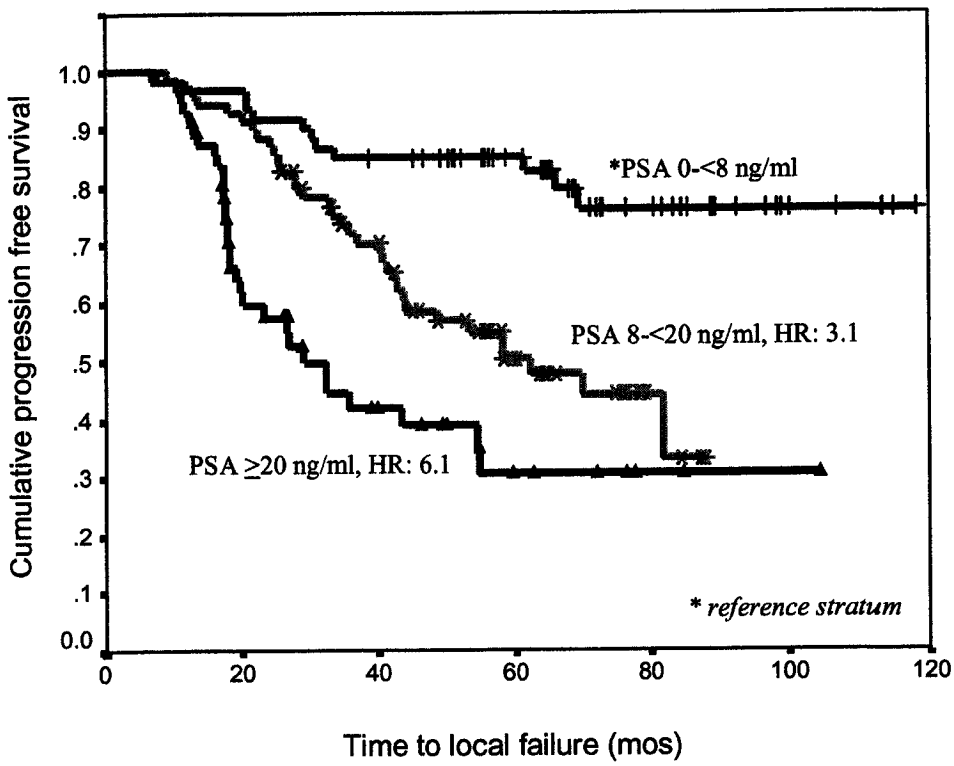
Figure 9: Kaplan Meier estimate of time to local failure by Gleason score



Pre-treatment PSA levels are highly correlated with the risk of local recurrence but the relationship is not linear (Figure B-1). The lack of risk discrimination for local recurrence within values larger than 20 ng/ml is likely related to the increased risk of the competing outcome, distant progression, in that population (Table B-1). In these patients, local

progression would have been censored at the time of distant progression. Eighty six percent (86%) of all distant recurrences occur amongst patients with pre-treatment PSA levels > 20.0 ng/ml. Combining values between 0 and <8 ng/ml, and between 8 and 20 ng/ml forms groups of relatively homogeneous local recurrence risk levels. In that form, PSA levels 8-20 ng/ml and >20 ng/ml are significantly associated with higher risk of local recurrence with HR of 3.1 (95% CI: 1.6-6.1) and 6.1 (95% CI: 3.1-12.0), respectively (Figure 10).

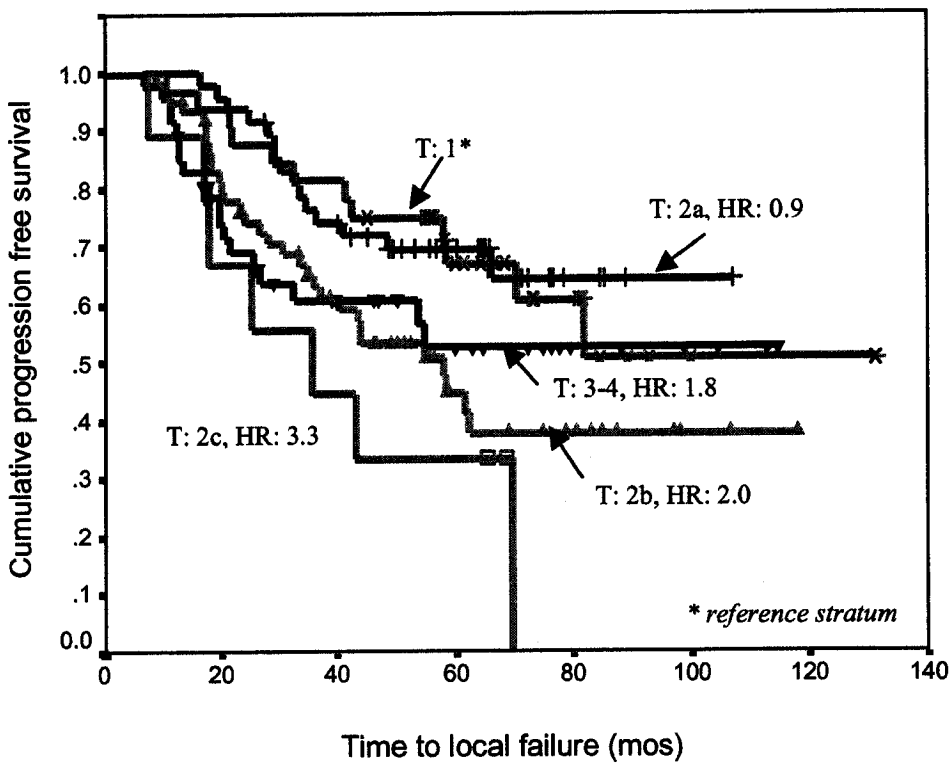
Figure 10: Kaplan Meier estimate of time to local failure by PSA group



Kaplan Meier curve depiction of time to local progression for clinical stage is shown in Figure 11. Patients with extracapsular disease (T3-4) were combined into one group. Patients with tumor involving less than 50% of one lobe (T2a) had similar risk level (HR: 0.9) to those with inapparent tumor (T1). Patients with stages 2b and 2c were more likely to recur locally (HR: 2.0, 95% CI: 1.2-3.8; and HR: 3.3, 95% CI: 1.4-7.6, respectively), whereas stages 3 or 4 were associated with an intermediate risk (HR: 1.7, 95% CI: 1.0-3.2).

This is again consistent with the documented higher risk of developing the competing outcome, distant recurrence, amongst patients with stages 3-4 (Table B-2). Seventy one percent (71%) of distant recurrences occurred amongst patients with stages 3-4.

Figure 11: Kaplan Meier estimate of time to local failure by t stage



There was no apparent relationship between age and risk of local progression (Figure B-2), and attempts to dichotomize the variable at different cut offs ($\geq$ 70 or 75) did not produce statistically significant results.

In summary, pre-treatment PSA levels, clinical T stage, and Gleason score were significant determinant of the risk of local progression, but age was not (Table 8).

Table 8: Traditional factors and local progression – Univaraite Cox regressions

Variable	n	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
Age	195	0.85	1.002	0.97-1.03
Gleason score		0.040		
4-5	24	Ref	1	
6-7	146	0.097	1.94	0.89-4.25
8-9	25	0.013	3.15	1.28-7.75
PSA (ng/ml)		<0.00001		
0-<8	60	Ref	1	
8-<20	69	0.0007	3.14	1.62-6.08
≥20	56	<0.0001	6.12	3.11-12.04
Clinical T stage		0.0079		
1-2a	79	Ref	1	
2b	60	0.010	1.97	1.17-3.81
2c	9	0.0047	3.32	1.44-7.64
3 or 4	47	0.054	1.78	0.99-3.21

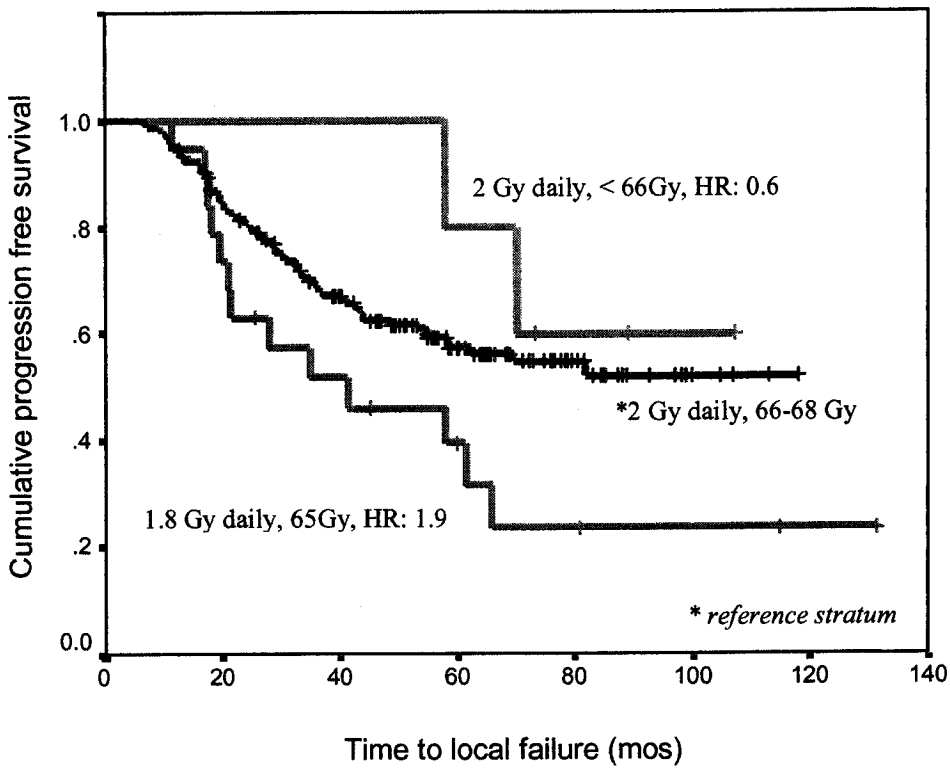
4.4.1.2 Treatment

Treatment consisted of EBRT alone or with ADT. EBRT was categorized according to the daily fraction dose (1.8 Gy vs 2.0 Gy) and total dose delivered (<66 Gy vs ≥ 66Gy). Patients prescribed daily doses of 1.8 Gy were 1.9 (95% CI: 1.1-3.5) times more likely to recur locally than those treated with 2.0 Gy daily doses for 33-35 fractions (Figure 12). The risk level for the five patients treated with 2.0 Gy daily for <33 fractions was not different from those receiving the larger number of fractions.

ADT was not found protective of local recurrence (p=0.48) in this setting because its effect is obscured by the higher risk level associated with the population for whom it is prescribed. Adjusting for disease severity factors will be considered in the multivariate analysis. The duration of ADT ranged from 1-10 months (4 patients with longer treatment duration were excluded). In no case was hormone therapy discontinued because of disease progression. Duration of ADT was marginally predictive of a higher, rather than lower, risk

of recurrence (Figure B-3) (p=0.098), likely due to the fact that prolonged ADT duration is prescribed to patients at higher risk of recurrence.

Figure 12: Kaplan Meier estimate of time to local failure by dose of radiotherapy



The relationship between treatment and risk of local recurrence is summarized in Table 9.

Table 9: Treatment and local progression – Univariate Cox regressions

Variable	N	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
ADT		0.48		
Not given	128	Ref	1	
Given	67		0.85	0.53-1.35
Duration of ADT	65	0.098	1.17	0.97-1.41
EBRT		0.074		
Daily 2.0 Gy x 33-35	171	Ref	1	
Daily 2.0 Gy x 30-32	5	0.53	0.64	0.16-2.60
Daily 1.8 Gy x 36	19	0.031	1.91	1.06-3.47

4.4.1.3 Biomarkers

The value of biomarkers Bax, BCL2, MVD, MIB-1 and P-53 in predicting local progression was studied. Since other investigators had reported that the relationship between some biomarkers and outcome may be different amongst those patients receiving ADT than amongst ADT naïve patients, the effect modifying properties of ADT on the biomarkers was investigated.

The presence of the apoptosis inducer Bax (Bax positivity) was not significantly associated with local recurrence (HR: 1.6, 95% CI: 0.9-2.6) (Figure 13). The apoptosis inhibitor, BCL2, also showed no association with outcome (Figure 14). Patients with combination Bax positive and/or BCL2 negative immunoreactivity appeared more likely to progress locally but this was not statistically significant (HR: 1.84, 95% CI: 0.8-4.3) (Figure 15). ADT did not modify the effect of Bax or BCL2 (p=0.54 and 0.94, respectively).

Figure 13: Kaplan Meier estimate of time to local failure by Bax status

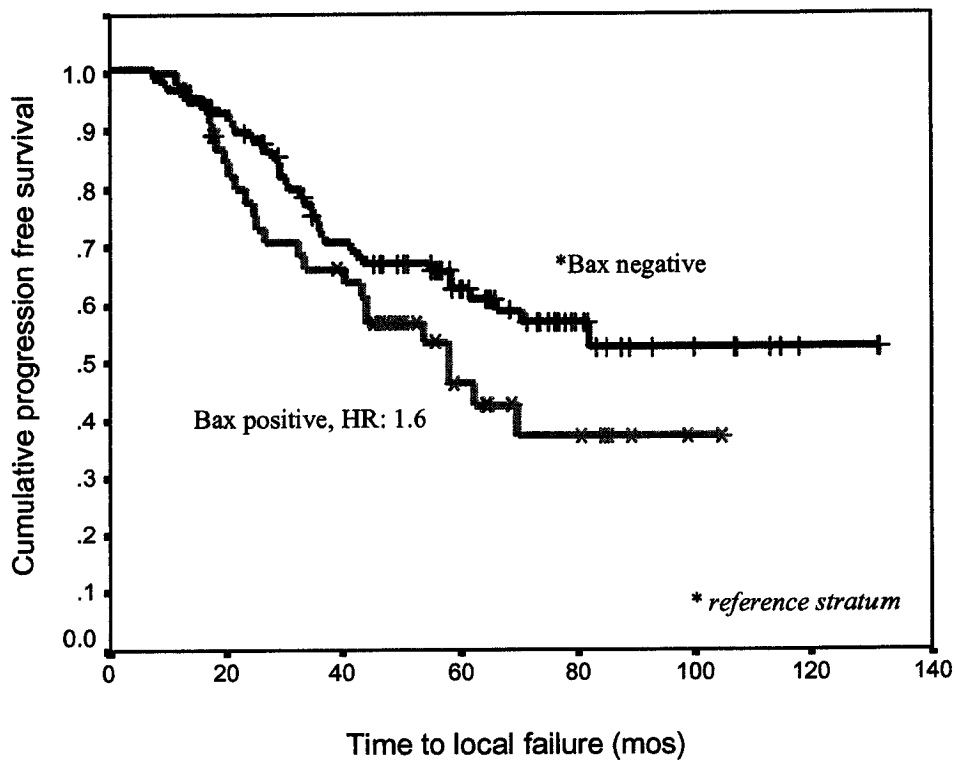


Figure 14: Kaplan Meier estimate of time to local failure by BCL2 status

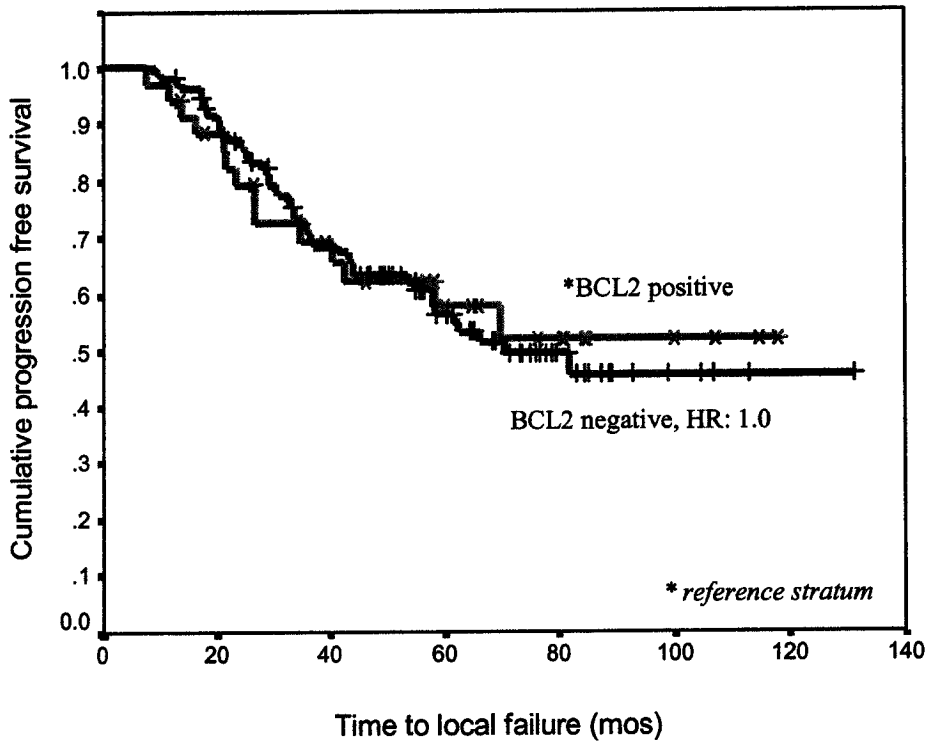
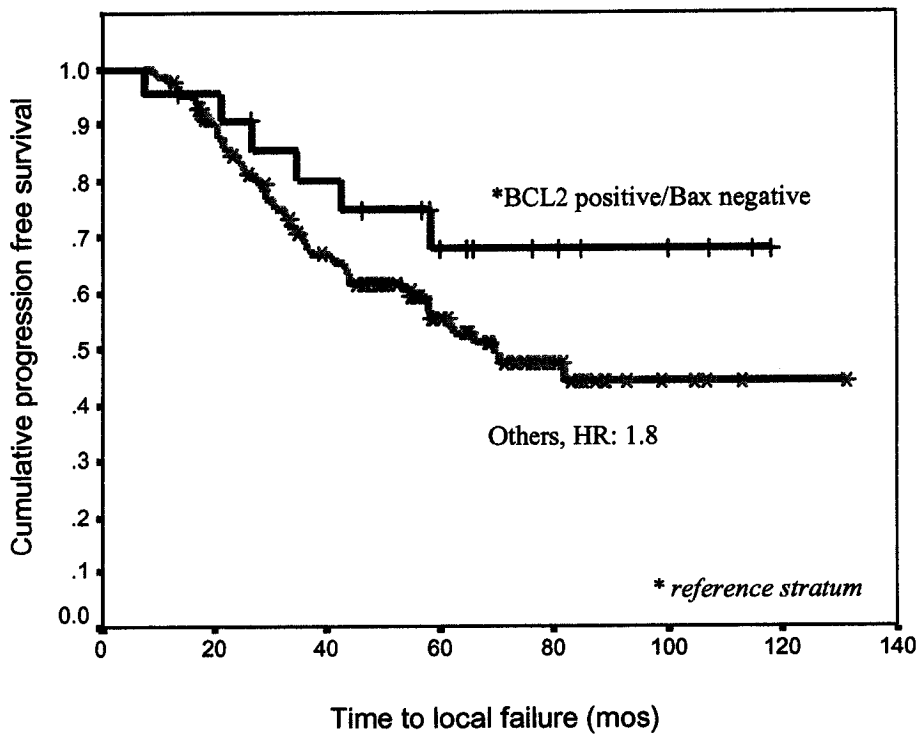
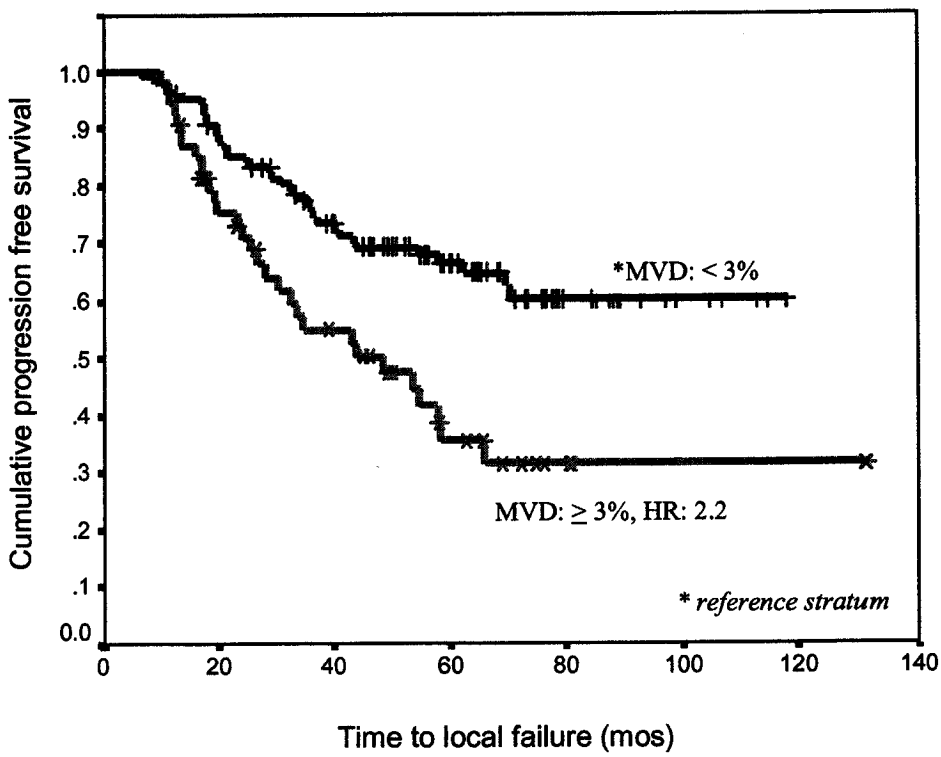


Figure 15: Kaplan Meier estimate of time to local failure by Bax/BCL2 combination



MVD was strongly related to the risk of local recurrence but the relationship was non-linear (Figure B-4). That is, the risk associated with each 1% rise in density is not equal throughout the MVD range. For that reason, the variable was dichotomized into groups with $< 3\%$ and $\geq 3\%$ vascular densities, and found highly predictive of local progression. Patients with more highly vascularized tumors had 2.2 times (95% CI: 1.4-3.6) the risk of local recurrence (Figure 16). There was no apparent interaction between ADT and risk of progression associated with MVD (p=0.58).

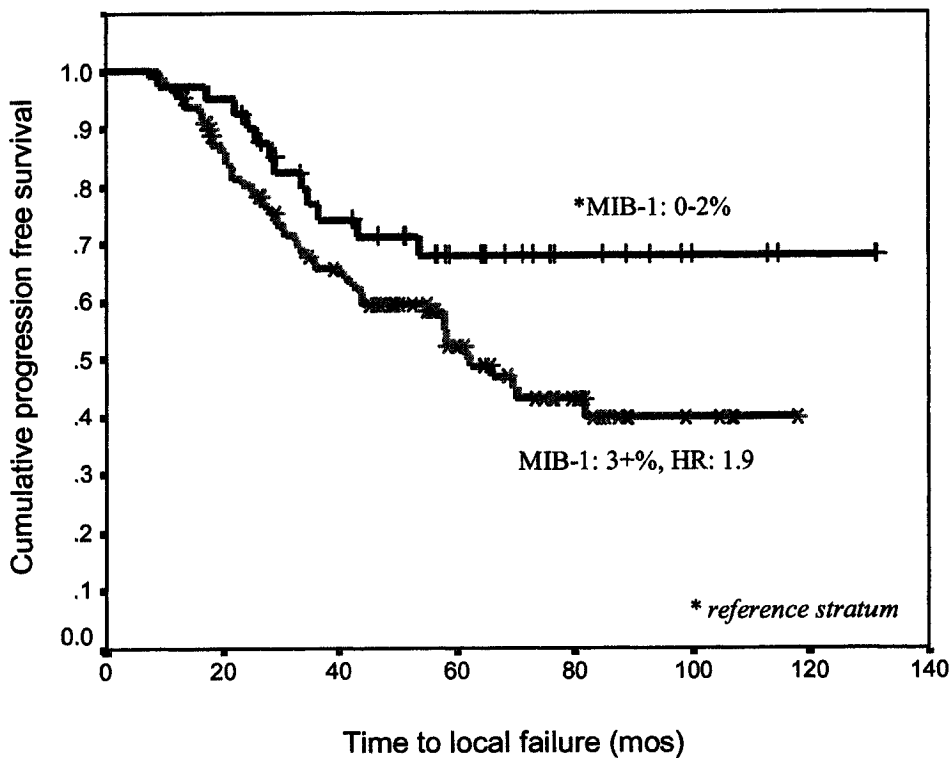
Figure 16: Kaplan Meier estimate of time to local failure by MVD (%) categorized



MIB-1 overexpression was linearly related to outcome for values up to 9%, after which there was a loss of correlation (Figure B-5). Amongst the categorizations and linear log transformation studied, MIB dichotomized at 0-2% vs $\geq$3% was found most the significant predictor of local recurrence (Figure 17). Patients expressing MIB-1 in more than 2% of their nuclei were at 1.9 times (95% CI: 1.0-3.6) the risk of recurring locally. ADT exposure

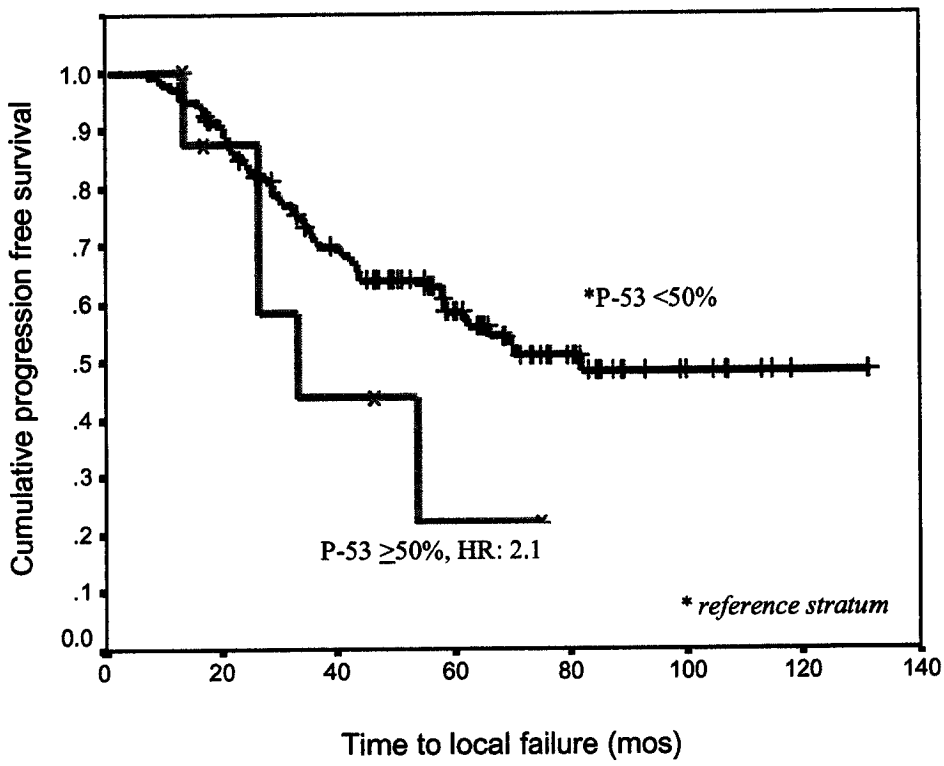
had no impact on the risk associated with MIB-1 (p=0.89).

Figure 17: Kaplan Meier estimate of time to local failure by MIB-1 (%) categorized



There was no apparent relationship between P-53 values and risk of local progression (Figure B-6). Specimens expressing immunoreactivity in more than 50% of their nuclei were associated with an apparently higher risk of recurrence, but this was not statistically significant (Figure 18) (HR: 2.08, 95% CI: 0.8-5.2). The ADT stratified analysis was not performed because of the small number of patients (9) in the P-53 $\geq$ 50% group.

Figure 18: Kaplan Meier estimate of time to local failure by P-53 (%) categorized



Results of the Cox regression analyses evaluating the relationship between the biomarkers and local progression is shown in Table 10, and results of the ADT stratified analysis and interaction term analysis are shown in Table 11. In summary, MVD was highly predictive of increase risk of local recurrence, MIB-1 was marginally predictive, and Bax, BCL2 and P-53 were not significantly associated with the risk of local recurrence.

Table 10: Biomarkers and local progression – Univariate Cox regression

Variable		N	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
Bax			0.082		
	Negative	100	Ref	1	
	Positive	46		1.58	0.94-2.64
BCL2			0.93		
	Negative	111	Ref	1	
	Positive	35		0.97	0.54-1.76
Bax -ve and BCL2 +ve			0.15		
	True	22	Ref	1	
	False	125		1.84	0.79-4.28
% MVD			0.0012		
	0-<3.0	108	Ref	1	
	≥ 3.0	54		2.22	1.37-3.60
% MIB-1			0.049		
	0-2	41	Ref	1	
	≥3	111		1.92	1.02-3.59
% P-53			0.12		
	0-49	136	Ref	1	
	50+	9		2.08	0.83-5.22

Table 11: Biomarkers and ADT interaction in local progression

Variable		N	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
Bax	Interaction term		0.54		
No ADT	Negative	77	Ref	1	
	Positive	25	0.048	1.87	1.01-3.49
ADT given	Negative	23	Ref	1	
	Positive	21	0.63	1.28	0.47-3.43
BCL2	Interaction term		0.93		
No ADT	Negative	75	Ref	1	
	Positive	27	0.94	0.97	0.49-1.92
ADT given	Negative	36	Ref	1	
	Positive	8	0.97	0.98	0.28-3.34
% MVD	Interaction term		0.58		
No ADT	0.<3.0	72	Ref	1	
	≥3.0	36	0.0020	2.44	1.37-4.36
ADT given	0.< 3.0	36	Ref	1	
	≥3.0	18	0.21	1.78	0.72-4.36
% MIB-1	Interaction term		0.89		
No ADT	0-2	9	Ref	1	
	>2	44	0.11	1.88	0.97-10.5O
ADT given	0-2	17	Ref	1	
	>2	83	0.23	1.96	0.31-5.94

4.4.2 Model building: Traditional factors in local progression

4.4.2.1 Traditional factors: Variable selection

One hundred and eighty five (185) patients were eligible for inclusion in the multivariate analysis. Ten (10) were excluded because of missing pre-treatment PSA information. The traditional factor model was built in a stepwise fashion as described in section 3.7.4.1. Using this approach, in the first step, all traditional factors are considered for their ability to predict local progression, and the most statistically significant variable is incorporated in the model first. In the second step, each of the remaining variables is evaluated for its contribution in predicting for local recurrence while that first variable is in the model. These steps are repeated until all variables are entered as shown in Table B-3. The selected model is one for which all variables entered produced a change in the likelihood ratio that resulted in a p value < 0.10. Exceptionally, the three main traditional factors (pre-treatment PSA, Gleason score, and clinical stage) would be included in the model, regardless of their statistical significance. In this case, the model resulting from step #5 and containing pre-treatment PSA levels, clinical stage, EBRT fraction size and ADT status would be selected. Gleason score showed a trend for higher risk of local recurrence with lower grade, but this was not statistically significant. However, since Gleason score is one of the three main traditional factors found predictive of outcome in the literature, Gleason score categorization was re-evaluated and forced into the equation.

Details of the resulting model are shown in Table 12. PSA categories are highly predictive of local progression. The relationship between stage and the risk of local progression observed in the univariate setting is maintained. That is, the risk increases with stage 2b and 2c, but not for stage 3-4. As expected, ADT is demonstrated protective after

adjusting for measures of disease severity (HR: 0.54, 95% CI: 0.3-0.99). Lower daily fraction doses (1.8 Gy) remains associated with a higher risk of local recurrence (HR: 2.0, 95% CI: 0.9-4.3).

Table 12: Multivariate analysis of local progression using traditional factors

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
PSA (ng/ml)		<0.0001			
0-<8	60	Ref	0	1	
8-<20	69	0.0023	1.07	2.90	1.46-6.91
20+	56	<0.0001	1.93	6.91	3.33-14.33
Clinical T stage		0.0041			
1-2a	74	Ref	0	1	
2b	57	0.073	0.52	1.68	0.95-2.97
2c	9	0.0024	1.50	4.50	1.46-5.77
3-4	45	0.99	0.01	1.01	0.49-2.06
ADT		0.047			
Not given	121	Ref	0	1	
Given	64		-0.61	0.54	0.30-0.99
EBRT		0.072			
2.0 Gy daily	172	Ref	0	1	
1.8 Gy daily	13		0.70	2.01	0.94-4.30
Gleason score		0.22			
<6	22	Ref	0	1	
≥6	163		0.53	1.69	0.73-3.92

4.4.2.2 Traditional factors: Linearity

The scale of PSA was re-evaluated in the multivariate setting using visual examination and fractional polynomials as described in section 3.7.4.2 (Figure B-7 and Table B-4). The relationship between PSA and local progression was linear for values <30 ng/ml only. Amongst the three potential transformations suggested by the fractional polynomial analysis (ln (PSA), 1/sqrt (PSA), categorized PSA), the continuous forms (ln (PSA) and 1/sqrt (PSA)) had the best LR but both showed a loss of correlation with higher PSA values (Figures B-8 and B-9). Ln (PSA) was selected because the term is easier to interpret and other published models had used that transformation. The resulting adjusted model for local

progression shown below suggests that PSA transformation had little impact on the risk associated with other variables (Table 13).

Table 13: Multivariate analysis of local progression using traditional factors and transformed PSA

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
Ln (PSA) ng/ml	185	<0.0001	0.687	1.99	1.49-2.65
Clinical T stage		0.0043			
1-2a	74	Ref	0	1	
2b	57	0.11	0.46	1.59	0.90-2.81
2c	9	0.0041	1.42	4.13	1.57-10.87
3-4	45	0.74	-0.13	0.88	0.41-1.88
ADT		0.024			
Not given	121	Ref	0	1	
Given	64		-0.70	0.50	0.27-0.91
EBRT		0.030			
2.0 Gy daily	172	Ref	0	1	
1.8 Gy daily	13		0.83	2.29	1.08-4.85
Gleason score		0.16			
<6	22	Ref	0	1	
≥6	163		0.57	1.77	0.79-3.98

4.4.2.3 Traditional factors: interactions

To investigate the presence of interaction between the traditional factors, interaction terms were considered as described in section 3.7.4.3. The principal interaction of interest was for effect modifying properties of ADT. Since the majority of patients having received ADT had a Gleason score ≥6 and all had received a 2.0 Gy daily dose of EBRT, these interactions could not be studied. The relationship between ADT, PSA, Gleason score and stage were found to be additive (Table B-5), that is, one variable did not significantly modify the risk of local recurrence associated with another variable.

The preliminary traditional factor statistical model to which biomarkers may be added is therefore unchanged from Table 13. The coefficients associated with each variable are shown in Table B-6.

4.4.3 Model building: Biomarkers in local progression

The traditional factor statistical model developed in the previous section was extended to include independently significant biomarkers. The process only permitted new variables to be entered but did not allow variables contained in the original traditional factor model to be modified or removed. Since that model was determined to be the best equation predicting for local progression, maintaining it static was required to evaluate whether biomarkers supplemented the traditional factor information. For example, if traditional factors were removed, significant contribution of a biomarker could have been a result of having supplanted, not supplemented, the information contained in the traditional factors.

4.4.3.1 Biomarkers: Variable selection

The process of variable selection was performed as described earlier. However, since the sample size available for the analysis of each biomarker varied according to the population in which the biomarker was sampled, each step was performed manually to allow the maximum number of samples to be included. Results of the stepwise analysis are shown in Table B-7. In the first step, MVD was found the most significant independent predictor of local progression. Adding MVD to the equation produced a change in likelihood ratio associated with a highly significant p value (p=0.0096). In contrast, Bax/BCL2 was marginally predictive (p=0.076) (Table B-7). MVD was therefore incorporated in the model and a second stepwise selection of biomarkers was carried out (Table B-8). In that analysis

the combination of Bax/BCL2 was found to be an independent predictor of local progression (p=0.011). Since the sample size on which this equation is based is small (115), and only a small number of patients fall into the reference category for Bax/BCL2, these results may be aberrant and a result of overfitting rather than reflect true biology. For that reason, separate models will be investigated, one containing MVD only, and one containing MVD and the Bax/BCL2 combination term. The two models are represented in Tables 14 and 15.

Table 14: Multivariate model of local progression containing MVD (%) (n=153)

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
Ln (PSA) ng/ml	153	0.0007	0.564	1.758	1.26-2.43
Clinical T stage		0.019			
1-2a	59	Ref	0	1	
2b	45	0.039	0.71	2.03	1.03-3.96
2c	7	0.046	1.16	3.20	1.02-10.01
3-4	42	0.95	-0.03	0.97	0.41-2.30
ADT		0.0708			
Not given	102	Ref	0	1	
Given	51		-0.62	0.54	0.28-1.05
EBRT		0.020			
2.0 Gy daily	143	Ref			
1.8 Gy daily	10		1.13	3.09	1.19-8.00
Gleason score		0.060			
<6	14	Ref	0	1	
≥6	139		1.17	3.25	0.95-11.15
% MVD		0.0083			
<3	103	Ref	0	1	
≥3	50		0.72	2.06	1.20-3.51

Table 15: Multivariate model of local progression containing MVD (%) and Bax/BCL2 (n=115)

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
Ln (PSA) ng/ml	115	0.023	0.447	1.56	1.06-2.30
Clinical T stage		0.088			
1-2a	43	Ref	0	1	
2b	35	0.19	0.55	1.74	0.76-3.97
2c	6	0.10	1.04	2.84	0.81-9.94
3-4	31	0.76	-0.17	0.84	0.27-2.59
ADT		0.23			
Not given	78	Ref	0	1	
Given	37		-0.47	0.62	0.29-1.34
EBRT		0076			
2.0 Gy daily	107	Ref	0	1	
1.8 Gy daily	8		1.46	4.29	1.47-12.47
Gleason score		0.13			
<6	13	Ref	0	1	
≥6	102		1.18	3.24	0.71-14.75
% MVD		0.0006			
<3	74	Ref	0	1	
≥3	41		1.13	3.09	1.62-5.88
Bax/BCL2		0.0585			
Bax-ve/BCL2+ve	12	Ref	0	1	
Others	103		1.95	7.00	0.93-52.56

4.4.3.2 Biomarkers: Verifying linearity

The scale of MVD was re-examined to determine the best transformation in this multivariate setting using visual examination and fractional polynomials (Figure B-10 and Table B-9). The results suggest that MVD was linear for values up to 4% only. When categorized forms were investigated, a dichotomized variable separating patients with MVD lower than 3% from those with ≥3% ($MVD_{\geq 3\%}$) had superior predictive value, as estimated by its higher likelihood ratio, and will be considered in the models.

4.4.3.3 Biomarkers: Hormone therapy interactions

There was no interaction between $MVD_{\geq 3\%}$ and ADT (p=0.59). Interaction between

hormone therapy and Bax/BCL2 could not be evaluated because only 2 patients with Bax-ve/BCL2 +ve had also received ADT.

Two biomarker models will be investigated, one containing $MVD_{\geq 3\%}$ only and one containing $MVD_{\geq 3\%}$ and the Bax/BCL2 combination term. The coefficients associated with these preliminary biomarker statistical models are shown in Table B-10.

4.4.4 Verifying the proportional hazard

The proportional hazard assumption for each variable contained in the models was evaluated using the two approaches described in section 3.7.5. Results of the –ln (-ln(S)) plots (Figures B-11 through B-16) suggest that, except for stage 3-4, all variables had tolerable patterns of hazard, and the proportionality of hazard could be assumed. This deviation from the PH assumption for stages 3-4 was confirmed in the results of the extended Cox models (Table B-11). Since stage 1-2a and 3-4 are associated with similar risk of local progression, the two strata were collapsed. The new variable coding for stage then met the assumption of proportional hazard.

4.4.5 Final traditional and biomarker models

The three models (traditional factor, MVD, and MVD + Bax/BCL2) were regenerated using the collapsed term stages 1-2a and 3-4 to correct for the hazard non proportionality for stages 3-4. This had minimal impact on the coefficient of the variables, as shown in Table B-12.

All models having been developed to predict progression in prostate cancer patients undergoing radical prostatectomy or external beam radiotherapy have been depicted graphically to allow the readers, usually clinicians, to obtain a more clinically relevant

representation of the equation or nomogram. While caution is given against using the disparity between curves within each model to visually compare the discrimination potential between models, I do believe that the practice of representing the model graphically is useful. Accordingly, the hazard ratios obtained with each equation (model) were categorized into quartiles of equal sizes to produce 4 risk categories as described in section 3.7.6. A descriptive summary of the hazard ratios derived from each model is given in Table B-13, and the stratification groups are defined in Table B-14. Curves derived from the traditional model (Figure B-17), MVD $\geq$ 3% model (Figure B-18) and MVD $\geq$ 3% + Bax/BCL2 model (Figure B-19) are represented in the appendices.

4.4.6 Comparing models: Local progression

To determine whether the addition of $MVD_{\geq 3\%}$ or $MVD_{\geq 3\%}$ and Bax/BCL2 improved the predictive value and discrimination potential of the model based on traditional factors only, the two were compared using the AICc and c index as described in section 3.7.7.

4.4.6.1 Information criteria

Results of the information criteria estimates are shown in Table 16. The Akaike information criterion (AIC) is an estimate of the explanatory power of the model that is based on the –2 log likelihood (-2LL) value. Since the –2LL increases monotonically with number of independent variables in the model, the AIC incorporates a penalty related to the number of variables in the model to enforce parsimony. The Bayesian information criterion (BIC) and corrected Akaike information criterion (AICc) are directly related to the AIC but make further adjustment for small sample sizes (BIC) and models in which the sample size to parameter ratio is small (AICc). In all three, smaller values reflect a model with a

superior predictive value, one with more explanatory value. As described in section 3.7.7, the AICc is determined to be the least biased estimate of goodness of fit for the current study. Based on that estimate, the model containing both $MVD_{\geq 3\%}$ and Bax/BCL2 has more predictive value because it produces a considerably smaller AICc value (difference of 11.4).

Table 16: Information criteria for the models predicting local progression.

Patient subset	Model	-2LL	K	n	AIC	AICc	BIC
153 with MVD							
Traditional factor model		530.1	6	153	542.0	547.6	560.2
Biomarker model: $MVD_{\geq 3\%}$		522.8	7	153	536.8	543.5	558.0
Difference in information criterion					**5.2**	**4.1**	**2.2**
115 with MVD & Bax/BCL2							
Traditional factor model		362.7	6	115	374.7	380.4	391.2
Biomarker model: $MVD_{\geq 3\%}$ +Bax/BCL2		344.7	8	115	360.7	369.0	382.7
Difference in information criterion					**14.0**	**11.4**	**8.5**

Each model is compared to the traditional model generated in the same subset of patients to produce difference in information criterion.

4.4.6.2 Concordance (c) index

The c index was computed for all evaluable pairs. A higher c index denotes a model with superior predictive discrimination potential. The c index of each biomarker model was compared to the c index of the traditional model derived from the same subpopulation (Table 17). The traditional factor model c index for the entire population is also shown to demonstrate that results are consistent with those obtained in the subgroups. The results suggest that the addition of MVD to the model improves discrimination, and that the variable Bax/BCL2 adds further discrimination potential. The biomarker model based $MVD_{\geq 3\%}$ could predict local progression with 2.4% more accuracy than the model based on traditional factors only, while the model containing $MVD_{\geq 3\%}$ and Bax/BCL2 improved accuracy by 7.0%.

Table 17: c indices computations for models predicting local progression

	Traditional factor model in all patients	Traditional factor model in patients with MVD data	Biomarker model: MVD 3%	Traditional factor model in patients with MVD & Bax/BCL2	Biomarker model: MVD 3% & Bax/BCL2
All pairs	17020	11628	11628	6555	6555
Unusable pairs	7012	5009	5009	2961	2961
Total evaluable	10008	6619	6619	3594	3594
Count	7046	4643	4804	2492	2742
c index	70.4%	70.2%	72.6%	69.3%	76.3%
Difference			**2.4%**		**7.0%**

c indices for the traditional factor model were estimated in the entire patient population and subpopulations in which the biomarker models were developed. Differences in c indices are estimated from c indices computed in the same population.

4.4.7 Summary

The present analysis suggests that MVD and Bax/BCL2 both provide supplemental information to traditional factors. Adding MVD to the model composed of traditional factors improves the predictive discrimination of that model. Further addition of the Bax/BCL2 combination term results in more enhanced discrimination ability.

4.5 Distant progression analysis

In this section, a similar approach to section 4.4 (local progression analysis) is used to establish a model predicting for distant progression that is based on traditional factors only and one that incorporates biomarkers, and to determine the one with best predictive discrimination value.

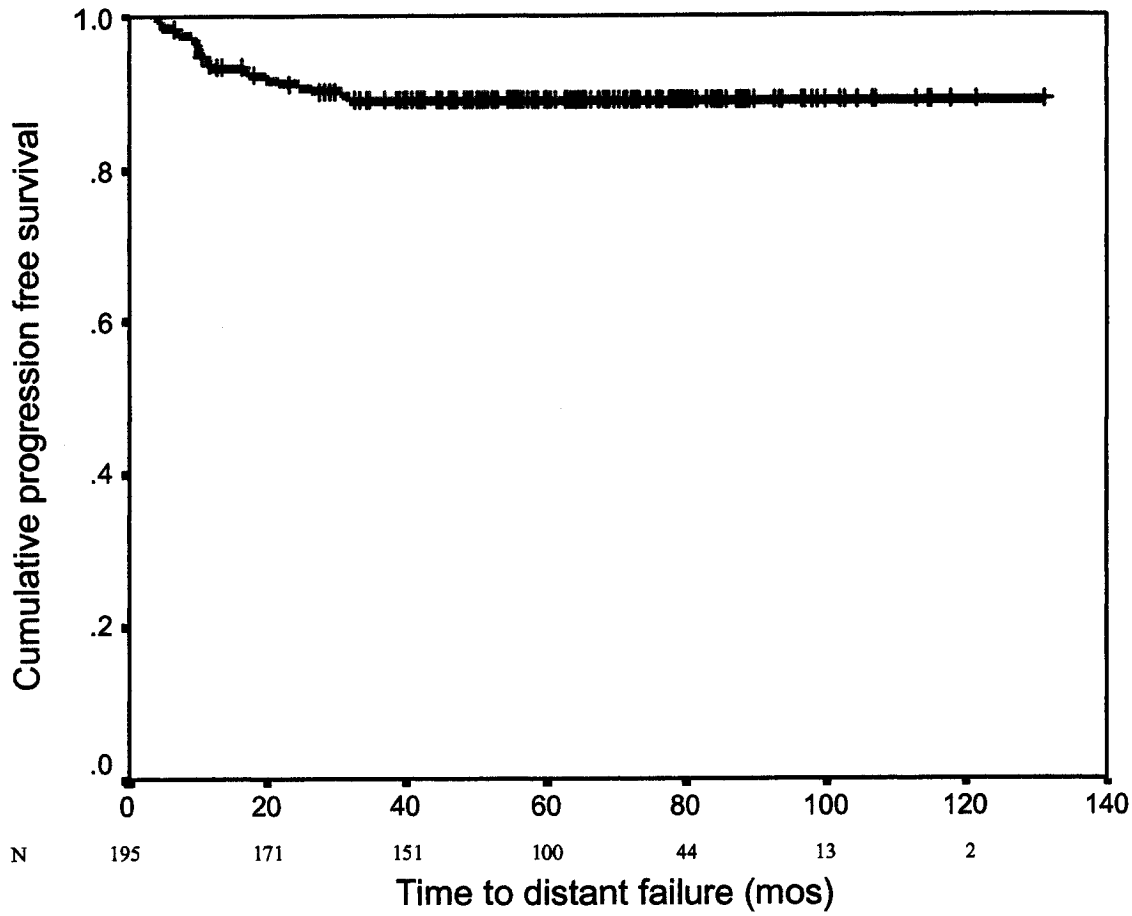
4.5.1 Univariate analysis of distant progression free survival

At the time of analysis, 22 patients (11%) had a documented disseminated disease, and another 8 (4%) were imputed to have had a distant progression based on the PSA doubling rate. Amongst these 30 patients, 18 (9%) had documented or imputed metastatic disease without prior local failure, and 3 are had local and distant progression documented within three months of one another. These 21 events are the subject of this analysis. The remaining 9 patients had progressed within the prostate before metastatic disease was detected, and their follow-up for distant recurrence was censored at the time of local progression. All events had occurred within the first 3 years following the end of EBRT. The 2 and 3 year PFS were 92% and 89%, respectively (Figure 19).

The overall distant progression free survival was 89%. To permit better visualization of risk levels separation between categories of the variables studied, the y axes of all subsequent cumulative PFS curves are truncated at 40%. This approach provided a better visual separation of the curves associated with different categories of each variable, which should not be interpreted as larger hazard ratios. Also, because there were few events in this analysis, to permit the reader to gauge the power of detecting a difference between the categories of patient groups in tables representing the relationship between individual factors

and distant progression, the number of events is provided alongside the sample size for each category.

Figure 19: Kaplan Meier estimate of distant progression free survival for all patients



Number of patients remaining at risk is indicated below each time interval

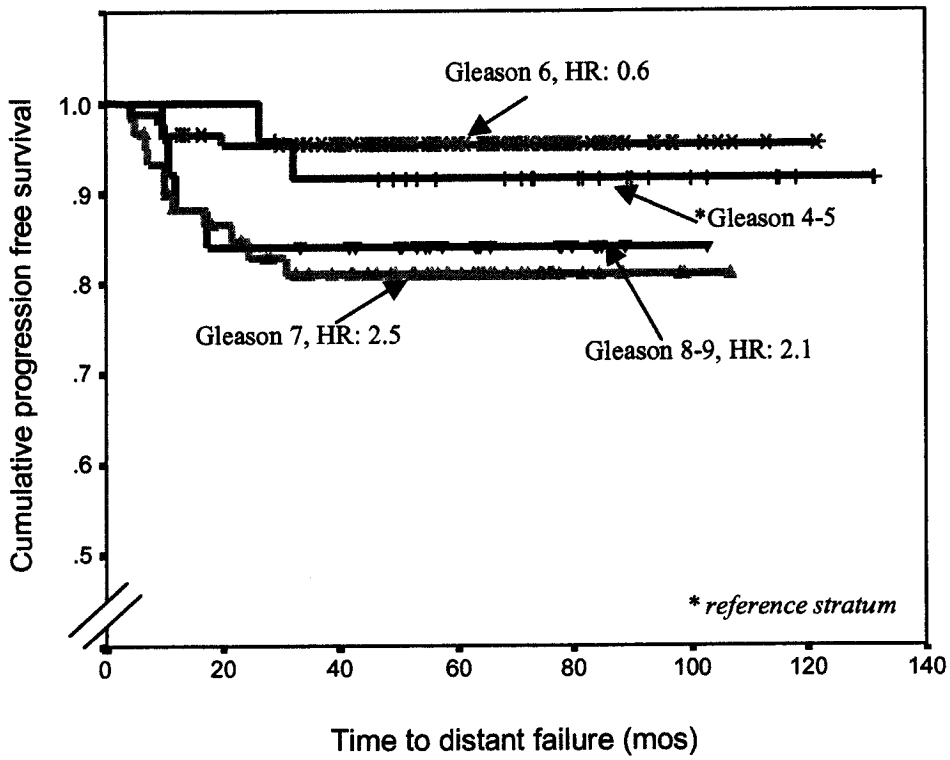
4.5.1.1 Traditional factors

Amongst the traditional factors studied, only age was not related to the risk of distant recurrence (Figure C-1). Individuals older than 70 were somewhat more likely to progress distantly but this was not statistically significant (p=0.11).

The Kaplan Meier estimate of distant progression free survival by Gleason score is shown in Figure 20. Patients with Gleason scores of 6 did not have a higher risk of

developing distant progression compared to patients with scores of 4-5 (HR: 0.6), and these groups were collapsed. Similarly, patients with Gleason scores 8-9 (HR: 2.1) did not have a higher risk of recurring distantly than patients with Gleason score of 7 (HR: 2.7), and these two groups were collapsed. For future analyses of distant progression, Gleason scores were dichotomized into 4-6 and 7-9. Gleason scores 7-9 were strongly associated with a higher risk of distant recurrence compared to scores 4-6 (HR: 3.5, 95% CI: 1.4-9.1).

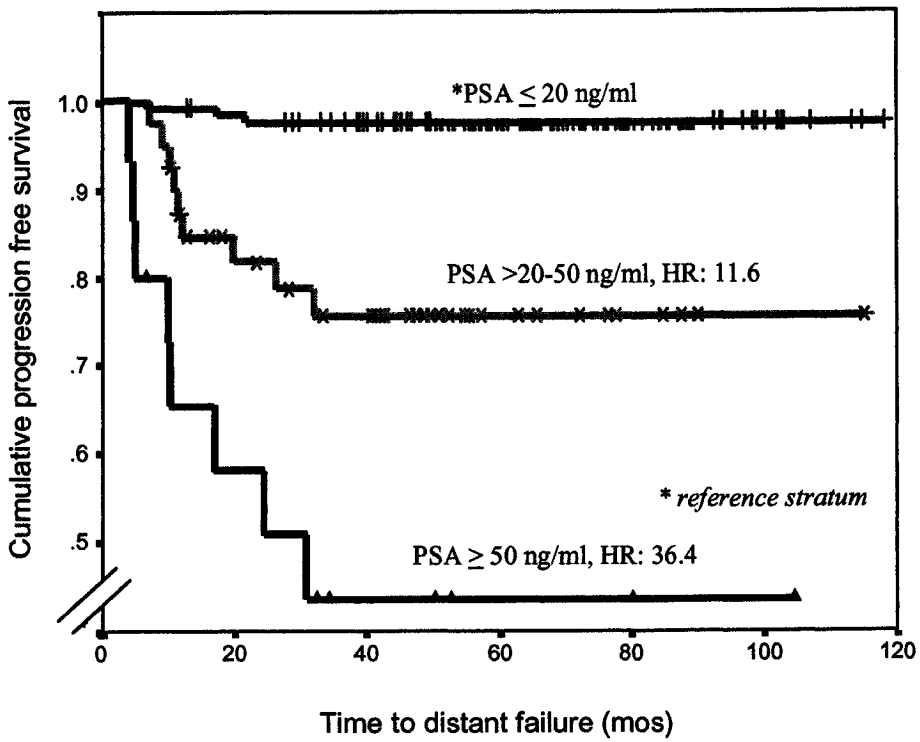
Figure 20: Kaplan Meier estimate of time to distant failure by Gleason score



Only 2 distant events were observed amongst the 129 patients with PSA values <20 ng/ml. For the remaining 56 subjects, risk levels appeared clustered for PSA levels ranging between 20 ng/ml and 50 ng/ml and above 50 ng/ml (Figure C-2). These PSA categories were significantly predictive of distant recurrence. Patients with pre-treatment PSA levels <20 ng/ml had a 3 year PFS of 98% compared to 76% and 44% for patients with PSA 20-50

ng/ml and >50 ng/ml, respectively (Figure 21). The hazard ratios associated with these categories were 11.6 (95% CI: 3.1-42.9) and 36.4 (95% CI: 9.6-137.6), respectively.

Figure 21: Kaplan Meier estimate of time to distant failure by PSA (ng/ml) group



Patients with stages 1-2a, 2b and 2c had similar risks of distant progression (Figure 22). In comparison, patients with stage 3-4 had a significantly elevated risk (HR: 9.6, 95% CI: 3.7-24.7).

The value of age, Gleason score, clinical T stage, pre-treatment PSA levels and treatment in predicting distant progression are summarized in Table 18. Because few patients had a distant progression, the number of events associated with each strata is shown to allow the reader to appraise the power of each analysis.

Figure 22: Kaplan Meier estimate of time to distant failure by t stage

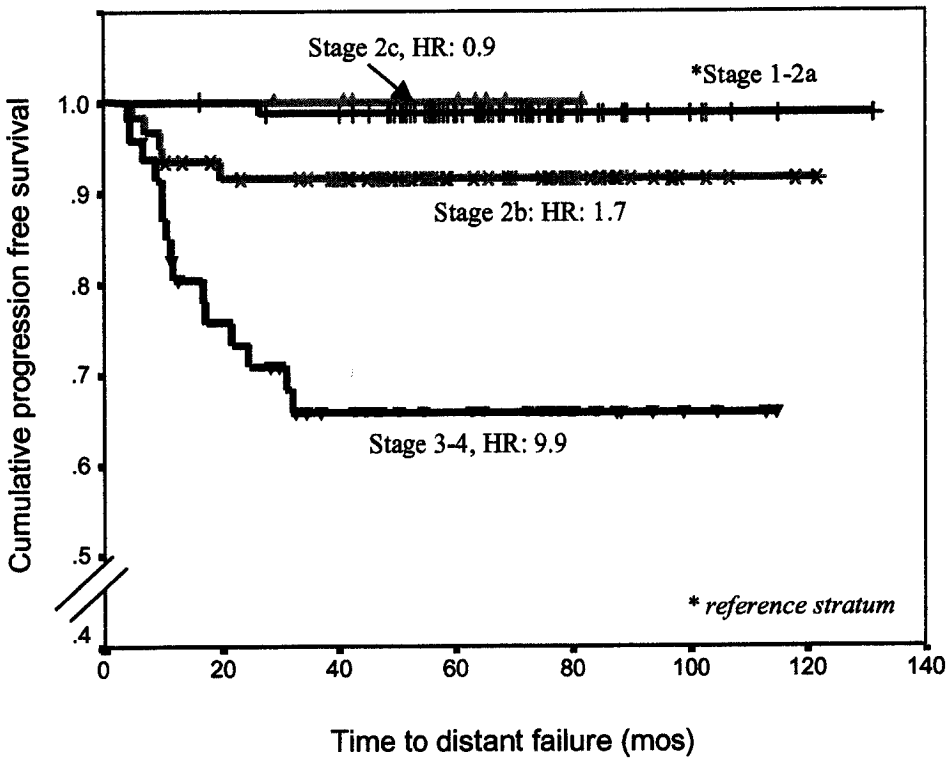


Table 18: Traditional factors and distant progression – Univariate Cox regressions

Variable		n	n events	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
Age				0.11		
	<70	106	8	Ref	1	
	≥ 70	89	13	0.11	2.03	0.84-4.91
Gleason score				0.0055		
	4-6	110	6		1	
	7-9	85	15	0.0090	3.53	1.37-9.11
PSA (ng/ml)				<0.00001		
	0-≤20	130	3	Ref	1	
	>20-50	40	9	0.0002	11.59	3.13-42.87
	>50	15	8	<0.0001	36.35	9.61-137.55
Clinical T stage				<0.0001		
	1-2a, 2b, 2c	148	6	Ref	1	
	3 or 4	47	15	<0.0001	9.58	3.71-24.73

4.5.1.2 Treatment

Radiotherapy was grouped into daily fraction size of 1.8 Gy vs 2.0 Gy. There was no significant difference in the risk of distant recurrence with fraction size (Figure 23). ADT status was also not significantly associated with outcome. These results are summarized in Table 19.

Figure 23: Kaplan Meier estimate of time to distant failure by dose of radiotherapy

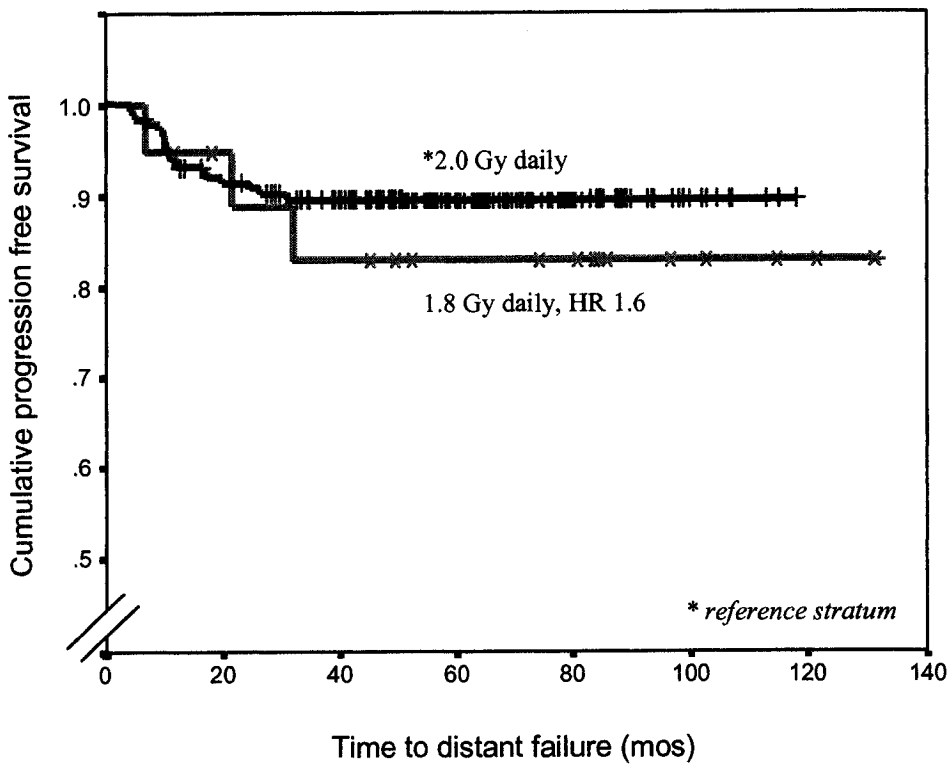


Table 19: Treatment and distant progression – Univariate Cox regressions

Variable		n	n events	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
ADT				0.95		
	Not given	128	14	Ref	1	
	Given	67	7		0.98	0.39-2.42
EBRT				0.46		
	2.0 Gy daily	176	18	Ref	1	
	1.8 Gy daily	19	3		1.58	0.47-5.36

4.5.1.3 Biomarkers

Bax positivity was not a predictor of distant outcome (Figure 24), whereas BCL2 positivity was marginally associated with an increased risk (HR 2.4, 95% CI: 0.9-6.2) (Figure 25). The combination term representing Bax negative and BCL2 positive was not superior to BCL2 alone (HR: 1.8, 95% CI: 0.6-5.5) (Figure 26). In the ADT stratified analysis, the effect of Bax immunopositivity was associated with a reduced risk of distant recurrence amongst individuals receiving ADT (HR 0.21, 95% CI: 0.02-1.8), whereas in ADT naïve patients, it was associated with a higher risk (HR: 1.8, 95% CI: 0.6-6.4). Consistent with this observation, the Bax ADT combination term approached statistical significance (p=0.055). There was no interaction between ADT and the combination term Bax negative and BCL2 positive (p=0.40). ADT BCL-2 interaction could not be evaluated because of the small number of patients in some categories.

Figure 24: Kaplan Meier estimate of time to distant failure by Bax status

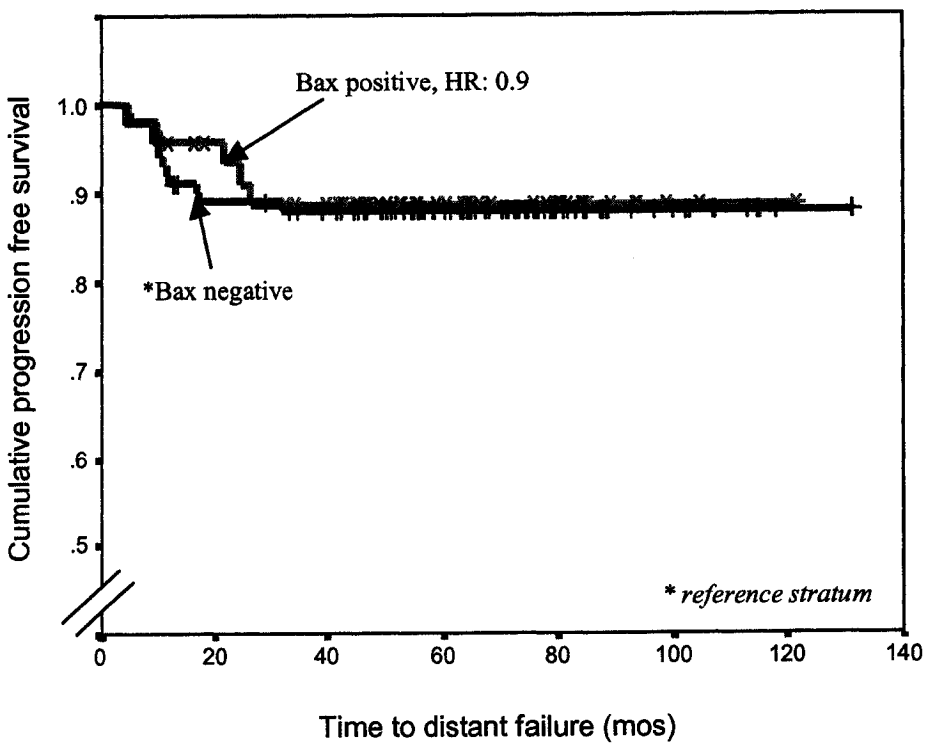


Figure 25: Kaplan Meier estimate of time to distant failure by BCL2 status

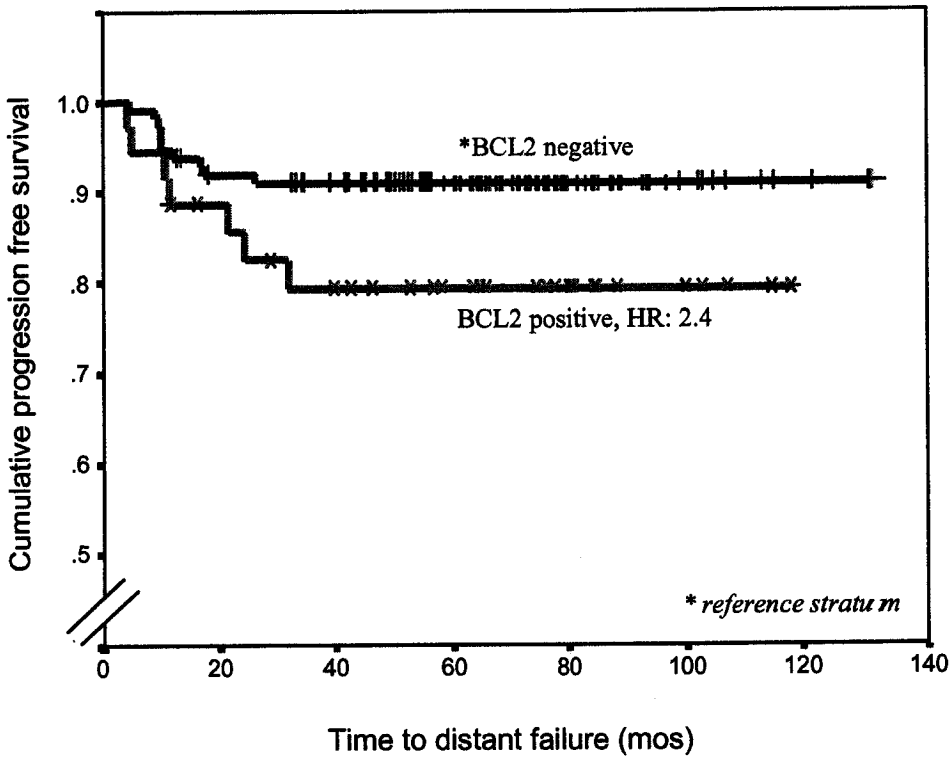
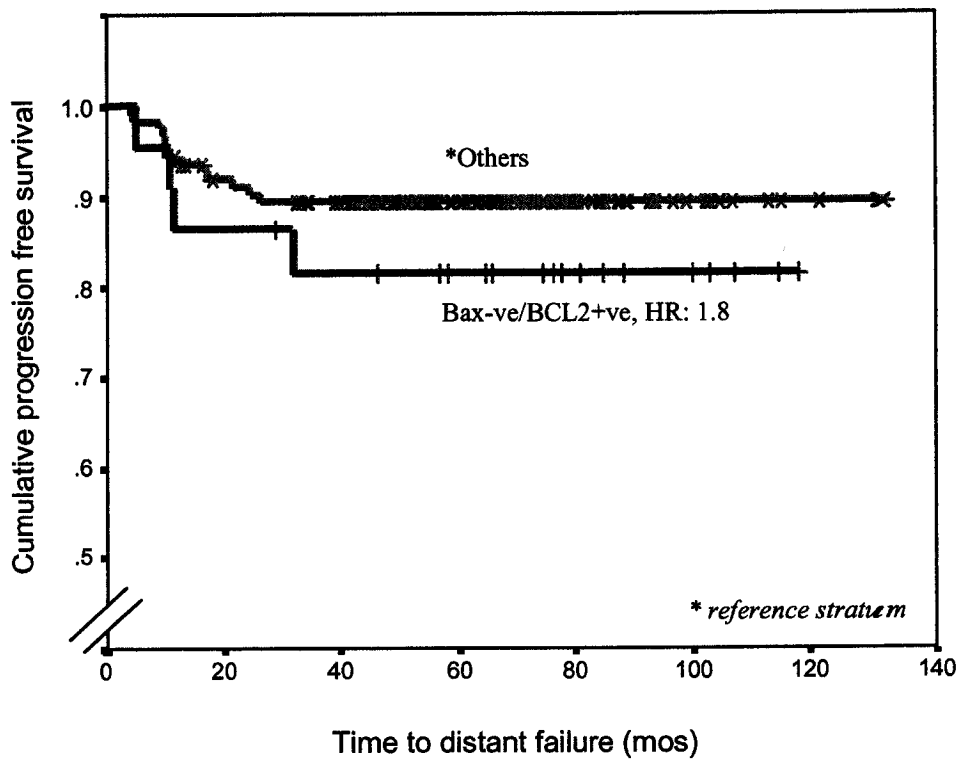
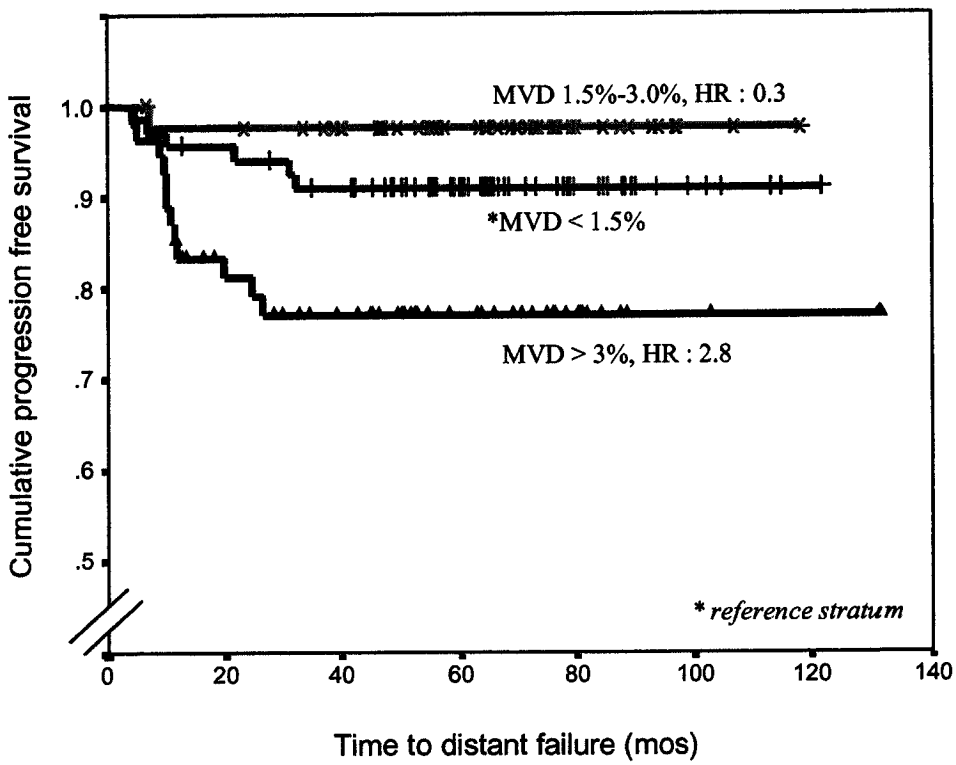


Figure 26: Kaplan Meier estimate of time to distant failure by Bax/BCL2 combination



MVD did not have a linear relationship with the risk of distant progression (Figure C-3). The risk of distant recurrence in patients with MVD values between was apparently lower amongst patients with MVD levels between 1.5% and 3.0%(Figure 27). This may be an aberration related to our small sample size or may reflect true biology. Since the risk level associated with these MVD values was not statistically significant(p=0.23), it was combined with the reference strata (MVD 0-1.5%). Patients with more densely vascularized tissue (MVD ≥3%) had a significantly increased risk of developing distant recurrence (HR: 3.9, 95% CI: 1.5-9.9). There was no apparent effect modifying properties of ADT on MVD (p=0.85).

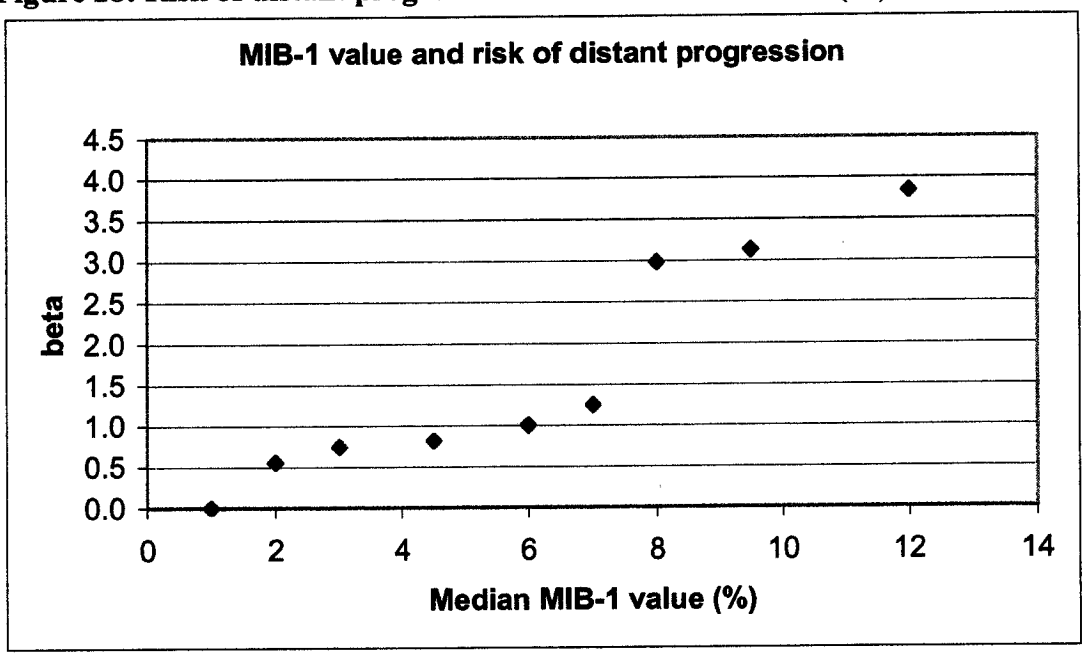
Figure 27: Kaplan Meier estimate of time to distant failure by MVD (%) categories



MIB-1 immunoreactivity level was strongly related to the risk of distant progression (p=0.0009). The relationship between MIB-1 and distant progression appears linear and was

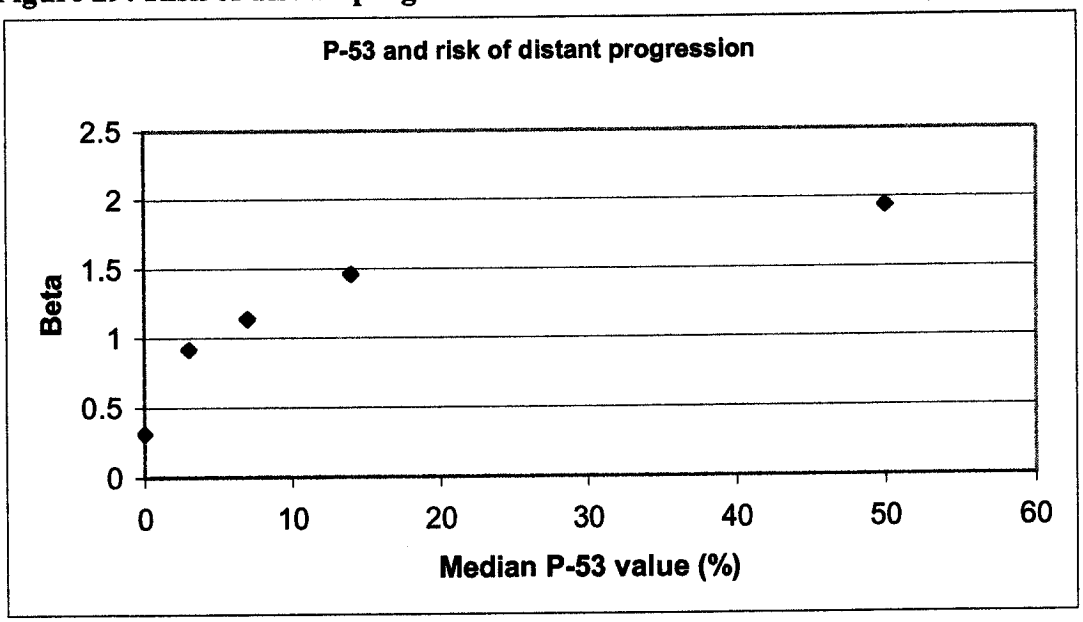
therefore not categorized. To depict that relationship, MIB-1 values were grouped and regressed against distant progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of MIB-1 (Figure 28). The results suggest that for each 1% increase in MIB-1 value, the risk of distant recurrence rises by 7%. ADT did not significant modify the effect of MIB-1 on distant recurrence (p=0.40).

Figure 28: Risk of distant progression associated with MIB-1 (%) values



Higher P-53 immunoreactivity levels were marginally associated with increased risk of distant progression, but this was not statistically significant (p=0.32). Figure 29 represents that relationship between these two variables using the same approach as described for MIB-1. There was no apparent effect modifying properties of ADT on the risk associated with P-53 (p=0.92).

Figure 29: Risk of distant progression associated with median P-53 (%) values



A summary of the value of biomarkers in predicting distant progression and their interaction with ADT is shown in Tables 20 and 21, respectively. Because of the small number of distant events only a drastic interaction would have detectable.

Table 20: Biomarkers and distant progression – Univariate Cox regressions

Variable		n	n events	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
Bax				0.85		
	Negative	100	12	Ref	1	
	Positive	46	5		0.91	0.32-2.57
BCL2				0.082		
	Negative	111	10	Ref	1	
	Positive	35	7		2.36	0.90-6.20
Bax-/BCL2+				0.31		
	Others	125	13	Ref	1	
	True	22	4		1.78	0.58-5.46
% MVD				0.0045		
	0.-<3.0	108	7	Ref	1	
	≥3.0	54	12		3.87	1.52-9.85
% MVD				0.019		
	0-<1.5	67	6	Ref	1	
	1.5-<3.0	41	1	0.23	0.27	0.03-2.26
	≥3.0	54	12	0.039	2.80	1.05-7.48
% MIB-1		152	17	0.0009	1.066	1.027-1.108
% P-53		145	17	0.32	1.011	0.99-1.03

Table 21: Biomarker and ADT interaction in distant progression

Variable		N	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
Bax	Interaction term		0.055		
No ADT	Negative	77	Ref	1	
	Positive	25	0.32	1.87	0.55-6.40
ADT given	Negative	23	Ref	1	
	Positive	21	0.15	0.21	0.024-1.77
Bax/BCL2	Interaction term		0.40		
No ADT	Negative	75	Ref	1	
	Positive	27	0.52	0.65	0.17-2.43
ADT given	Negative	36	Ref	1	
	Positive	8	0.15	0.20	0.023-1.75
%MVD	Interaction term		0.81		
No ADT	0.-<3.0	72	Ref	1	
	≥3.0	36	0.026	3.57	1.17-10.93
ADT given	0.-<3.0	36	Ref	1	
	≥3.0	18	0.086	4.42	0.81-24.18
%MIB-1	Interaction term		0.40		
No ADT	Cont	100	0.036	1.055	1.004-1.109
ADT given	Cont	52	0.005	1.103	1.030-1.181
%P-53	Interaction term		0.92		
No ADT	Cont	100	0.45	1.012	0.982-1.042
ADT given	Cont	45	0.63	1.008	0.976-1.040

4.5.2 Model building: Traditional factors in distant progression

4.5.2.1 Traditional factors: Variable selection

One hundred and eighty five (185) patients were included in the multivariate analysis. Ten (10) were excluded because of missing pre-treatment PSA information. Stepwise selection of variables was performed as described in section 3.7.4 .1 and the results are shown in Table C-1. Pre-treatment PSA levels, clinical T stage, ADT status and Gleason score were all independent predictors of distant progression. Details of the resulting equation are shown in Table 22. PSA categories 20-50 ng/ml and >50 ng/ml were associated with risk levels 8.3 times (95% CI: 2.2-32.1) and 16 times (95% CI: 3.8-68.0) higher than the reference group. Clinical stage and Gleason score also imparted significant

prognostic information, identifying patients at 4.6 (95% CI: 1.6-13.6) and 3.0 (95% CI: 1.1-9.4) times, respectively, higher risk of progression. As expected, hormone therapy is demonstrated protective in this multivariate setting.

Table 22: Multivariate analysis of distant progression using traditional factors

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
PSA (ng/ml)		0.0008			
0-<20	130	Ref	0	1	
20-≤50	40	0.0021	2.12	8.33	2.16-32.09
>50	15	0.0002	2.77	15.98	3.75-68.02
Clinical T stage		0.0054			
1-2c	140	Ref	0	1	
3-4	45	0.0061	1.53	4.62	1.57-13.58
ADT		0.016			
Not given	121	Ref	0	1	
Given	64	0.017	-1.23	0.29	0.11-0.80
Gleason score		0.040			
≤6	104	Ref	0	1	
>6	81	0.043	1.09	2.97	1.05-9.35

4.5.2.2 Traditional factors: Linearity

The scale of PSA was re-examined using visual examination and fractional polynomials as described in section 3.7.4.2 (Figure C-4 and Table C-2). The relationship between PSA and its associated risk remained non linear for higher PSA levels. Amongst the three potential transformations determined superior in the fractional polynomial analysis (1/PSA, 1/sqrt (PSA) or categorized), the categorized form was considered the best representation of the variable despite producing a somewhat lower likelihood ratio (19.3 compared to 22.1 and 24.5). This transformation was selected because it is easier to interpret and the risk associated with PSA values within each group (PSA 20 ng/ml, PSA 20-50 ng/ml and >50 ng/ml) visually appear similar (Figure C-4).

4.5.2.3 Traditional factors: interactions

To investigate the presence of interaction between the traditional factors, interaction terms were investigated as described in section 3.7.4.3. Too few events were present to permit an analysis between most variables except ADT and duration of ADT, and stage and Gleason score. These relationships were determined to be additive only ($p = 0.10$ and 0.56, respectively) (Table C-3).

The preliminary traditional factor statistical model to which biomarkers may be added is therefore unchanged from Table 22. The coefficients associated with each variable are shown in Table C-4.

4.5.3 Model building: Biomarkers in distant progression

4.5.3.1 Biomarkers: Variable selection

The stepwise variable selection process described earlier was repeated to build the model incorporating biomarkers. In the initial step, both Bax ($p=0.044$) and MVD ($p=0.052$) were marginally predictive of distant recurrence (Table C-5). Bax was added first to the model because it produced the largest likelihood ratio (4.0 vs 3.8). Following the inclusion of Bax, MVD was no longer significant and no further variables were added to the model (Table C-6). The biomarker model containing Bax is represented in Table 23.

Table 23: Multivariate analysis of distant progression with model containing Bax

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
PSA (ng/ml)		0.0018			
0-20	100	Ref	0		
>20-50	24	0.0028	2.46	11.66	2.33-58.52
>50	13	0.0004	3.18	24.03	4.09-141.16
Clinical T stage		0.0058			
1-2	103	Ref	0	1	
3-4	34		1.85	6.34	1.70-23.55
ADT		0.15			
Not given	102	Ref	0	1	
Given	51		-0.83	0.44	0.14-1.34
Gleason score		0.033			
<7	77	Ref	0	1	
≥7	60		1.32	3.74	1.12-12.57
Bax		0.056			
Negative	93	Ref	0	1	
Positive	44		-1.16	0.31	0.09-1.03

4.5.3.2 Biomarker: Hormone therapy interaction

In section 4.5.1.3, a univariate analysis had suggested that ADT may be an effect modifier for Bax. However, after adjusting for other covariates, no significant interaction between these two variables was detected ($p = 0.18$).

The coefficients associated with the preliminary biomarker statistical model that contains Bax are shown in Table C-7.

4.5.4 Verifying the proportional hazard

The proportionality hazard for each variable contained in the models was evaluated using the two approaches described in section 3.7.5. Results of the –ln (-ln(S)) plots shown in Figures C-5 through C-8 demonstrate that all variables, except Bax, meet the PH assumption. Results of the extended Cox model (Table C-8), however, did not reveal a

significant departure from proportionality for Bax or any other variable. Therefore, no model adjustment was carried out.

4.5.5 Final traditional and biomarker models

The final models are unchanged from those depicted in Tables C-4 and C-7.

The hazard ratios obtained with each equation (model) were used to produce a graphical representation of the models. The hazard ratios were categorized into quartiles of equal sizes to produce 4 risk categories as described in section 3.7.6. A descriptive summary of the hazard ratios derived from each model is given in Table C-9, and the stratification groups are defined in Table C-10. Curves derived from the traditional model and Bax biomarker model are represented in Figures C-9 and C-10, respectively.

4.5.6 Comparing models: distant progression

To determine whether the addition of Bax to a model containing traditional factors only improved its predictive value and discrimination potential, the two nested models were compared using the AICc and c index as described in section 3.7.7.

4.5.6.1 Information criteria

Results of the information criteria estimates are shown in Table 24. Bax does not add predictive discrimination to the traditional factor model (AICc difference =0.86).

Table 24: Information criteria for the models predicting distant progression

Patients with Bax data	-2LL	K	n	AIC	AICc	BIC
Traditional factor model	109.81	5	137	119.81	124.25	134.41
Bax model	105.77	6	137	117.77	123.38	135.29
Difference in information criterion				**2.047**	**0.86**	**-0.88**

The biomarker model is compared to the traditional model generated in the same subset of patients to produce difference in information criterion.

4.5.6.2 Concordance (c) index

The c index analysis demonstrates that the model based on the traditional factors only has very good discrimination potential. That model could accurately identify between two patients the one most likely to develop a distant progression 92% of the time. Incorporating Bax into the model did not improve its predictive value (Table 25). The c index estimated in the entire group (91.6%) is included to show consistency between the two populations.

Table 25: c indices computations for models predicting distant progression

	Traditional factor model in all patients	**Traditional factor model in patients with Bax data**	**Biomarker model: Bax**
All pairs considered	17020	9316	9316
Unusable pairs	13676	7319	7300
Total evaluable	3344	2026	2026
Count	3117.5	1877.5	1864
c index	91.6%	92.7%	92.0%
Difference			**-0.7%**

C indices for the traditional factor model were estimated in the entire patient population and subpopulations in which the biomarker model was developed. Differences in c indices are estimated from c indices computed in the same population.

4.5.7 Summary

In summary, while the addition of Bax to the traditional factor model produced a statistically significant improvement in the likelihood ratio, the resulting model did not have a superior predictive and discrimination potential.

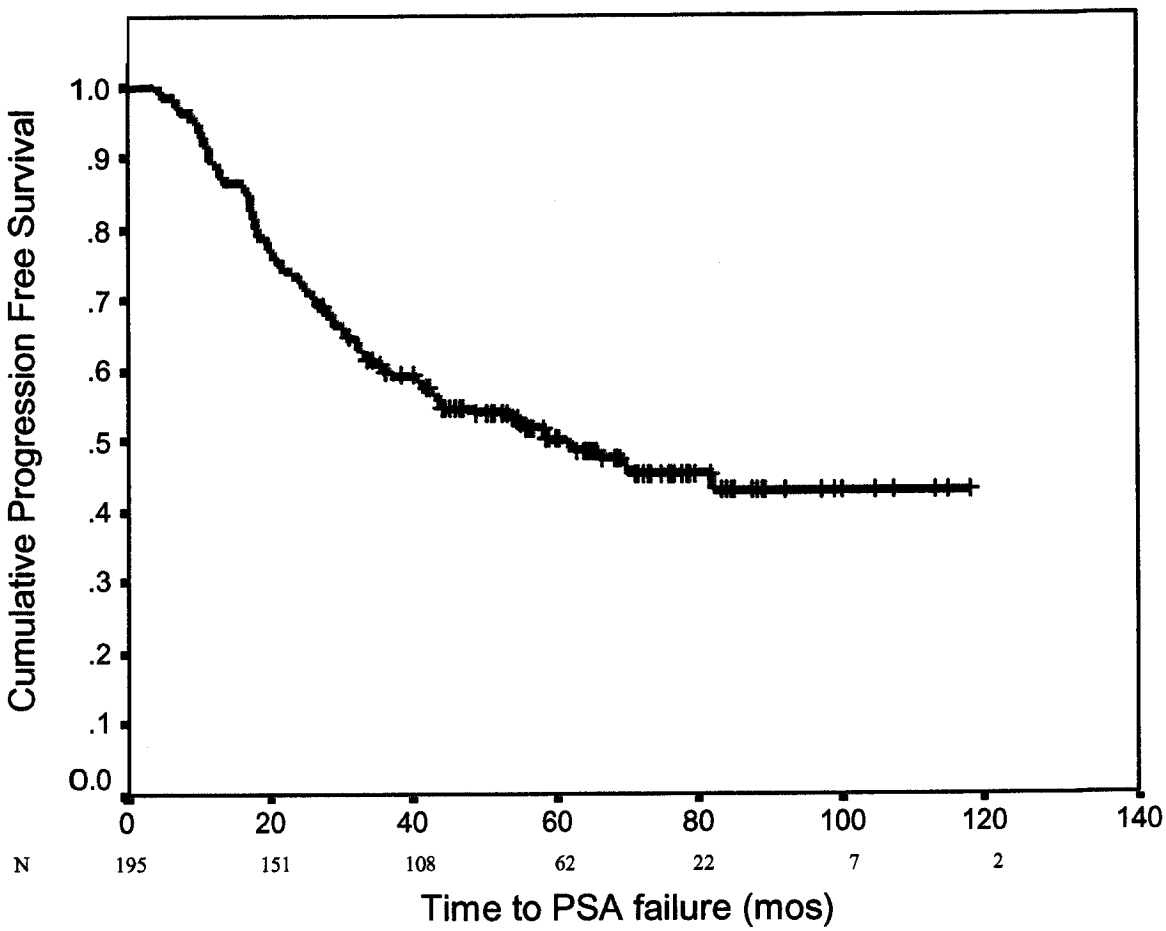
Note: Since the significance level of MVD was very similar to that of Bax, the analysis was repeated allowing MVD instead of Bax to be contained in the biomarker model. MVD did not improve the predictive value of the model based on traditional factors only (results not shown).

4.6 Biochemical progression

4.6.1 Univariate analysis of biochemical progression

At the time of analysis, 104 patients (52%) had a documented biochemical progression. Median time to progression was 61 months, and the 2, 5 and 8 year progression free survival were 73%, 50%, and 43%, respectively (Figure 30).

Figure 30: Kaplan Meier estimate of biochemical progression free survival for all patients



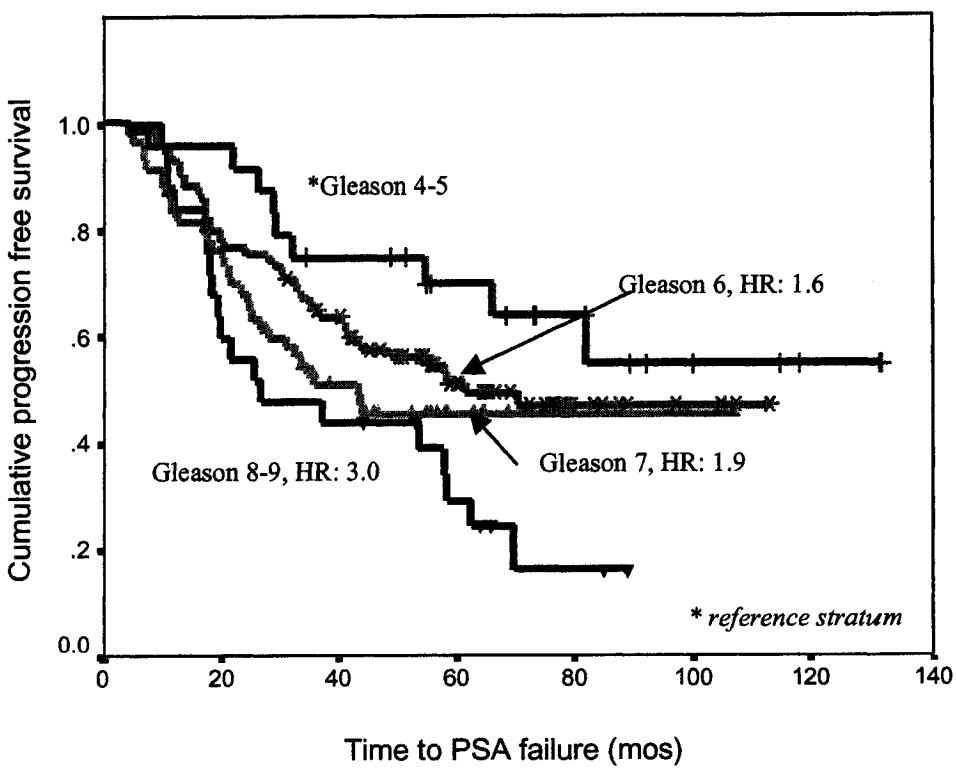
Number of patients remaining at risk is indicated below each time interval

In this section, the ability of biomarkers to improve the prediction of the combined endpoint, biochemical progression, was studied. The procedures carried out for developing and comparing models based on traditional factors only and incorporating biomarkers which were described earlier were repeated for this endpoint.

4.6.1.1 Traditional factors

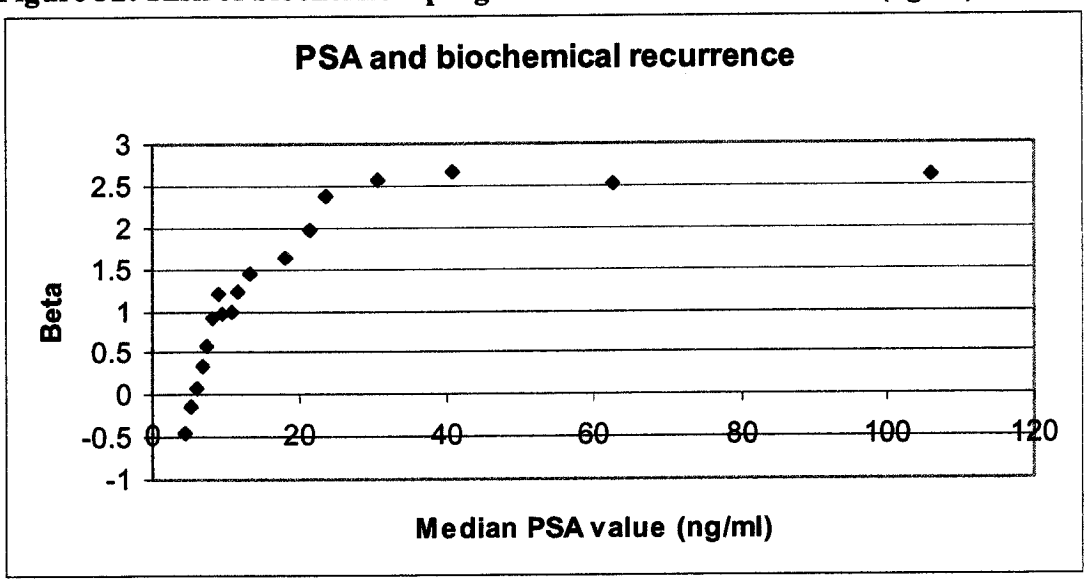
The Kaplan Meier estimate of biochemical PFS by Gleason score is shown in Figure 31. Gleason scores 6 (HR: 1.6) and 7 (HR: 1.9) were associated with similar risk levels and were combined. Compared to patients with Gleason scores 4-5, those with scores 6-7 and 8-9 had hazard ratios of HR: 1.7 (95% CI: 0.9-3.4) and 3.0 (95% CI: 1.3-6.6), respectively.

Figure 31: Kaplan Meier estimate of biochemical progression free survival by Gleason score



Pre-treatment PSA levels were strongly predictive of biochemical recurrence, although the relationship was not linear, especially for PSA values > 25 ng/ml. Since the variable is highly significant and there were no apparent risk clusters, the variable was not categorized. Transformation to achieve linearity will be evaluated in the multivariate setting. The relationship between the untransformed variable and the risk of biochemical progression is shown in Figure 32. To depict that relationship, PSA was categorized and regressed against biochemical progression. The resulting beta values (running averages of three neighboring groups) were plotted against the median values of PSA groups to demonstrate the relationship between the two variables.

Figure 32: Risk of biochemical progression associated with PSA (ng/ml) levels



The relationship between stage and biochemical progression is shown in Figure 33. Stage was grouped into 3 categories containing patients with similar risk levels. Compared to patients with stages 1-2a, patients with stages 2b and 2c-3-4 had a significantly elevated risk of biochemical recurrence (HR: 2.1, 95% CI: 1.3-3.5; and 3.1, 95% CI: 1.9-5.1, respectively) (Figure 33).

Finally, age was associated with the risk of biochemical progression (p=0.58) (Figure 34).

Figure 33: Kaplan Meier estimate of time to biochemical failure by t stage

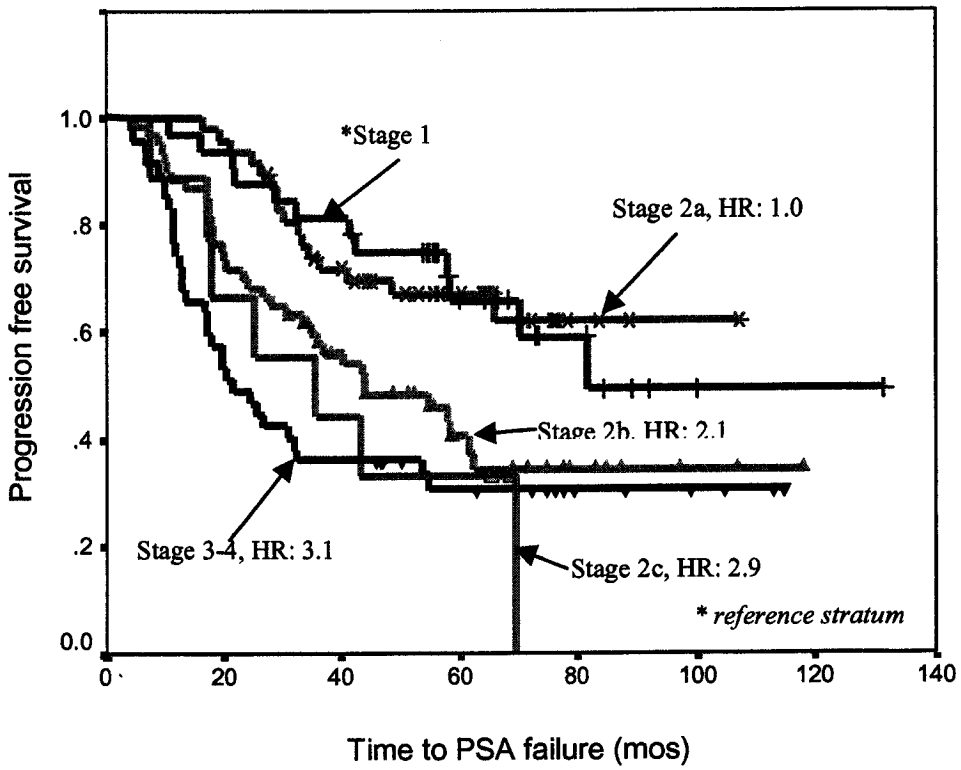
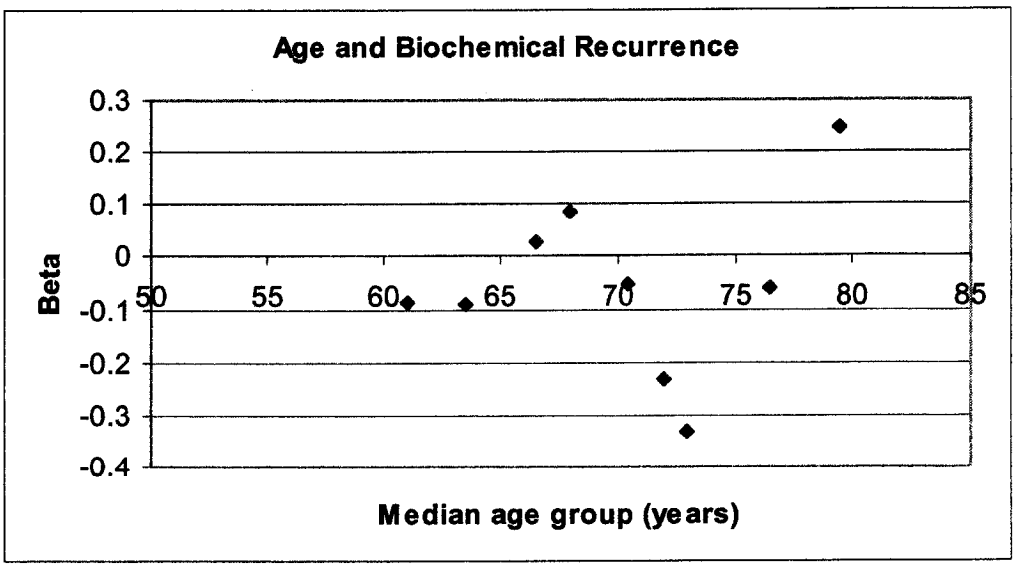


Figure 34: Risk of biochemical progression associated with age groups



A summary of the relationship between traditional factors and the risk of biochemical recurrence is shown in table 26.

Table 26: Traditional factors and biochemical progression – Univariate Cox regressions

Variable		n	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
Age			0.58	1.008	0.98-1.04
Gleason score			0.018		
	4-5	24	Ref	1	
	6-7	146	0.123	1.71	0.85-3.42
	8-9	25	0.0073	2.97	1.34-6.59
PSA (ng/ml)			<0.00001	1.011	1.007-1.015
Clinical T stage			<0.0001		
	1-2a	79	Ref	1	
	2b	60	0.0031	2.12	1.29-3.49
	2c, 3 or 4	56	<0.0001	3.13	1.92-5.10

4.6.1.2 Treatment

ADT status was not a significant predictor of outcome in the univariate setting (p=0.46). However, a smaller daily radiotherapy fraction size (1.8 Gy) (Figure 35) was associated with a higher risk of biochemical recurrence (HR: 1.9, 95% CI: 1.1-3.3). The relationship between treatment and the risk of biochemical recurrence is summarized in Table 27.

Figure 35: Kaplan Meier estimate of time to biochemical failure by dose of radiotherapy

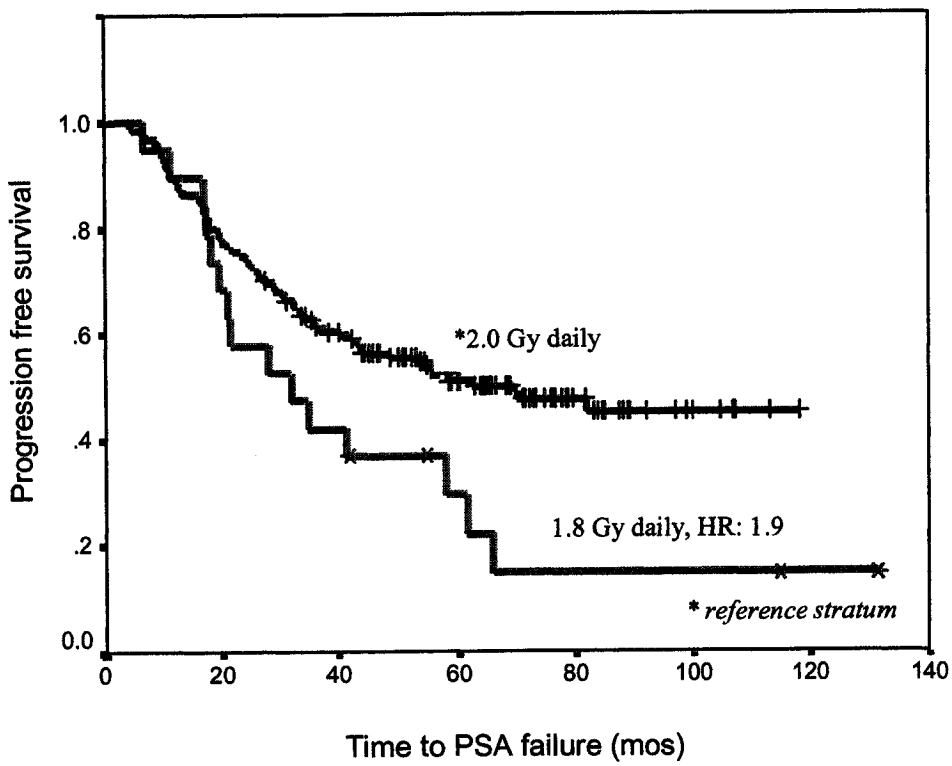


Table 27: Treatment and biochemical progression – Univariate Cox regressions

Variable		n	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
ADT			0.46		
	Not given	128	Ref	1	
	Given	67		0.85	0.56-1.30
Duration of ADT			0.67	0.98	0.89-1.07
EBRT			0.0224		
	2.0 Gy daily	176	Ref	1	
	1.8 Gy daily	19		1.90	1.09-3.28

4.6.1.3 Biomarkers

Bax (Figure 36, p=0.18) and BCL2 (Figure 37, p=0.40) were not statistically significantly related to the risk of biochemical progression. The combination term Bax+ve/BCL2-ve did not warrant investigation. There was no apparent interaction between ADT and Bax (p=0.17). ADT and BCL2 interaction could not be evaluated because of the small number of patients in one category.

Figure 36: Kaplan Meier estimate of time to biochemical failure by Bax status

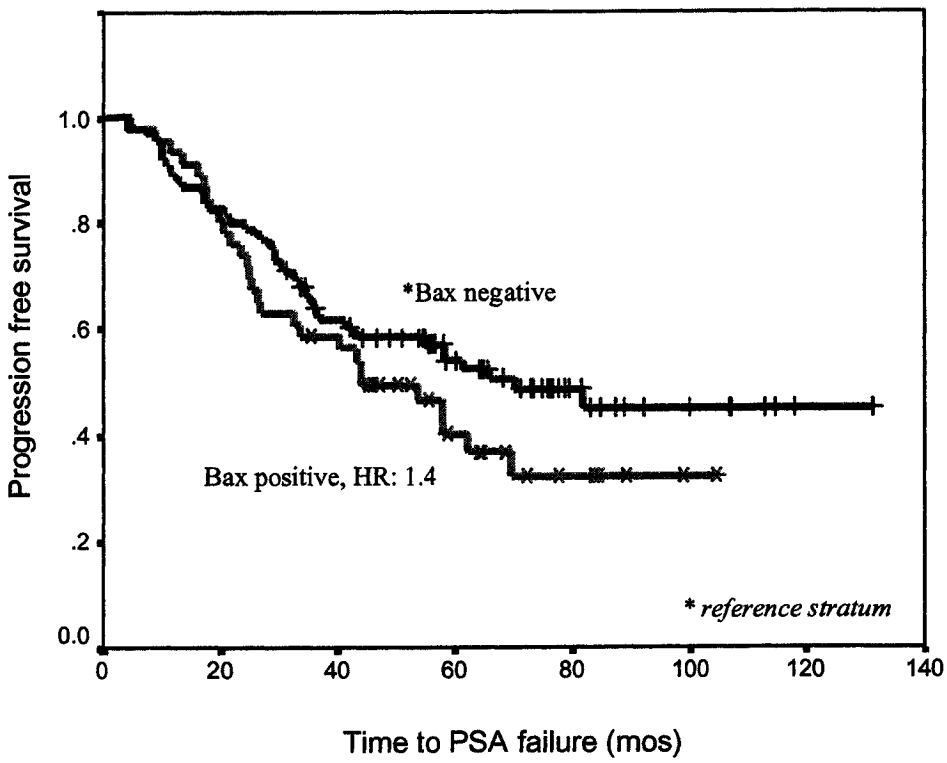
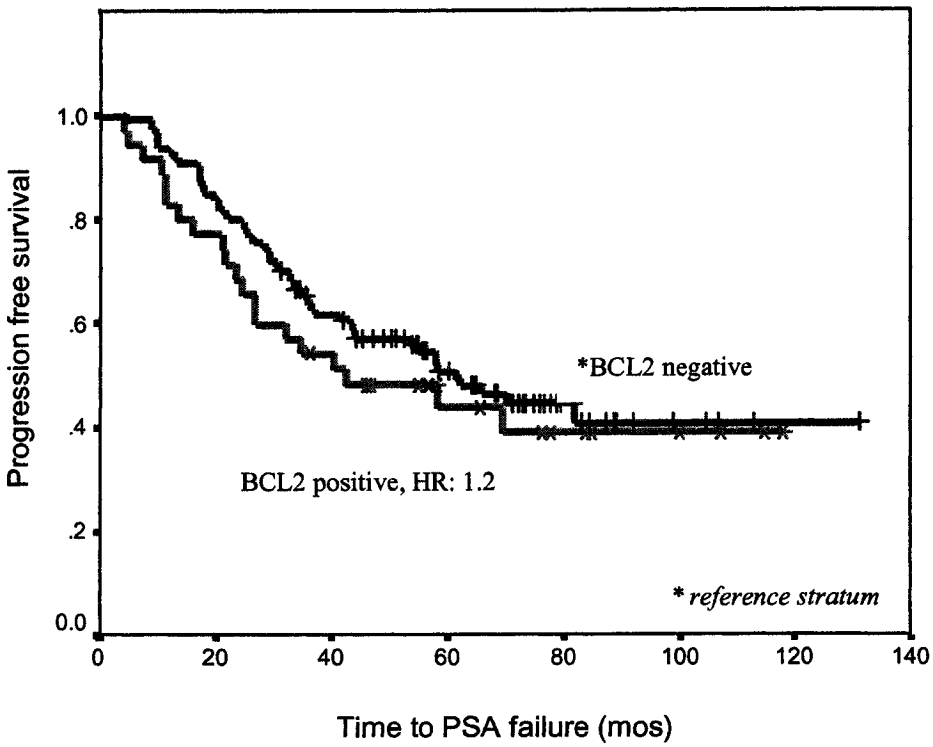


Figure 37: Kaplan Meier estimate of time to biochemical failure by BCL2 status



MVD was positively associated with outcome, for values < 4% only (Figure D-1). Dichotomizing the variable into densities <3% and ≥3% identified patients at increased risk biochemical recurrence (HR: 2.6, 95% CI: 1.7-4.0) (Figure 38). There was no significant interaction between ADT and MVD levels (p=0.56).

The rate of proliferation measured by MIB-1 immunoreactivity was significantly positively associated with outcome (p=0.0075). The relationship between risk of biochemical progression and the untransformed variable is depicted in Figure 39. The risk associated with MIB-1 immunoreactivity was somewhat higher in patients treated with ADT (HR: 1.084) compared to ADT naïve patients (HR: 1.029), but the corresponding interaction term was not statistically significant (p=0.16).

Figure 38: Kaplan Meier estimate of time to biochemical failure by MVD (%) level

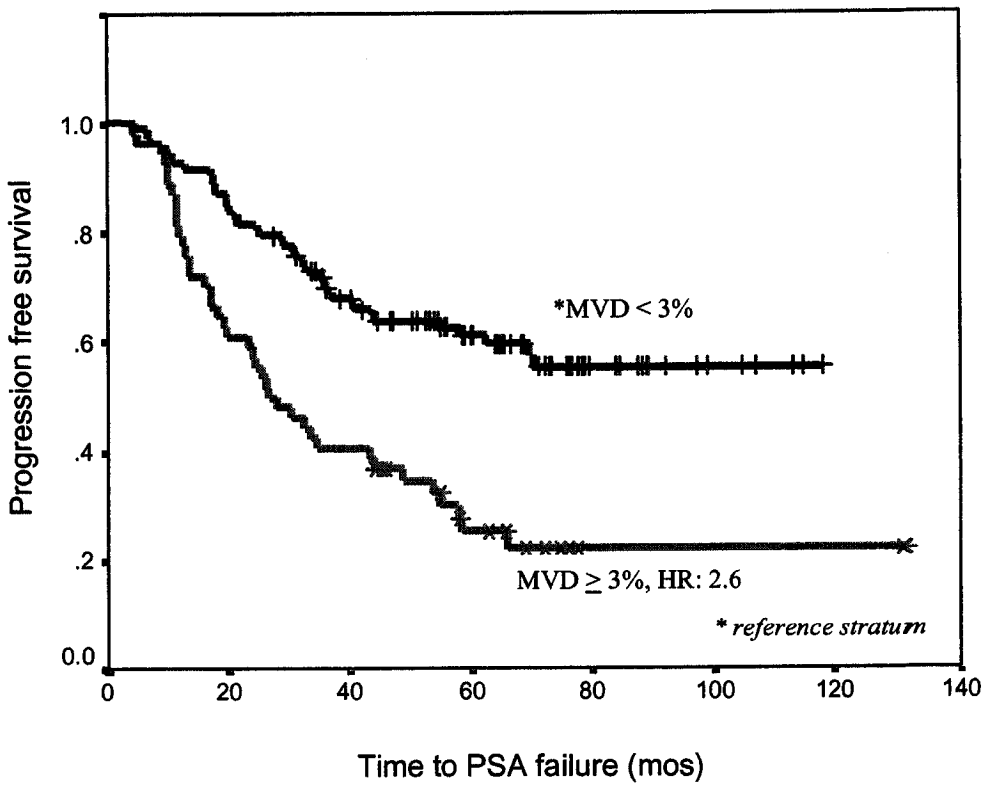
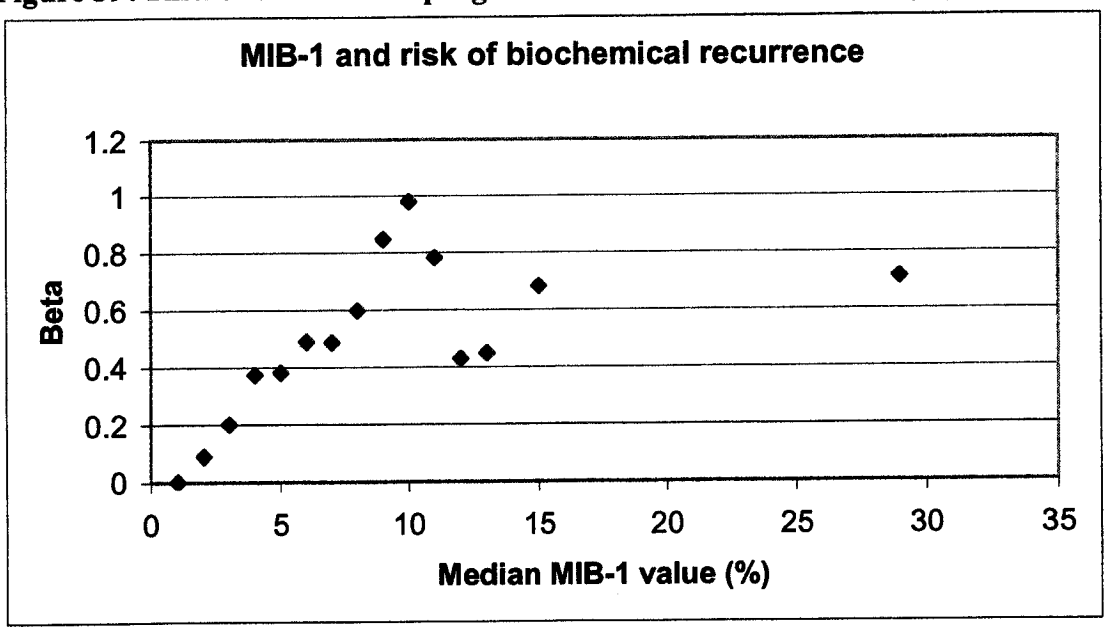
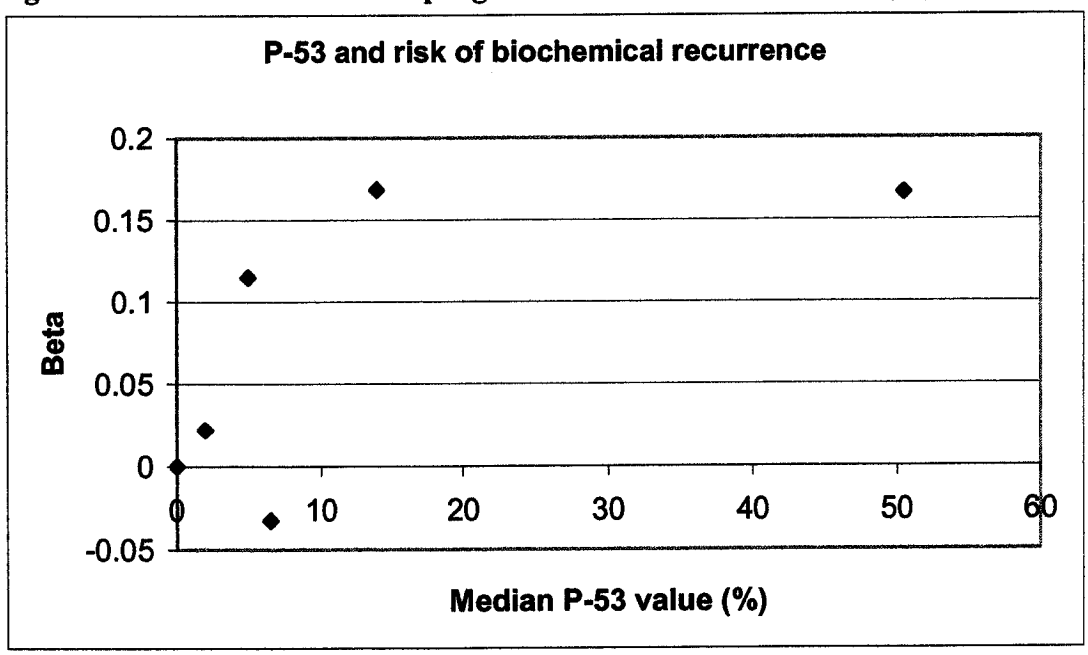


Figure 39: Risk of biochemical progression associated with MIB-1 (%) levels



Specimens in which higher levels of mutated P-53 were detected had a significantly elevated risk of biochemical recurrence (p=0.031) (Figure 40). The relationship between P-53 and outcome was not linear, but since no apparent risk clustering was observed, the variable was not categorized. Transformation will be considered in the multivariate setting. Hormone exposure had no apparent effect on P-53 risk (p=0.85).

Figure 40: Risk of biochemical progression associated with P-53 (%) levels



The value of biomarkers in predicting biochemical progression and their interaction with hormone therapy is summarized in Tables 28 and 29, respectively.

Table 28: Biomarkers and biochemical progression – Univariate Cox regressions.

Variable		N	Wald statistics P value	Hazard ratio	Hazard ratio 95% CI
Bax			0.175		
	Negative	100	Ref	1	
	Positive	46		1.38	0.87-2.20
BCL2			0.399		
	Negative	111	Ref	1	
	Positive	35		1.24	0.75-2.07
% MVD			<0.0001		
	0-<3.0%	108	Ref	1	
	≥3.0%	54		2.58	1.68-3.98
% MIB-1		152	0.0075	1.036	1.009-1.063
% P-53		145	0.031	1.0129	1.001-1.025

Table 29: Biomarker and ADT interaction in biochemical progression

Variable		N	P value	Hazard ratio	Hazard ratio 95% CI
Bax	Interaction term		0.17		
No ADT	Negative	77	Ref	1	
	Positive	25	0.038	1.82	1.03-3.19
ADT given	Negative	23	Ref	1	
	Positive	21	0.73	0.86	0.37-2.03
%MVD	Interaction term		0.56		
No ADT	0.-<3.0	72	Ref	1	
	≥3.0	36	0.0001	2.84	1.69-4.77
ADT given	0.-<3.0	36	Ref	1	
	≥3.0	18	0.068	2.09	0.95-4.62
%MIB-1	Interaction term		0.16		
No ADT	Cont	100	0.060	1.03	0.999-1.061
ADT given	Cont	52	0.008	1.08	1.021-1.151
%P-53	Interaction term		0.85		
No ADT	Cont	100	0.068	1.01	0.999-1.029
ADT given	Cont	45	0.25	1.01	0.992-1.030

4.6.2 Model building: Traditional factors biochemical progression

4.6.2.1 Traditional factors: Variable selection

One hundred and eighty five (185) patients were included in the multivariate analysis. Ten (10) were excluded because of missing pre-treatment PSA information. Stepwise selection of variables was carried out as described in section 3.7.4.1 and the results are shown in Table D-1. Pre-treatment PSA levels, clinical stage, ADT status, Gleason score, and EBRT daily fraction size were all significant independent predictors of biochemical recurrence. Details of the resulting equation are shown in Table 30.

Table 30: Multivariate analysis of biochemical progression using traditional factors

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
PSA (ng/ml)	185	<0.0001	0.0111	1.0111	1.006-1.0164
Clinical T stage		0.0016			
1-2a	79	Ref	0	1	
2b	60	0.0069	0.73	2.08	1.22-3.55
2c, 3-4	56	0.0005	1.04	2.82	1.57-5.05
ADT		0.0051			
Not given	121	Ref	0	1	
Given	64		-0.76	0.47	0.28-0.81
Gleason score		0.028			
4-5	24	Ref	0	1	
6-7	146	0.18	0.50	1.65	0.79-3.44
8-9	25	0.012	1.11	3.03	1.27-7.21
EBRT		0.030			
2.0 Gy daily	176	Ref	0	1	
1.8 Gy daily	19		0.77	2.16	1.08-4.33

4.6.2.2 Traditional factors: Linearity

The scale of PSA was re-evaluated in the multivariate setting using visual examination (Figure D-2) and fractional polynomial analysis (Table D-2). The relationship appears linear for values <30 ng/ml only. PSA transformation into ln (PSA) produced the best likelihood ratio, and this form was confirmed to meet the linearity assumption (Figure D-3). Substituting the ln transformed PSA for untransformed PSA into the equation lead to a decrease in the risk associated with stage and Gleason score, likely because transformed PSA had better predictive value than the untransformed variable. This resulted in little risk difference between stages 2b (HR: 1.5) and 2c (HR 1.8) (Table D-3), and these strata were collapsed. The revised model is shown in Table 31.

Table 31: Multivariate analysis of biochemical progression using traditional factors with PSA transformed and stage dichotomized

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
ln (PSA) ng/ml	185	<0.0001	0.806	2.24	1.76-2.85
Clinical T stage		0.062			
1-2a	79	Ref	0	1	
2b-c, 3-4	116		0.49	1.63	0.97-2.73
ADT		0.0015			
Not given	121	Ref	0	1	
Given	64		-0.82	0.44	0.26-0.796
Gleason score		0.078			
4-5	24	Ref	0	1	
6-7	146	0.27	0.41	1.51	0.73-3.12
8-9	25	0.036	0.92	2.50	1.06-5.89
EBRT		0.016			
2.0 Gy daily	176	Ref	0	1	
1.8 Gy daily	19		0.84	2.31	1.17-4.56

4.6.2.3 Traditional factors: interactions

To investigate the presence of interaction between the traditional factors, interaction terms were evaluated as described in section 3.7.4.3. Tumor grade and stage interaction was found to be statistically significant (p=0.035) (Table D-4). Results of the Cox regression incorporating that term is shown in Table D-5, and the coefficients are interpreted in Table D-6. The results suggest that the increased risk associated with Gleason score is only present amongst patients with stage 2b+. A Cox regression analysis stratified by stage supports this notion (Table D-7). Gleason scores 6-7 and 8-9 are associated with a higher risk of biochemical recurrence only amongst patients with Stages 2b+. Together these results suggest that only patients with stages 2b+ and Gleason scores 6-7 or 8-9 have a higher risk and should be considered separately to support model parsimony. A cross tabulation between various Stage*Gleason score groups and the occurrence of biochemical progression is also in keeping with this concept (Table D-8). Therefore, the six groups

forming the stage*Gleason interaction were collapsed into 3 groups representing all patients with stages 1-2a, those with stages 2b-4 and Gleason scores 6-7 and those with stages 2b-4 and Gleason scores 8-9. The resulting preliminary traditional factor model in which the combination term is included is shown in Table 32, and the coefficient associated with each variable is shown in Table D-9.

Table 32: Traditional factor model incorporation ln PSA and Stage*Gleason score interaction

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
ln (PSA) ng/ml	185	<0.0001	0.79	2.19	1.74-2.77
Stage * Gleason score		0.0022			
[Stage 1-2a (all patients) + Stage 2b-4 & Gleason 4-5]	83	Ref	0	1	
Stage 2b-4 & Gleason 6-7	86	0.0084	0.69	1.98	1.19-3.30
Stage 2b-4 & Gleason 8-9	16	0.0009	1.14	3.12	1.59-6.09
ADT		0.0010			
Not given	121	Ref	0	1	
Given	64		-0.86	0.42	0.26-0.71
EBRT		0.011			
2.0 Gy daily	176	Ref	0	1	
1.8 Gy daily	19		0.86	2.37	1.22-4.60

4.6.3 Model building: Biomarkers in biochemical progression

4.6.3.1 Biomarkers: Variable selection

The stepwise variable selection was performed as described earlier. Only MVD was a significant independent predictor of biochemical recurrence (p=0.0046) (Tables D-10 and D-11). The model incorporating MVD is shown in Table 33.

Table 33: Multivariate analysis of biochemical progression with model containing MVD

Variable	N	Wald statistics P value	Beta value	Hazard ratio	Hazard ratio 95% CI
ln (PSA) ng/ml	153	<0.0001	0.73	2.07	1.59-2.68
% MVD		0.0046			
<3	103	Ref	0	1	
≥3	50		0.68	1.97	1.23-3.15
Stage * Gleason score		0.0024			
[Stage 1-2a (all patients) + Stage 2b-4 & Gleason 4-5]	65	Ref	0	1	
Stage 2b-4 & Gleason 6-7	76	0.0214	0.72	2.05	1.11-3.79
Stage 2b-4 & Gleason 8-9	12	0.0006	1.40	3.82	1.77-8.24
ADT		0.0026			
Not given	102	Ref	0	1	
Given	51		0.87	0.42	0.24-0.74
EBRT		0.0026			
2.0 Gy daily	143	Ref	0	1	
1.8 Gy daily	10		1.02	2.77	1.27-6.04

4.6.3.2 Biomarkers: Verifying linearity

The scale of MVD was re-examined to determine the best transformation in this multivariate setting using visual examination (Figure D-4) and fractional polynomial analysis (Table D-12). MVD was not linearly related to the risk of biochemical progression, and the dichotomized form (MVD ≥3%) produced the best likelihood ratio.

4.6.3.3 Biomarker: Hormone therapy interactions

There was no significant interaction between MVD and ADT (p= 0.36).

The preliminary biomarker factor statistical model is unchanged from Table 33, and the associated coefficients are shown in Table D-13.

4.6.4 Verifying the proportional hazard

The proportionality hazard for each variable contained in the models was evaluated using the two approaches described in section 3.7.5. Results of the –ln (-ln(S)) plots (Figures D-5 through D-8), and extended Cox models (Table D-14) suggest that all variables, except the stratum combining stage 2b+ & Gleason score 7-8, meet the PH assumption (p=0.021). For that stratum, the risk is higher than the reference stratum before 40 months after which, the curves approach suggesting that the hazard rate is lower in that strata than the reference strata. Therefore, an extended Cox model was developed in which a variable coding for time was incorporated into the Stage2b+/Gleason score 6-7 group as shown below. That variable meets the PH (Wald statistics p value of ln (time) interaction = 0.68). The resulting interaction term is interpreted for the traditional model and the biomarker models separately in Tables D-15 and D-16, respectively.

4.6.5 Final traditional and biomarker models

The models were therefore each regenerated using the extended Cox model in which a time-dependent covariate interaction with Gleason and Stage is incorporated. The coefficients associated with the final models predicting for biochemical recurrence are represented in Table D-17.

The hazard ratios obtained with each equation (model) were used to produce a graphical representation of each model. The hazard ratios were categorized into quartiles of equal sizes to produce 4 risk categories as described in section 3.7.6. A descriptive summary of the hazard ratios derived from each model is given in Table D-18, and the stratification groups are defined in Table D-19. Curves derived from the traditional model and MVD biomarker model are represented in Figures D-9 and D-10, respectively.

4.6.6 Comparing models: biochemical recurrence

To determine whether the addition of $MVD_{\geq 3\%}$ improved the predictive value and discrimination potential of the model based on traditional factors only, the two were compared using the AICc and c index as described in section 3.7.7.

4.6.6.1 Information criteria

Results of the information criteria estimates are shown in Table 34. Based on the AICc, a marginal improvement in predictive advantage of the model containing MVD over the traditional factor model is observed. The AICc of the biomarker was smaller than the traditional factor model by 1.46.

Table 34: Information criteria for the models predicting biochemical progression.

Patients with MVD	-2LL	K	n	AIC	AICc	BIC
Traditional factor model	593.7	6	153	605.70	611.25	623.88
Biomarker model: MVD ≥3%	589.06	7	153	603.06	609.79	624.27
Difference in information criterion				**2.64**	**1.46**	**-0.39**

The biomarker model is compared to the traditional factor model generated in the same subset of patients to produce difference in information criterion.

4.6.6.2 Concordance (c) index

The c index derived from the biomarker model is compared to the c index obtained with the traditional model evaluated in the same subpopulation (Table 35). The predictive value of the traditional model is high (82%), and adding MVD only marginally improved the model's discrimination (change in c index = 1%).

Table 35: c indices computations for models predicting biochemical progression

	All TF	TF with MVD	MVD
All pairs considered	17020	11628	11628
Unusable pairs	4567	3209	3209
Total evaluable	12453	8419	8419
Count	10259.5	7019.5	7107.5
c index	82.4%	83.4%	84.4%
Difference			**1.0%**

c indices for the traditional factor model were estimated in the entire patient population and subpopulations in which the biomarker models were developed to demonstrates consistency. The difference in the c indices is computed from the patients with MVD data.

4.6.7 Summary

MVD was a significant independent predictor of biochemical recurrence ($p<0.0001$). However, it only marginally improved the overall predictive value of the model based on traditional factors.

5. DISCUSSION

5.1 Overview

The biological foundation of cell regrowth within the field of radiotherapy and for micro-dissemination is different. Deficits or anomalies in the cellular pathways involved in the biological response to radiotherapy may be responsible for radioresistance and reduce the probability of achieving local control, whereas conditions favorable for the dissemination and survival of tumor cells may increase the risk of developing metastatic disease. It follows that biomarkers that confer increased risk of radio-resistance may not necessarily also be associated with an elevated risk of dissemination, and vice versa. For that reason, biomarkers were studied for their value in quantifying risk associated with local and distant progression separately in a cohort of patients undergoing radical EBRT. Individual mathematical models were developed to predict local and distant progression that were based on traditional factors alone, and to which biomarkers could be included. Since biochemical recurrence is the benchmark endpoint on which previous models had been developed, that outcome was also studied. This study considered: apoptosis associated proteins Bax (inducer) and BCL-2 (inhibitor); cell proliferation marker, MIB-1; cell cycle regulator, P-53; and a measure of microvessel density (MVD) for their value in improving the accuracy of risk level measurements obtained from the traditional factor model. The cohort of patients in whom the biomarkers were evaluated had received standard curative radiotherapy in combination with adjuvant hormone therapy, where indicated. The subjects pre-treatment profile with respect to Gleason score, tumor stage and pre-treatment PSA

levels were grossly similar to those of other cohorts on which prediction models were based (12,13,18,67).

In this cohort, P-53 was not a significant predictor of local or distant progression but was associated with the combined measure of biochemical progression. The findings suggest that higher proliferation levels measured with MIB-1 antibodies were related to a higher risk of local, overall (biochemical) and, to a lesser extent, distant recurrence when studied alone, but not after adjusting for traditional factors.

Bax and BCL-2 immunopositivity were associated with conflicting risk direction in local compared to distant progression. In the univariate setting, Bax immunopositivity was marginally correlated with an elevated risk of local recurrence. In the presence of traditional factors, it was associated with a reduced risk of distant recurrence, and was therefore incorporated into the biomarker model predicting for distant recurrence. However, the larger model containing Bax did not provide better risk discrimination over the model based on traditional factors alone. BCL-2 immunopositivity was marginally correlated with a higher risk of distant progression in the univariate analysis but not after adjusting for traditional factors. A combination term identifying patients with tissue immunopositive to Bax and/or immunonegative to BCL2 was associated with a statistically significant reduced rate of local control, and was therefore incorporated into the biomarker model predicting for local recurrence.

Tumor microvessel density was found significantly associated with poor disease control in general. Higher MVD values were associated with elevated risks of local recurrence, hematogeneous spread of tumor cells and overall biochemical recurrence. MVD was also an independent predictor of the risk of local and biochemical recurrences and was therefore

incorporated in both models. That quantitative estimate of intratumor microvessel density in combination with the interaction term Bax/BCL2 provided a statistically independent and clinically valuable supplemental measure of the risk estimate for local progression. The model incorporating the biomarkers could discriminate with a 7% higher accuracy patients' risk levels. That model also had a lower AICc index than the traditional factor model, suggesting it contained more explanatory potential. When contained in the the model predicting for biochemical progression, MVD only provided a marginal discrimination benefit.

5.2 Methodological approach

5.2.1 Imputing the site of disease progression

Biochemical progression was documented in 52% of patients. In 13%, the site of progression giving rise to the elevated PSA was not known and PSA kinetics was used to impute that site. The PSA doubling time at the time of disease progression had previously been demonstrated to be longer in patients with local progression compared to distant progression (116-118). A receiver operating characteristic curve was used to determine a cut off point that would allow us to assign site of failure. Based on that analysis, a PSA doubling time larger than 5.0 months was determined to provide a sensitive measure (92%) of detecting local progression and was adequately specific (91%). Two other studies have estimated the PSA doubling velocity discrimination level between local and distant progression. Both studies were conducted in prostatectomy patients and determined that a 6.0 month doubling time was an appropriate discriminator of progression site(127,128). Using the PSA cut off mark of 5.0 months, 18 biochemical recurrences were assigned to be

local recurrences. Only one more patient would have been included in that group had a 6-month cut off level been used. The result of incorrectly imputing the site of recurrence would have been to reduce the ability of the analysis to detect an association that might have been present between a factor and outcome, resulting in an effect size estimate with a tendency to migrate towards the null effect. Since the analyses detected profound associations between some factors and outcome, the potential bias introduced in imputing sites of progression was likely minimal.

5.2.2 Outcome assignment

Because the study required that the risk associated with each type of progression be measured independently, separate models were developed to predict local and distant progression. Since the two risks are likely not independent, a competing risk analysis was performed in which patient follow-up for progression at one site was stopped if progression at the other site was documented. The association between local and distant progression has also been demonstrated in other disease sites like lung and breast cancer. A small number of the studies performed a competing risk analysis to evaluate the association between a factor and outcome using approaches documented by Kalbfleish and Prentice or Lagakos (129,130). These are sophisticated methods in which the correlation between a variable and the site and time to progression is evaluated simultaneously. The advantage these approaches provide over standard Cox regression modeling is that risk measurement for variables that are related to both outcomes is based on a larger number of events. The disadvantage is that the risk estimate for covariates associated with local and distant outcomes is a combined one. Therefore, a variable that would have been significantly associated with twice the risk of local progression and 5 times the risk of distant progression

would generate an intermediate risk level. In this study, individual covariates were found statistically significant in predicting both outcomes, but in some cases, were associated with different levels and even direction of risk. For example, in the present analysis, using patients with PSA levels 0-20 ng/ml as a reference, patients with levels >20-50 and > 50 ng/ml were found to have a 12 and 36 times higher risk of distant recurrence, respectively (Table 18). In contrast, that PSA categorization was not appropriate for estimating the risk of local progression because the risk level with the reference strata were very heterogeneous (Table B-1). Had these categories been used, the estimated risk level associated with levels >20-50 and > 50 ng/ml would have been estimated at 3 and 2 times the reference category, respectively (results not shown). For that reason, the Kalbfleish and Prentice or Lagakos approach to competing risk was not selected. Instead, separate analyses were performed for estimating the risk of local and distant progression, and the outcome was assigned from the first observed event.

5.3.3 Biomarker distribution

Findings of biomarkers expression levels consistent with the literature lend credence to the results. Unusual deviations might suggest poor performance of antibodies used or inadequate immunohistochemical techniques. In this section, I briefly compare the results obtained in the present study with previous reports.

Since higher biomarker immunoreactivity may be associated with more aggressive tumor phenotype, their expression in the population studied will be dependent on the profile of that cohort. The present cohort included patients with any T stage (T1 - T4) and without clinical evidence of nodal involvement or metastatic disease. The comparison of the

biomarker expression level in the present study to the literature was therefore limited to studies of patients with disease confined to the prostate.

Mutated P-53 phenotypic expression was found in 47% of tissues that were analyzed. These results are lower than those reported from studies of prostatectomy specimens (57% to 97%)(92,96,99,100,131), but are consistent with biopsy evaluations in which immunoreactivity is detected in 16%-66% of tissues(92,100,132-134). The present estimates of the extent of cells expressing mutated P-53 within immunoreactive specimens are also consistent with other reports of biopsy specimen studies. In this cohort, 10% of the tissue expressed mutated P-53 in more than 20% of their nuclei, consistent with values of 7%-18% established in previous studies, including an RTOG trial (35,135).

Proliferative cellular activity was detected in most tissues. The median proportion of cells immunoreactive to MIB-1 was 5% in our cohort, an estimate that is consistent with studies of prostatectomy and biopsy specimens that report the median proportion of immunoreactive cells at 7% and 8%, respectively(94,133).

BCL-2 and Bax immunoreactivity was determined to be positive in 25% and 33% of patients, respectively. These results are grossly consistent with prostatectomy and biopsy studies in which these apoptosis regulators were detected in 12%-45% of tissue (92,96,99,100,134).

CD-34 monoclonal antibody was used to evaluate the extent of angiogenesis (MVD) because it reported to be better associated with outcome than other antibodies(93). Reports of MVD suffer from heterogeneity in their approach to quantifying not visualizing the vascularity. MVD can represent the manually or digitally estimated count of vessels within a predefined microscope field size (22,78,136,137) or the digitally estimated absolute count

of involved pixels on a computer screen within a predefined field size(138). While the digitally derived estimate correlates very well with manually obtained counts (r=0.95, unpublished results), the results would be dependent on the screen resolution and make comparison with other studies difficult. In the present study, the proportion (and not absolute number) of digitally estimated pixels involved with CD-34 stain relative to the total number of pixels was reported. Since this percentage is independent of field size and screen resolution (except in the amount of random variability expected), it would permit better comparison across studies in the future. The MVD measures obtained in this study could not be directly compared to those of other studies in which variant approaches were used.

5.3 Biomarkers analysis

5.3.1 Correlation with Traditional factors

The primary purpose of evaluating the relationship between biomarker expression levels and traditional factors was to detect associations that could help explain a loss of correlation with progression from univariate to multivariate analysis. Results obtained in this analysis are in general agreement with previously published findings. Denser vascularization was found in specimens with higher Gleason scores(26,32,139,140) and higher stage(141,142). A positive association between the proliferation marker, MIB-1, and stage(96,102), but, surprisingly, not Gleason score, was observed. Gleason score is a measure of tissue differentiation and, therefore, potential for proliferation. Other studies had reported a correlation between Gleason score and MIV-1 immunopositivity levels(96,102,133). The lack of correlation with tumor grade in this study is likely not due to the inherent variability of Gleason scores derived from biopsy specimens, since Gleason score was strongly

associated with MVD and P-53, consistent with multiple previous reports(28,28-33,85,143). Bax and BCL-2 immunoreactivity were marginally associated with stage only. These results are consistent with other reports that failed to detect a relationship between BCL-2 and other measures of disease severity (31,96,133,144). No correlative studies of Bax with traditional factors were found in the literature.

5.3.2 Predictors of outcome

I postulated that biomarkers associated with a generally more aggressive phenotype would contribute to the overall risk of disease recurrence, regardless of site, whereas those conferring radioresistance would identify patients at risk of local but not necessarily distant recurrences, and those facilitating dissemination and survival of metastatic seeds would be associated with a higher risk of distant progression.

5.3.2.1 Local progression

MVD was significantly associated with the risk of local progression. Using a density measure cut off of 3%, one third of the population could be identified that had a significantly increased risk of local progression alone (HR 2.2) and after adjusting for other variables, including Bax/BCL-2 (HR 2.1). One other study reported on the risk associated with MVD amongst prostate cancer patients treated with EBRT. Hall found generally higher risk of recurrence with higher MVD levels, including local recurrence, but the risk level was not quantified(26). MVD has been studied more extensively in prostate cancer patients undergoing prostatectomy. In that population, it was found to be significantly associated with a worse clinical outcome in the majority of studies(22,78-81,83,145,146). Studies of MVD relationship to outcome in other patient populations receiving radiotherapy reported

very significant, albeit conflicting, correlation with local control. In one study of laryngeal carcinoma, elevated MVD levels were found associated with increased radiosensitivity (147). However, consistent with the present report, results of others investigations in oropharyngeal and cervical cancer reported MVD to be associated with an elevated risk of local failure(23-25). How MVD mediates its effect on outcome remains unclear. It is well established that tumor growth depends on neovascularization for its nutritional and oxygenation requirements. We also know that better oxygenated tissue is more radiosensitive(148). Therefore, if MVD measures had reflected enhanced tissue perfusion, and therefore higher oxygenation, MVD levels would have been expected to correlate with superior, not reduced, local control. In a study of oral carcinoma, histopathological evaluation of the response to radiotherapy also showed no correlation between MVD levels and improved tissue response(23). MVD is therefore not likely measuring oxygen perfusion levels. West et al examined the relationship between two measures of vascularity determined immunohistochemistry, and tissue hypoxia measured from Eppendorf needle electrodes(25). The first measure of vascularity was the microvessel density and the second was a measure of interacapillary distance. He showed that high intercapillary distance was related to lower tissue oxygen concentration, whereas MVD was not associated with oxygen levels(25). He further demonstrated that larger intercapillary distances and higher MVD levels were both significantly and independently associated with a higher risk of local recurrence. Because vascular endothelial cell density is not associated with tissue perfusion, the vessels identified with CD-34 stain are likely not structurally or functionally sound. These results suggest that MVD reflects a generally more aggressive phenotype, rather than

a predictor of response to radiotherapy. The elevated risk of distant recurrence observed with higher MVD values is consistent with that hypothesis.

Most studies(34,42,149,150), but not all(36,37), have reported reduced radiosensitivity in P-53 overexpressing specimens. In the present cohort, elevated levels of mutated P-53 phenotypic expression were not significantly associated with the risk of local recurrence.

Bax positivity was associated with an apparently elevated risk of local progression in the univariate setting (HR:1.6) but this was not statistically significant. BCL-2 status alone was also not significantly related to the risk of local recurrence. However, in the multivariate model predicting for local progression, the combination term identifying patients in whom Bax immunoreactivity was found positive and/or BCL-2 negative, was significantly associated with an elevated risk of developing a recurrence within the irradiated field (HR: 7.0). Since Bax is an apoptosis inducer and BCL-2 an apoptosis suppressor, the results are contrary to expectations based on present knowledge. Another study has reported that patients with BCL2 negative phenotypic expression had a significantly reduced cancer specific survival following radiotherapy(39), while two other studies have found higher BCL-2 levels to be associated with a poorer outcome. BCL2 levels were elevated in locally recurrent tumors compared to pretreatment specimens in one study(42), and were predictive of an increased risk of local progression in interaction with Bax in another(38). Since only 12 patients were contained in one stratum of the combination term, the results in this analysis may be an aberration of the small sample size. The potential effect of Bax and BCL-2 on local control requires further study.

In this cohort, MIB-1 labeling index was strongly correlated with risk of local recurrence before adjusting for other variables only. The loss of correlation with outcome in the

presence of traditional factors may be due to the observed association between MIB-1 levels and clinical T stage. There were no reports directly correlating MIB-1 activity with disease control in EBRT. However, MIB-1 immunoreactivity is indirectly linked to outcome in reports showing that levels of MIB-1 immunoreactivity is higher in the prostate biopsy specimens obtained at the time of local failure following EBRT than the pre-treatment specimens from the same patient(41,42).

5.3.2.2 Distant progression

The proliferation index measured with MIB-1 and MVD were strongly associated with the risk of distant recurrence alone, but not after adjusting for traditional factors (MVD was marginally significant). The apparent association between both biomarkers and stage may be responsible for the observed loss of correlation. MVD and MIB-1 proliferation rate were both significantly associated with local and distant progression, suggesting that these markers may reflect generally a more aggressive tumor phenotype(40,77).

Addition of Bax status to the traditional factor model produced a statistically significant improvement in the equation. After adjusting for traditional factors, patients with Bax positive phenotypes were found to have a reduced risk of developing distant metastases (HR:0.31, 95% CI: 0.1-1.0). The association between higher Bax immunoreactivity and improved outcome may be a result of the apoptosis inducing activity attributed to Bax.

An RTOG trial in which the association between P-53 association and outcome was evaluated reported that, amongst patients treated with adjuvant ADT, those with tumors overexpressing P-53 were more likely to develop distant metastasis(151). Results of studies conducted in patients treated palliatively with ADT also suggest the presence of a P-53

interaction with ADT(152,153,153-156,156,157). In the present study, mutated P-53 levels correlated poorly with risk of distant metastasis, and no interaction with ADT was detected.

5.3.2.3 Biochemical progression

MVD measure of tissue vascularity, MIB-1 index and P-53 were all significantly associated with an elevated risk of biochemical recurrence. Their impact on risk was not significantly modulated by ADT. Only MVD was an independent predictor of outcome.

The current knowledge on the role of biomarkers in predicting disease recurrence following EBRT in patients with prostate cancer was reviewed in the previous sections, in the context of the site of progression.

5.4 Supplemental value of biomarkers

The biological basis for tumor regrowth at the site of original disease (local failure) and for dissemination of disease (distant progression) are different. I believe that combining these two outcomes into a single category (biochemical failure) confounds the effect of a marker on one outcome by its effect (or lack of) on the other outcome. I therefore studied the association between biomarker expression levels and the risk of local or distant progression separately. Independent models were built for the prediction of local recurrence and distant progression. Since all nomograms reported in the literature were developed exclusively to measure the risk of biochemical failure, that endpoint was also studied. The aim was to determine whether the addition of biomarkers to a model based on traditional prognostic factors only would improve its predictive discrimination.

Several studies have quantified the value of traditional factors in predicting recurrence risk levels amongst patients treated with radical EBRT(65,68,158-161), but few provide a

complete nomogram incorporating all three basic factors: PSA, tumor grade, and stage (12-20). Unfortunately, all models were developed to measure the risk of biochemical failure, and could therefore only be directly compared to the results of the biochemical progression model developed in this study.

5.4.1 Local recurrence

The main objective of the present analysis was to determine whether biomarkers add prognostic value beyond that already contained in traditional factors. The fact that a biomarker is independently statistically significant (i.e. in the presence of traditional factors) does not necessarily demonstrate added value. For example, in the distant recurrence model, Bax was a significant independent variable, but did not improve the predictive value of that model. The AICc and c index were used to quantify the change in predictive ability. The AICc is an estimate of the explanatory value of the model founded on the –2LL value but that introduces a penalty proportional to the number of variables (enforcing parsimony) and another for small numbers of events. Essentially, the AICc is a value that represents a trade-off between underfitting and overfitting the sample data. Comparing the AICc values for two models estimated in the same patient population provides an objective estimate of the true difference in explanatory value between the models. The c index is a measure of a model's ability to discriminate between those patients who will benefit from treatment and those more likely to relapse. The c index is derived by determining, for each pair of patients, whether the clinical outcome of the two patients is concordant to the model's prediction for these patients.

In the local progression model, the addition of MVD and Bax/BCL2 was found to improve the predictive ability of the model based on traditional factors alone. When the –

2LL associated with these models were compared, the biomarker model provided more explanation of the variance, even after penalties were introduced to correct for the biomarker model's larger size and small sample size to parameter ratio. The AICc was reduced by 11.4 points in the biomarker model compared to the traditional factor model. The biomarker model could also distinguish with 7% higher accuracy individual patient risk levels (i.e. increase in c index by 7%). Because one strata of the Bax/BCL2 interaction term contained only 12 patients, it raised the concern that the model may be overfitting idiosyncrasies in the data rather than predicting true biological associations. For that reason, a biomarker model based on MVD and excluding the combination term Bax/BCL2 was also evaluated. That model was also found superior to the traditional factor model using the AICc estimate and c index. However, the improvement was not as impressive in the absence of Bax/BCL2.

The present study did not attempt to produce a clinical decision tool upon which risk level determination could be based and from which treatment decision could be made. The goal was to determine, using the strictest criteria, whether biomarkers provided supplemental value to the currently used factors. For example, during model development, I strived to optimize the predictive value of traditional factors so that improvement obtained from adding biomarkers would represent truly complementary information. The results suggest that biomarkers do contain supplemental information to traditional factors. We plan to validate the results obtained in this study in a separate cohort. If the biomarkers value is confirmed, a clinical decision tool may be developed that incorporates biomarkers. A cost-effectiveness analysis could then help determine whether incorporating biomarker evaluation in standard clinical practice is warranted.

The traditional factors were less reliable in predicting local progression (c index = 70%) than in predicting distant progression (c index = 92%). Biomarkers were more contributory to local than distant progression risk estimates. This is likely due to generally weaker correlation of traditional factors with local compared to distant progression, leaving more of the variance to be explained. In the univariate setting, PSA categories were associated with smaller hazard ratios for local (HR: 3 and 6) compared to distant progression (HR: 12 and 36). Gleason score was only marginally significant at predicting local progression in the univariate setting and was not an independent predictor after adjusting for PSA and stage. It was, however, more strongly associated with distant progression. Other studies conducted in patients undergoing radiotherapy reported similar loss of correlation with tumor grade after the addition of PSA, supporting the notion that the poor correlation observed in the present cohort is not a result of poor variable definition(67,68). Because of the same approach was used to develop the local and distant model and because every effort was made to optimize the variable, reduced reliability of the local progression model is likely not due to sub-optimal performance of the traditional factor resulting from the analytical approach.

5.4.2 Distant recurrence

The model based on traditional factors alone had very good discrimination. That model could correctly identify between two individuals the one with the higher risk of developing distant recurrence 9 out of 10 times (c index 92%). The addition of Bax to the traditional factor model did not add predictive value.

In this cohort, all traditional variables were strongly associated with the risk of distant progression. Pre-treatment PSA level was the single most influential measurement in

predicting distant recurrences. Based on PSA levels alone, a cohort of patients making up 70% of the population (PSA $\leq$20 ng/ml) and for whom the risk of distant recurrence was negligible could be identified. The remaining 30% could also be divided into cohorts with 12 and 36 times higher risk levels. Gleason scores (HR: 3.0) and stage (HR: 4.6) were also powerful determinants of distant progression. Although Bax was an independently significant variable associated with a HR of 0.3, it did not contribute supplemental information to the model.

MVD and Bax were both independent predictors of distant progression. Bax was selected for inclusion because it was associated with a marginally better likelihood ratio than MVD. Because of the exceptional performance of the traditional factor model, any improvement in discrimination with the addition of biomarker could likely only be demonstrated in larger patient cohorts.

5.4.3 Biochemical

MVD was a significant independent predictor of biochemical progression but added little prognostic value to the model. The model containing MVD produced a marginally smaller AICc (1.5) and a 1% increase in concordance index. I postulate that because the model based on traditional factors alone has a high performance (c index = 82%), the additional value of biomarkers was minimal.

All previously published models were based on the prediction of biochemical recurrence. Comparing the predictive discrimination of the current model to those in the literature is problematic when correlative indices or other measures of discrimination are not provided. The Kaplan Meier curves built from strata derived from a model are a useful visual representation of the model. They facilitate clinical interpretation of the model. A

model that can identify a group of individuals with very little risk of recurrence and another with almost certain recurrence is attractive. However, that separation between the curve of different strata cannot be used to compare models since the number of strata with which a model is depicted, number of patients in each stratum and overall progression free survival in the cohort varies between studies. That variability prohibits direct comparisons.

Models can only be compared when an objective measure of discrimination is reported. Kattan evaluated the validity and accuracy of a model developed by his group(12) to the main nomograms developed in EBRT(13,14,17,18,87,88) by computing the nomograms' Somer's D indices within a separate validation cohort. Somer's D is a measure of concordance related to the c index. Somers' D indices reported in Kattan's study were converted to c indices by dividing the measure by two and adding 0.5 as described by Harrell and colleagues (126) to allow comparison with the present results. Derived c indices ranged from 65%(87) to 73%(14) for the nomograms evaluated in that study. Kattan's model estimated c index was 76% (Somers' D = 0.52). In the present study, the c index estimated from the biochemical risk model was 82%. Although this estimate likely represent an inflated measure of correlation because it was estimated in the same population in which it was developed whereas the estimates obtained in Kattan's study were all externally validated measures, it may be useful in explaining the minimal contribution of biomarkers.

The present study is the first to report a model that includes biomarkers for the prediction of outcome after EBRT. Two models developed to predict disease control following prostatectomy and incorporating biomarkers have been reported(97,104). Leibovich et al studied the added value of mutated P-53 expression, and found that it

provided 5% increase in the c index over a model containing traditional factors only (reported c index increased from 66% to 71%)(97). Bauer and colleagues considered MIB-1, P-53 and BCL-2 in the prediction of biochemical failure. Using a backward elimination model building approach in which variables were excluded based on their significance level, they produced a model containing P-53, BCL-2, PSA and one measure of stage but not tumor grade. The ensuing predictive hazards were used to create three risk groups. The authors depict the results graphically with Kaplan Meier estimate and conclude that the model has good discrimination, but did not quantify the added benefit of the biomarkers(104).

5.5 Conclusions and future work

Traditional factors, clinical stage, PSA levels and Gleason scores are routinely available clinical parameters that are highly predictive of disease control following radiotherapy. Several biomarkers were also correlated with outcome, and in some settings provided a significant improvement in predictive power over traditional factors alone. Clinical decision tools incorporating biomarkers may be useful in categorizing patient risk level and identifying those potentially eligible for biomodulator or gene therapy. For example, if MVD is confirmed useful in identifying patients at higher risk of recurrence, MVD may be incorporated into a model to stratify patients into risk categories and may provide a rational for anti-angiogenic therapy in the population with elevated MVD. In athymic mice carrying prostate cancer, treatment with a monoclonal antibody to human angiogenin, the peptide responsible for angiogenesis, was associated with a reduced rate of distant metastases(162). Clinical studies of thalidomide and other drugs with angiogenic properties are underway,

and preliminary results also suggest promising activity(58,59). Biomarkers warrant further investigations in patients receiving EBRT.

The results obtained in the present study suggest that MVD, Bax and BCL2 may provide supplemental information to traditional factors in identifying patients with higher risk of local progression. Further investigation is warranted.

5.6 Limitations

Outcome prediction models are useful only if they predict a clinically relevant endpoint and provide a reliable and discriminating measure of risk. The present models predict two clinically significant endpoints (local and distant recurrences) as well as a combined measure of biochemical failure. The biomarker models also have good predictive discrimination in the cohort of patients in whom they were developed (c indices 72%-92%) but these results remain to be validated in a separate cohort. Because of the small number of patients for whom biomarker results were available, data splitting techniques could not be used to develop the model. In that approach, the data set is divided into a training sample in which the model is developed, and a validation sample in which the generalizability of the model developed is measured(163). I plan to validate the present models in a separate dataset on which biomarker assessments are currently being performed.

In developing the models, other known measures of disease severity related to tumor volume such as number of biopsy core involved and percentage of tissue involved within positive biopsies were not considered. Although these measures are readily available today, for the cohort of patients studied, these results were not consistently documented.

Biomarker studies are a relatively new field of research. The field lacks consistency in methodological approach and reporting. Current reports are based on analyses carried out

using a variety of techniques, antibodies with increasing sensitivity in more recent years, and are reported in the absence of standardized scales. These disparities make comparisons between studies less reliable. Despite this, results between studies are largely congruent, at least in prostatectomy specimens where multiple foci of immunoreactivity may be sampled. Prognostic models in EBRT rely on biopsy estimates of biomarkers and Gleason score, both of which have been shown to have reduced reliability when measured in biopsy specimens. Prostate cancer samples are heterogeneous in their biomarker P-53 and BCL-2 expression(164) and measures obtained from needle biopsy apparently underrepresent the actual level of biomarker expression. In fact, some studies have found that in the same patient population where P-53 and BCL-2 activity evaluated from prostatectomy specimens were found to be independent predictors of outcome, the biopsy correlates of these studies were not(103,133). To compensate for lower accuracy of measurements obtained from biopsy specimens, models predicting for outcome following EBRT should be developed in large cohorts of patients where biologically meaningful association may be statistically separated from the inherent variability and idiosyncrasies of the data. The cohort available for model development in this study was relatively small. To avoid overfitting the data, data reduction techniques were used, limiting the number of parameters permitted in the model to no more than 1 in 10 events for local and biochemical progression by setting the significance level to $p<0.10$. The numbers of parameters could not be limited to one tenth of the events in the distant progression models because of the small number of events observed.

Each biomarker expression level was evaluated in a subset of patients (149-166 patients) either because we had insufficient tissue or because tissue was preserved for future analyses. This could have introduced a bias in the estimated effect of the biomarkers if there was a

non-random exclusion of tissues from a biomarker analyses. For example, insufficient tissue availability may be due to smaller tumor volumes and may therefore be associated with stage. This potential preferential exclusion of patients with lower stage from some analyses could have produced a biased estimate of biomarker effect only if that effect was dependent on stage. The present study did not reveal this type of interaction.

The limitations discussed above, taken overall, do no invalidate the findings in this study.

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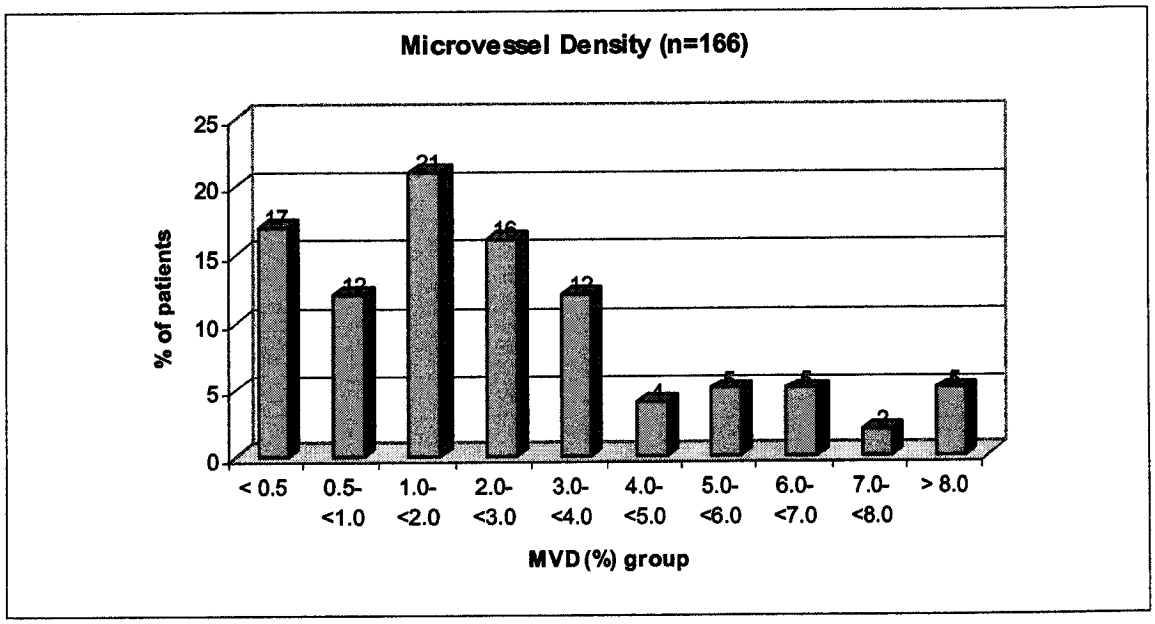
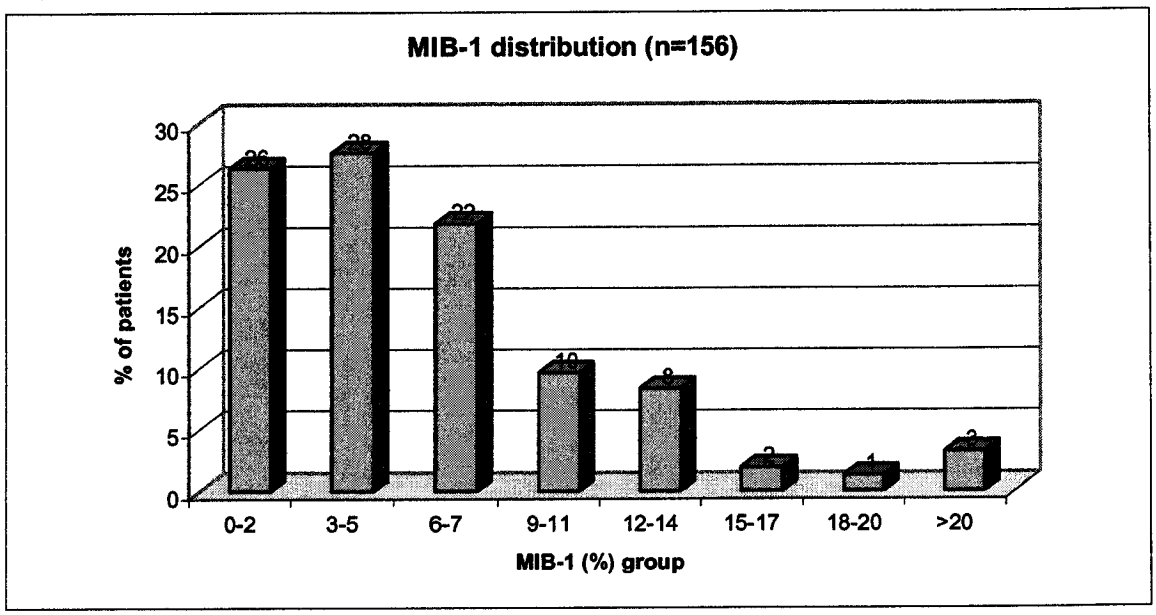
APPENDICES A

Figure A-1: Frequency histogram of grouped MVD (%) scores



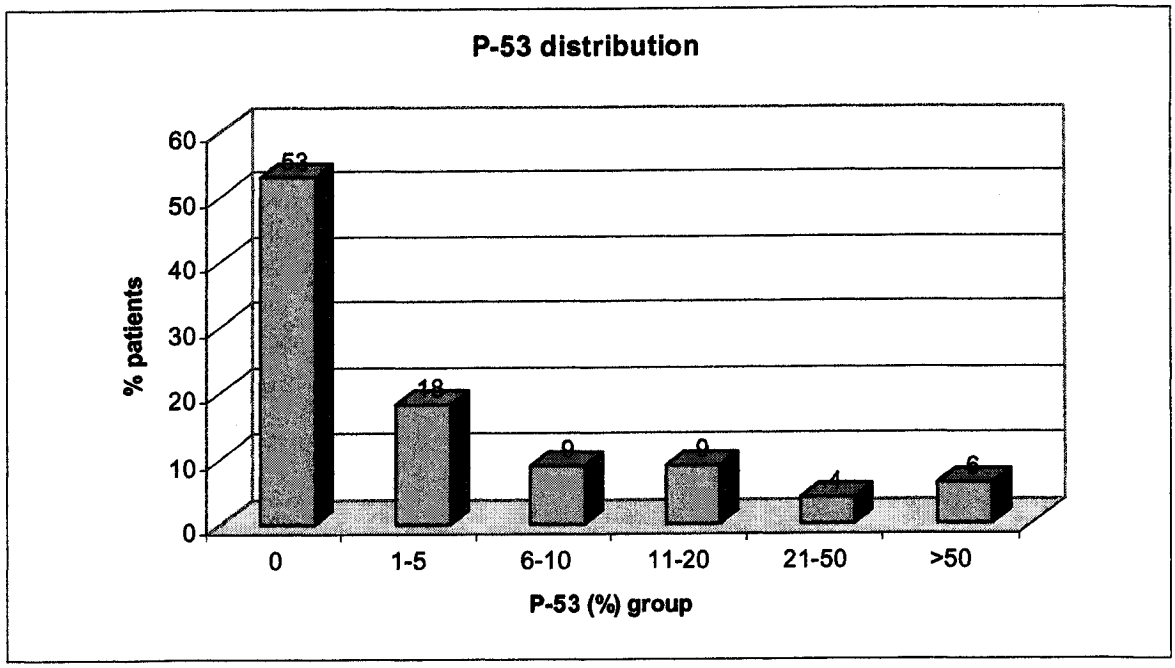
Numbers at the top of each bar represent the percentage of patients in that MVD range

Figure A-2: Frequency histogram of grouped MIB-1 (%) scores



Numbers at the top of each bar represent the percentage of patients with that MIB-1 range.

Figure A-3: Frequency histogram of grouped P-53 (%) scores

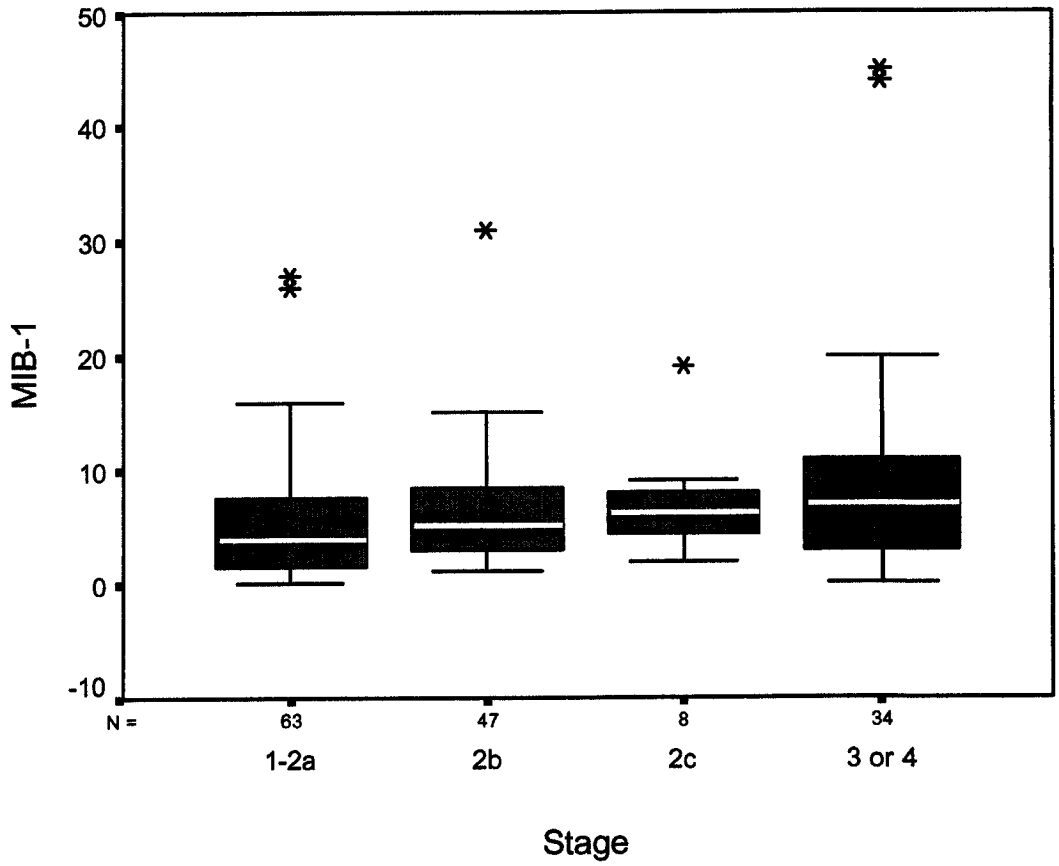


Numbers at the top of each bar represent the percentage of patients with that P-53 group.

Table A-1: Imputed outcome based on PSA doubling time cut off value of 5.0 months

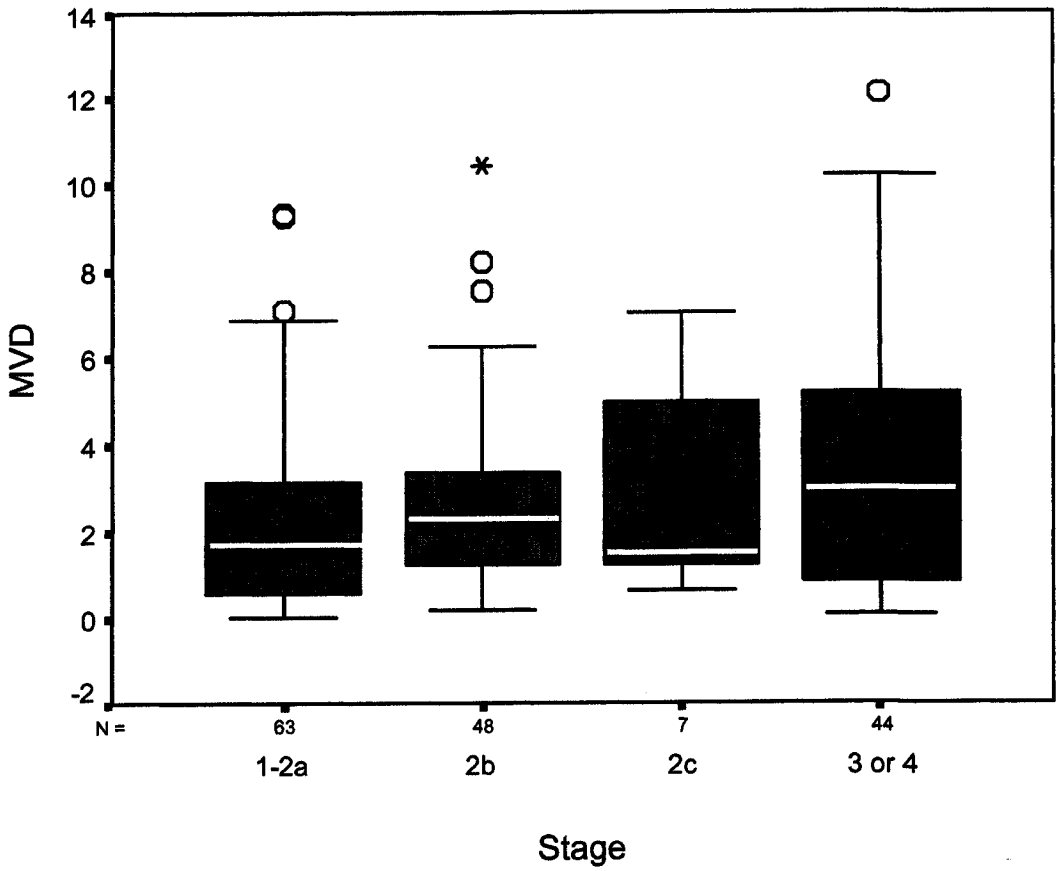
PSA doubling time (months)	Status	Revised status
1.7	Unknown	Distant
3	Unknown	Distant
3.2	Unknown	Distant
3.6	Unknown	Distant
3.8	Unknown	Distant
4.3	Unknown	Distant
4.8	Unknown	Distant
5.5	Unknown	Local
6.3	Unknown	Local
7.5	Unknown	Local
7.6	Unknown	Local
8.3	Unknown	Local
8.7	Unknown	Local
10.2	Unknown	Local
11.5	Unknown	Local
12.8	Unknown	Local
14.6	Unknown	Local
15	Unknown (then distant)	Local (then distant)
18	Unknown	Local
19.2	Unknown	Local
21.4	Unknown	Local
21.4	Unknown	Local
25.1	Unknown	Local
27.7	Unknown	Local
41.9	Unknown	Local

Figure A-4: Box plot of the MIB-1 (%) summary information for stage



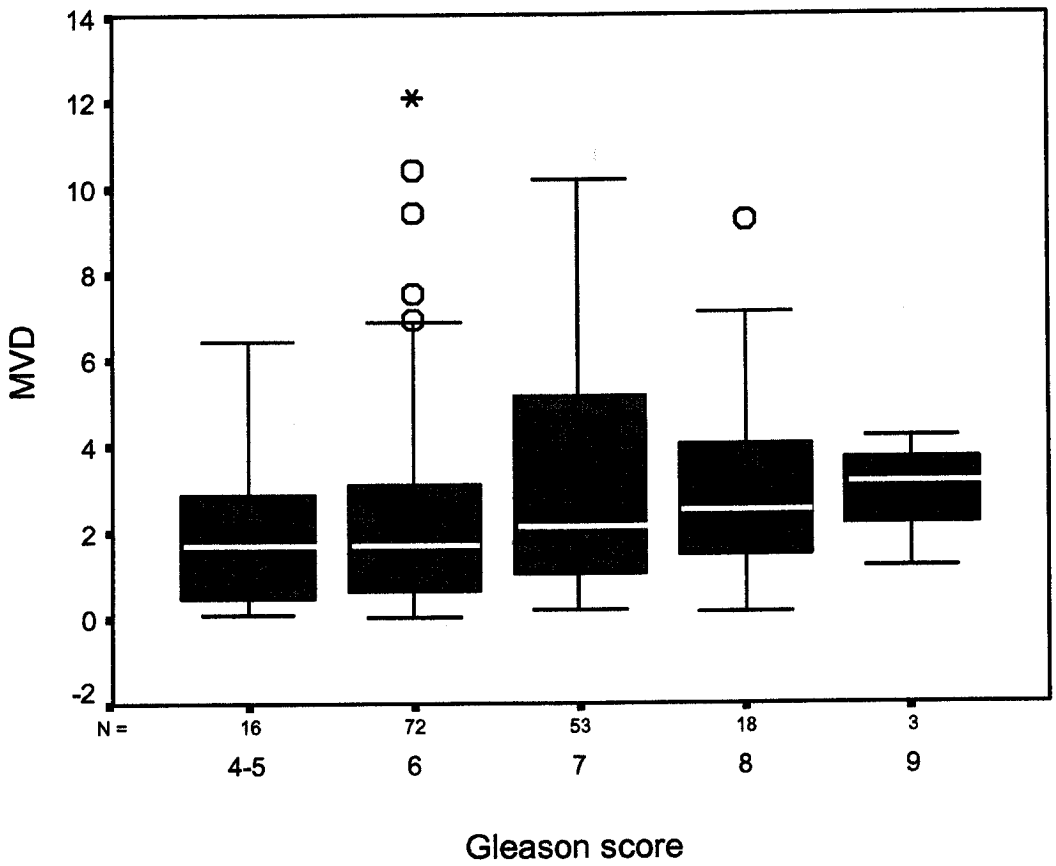
The boxes represent the interquartile range, the dark line inside each box is the median, the whiskers are the ranges excluding outliers and extreme values, the symbols outside the whiskers are values >1.5 the box length. Sample sizes are reported above each category.

Figure A-5: Box plot of the MVD (%) summary information for stage



The boxes represent the interquartile range, the dark line inside each box is the median, the whiskers are the ranges excluding outliers and extreme values, the symbols outside the whiskers are values >1.5 the box length. Sample sizes are reported above each category.

Figure A-6: Box plot of the MVD (%) summary information for Gleason scores



The boxes represent the interquartile range, the dark line inside each box is the median, the whiskers are the ranges excluding outliers and extreme values, the symbols outside the whiskers are values >1.5 the box length. Sample sizes are reported above each category.

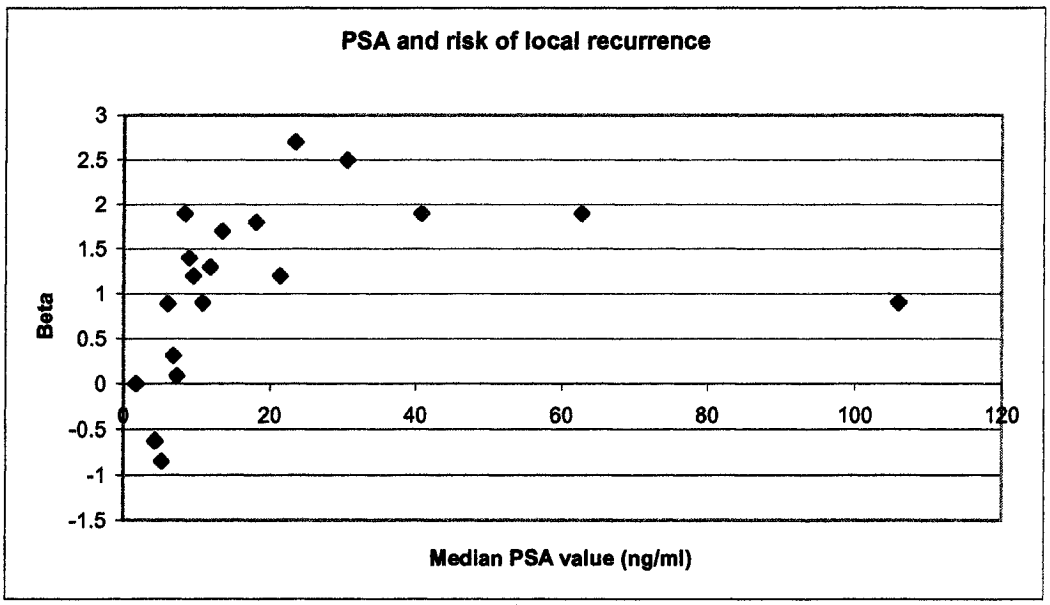
Table A-2: Cross tabulation between P-53 (%) groups and Gleason score

P-53 (%) range	% of patients in each Gleason score category				
	4-5	6	7	8	9
0	64	61	35	67	50
2-5	14	18	23	13	0
6-9	14	2	19	0	0
10-19	9	12	12	0	25
20+	0	8	12	20	25

Percentages of cases within each Gleason category are shown for each P-53 group.

APPENDICES B

Figure B-1: Risk of local progression associated with PSA (ng/ml) levels



PSA was categorized and regressed against local progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of PSA groups to demonstrate the relationship between the two variables and evaluate linearity.

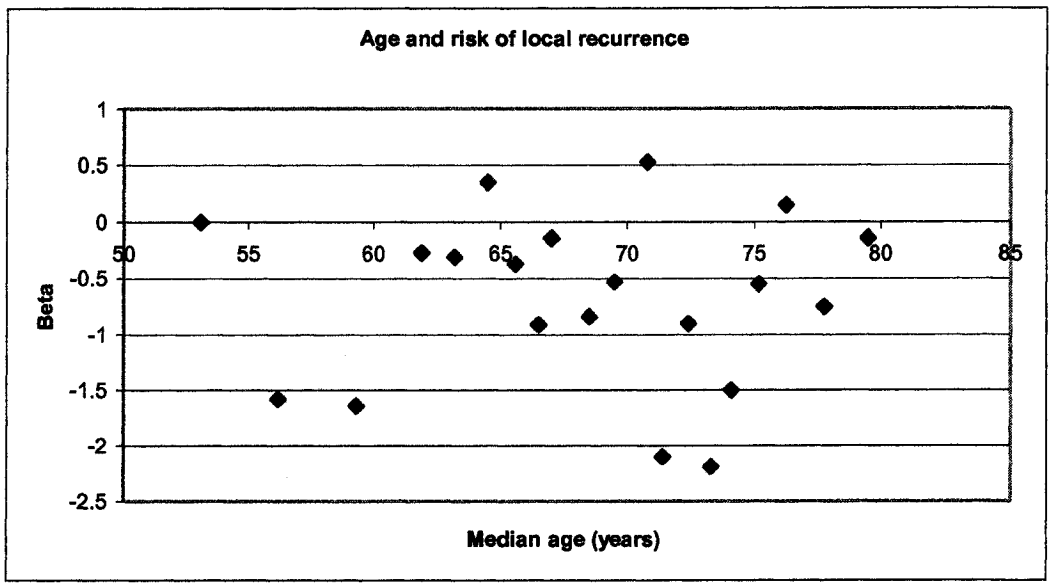
Table B-1: Median PSA (ng.ml) values and the proportion of distant progression

PSA (ng/ml) range	N	Number (%)with distant progression
0.9-10.0	88	0 (0)
10.1-20.0	40	2 (10)
20.1-218	57	18 (86)
Unknown	10	1 (5)
Total	195	21

Table B-2: Clinical stage and the proportion of distant progression

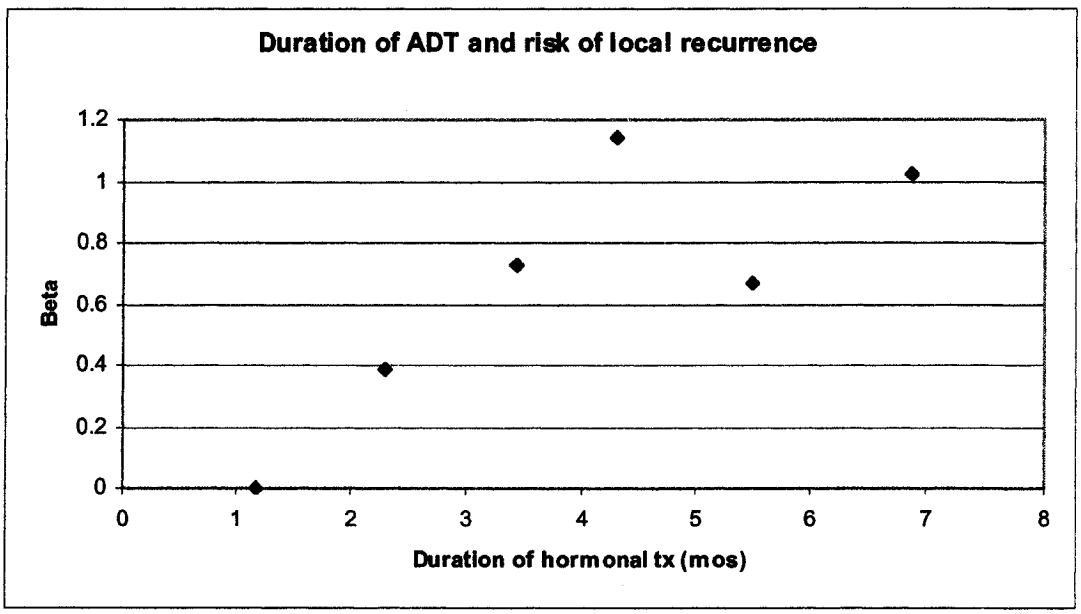
Clinical T stage	N	Number (%)with distant progression
1	32	0 (0)
2a	47	1 (5)
2b	60	5 (24)
2c	9	0 (0)
3 or 4	47	15 (71)
Total	195	21

Figure B-2: Risk of local progression associated with age (years)



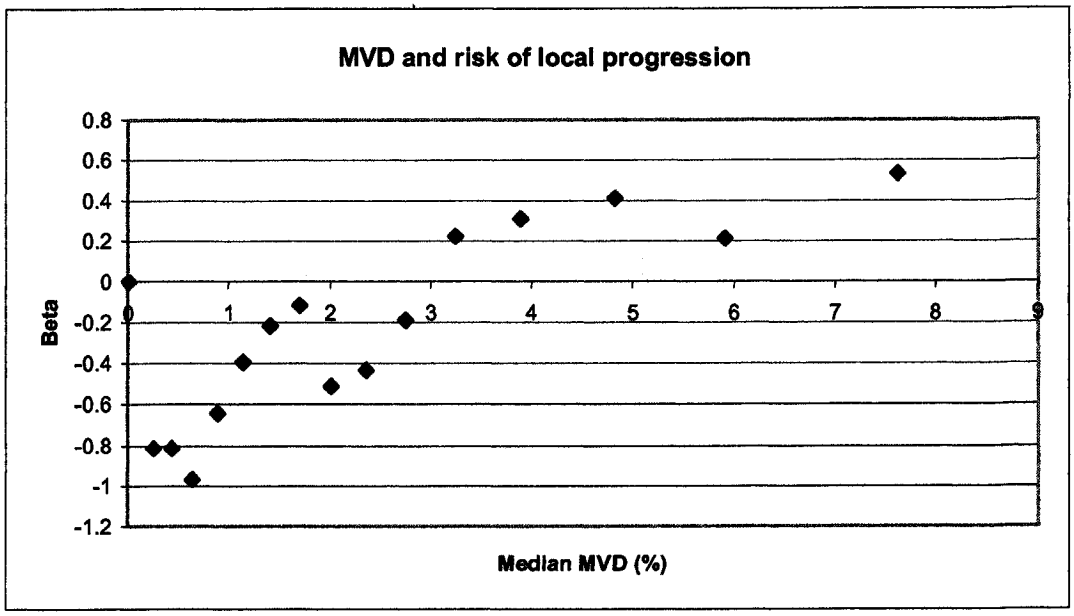
Age was categorized and regressed against local progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of age groups to demonstrate the relationship between the two variables and evaluate linearity.

Figure B-3: Risk of local recurrence associated with the duration of hormonal therapy (months) amongst patients having received hormonal therapy



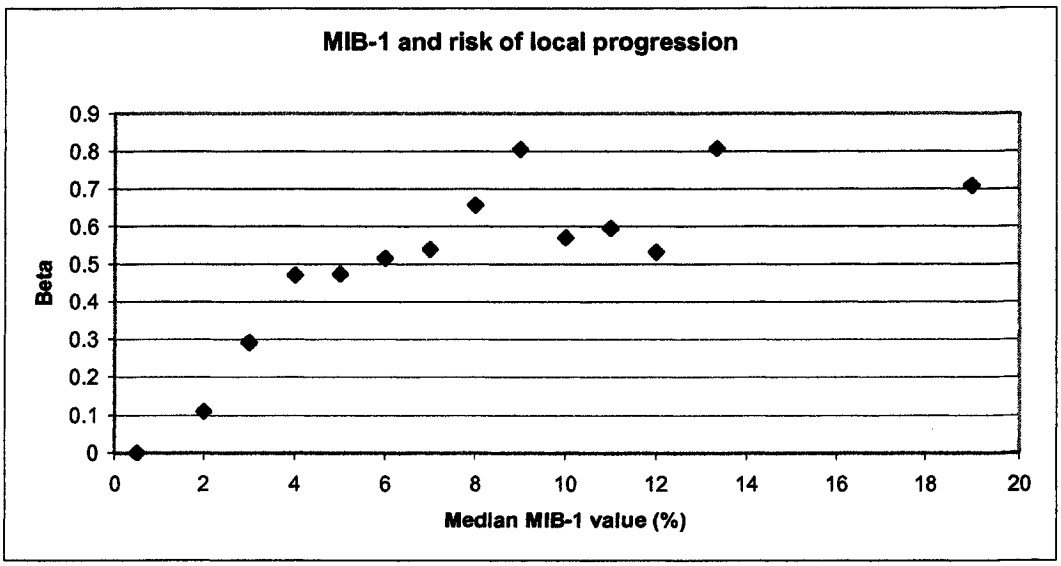
Duration of ADT was categorized and regressed against local progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of ADT treatment duration groups to demonstrate the relationship between the two variables and evaluate linearity.

Figure B-4: Risk of local progression associated with median MVD (%) values



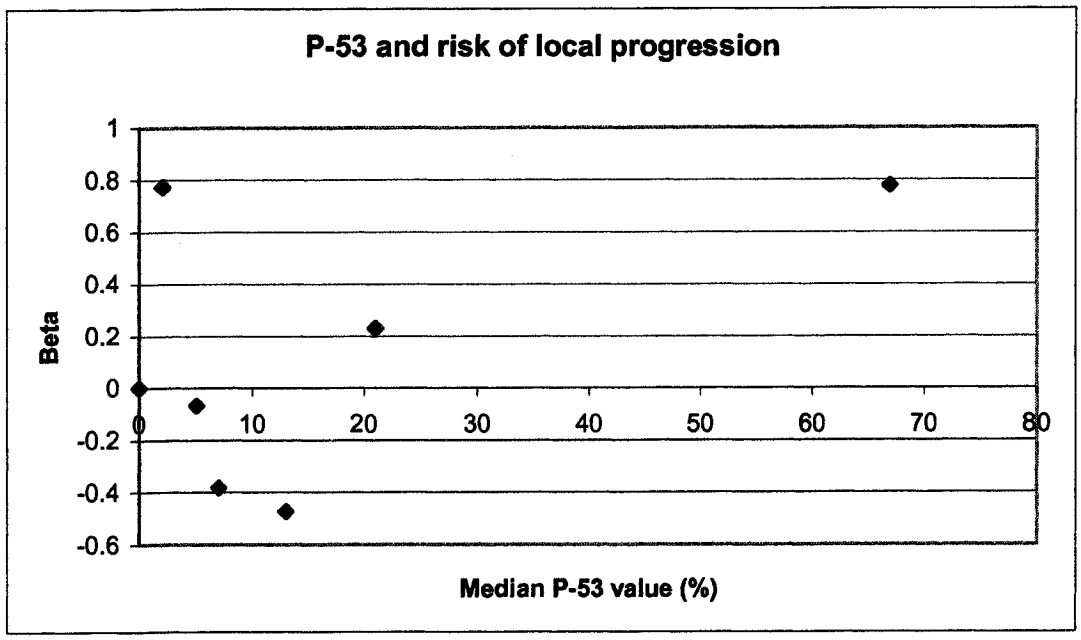
MVD was categorized and regressed against local progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of MVD groups to demonstrate the relationship between the two variables and evaluate linearity.

Figure B-5: Risk of local progression associated with MIB-1 (%) values



MIB-1 was categorized and regressed against local progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of MIB-1groups to demonstrate the relationship between the two variables and evaluate linearity.

Figure B-6: Risk of local progression associated with P-53 (%) values



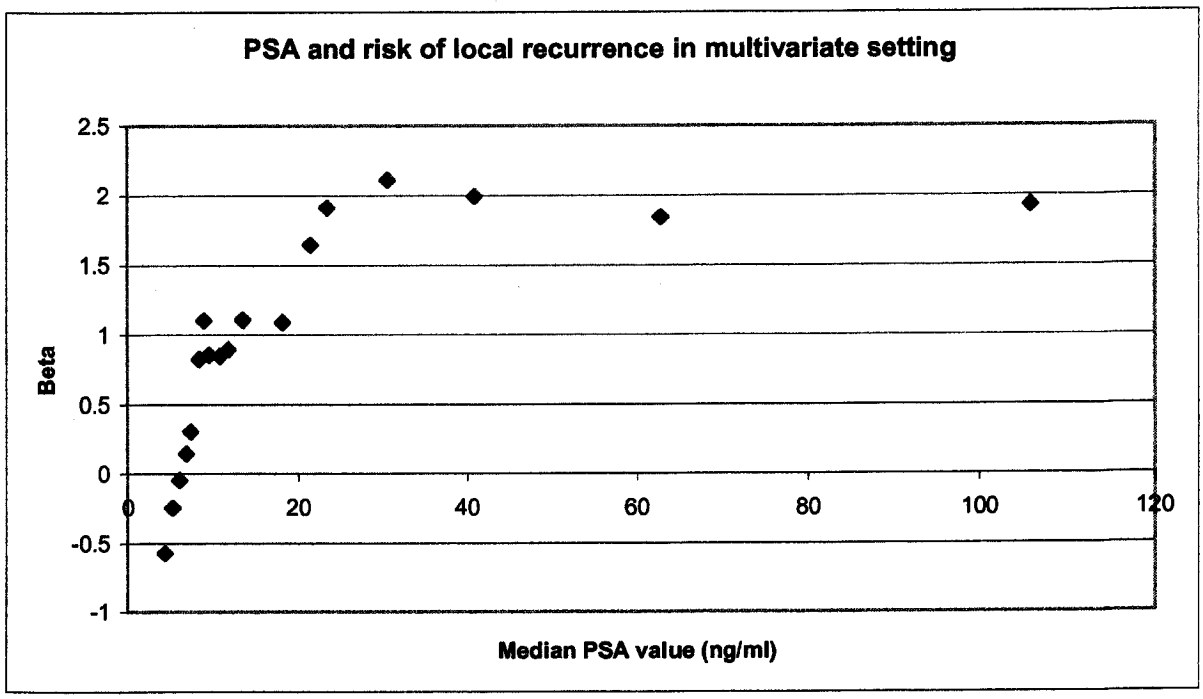
P-53 was categorized and regressed against local progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of P-53 groups to demonstrate the relationship between the two variables and evaluate linearity.

Table B-3: Variable stepwise selection of traditional factor for local progression model

Step	PSA (ng/ml)	Stage	XRT fraction	ADT	Gleason	Age	LR	df	P value
0	<0.0001	0.0065	0.023	0.46	0.143	0.85			
1	<0.0001	0.0053	0.021	0.079	0.55	0.83	32.47	2	<0.0001
2	<0.0001	0.0083	0.014	0.021	0.65	0.54	10.33	3	0.016
3	<0.0001	0.0068	0.017	0.077	0.39	0.69	4.65	1	0.032
5	<0.0001	0.0025	0.11	0.079	0.22	0.71	3.33	1	0.072
6	<0.0001	0.0041	0.072	0.047	0.22	0.72	1.67	1	0.20
7	<0.0001	0.0040	0.079	0.048	0.22	0.72	0.13	1	0.72

Variables were entered in sequence based on their significance level. For each step, the overall LR, degrees of freedom and corresponding p value for adding that variable is represented in the last three columns, respectively. P values of individual variables are represented in each box and for each step. P values found to the left of the bold line represent the Wald statistics for that variable that is entered in the equation. P values found to the right of the bold line represent the Score p value of the variable not in the equation. For example, in step two, the equation was composed of PSA and stage. The Wald statistics for these two variables were highly significant (<0.0001 and 0.0083, respectively). While these two variables are in the equation, the Score p value for radiotherapy, hormone therapy, Gleason and age are 0.0139, 0.0208, 0.6526, 0.5350, respectively. Based on inclusion of variables in order of significance, radiotherapy will be the next variable to enter the equation. To the right of the double line, the LR, corresponding degrees of freedom and p value for stage is indicated.

Figure B-7: Linearity verification of PSA (ng/ml) and local progression in the multivariate setting: Visual examination



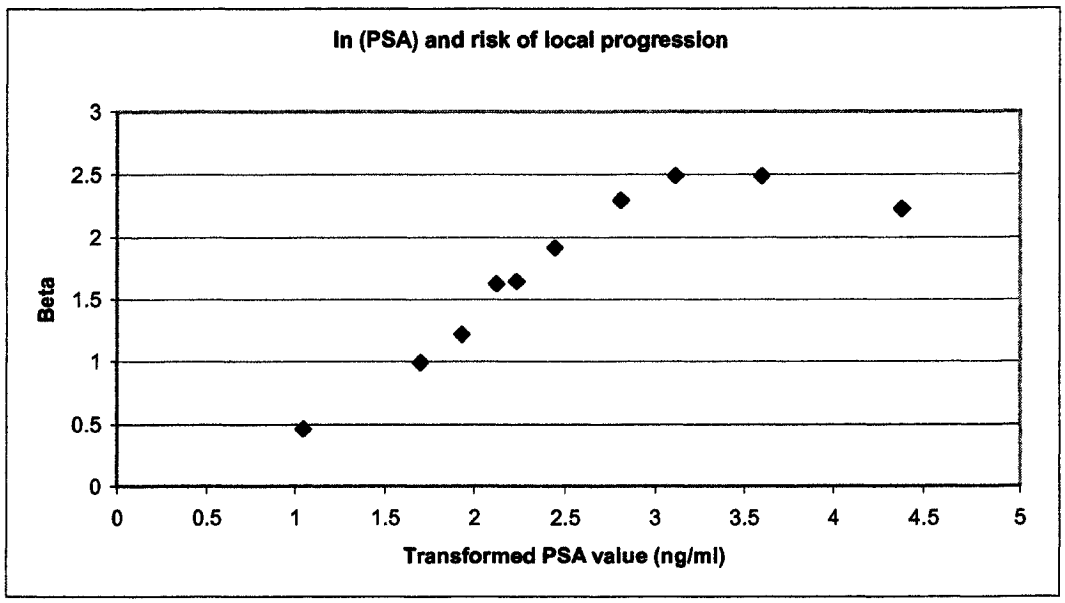
PSA was categorized and included in the multivariate Cox regression against local progression. The resulting beta values (running averages of three neighboring groups) are plotted against the median values of PSA groups to demonstrate the relationship between the two variables and evaluate linearity in the multivariate setting.

Table B-4: Linearity verification of PSA and local progression in the multivariate setting: Fractional polynomial analysis

Model	LR (1df)	LR p value	PSA Wald statistics
PSA1	7.54	0.0060	0.0020
PSA-3	0.29	0.5925	0.6271
PSA-2	2.07	0.1502	0.2636
PSA-1	15.61	0.0001	0.0015
PSA-0.5	22.81	<0.0001	<0.0001
PSA0	22.30	<0.0001	<0.0001
PSA0.5	14.89	0.0001	<0.0001
PSA2	2.29	0.1304	0.0746
PSA3	1.49	0.2222	0.1455
PSA0-<8, 8-20, >20	31.03 (2df)	<0.0001	<0.0001

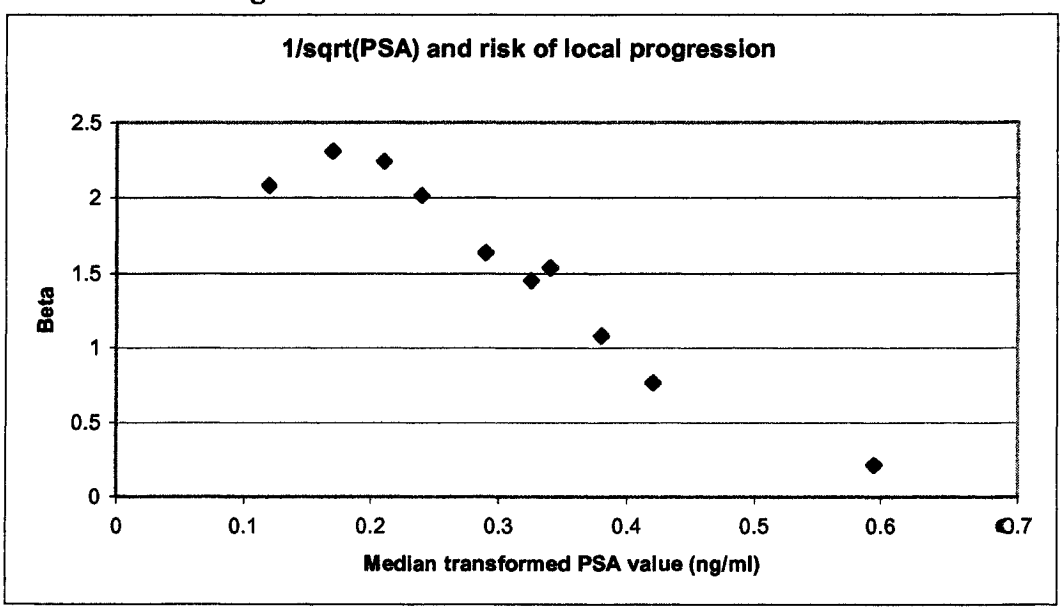
The change in –2LL from adding the transformed variable into the model is shown along with its associated p value.

Figure B-8: Linearity verification of ln PSA (ng/ml) and local progression in the multivariate setting: Visual examination



Ln PSA was categorized and included in the multivariate Cox regression against local progression. The resulting beta values (running averages of three neighboring groups) are plotted against the median values of ln PSA groups to demonstrate the relationship between the two variables and evaluate linearity in the multivariate setting

Figure B-9: Linearity verification of 1/sqrt PSA (ng/ml) and local progression in the multivariate setting: Visual examination



1/sqrt PSA was categorized and included in the multivariate Cox regression against local progression. The resulting beta values (running averages of three neighboring groups) are plotted against the median values of 1/sqrt PSA groups to demonstrate the relationship between the two variables and evaluate linearity in the multivariate setting.

Table B-5: Interaction between variables in the traditional factor local progression model

Factor #1	Factor #2	LR	P value
ADT	PSA transformed	0.422 (1df)	0.52
	Stage	2.242 (3df)	0.52
	ADT treatment duration	0.7541 (1df)	0.37
Ln PSA (ng/ml)	Gleason	1.196 (1df)	0.27
	Stage	5.146 (1df)	0.16
Stage	Gleason	0.585 (3df)	0.90

Table B-6: Preliminary local progression traditional factor model

Variable	Gleason	XRT	Stage			ln PSA	ADT
Category	6+	1.8Gy	2b	2c	3-4	ng/ml	given
Beta	0.574	0.829	0.464	1.418	-0.131	0.6871	-0.702

Table B-7: Variable stepwise selection of biomarkers in the local progression model

Since biomarkers were measured in different subsets of patients, stepwise selection was carried out manually with each biomarker evaluated separately. Each variable was added to the model containing the traditional model and the corresponding LR p value recorded. Results of the analysis are shown below and the number of available records for each analysis is indicated in parenthesis.

Steps 0 and 1:

MVD (n=153)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	MVD 3%	LR	df	P value
0	<0.0001	0.032	0.027	0.024	0.028	0.0074			
1	0.0007	0071	0.020	0.019	0.061	0.0083	6.700	1	**0.0096**

P-53 (n=136) (similar results were obtained with P-53 cut off at 20)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	P-53: 50%	LR	df	P value
0	0.0045	0.042	0.076	0.018	0.17	0.084			
1	0.0042	0.034	0.058	0.010	0.22	0.093	2.379	1	0.12

MIB-1 (n=143) (similar results were obtained with other forms of MIB, including log transformed)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	MIB: 2%	LR	df	P value
0	0.0003	0.17	0.022	0.0091	0.21	0.44			
1	0.0004	0.20	0.029	0.011	0.27	0.44	0.600	1	0.44

Bax (n=137)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	Bax	LR	df	P value
0	0.0017	0.029	0.046	0.0085	0.12	0.10			
1	0.0014	0.016	0.028	0.011	0.17	0.10	2.552	1	0.11

BCL2 (n=137)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	BCL2	LR	df	P value
0	0.0020	0.079	0.048	0.015	0.232	0.68			
1	0.0019	0.077	0.046	0.015	0.025	0.67	0.185	1	0.67

Bax/BCL2 (n=137)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	Bax/BCL2	LR	df	P value
0	0.0016	0.065	0.049	0.014	0.23	0.10			
1	0.0012	0.054	0.031	0.017	0.40	0.11	3.141	1	**0.076**

Table B-8: Variable stepwise selection of biomarkers in the local progression model following inclusion of MVD

Each variable was added to the model containing the traditional variables and MVD, and the corresponding LR p value recorded.

Steps 1 and 2: Models with MVD

P-53 (n=114)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	MVD 3%	P-53%	LR	df	P value
1	0.074	0.24	0.012	0.051	0.044	0.0037	0.89			
2	0.081	0.26	0.012	0.064	0.043	0.0046	0.89	0.021	1	0.89

Bax (n=115)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	MVD: 3%	Bax	LR	df	P value
1	0.028	0.36	0.0075	0.036	0.060	0.0014	0.32			
2	0.026	0.26	0.0068	0.045	0.068	0.0027	0.31	1.011	1	0.32

BCL2 (n=114)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	MVD: 3%	BCL2	LR	df	P value
1	0.034	0.41	0.0071	0.034	0.059	0.0015	0.35			
2	0.031	0.42	0.0074	0.050	0.072	0.0009	0.35	0.917	1	0.34

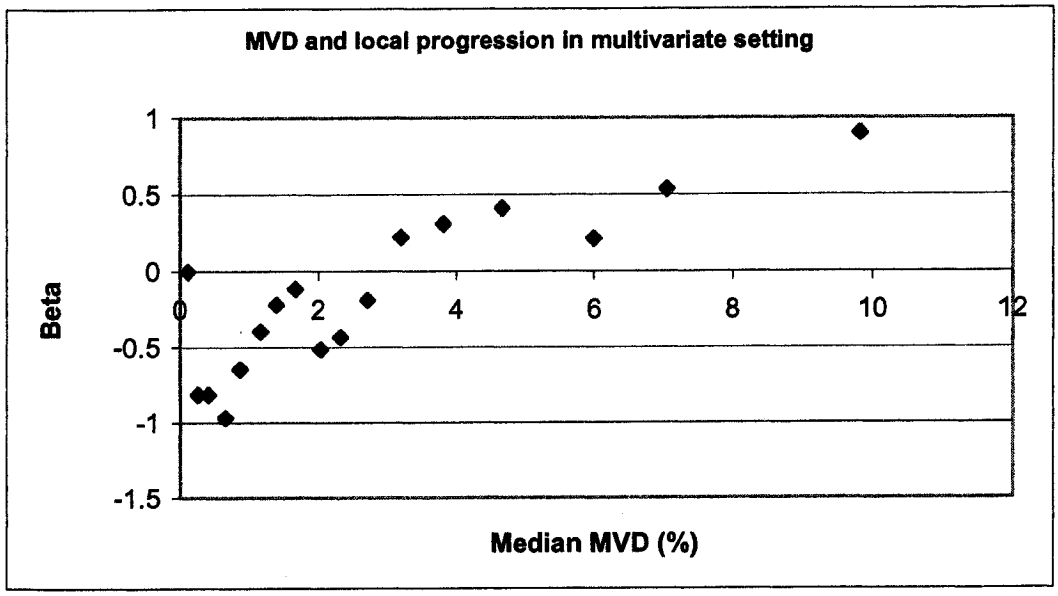
Bax/BCL2 (n=115)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	MVD: 3%	Bax/ BCL2	LR	df	P value
1	0.028	0.36	0.0075	0.036	0.060	0.0014	0.029			
2	0.023	0.23	0.0076	0.088	0.13	0.0006	0.059	6.531	1	**0.0106**

MIB-1 (n=114)

	Ln PSA (ng/ml)	ADT	XRT fraction	Stage	Gleason	MVD: 3%	MIB-1%	LR	df	P value
1	0.093	0.50	00068	0.092	0.077	0.0012	0.97			
2	0.095	0.50	0.0080	0.092	0.082	0.0013	0.97	0.002	1	0.97

Figure B-10: Linearity verification of MVD (%) and local progression in the multivariate setting: Visual examination



MVD was categorized and included in the multivariate Cox regression against local progression. The resulting beta values (running averages of three neighboring groups) are plotted against the median values of MVD groups to demonstrate the relationship between the two variables and evaluate linearity in the multivariate setting.

Table B-9: Linearity verification of MVD (%) and local progression in the multivariate setting: Fractional polynomial analysis

Model	LR (1df)	LR p value	Wald statistics
MVD1	5.48	0.0193	0.0204
MVD-3	0.75	0.3882	0.7476
MVD-2	0.34	0.5550	0.6363
MVD-1	0.43	0.5117	0.5305
MVD-0.5	1.51	0.2185	0.2092
MVD0	4.19	0.0408	0.0467
MVD0.5	5.14	0.0234	0.0218
MVD2	5.29	0.0214	0.0231
MVD3	6.37	0.0264	0.0131
MVD cut off 3%	6.70	0.0096	0.0083

The change in –2LL from adding the transformed variable into the model is shown along with its associated p value.

Table B-10: Preliminary local progression biomarker models

Variable	Gls[1]	EBRT		Stage		ln PSA	ADT	MVD	B/B[2]
Category	6+	1.8Gy	2b	2c	3-4	ng/ml	given	≥3%	Other
Beta of Model									
MVD	+ 1.171	+ 1.130	+ 0.707	+ 1.162	- 0.030	+ 0.5642	- 0.618	+ 0.720	NA[3]
MVD + BB[2]	+ 1.179	+ 1.457	+ 0.547	+ 1.042	- 0.169	+ 0.4468	- 0.473	+ 1.130	+ 1.953

[1] Gls = Gleason score; [2] BB = Bax/BCL2 variable; [3] NA = Not applicable

Figures B-11 through B-16 represent the –log-log plot.
In these curves, each stratum should be compared to its reference stratum to evaluate the assumption of proportional hazard. For example, considering stage T1-2a to be the reference strata, all other strata appear to meet the assumption of proportionality (PH). That is, the distance between the curves appears proportional to the risk in the reference strata over time for stages 2b and 2c. For stages 3-4, the risk appears diminished after 3 years and the two curves (stage 1-2a and 3-4) approach, suggesting that the hazard is not proportional over time but rather that it is reduced for stages 3-4 after year 3. The variable coding for dose of radiotherapy meets the PH. The curves for MVD and Gleason intersect very early on. This is deemed a tolerable pattern, not violating the PH. The hormone therapy variable is not predictive in the univariate and the two curves overlap. The PH assumption of this variable can only be tested in the adjusted multivariate setting. Therefore, based on the graphical representation, all variables, except hormone therapy and stage 3-4, meet the PH assumption.

Figure B-11: Verification of PH assumption for daily fraction dose (Gy) in the equations predicting for local progression: Univariate evaluation of -log log plot

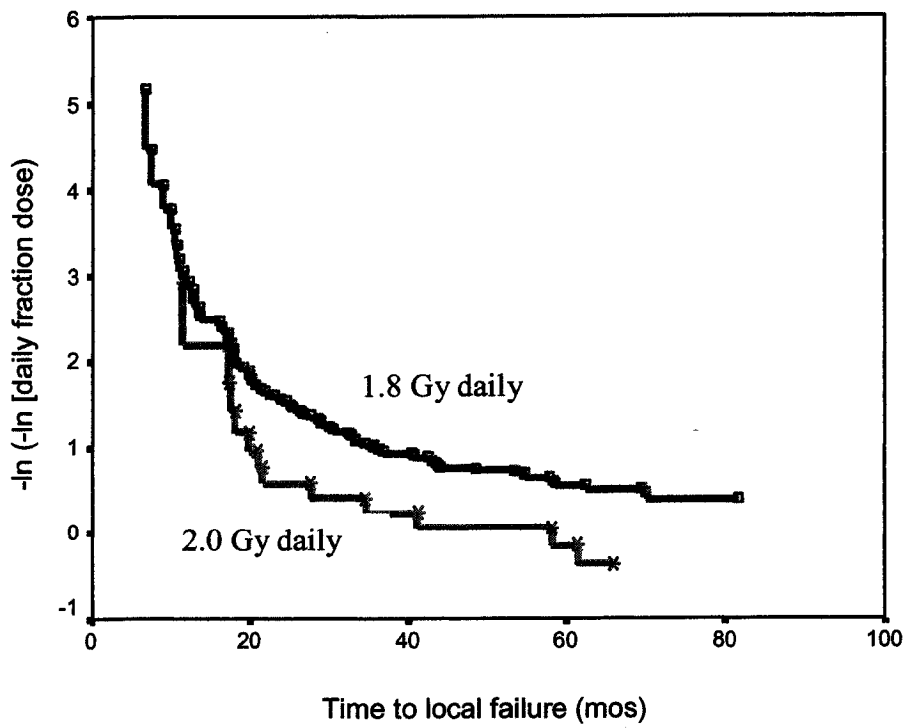


Figure B-12: Verification of PH assumption for MVD (%) in the equation predicting for local progression: Univariate evaluation of -log log plot

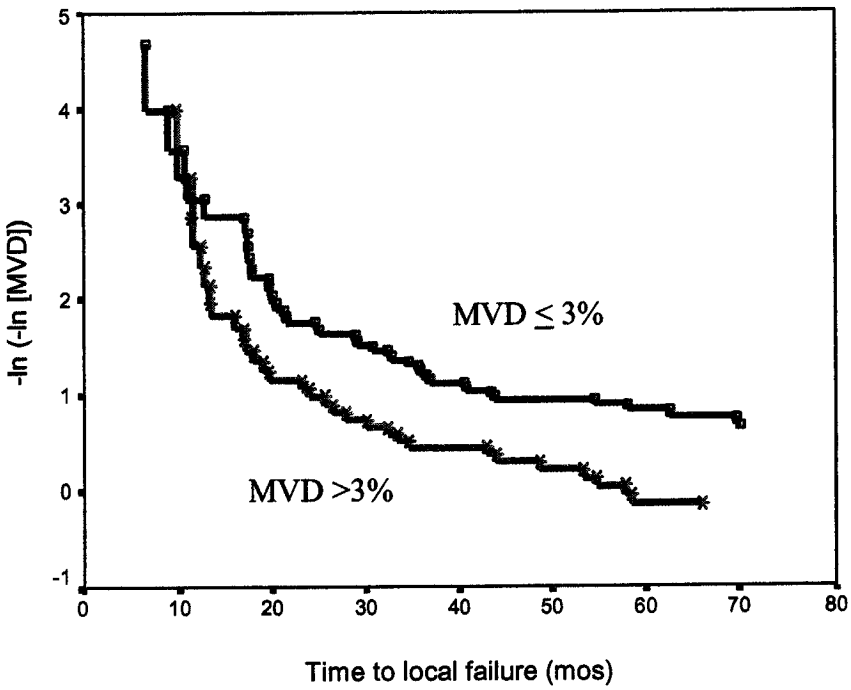


Figure B-13: Verification of PH assumption for Gleason score in the equation predicting for local progression: Univariate evaluation of -log log plot

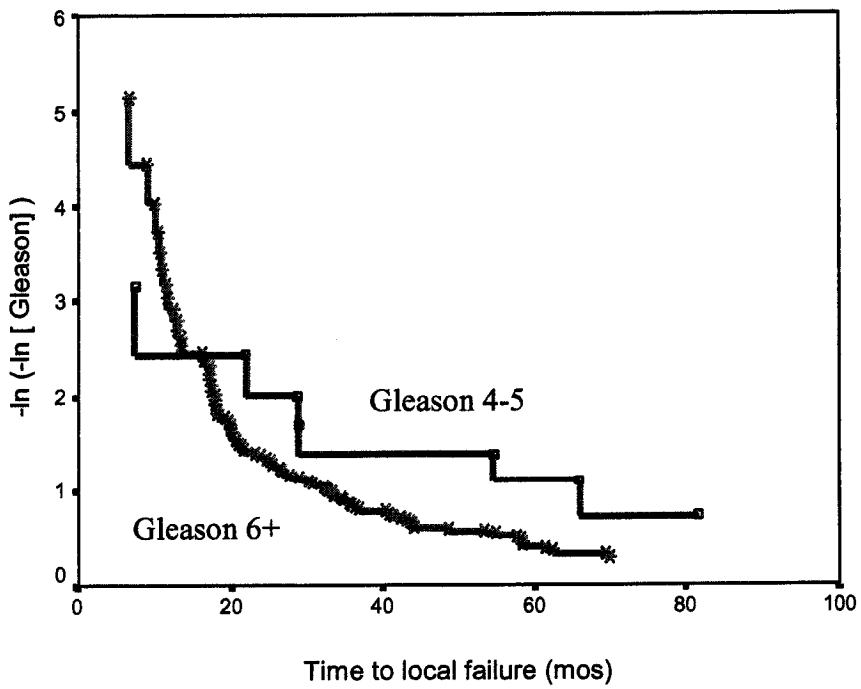


Figure B-14: Verification of PH assumption for stage in the equation predicting for local progression: Univariate evaluation of -Log log plot

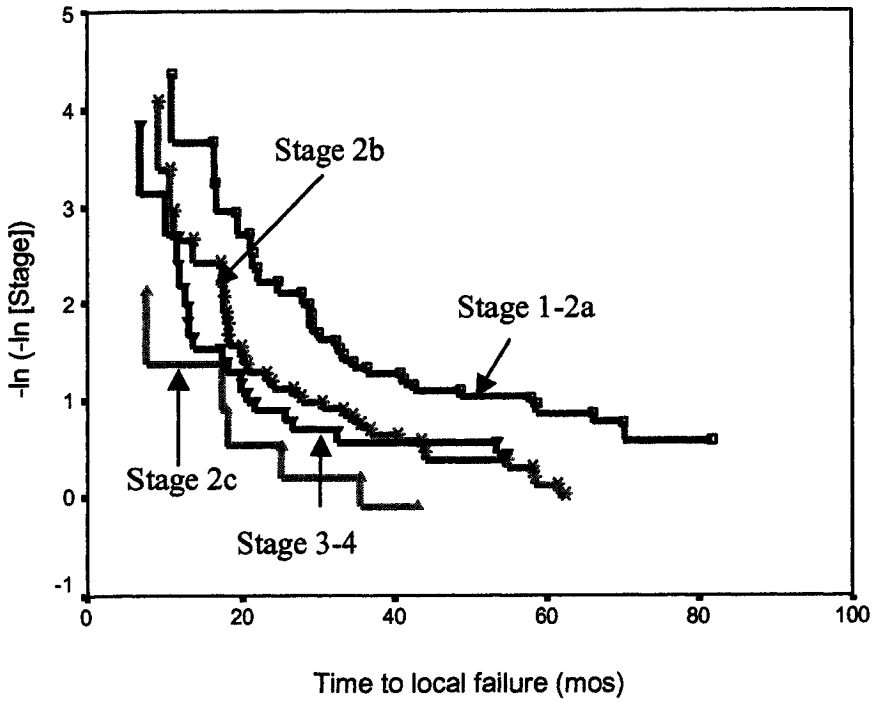


Figure B-15: Verification of PH assumption for Bax/BCL2 in the equation predicting for local progression: Univariate evaluation of -log log plot

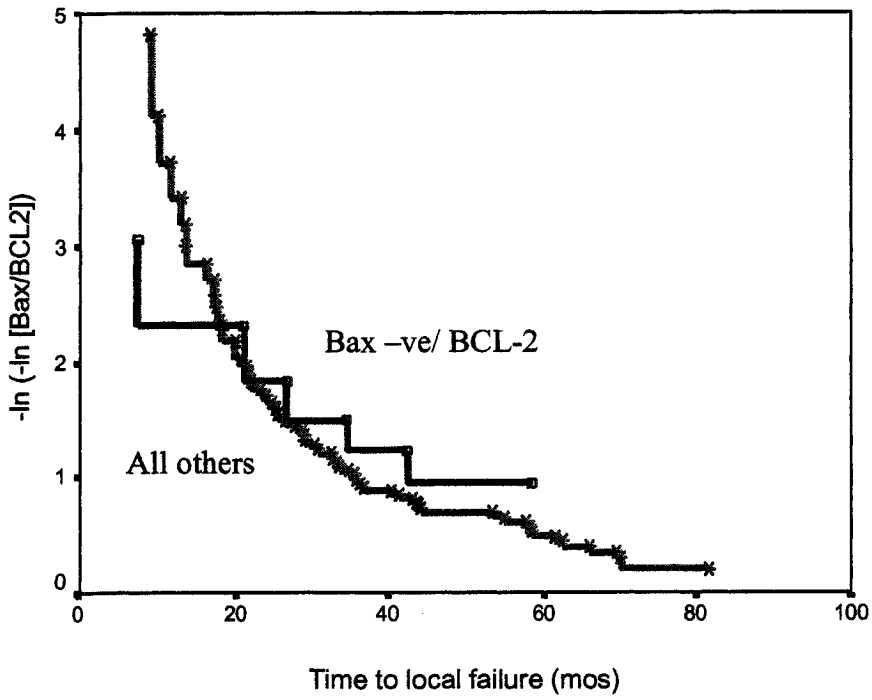


Figure B-16: Verification of PH assumption for ADT status in the equation predicting for local progression: Univariate evaluation of -log log plot

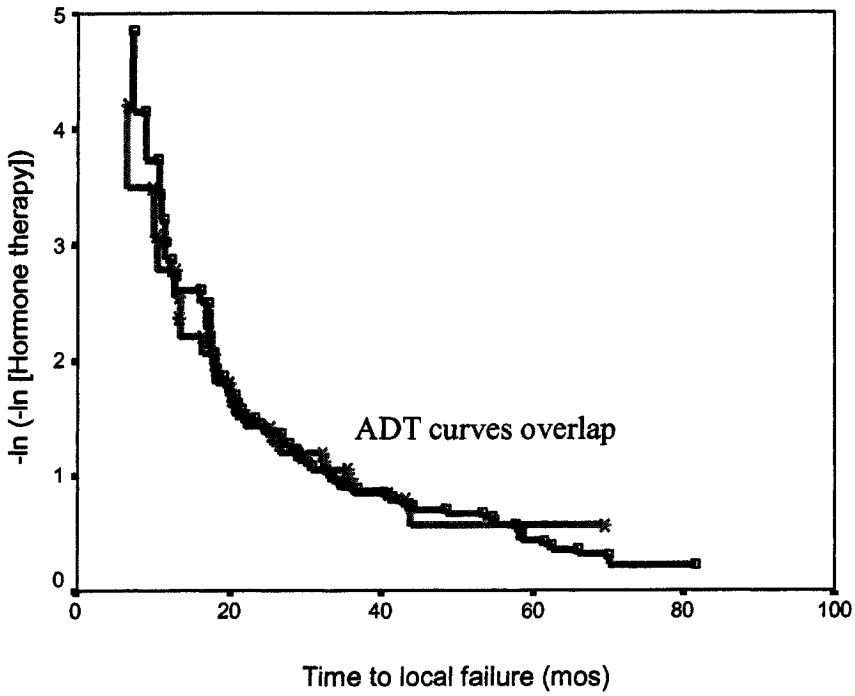


Table B-11: Verification of PH assumption for variables in the equations predicting for local progression: Extended Cox model

Interaction term	Wald statistics
Ln (time) * Gleason	0.61
Ln (time) * Radiotherapy fraction size	0.18
Ln (time) * Prior hormone therapy	0.15
Ln (time) * ln PSA	0.18
Ln (time) * Stage 2b	0.46
Ln (time) * Stage 2c	0.58
Ln (time) * Stage 3 or 4	**0.017**
Ln (time) * Stage 1-2a + 3-4	0.31
Ln (time) * MVD 3%	0.14
Ln (time) * Bax/BCL2	0.86

*Results of the extended Cox model in which a product term built between a time dependent variable (ln time) and, in turn, each variable is incorporated in the model. The tests were used to evaluate deviation from the PH assumption. A significant product term suggests that the effect of the variable changes over time and the PH assumption is not met. The results suggest that only stage 3-4 does not meet the proportionality assumption. Since patients with stage 3-4 had a similar risk of local recurrence to the reference strata stage 1-2a in the multivariate setting (HR 0.96), the two strata were collapsed. When these are combined, the product term (ln (time) * Stage 1-2a, 3,4 is not significant. All other variables met the PH assumption. The PH assumption for the traditional factors was investigated in the traditional factor model and for MVD and Bax/BCL2 in the biomarker model.*

Table B-12: Final local progression traditional factor and biomarker models

Variable	Gleason	EBRT	Stage		ln PSA	ADT	MVD	B/B[1]
Category	6+	1.8Gy	2b	2c	(ng/ml)	given	≥3%	Other
Beta of model								
Traditional	+ 0.581	+ 0.806	+ 0.517	+ 1.487	+ 0.664	- 0.728	NA[2]	NA[2]
MVD	+ 1.182	+ 1.122	+ 0.719	+ 1.180	+ 0.559	- 0.626	+ 0.719	NA[2]
MVD + BB[2]	+ 1.165	+ 1.456	+ 0.633	+ 1.144	+ 0.416	- 0.508	+ 1.105	+ 1.938

[1] BB = Bax/BCL2 variable; [2] NA = Not applicable

Table B-13: Summary information of hazard ratios derived from the 3 models predicting for local progression

	Traditional factor model	MVD ≥3% biomarker model	MVD ≥3% & BaxBCL2 biomarker model
Mean	.88	2.98	34.49
Median	.60	1.80	14.99
Range			
Minimum	.01	0.05	0.79
Maximum	8.44	35.57	626.04
Percentile			
10	.19	0.56	3.95
20	.31	0.83	6.13
30	.42	1.15	9.28
40	.53	1.33	11.37
50	.60	1.80	14.99
60	.73	2.39	20.38
70	.99	2.93	31.20
80	1.28	4.21	41.48
90	1.84	5.23	62.74

Table B-14: Hazard ratios derived from equations predicting for local progression categorized into quartiles

Quartile # and figure label	Traditional factor model	MVD 3% biomarker model	MVD 3% & BaxBCL2 biomarker model
First quartile = ♦	0.01-0.37	0.05-1.19	0.79-7.55
Second quartile = ▲	0.38-0.60	1.22-2.13	7.91-14.44
Third quartile = ▼	0.61-1.14	2.14-3.90	14.99-34.36
Fourth quartile = ■	1.17-8.44	4.15-36.18	34.95-626.04

Patients were grouped into quartiles based on their hazard ratio to evaluate the spread provided by the models. The table lists the hazard ratio intervals falling within each quartile.

Figure B-17: Kaplan Meier curves of the 4 strata derived from the traditional factor model in all patients

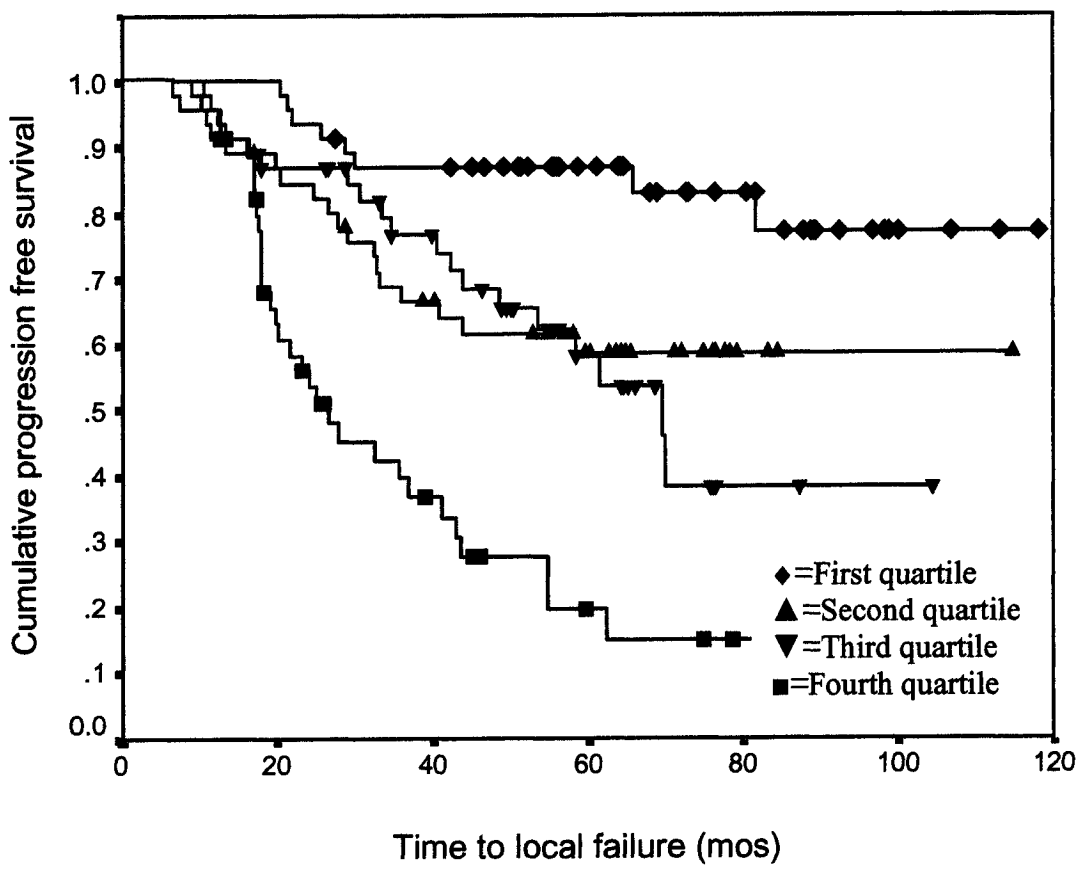


Figure B-18: Kaplan Meier curves of the 4 strata derived from the $MVD_{\geq 3\%}$ biomarker model

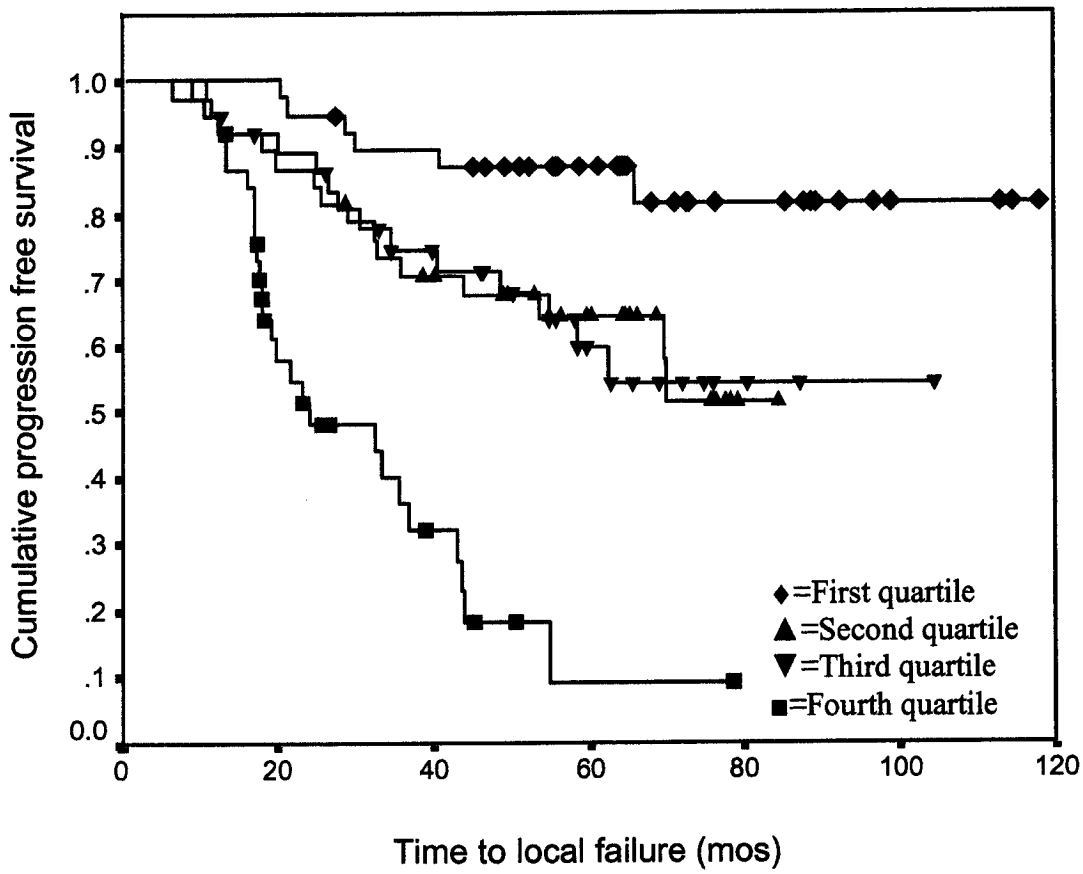
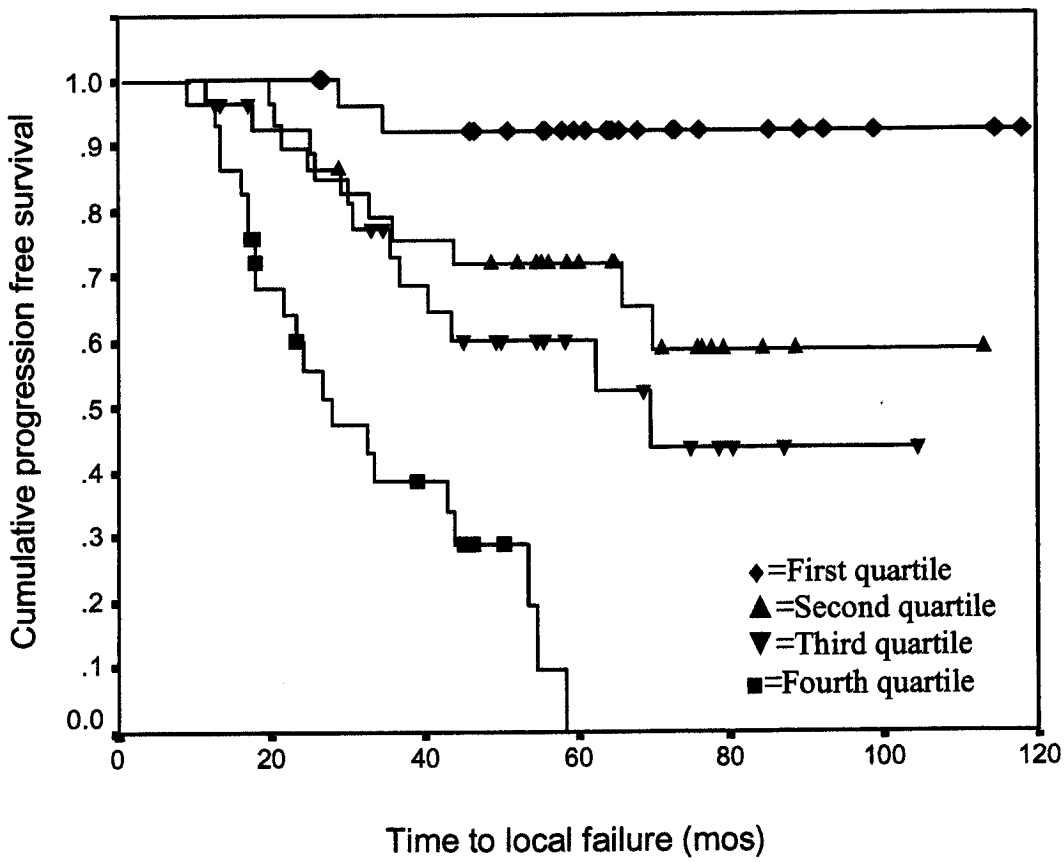
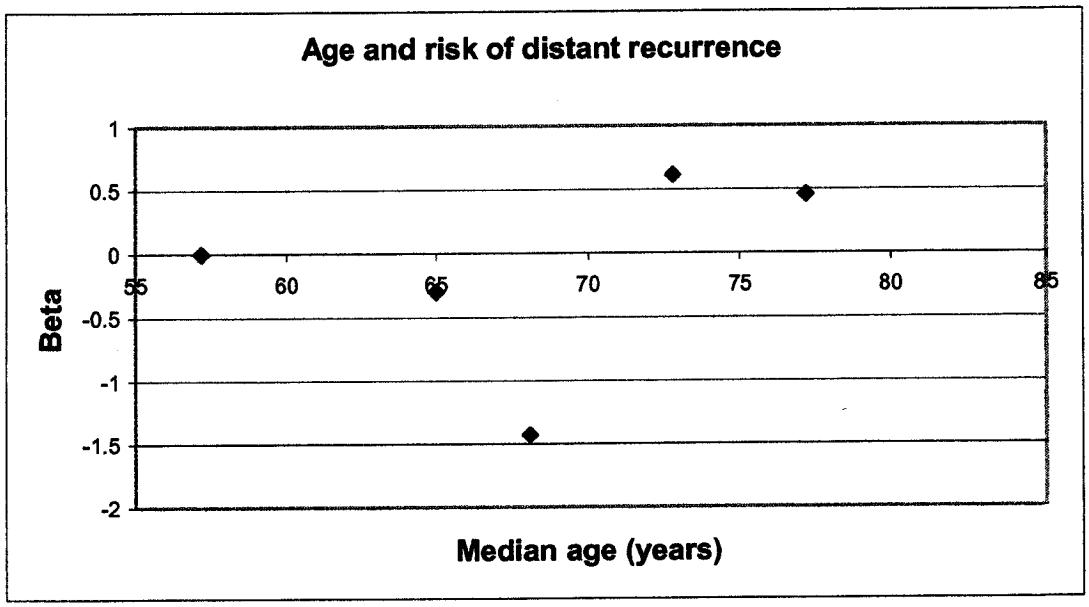


Figure B-19: Kaplan Meier curves of the 4 strata derived from the $MVD_{\geq 3\%}$ + Bax/BCL2 biomarker model



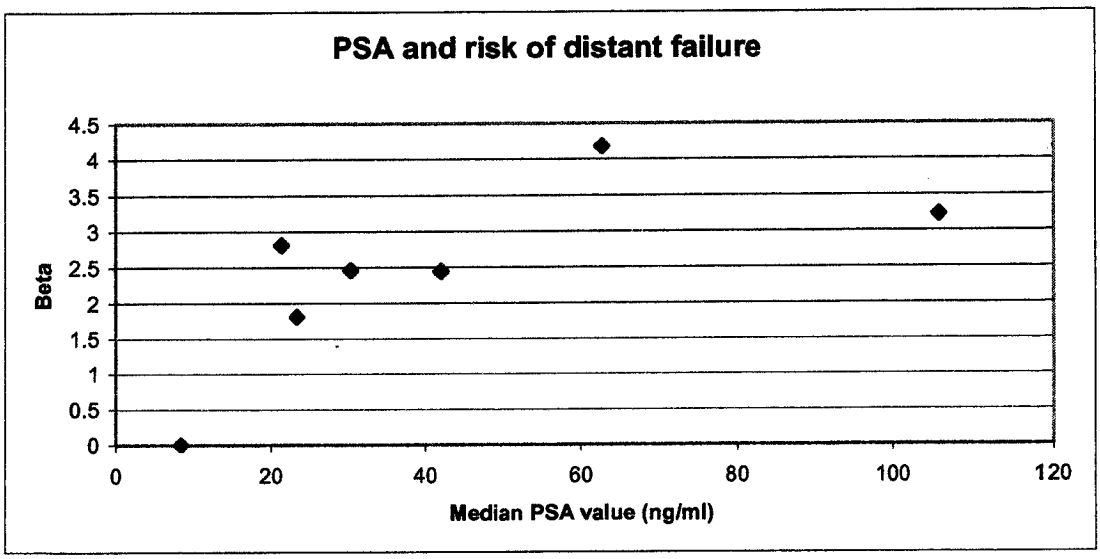
APPENDICES C

Figure C-1: Risk of distant progression associated with age (years)



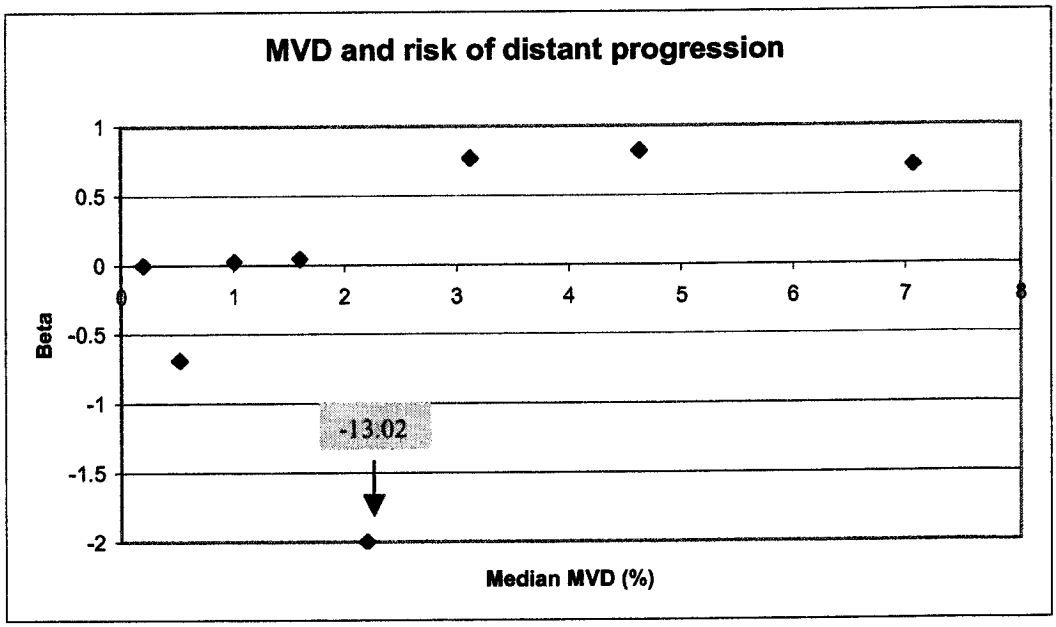
Age was categorized and regressed against distant progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of age groups to demonstrate the relationship between the two variables and evaluate linearity.

Figure C-2: Risk of distant progression associated with PSA (ng/ml) levels



PSA was categorized and regressed against distant progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of PSA groups to demonstrate the relationship between the two variables and evaluate linearity.

Figure C-3: Risk of distant progression associated with median MVD (%) values



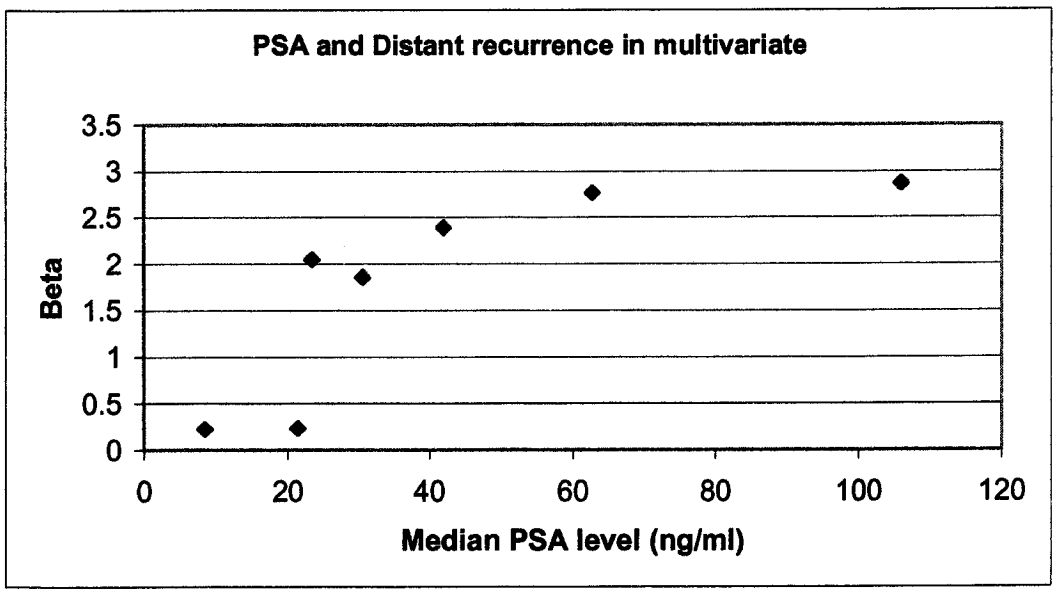
MVD was categorized and regressed against distant progression (Cox regression). The resulting beta values are plotted against the median values of MVD groups to demonstrate the relationship between the two variables and evaluate linearity. Exceptionally, running averages were not used to depict the beta values to allow the lack of events for MVD values between 1.75 and 3.0 to be visualized.

Table C-1: Variable stepwise selection of traditional factor for distant progression model

Step	PSA (ng/ml)	Stage	ADT	Gleason	Age	LR	df	P value
0	<0.0001	<0.0001	0.68	0.0022	0.083			
1	<0.0001	0.031	0.079	0.055	0.38	36.93	2	<0.0001
2	0.0009	0.036	0.018	0.048	0.60	4.57	1	0.033
3	0.0003	0.0095	0.024	0.032	0.45	5.59	1	0.01830
5	0.0008	0.0054	0.016	0.040	0.60	4.85	1	0.028
6	0.0010	0.0059	0.015	0.046	0.60	0.275	1	0.60

Variables were entered in sequence based on their significance level. For each step, the overall LR, degrees of freedom and corresponding p value for adding that variable is represented in the last three columns, respectively. P values of individual variables are represented in each box and for each step. P values found to the left of the bold line represent the Wald statistics for that variable that is entered in the equation. P values found to the right of the bold line represent the Score p value of the variable not in the equation.

Figure C-4: Linearity verification of PSA (ng/ml) and distant progression in the multivariate setting: Visual examination



PSA was categorized and included in the multivariate Cox regression against distant progression. The resulting beta values (running averages of three neighboring groups) are plotted against the median values of PSA groups to demonstrate the relationship between the two variables and evaluate linearity in the multivariate setting.

Table C-2: Linearity verification of PSA (ng/ml) and distant progression in the multivariate setting: Fractional polynomial analysis

Model	LR (1df)	LR p value	PSA Wald statistics
PSA1	5.09	0.0241	0.0141
PSA-3	Unstable		
PSA-2	Unstable		
PSA-1	24.49	<0.0001	0.0003
PSA-0.5	22.11	<0.0001	0.0001
PSA0	17.48	<0.0001	0.0001
PSA0.5	10.86	<0.0001	0.0007
PSA2	0.54	0.47	0.42
PSA3	0.003	0.96	0.96
PSA 0-<20, 20-50, >50 ng/ml	19.34 (2df)	0.0001	0.0008

The change in –2LL from adding the transformed variable into the model is shown along with its associated p value.

Table C-3: Interaction between variables in the traditional factor distant progression model

Factor #1	Factor #2	LR	P value
ADT	PSA	NA	
	Stage	NA	
	Gleason	NA	
	Duration of ADT	2.66	0.103
	Duration of ADT (<>5 months)	NA	
PSA (ng/ml)	Gleason	NA	
	Stage	NA	
Stage	Gleason	0.345 (3df)	0.56

Table C-4: Preliminary distant progression traditional factor model

Variable	Gleason	Stage	PSA (ng/ml)		ADT
Category	7+	3-4	20-50	>50	given
Beta	+ 1.087	+ 1.531	+2.120	+ 2.771	- 1.229

Table C-5: Variable stepwise selection of biomarkers in the distant progression model

Since biomarkers were measured in different subsets of patients, stepwise selection was carried out manually with each biomarker evaluated separately. Each variable was added to the model containing the traditional model and the corresponding LR p value recorded. Results of the analysis are shown below and the number of available records for each analysis is indicated in parenthesis.

Steps 0 and 1:

MVD 3% (n=153)

	PSA (ng/ml)	ADT	Stage	Gleason	MVD%: cont	▲-2LL	df	P value
0	0.0005	0.013	0.027	0.073	0.57			
1	0.0001	0.0039	0.0048	0.019	0.063	3.774	1	**0.052**

P-53 (n=136) (similar results were obtained with P-53 20 and P-53 50)

	PSA (ng/ml)	ADT	Stage	Gleason	P-53% cont	▲-2LL	df	P value
0	0.0034	0.075	0.011	0.039	0.88			
1	0.0036	0.092	0.011	0.041	0.88	0.024	1	0.88

MIB-1 (n=143) (similar results were obtained with other forms of MIB)

	PSA (ng/ml)	ADT	Stage	Gleason	MIB%: 2	▲-2LL	df	P value
0	0.0040	0.056	0.0067	0.036	0.75			
1	0.0054	0.061	0.0087	0.035	0.75	0.099	1	0.75

Bax (n=137)

	PSA (ng/ml)	ADT	Stage	Gleason	Bax	▲-2LL	df	P value
0	0.0043	0.073	0.0084	0.036	0.048			
1	0.0018	0.15	0.0058	0.033	0.056	4.046	1	**0.044**

BCL2 (n=137)

	PSA (ng/ml)	ADT	Stage	Gleason	BCL2	▲-2LL	df	P value
0	0.0043	0.073	0.0084	0.036	0.78			
1	0.0041	0.070	0.0098	0.038	0.78	0.082	1	0.78

Table C-6: Variable stepwise selection of biomarkers in the distant progression model following inclusion of Bax

Each variable was added to the model containing the traditional variables and Bax, and the corresponding LR p value recorded.

Steps 1 and 2: Models with Bax

MVD 3% (n=115) (similar results were obtained with other forms of MVD)

	PSA (ng/ml)	ADT	Stage	Gleason	Bax	MVD3%	▲-2LL	df	P value
1	0.0009	0.028	0.016	0.038	0.025	0.96			
2	0.0010	0.036	0.019	0.46	0.039	0.96	0.002	1	0.96

P-53 (n=133)

	PSA (ng/ml)	ADT	Stage	Gleason	Bax	P-53%	▲-2LL	df	P value
1	0.0014	0.17	0.0076	0.039	0.043	0.98			
2	0.0014	0.17	0.0076	0.044	0.044	0.98	0.001	1	0.98

Bcl2 (n=114)

	PSA (ng/ml)	ADT	Stage	Gleason	Bax	BCL2	▲-2LL	df	P value
1	0.0018	0.15	0.0059	0.0059	0.056	0.58			
2	0.0017	0.22	0.0088	0.054	0.053	0.58	0.305	1	0.58

MIB-1 (n=133) (similar results were obtained with other forms of MIB-1)

	PSA (ng/ml)	ADT	Stage	Gleason	Bax	MIB% cont	▲-2LL	df	P value
1	0.0014	0.17	0.0076	0.039	0.043	0.70			
2	0.0016	0.15	0.0067	0.043	0.042	0.70	0.151	1	0.70

Table C-7: Preliminary distant progression biomarker model

Variable	Gleason	Stage	PSA (ng/ml)		ADT	Bax
Category	7+	3-4	20-50	>50	given	positive
Beta	+ 1.320	+ 1.846	+2.457	+ 3.179	- 0.830	-1.164

Figures C-5 through C-8 represent the –log-log plot.
In these curves, each stratum should be compared to its reference stratum to evaluate the assumption of proportional hazard.

Figure C-5: Verification of PH assumption for Bax in the equation predicting for distant progression: Univariate evaluation of -log log plot

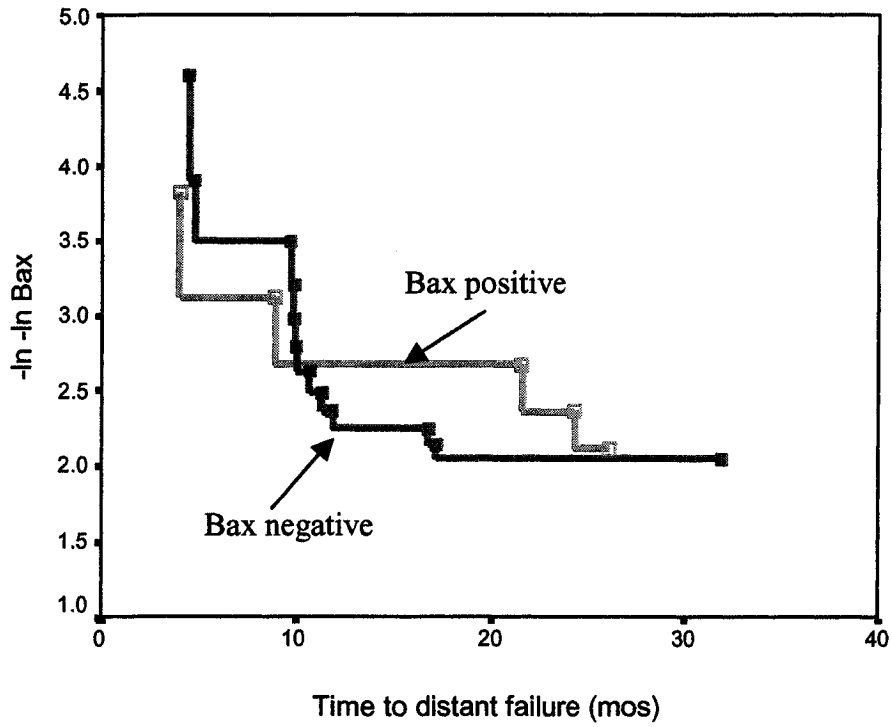


Figure C-6: Verification of PH assumption for Gleason in the equation predicting for distant progression: Univariate evaluation of -Log log plot

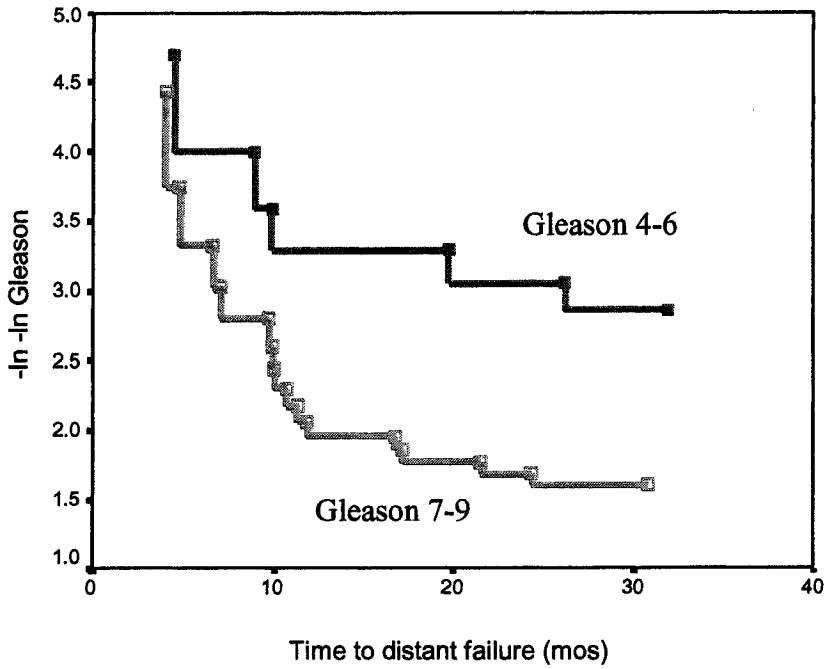


Figure C-7: Verification of PH assumption for stage in the equation predicting for distant progression: Univariate evaluation of -Log log plot

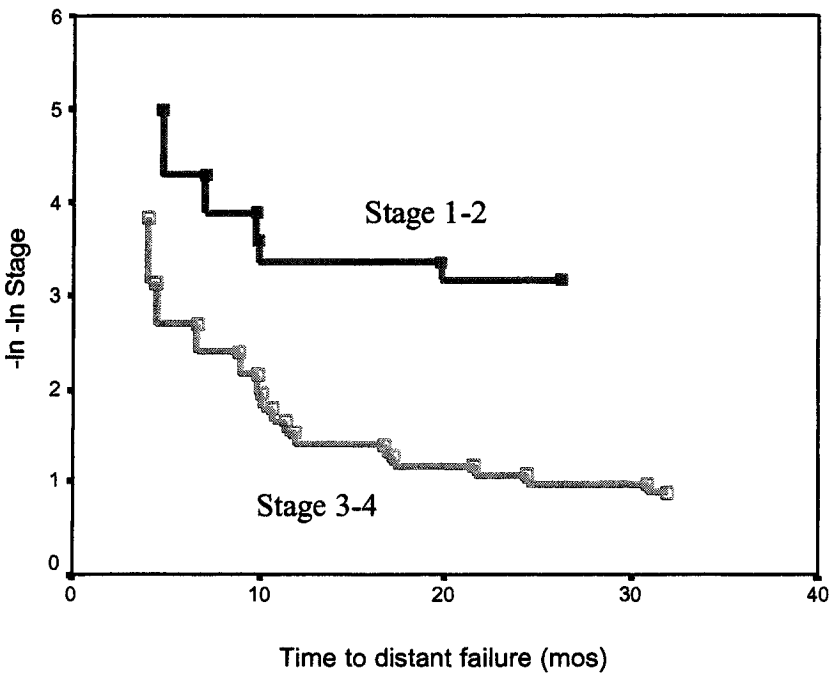


Figure C-8: Verification of PH assumption for PSA (ng/ml) in the equation predicting for distant progression: Univariate evaluation of -Log log plot

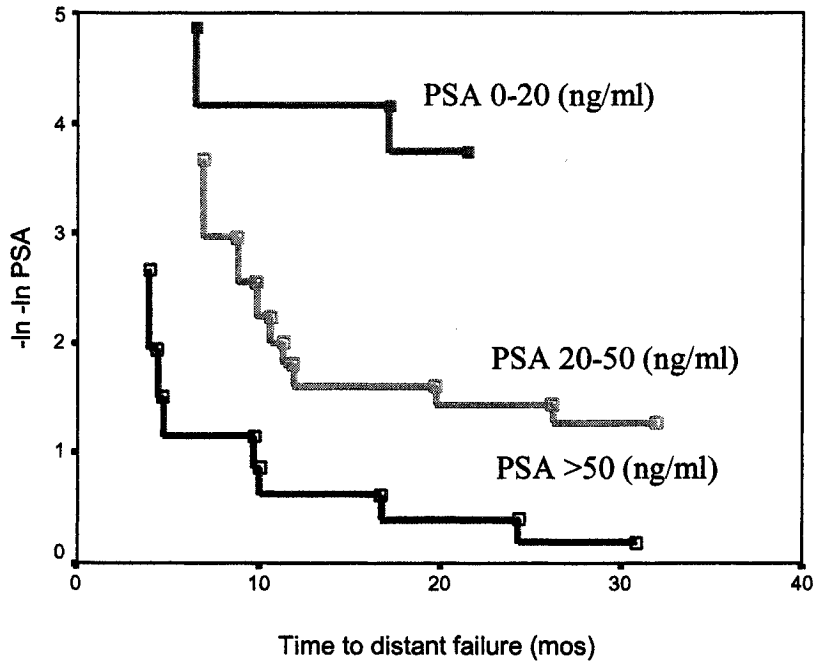


Table C-8: Verification of PH assumption for variables in the equations predicting for distant progression: Extended Cox model

Interaction term	Wald statistics
Ln (time) * Gleason	0.7487
Ln (time) * Prior hormone therapy	0.7524
Ln (time) * PSA (ng/ml)	0.3817
Ln (time) * Stage	0.7294
Ln (time) * Bax	0.9408

Results of the extended Cox model in which a product term built between a time dependent variable (ln time) and, in turn, each variable is incorporated in the model. The tests are used to evaluate deviation from the PH assumption. A significant product term suggests that the effect of the variable changes over time and the PH assumption is not met. All variables meet the PH assumption. The PH assumption for the traditional factors was investigated in the traditional factor model and for Bax in the biomarker model.

Table C-9: Summary information of hazard ratios derived from the 2 models predicting for distant progression

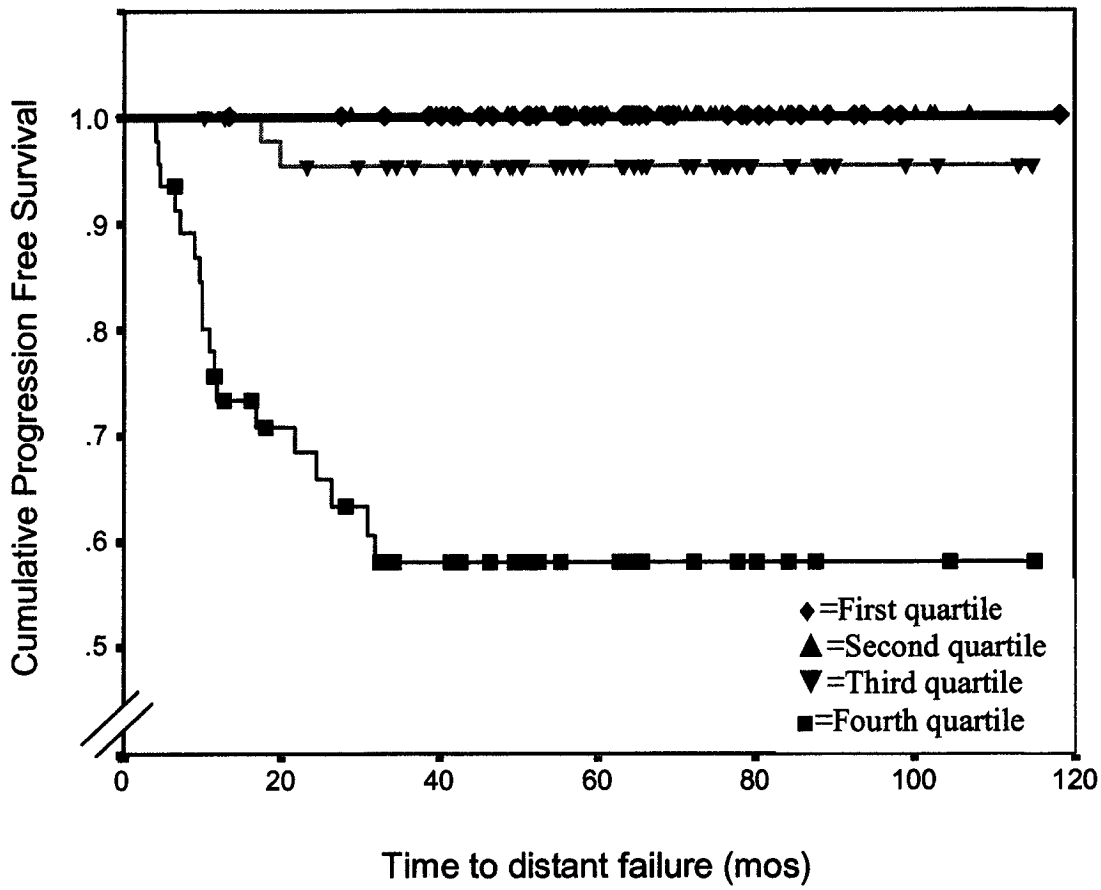
		Traditional factor model	Biomarker model
Mean		44.14	50.92
Median		10.13	2.68
Range			
	Minimum	1.00	0.31
	Maximum	748.45	1306.75
Percentile			
	10	1.00	1.00
	20	3.42	2.29
	30	3.42	2.29
	40	3.42	2.29
	50	10.13	2.68
	60	10.13	8.58
	70	17.58	16.00
	80	46.84	47.54
	90	121.12	169.47

Table C-10: Hazard ratios derived from equations predicting for distant progression categorized into quartiles

Quartile # and figure label	Traditional factor model	Biomarker model
First quartile = ♦	1.00-3.42	0.31-2.29
Second quartile = ▲	3.42-4.62	2.29-2.68
Third quartile = ▼	10.13-24.69	2.69-26.75
Fourth quartile = ■	28.45-748.45	31.28-1306.75

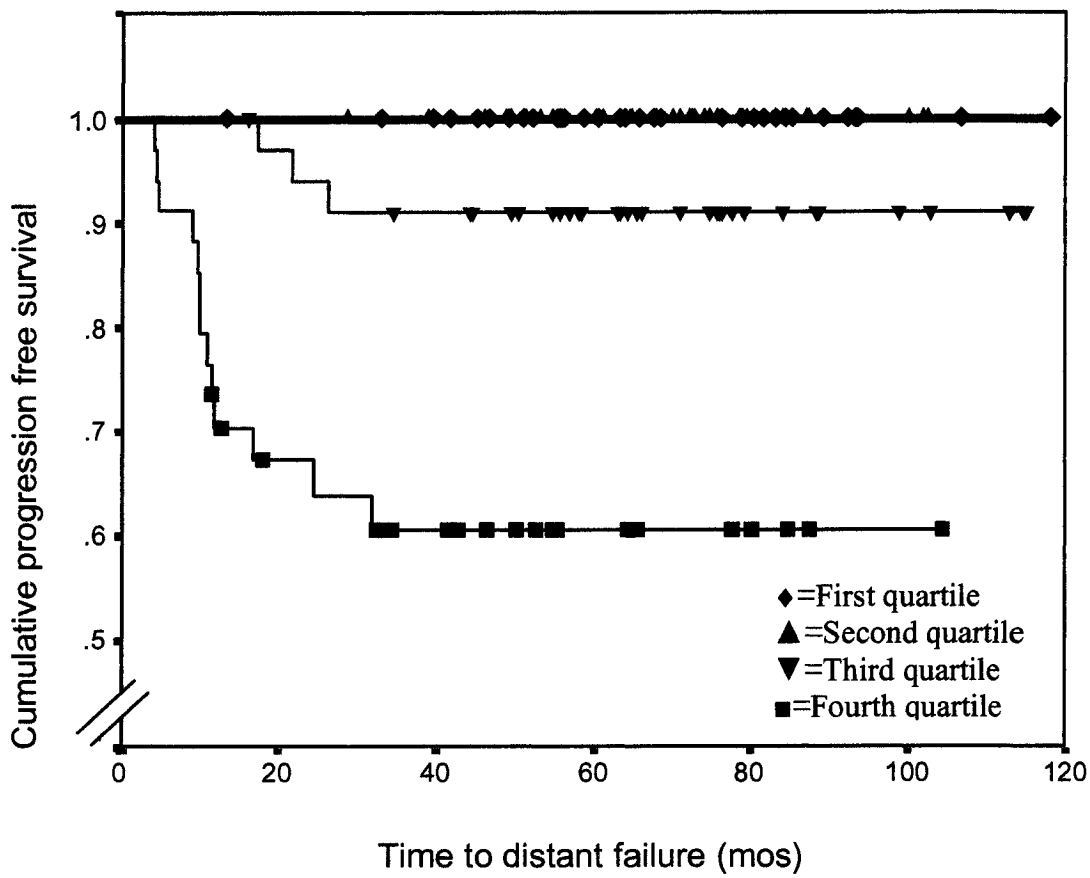
Patients were grouped into quartiles based on their hazard ratio to evaluate the spread provided by the models. The table lists the hazard ratio intervals falling within each quartile.

Figure C-9: Kaplan Meier curves of the 4 strata derived from the traditional factor model in all patients



No events were observed in the first and second quartiles. The two curves overlap.

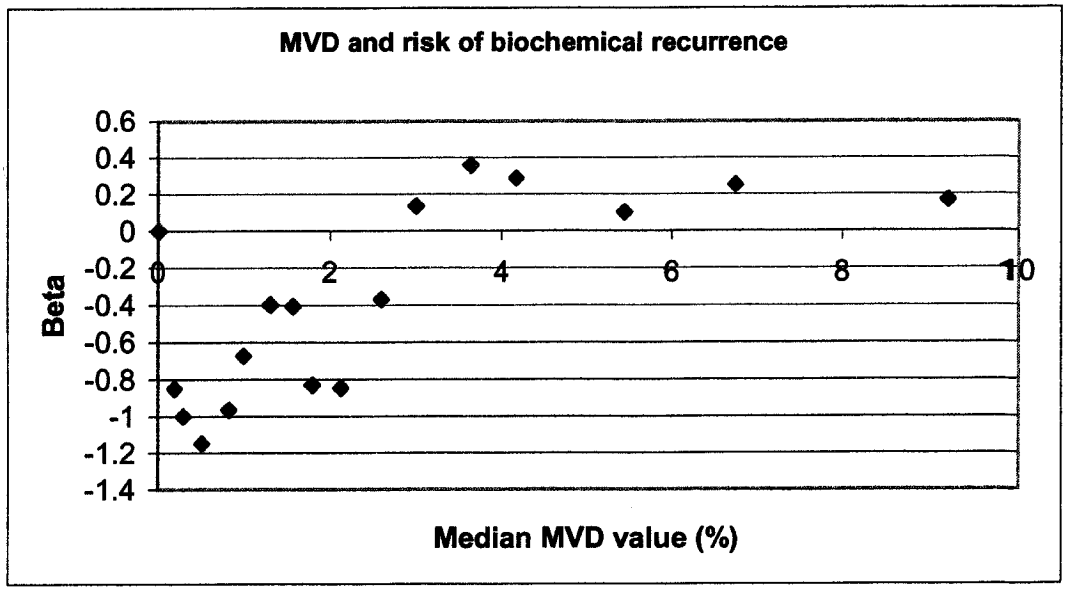
Figure C-10: Kaplan Meier curves of the 4 strata derived from the Bax model



No events were observed in the first and second quartiles. The two curves overlap.

APPENDICES D

Figure D-1: Risk of biochemical progression associated with MVD (%) levels



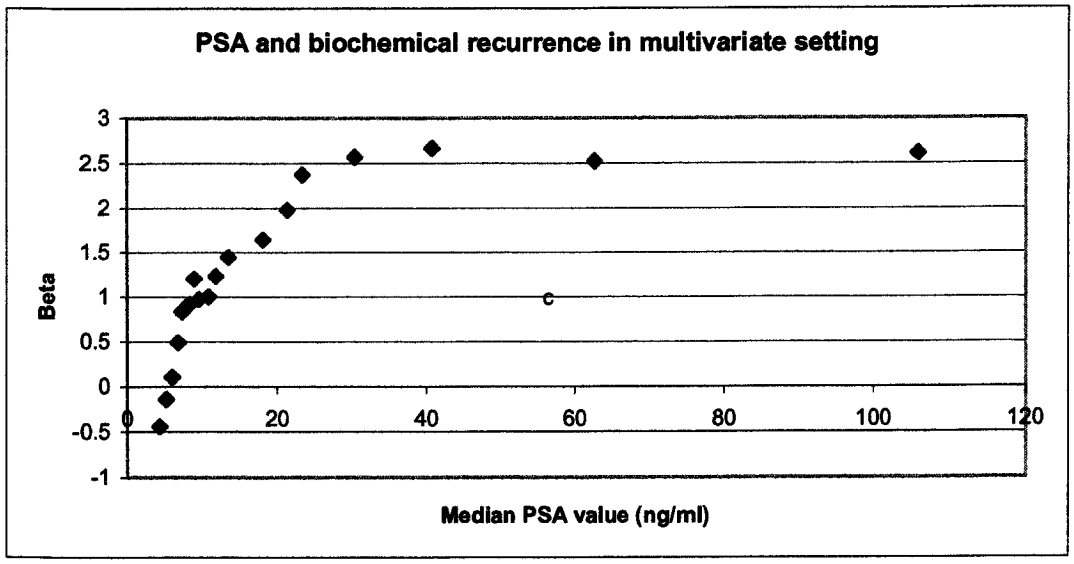
MVD was categorized and regressed against biochemical progression (Cox regression). The resulting beta values (running averages of three neighboring groups) are plotted against the median values of MVD groups to demonstrate the relationship between the two variables and evaluate linearity

Table D-1: Variable stepwise selection of traditional factor for biochemical progression (n=185)

Step	PSA (ng/ml)	Stage	ADT	Gleason	XRT fraction	-2LL	df	P value
0	<0.0001	<0.0001	0.44	0.037	0.0094			
1	<0.0001	0.0023	0.0339	0.035	0.0032	17.59	2	<0.0001
2	0.0013	0.0033	0.0014	0.097	0.011	12.28	2	0.0022
3	0.0001	0.0002	0.0017	0.067	0.13	10.81	1	0.0010
5	0.0001	0.0004	0.0011	0.072	0.027	4.92	2	0.086
6	<0.0001	0.0016	0.0051	0.028	0.030	4.08	1	0.44

Variables were entered in sequence based on their significance level. For each step, the overall LR, degrees of freedom and corresponding p value for adding that variable is represented in the last three columns, respectively. P values of individual variables are represented in each box and for each step. P values found to the left of the bold line represent the Wald statistics for that variable that is entered in the equation. P values found to the right of the bold line represent the Score p value of the variable not in the equation

Figure D-2: Linearity verification of PSA (ng/ml) and biochemical progression in the multivariate setting: Visual examination



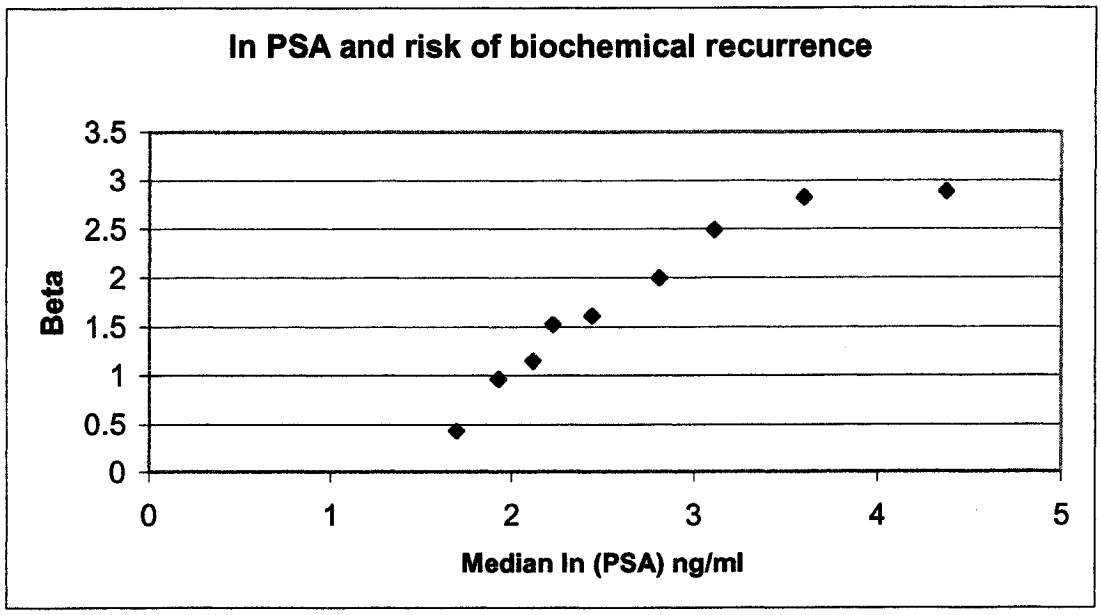
PSA was categorized and included in the multivariate Cox regression against biochemical progression. The resulting beta values (running averages of three neighboring groups) are plotted against the median values of PSA groups to demonstrate the relationship between the two variables and evaluate linearity in the multivariate setting.

Table D-2: Linearity verification of PSA (ng/ml) and biochemical progression in the multivariate setting: Fractional polynomial analysis

Model	LR (1df)	LR p value	PSA Wald statistics
PSA1	19.81	<0.0001	<0.0001
PSA-3	0.354	0.58	0.55
PSA-2	2.87	0.099	0.090
PSA-1	28.71	<0.0001	<0.0001
PSA-0.5	31.67	<0.0001	<0.0001
PSA0	40.27	<0.0001	<0.0001
PSA0.5	26.94	<0.0001	<0.0001
PSA2	2.96	0.075	0.086
PSA3	0.946	0.35	0.33

The change in –2LL from adding the transformed variable into the model is shown along with its associated p value.

Figure D-3: Linearity verification of ln PSA (ng/ml) and biochemical progression in the multivariate setting: Visual examination



ln PSA was categorized and included in the multivariate Cox regression against biochemical progression. The resulting beta values (running averages of three neighboring groups) are plotted against the median values of ln PSA groups to demonstrate the relationship between the two variables and evaluate linearity in the multivariate setting.

Table D-3: Multivariate analysis of biochemical progression using traditional factors stratified by PSA transformation

	Prior to PSA transformation			With PSA ln transformed		
Variable	Wald statistics P value	Beta value	HR	Wald statistics P value	Beta value	HR
PSA (ng/ml)	<0.0001	0.011	1.01	<0.0001	0.79	2.20
Clinical stage	0.0016			0.14		
1-2a	Ref	0	1	Ref	0	1
2b	0.0069	0.73	2.08	0.13	0.43	1.53
2c, 3-4	0.0005	1.04	2.82	0.053	0.61	1.84
ADT	0.0051			0.0013		
Not given	Ref	0	1	Ref	0	1
Given		-0.76	0.47		-0.88	0.41
Gleason score	0.027			0.065		
4-5	Ref	0	1	Ref	0	1
6-7	0.18	0.50	1.65	0.26	0.42	1.52
8-9	0.0124	1.11	3.03	0.0302	0.96	2.62
EBRT	0.0304			0.0228		
2.0 Gy daily	Ref	0	1	Ref	0	1
1.8 Gy daily		0.77	2.16		0.81	2.24

Transformation of PSA into ln PSA diminished the significance level of Gleason and stage

Table D-4: Interaction between variables in the traditional factor biochemical progression model

Factor #1	Factor #2	LR	P value
ADT	PSA	5.2638	0.2180
	Stage	0.8284	0.3627
	Gleason	2.2076	0.3316
	Dose of XRT	NA	
	Duration of ADT	0.0000	0.9967
PSA (ng/ml)	Gleason	0.6805	0.7116
	Stage	1.9055	0.1675
Stage	Gleason	6.6811	**0.0354**

Table D-5: Multivariate model of biochemical progression with Gleason*Stage interaction term

	n	With the interaction term		
		P value	Beta	HR
ln (PSA) ng/ml	185	<0.0001	0.82	2.27
ADT		0.0010		
Not given	79		0	1
Given	116		-0.86	0.42
Clinical stage		0.13		
1-2a	121	Ref	0	NA
2b-c, 3-4	64		-1.14	NA
Gleason score		0.36		
4-5	24	Ref	0	
6-7	146	0.24	-0.57	NA
8-9	25	0.90	0.092	NA
Gleason * Stage		0.058		
Stage 1-2a * Gls 4-5	93	Ref	0	
Gls 6-7*Stg 2b-4	86	0.017	1.88	NA
Gls 8-9*Stg 2b-4	16	0.080	1.70	NA
EBRT		0.0130		
2.0 Gy daily	176	Ref	0	1
1.8 Gy daily	19		0.8659	2.38

Table D-6: Interpretation of Gleason * Stage interaction term

Group	N	Hazard ratio
Stage 1-2a * Gleason 4-5	13	1
Stage 1-2a * Gleason 6-7	54	0.57
Stage 1-2a * Gleason 8-9	7	1.10
Stage 2b-4 * Gleason 4-5	9	0.32
Stage 2b-4 * Gleason 6-7	86	1.18
Stage 2b-4 * Gleason 8-9	16	1.91

Table D-7: Multivariate analysis of traditional factors in biochemical progression stratified by stage

		Stage 1-2a (n=74)			*Stage 2b+ (n=111)*		
Variable	*N*	*Wald statistics P value*	*Beta value*	*HR*	*Wald statistics P value*	*Beta value*	*HR*
ln (PSA) ng/ml	185	0.0001	1.498	4.47	<0.0001	0.7433	2.10
ADT		0.51			0.0023		
Not given	121	Ref	0	1		0	1
Given	64		-0.384	0.68		-0.86	0.43
Gleason score		0.29			0.037		
4-5	22	Ref	0	1	Ref	0	1
6-7	140	0.18	-0.35	0.50	0.043	1.26	3.51
8-9	23	0.97	0.031	1.03	0.012	1.71	5.50
EBRT		0.49			0.013		
2.0 Gy daily	176	Ref	0	1	Ref	0	1
1.8 Gy daily	19		0.54	1.72		0.87	2.38

Table D-8: Prevalence of biochemical progression amongst groups of Stage*Gleason

Gleason score category	Stage 1-2a	Stage 2b,c,3,4
4-5	6/15 (40%)	3/9 (33%)
6-7	18/56 (32%)	56/90 (62%)
8-9	4/8 (50%)	15/17 (88%)

Table D-9: Preliminary biochemical progression traditional factor model

Variable Category	Ln PSA ng/ml	XRT 1.8Gy	Stage (stg)* Gleason (Gls) Stg ≥2b&Gls 6-7	Stg ≥2b&Gls 8-9	ADT given
Beta	+ 0.7851	+ 0.864	+0.685	+1.136	- 0.856

Table D-10: Variable stepwise selection of biomarkers in biochemical progression model

Since biomarkers were measured in different subsets of patients, stepwise selection was carried out manually with each biomarker evaluated separately. Each variable was added to the model containing the traditional model and the corresponding LR p value recorded. Results of the analysis are shown below and the number of available records for each analysis is indicated in parenthesis.

Steps 0 and 1:

MVD (n=153)

	PSA (ng/ml)	ADT	Stg * Gls	XRT fraction	MVD: 3%	▲-2LL	df	P value
0	<0.0001	0.0016	0.0006	0.013	0.0041			
1	<0.0001	0.0026	0.0024	0.010	**0.0046**	7.89	1	0.0050

MIB-1 (n=143) (similar results were obtained with other forms of MIB)

	PSA (ng/ml)	ADT	Stg * Gls	XRT fraction	MIB%: cont	▲-2LL	df	P value
0	<0.0001	0.022	0.0079	0.032	0.66			
1	<0.0001	0.027	0.0076	0.0482	0.66	0.186	1	0.67

P-53 (n=136) (similar results were obtained with P-53 20 and P-53 50)

	PSA (ng/ml)	ADT	Stg * Gls	XRT fraction	P-53% : cont	▲-2LL	df	P value
0	<0.0001	0.0082	0.0069	0.049	0.18			
1	<0.0001	0.0074	0.012	0.041	0.18	1.576	1	0.21

Bax (n=137)

	PSA (ng/ml)	ADT	Stg * Gls	XRT fraction	Bax	▲-2LL	df	P value
0	<0.0001	0.0064	0.0061	0.032	0.65			
1	<0.0001	0.0059	0.0067	0.031	0.65	0.205	1	0.65

BCL2 (n=137)

	PSA (ng/ml)	ADT	Stg * Gls	XRT fraction	BCL2	▲-2LL	df	P value
0	<0.0001	0.015	0.0093	0.035	0.95			
1	<0.0001	0.015	0.0096	0.038	0.95	0.005	1	0.95

Table D-11: Variable stepwise selection of biomarkers in the biochemical progression model following MVD inclusion

Each variable was added to the model containing the traditional variables and MVD, and the corresponding LR p value recorded.

Steps 1 and 2: Models with MVD

MIB-1 (n=111) (similar results were obtained with other forms of MIB)

	PSA (ng/ml)	ADT	Stg * Gls	MVD 3%	XRT fraction	MIB%: cont	▲-2LL	df	P value
1	0.0001	0.066	0.014	0.0009	0.035	0.42			
2	0.0001	0.054	0.012	0.0009	0.12	0.42	0.634	1	0.43

P-53 (n=114) (similar results were obtained with P-53 20 and P-53 50)

	PSA (ng/ml)	ADT	Stg * Gls	MVD 3%	XRT fraction	P-53%: cont	▲-2LL	df	P value
1	0.0001	0.042	0.0072	0.0010	0.042	0.673			
2	0.0001	0.058	0.0065	0.0011	0.043	0.673	0.183	1	0.67

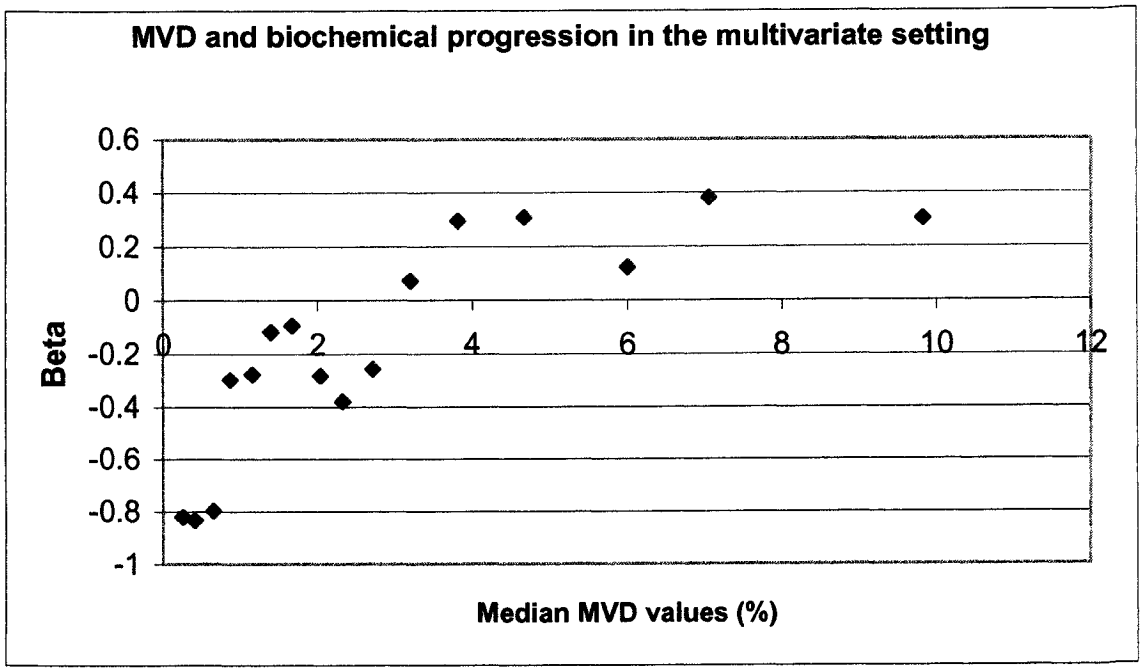
Bax (n=115)

	PSA (ng/ml)	ADT	Stg * Gls	MVD 3%	XRT fraction	Bax	▲-2LL	df	P value
1	<0.0001	0.062	0.0069	0.0003	0.028	0.69			
2	<0.0001	0.082	0.0065	0.0002	0.027	0.69	0.165	1	0.69

BCL2 (n=115)

	PSA (ng/ml)	ADT	Stg * Gls	MVD 3%	XRT fraction	BCL2	▲-2LL	df	P value
1	<0.0001	0.068	0.0068	0.0003	0.028	0.96			
2	<0.0001	0.069	0.0081	0.0004	0.029	0.971	0.002	1	0.97

Figure D-4: Linearity verification of MVD (%) and biochemical progression in the multivariate setting: Visual examination



MVD was categorized and included in the multivariate Cox regression against biochemical progression. The resulting beta values (running averages of three neighboring groups) are plotted against the median values of MVD groups to demonstrate the relationship between the two variables and evaluate linearity in the multivariate setting.

Table D-12: Linearity verification of MVD (%) and biochemical progression in the multivariate setting: Fractional polynomial analysis

Model	LR test (1df)	LR p value	PSA Wald statistics
MVD1	6.14	0.03	0.014
MVD-3	0.61	0.43	0.86
MVD-2	0.29	0.59	0.68
MVD-1	0.80	0.37	0.4679
MVD-0.5	1.97	0.16	0.15
MVD0	4.84	0.028	0.032
MVD0.5	6.04	0.014	0.015
MVD2	4.69	0.030	0.020
MVD3	5.75	0.017	0.018
MVD cut off 3%	8.26	0.0041	0.0046

The change in –2LL from adding the transformed variable into the model is shown along with its associated p value.

Table D-13: Preliminary biochemical progression biomarker model

Variable Category	ln PSA ng/ml	XRT 1.8Gy	Stage (stg)* Gleason (Gls) Stg ≥2b&Gls 6-7	Stg ≥2b&Gls 8-9	ADT given	MVD 3%
Beta	+ 0.7255	+ 1.019	+0.719	+1.340	- 0.866	- 0.679

Figures D-5 through D-8 represent the –log-log plot.

In these curves, each stratum should be compared to its reference stratum to evaluate the assumption of proportional hazard.

Figure D-5: Verification of PH assumption for radiotherapy fraction (Gy) dose in the equation predicting for biochemical progression: Univariate evaluation of -log log plot

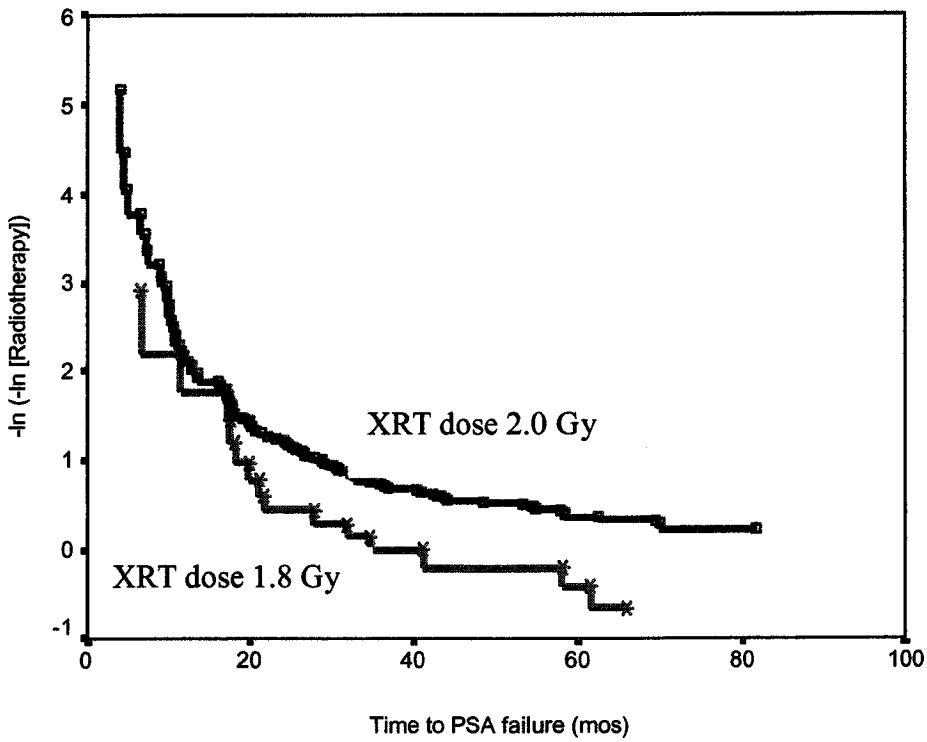


Figure D-6: Verification of PH assumption for Gleason score* stage interaction term in the equation predicting for biochemical progression: Univariate evaluation of -log log plot

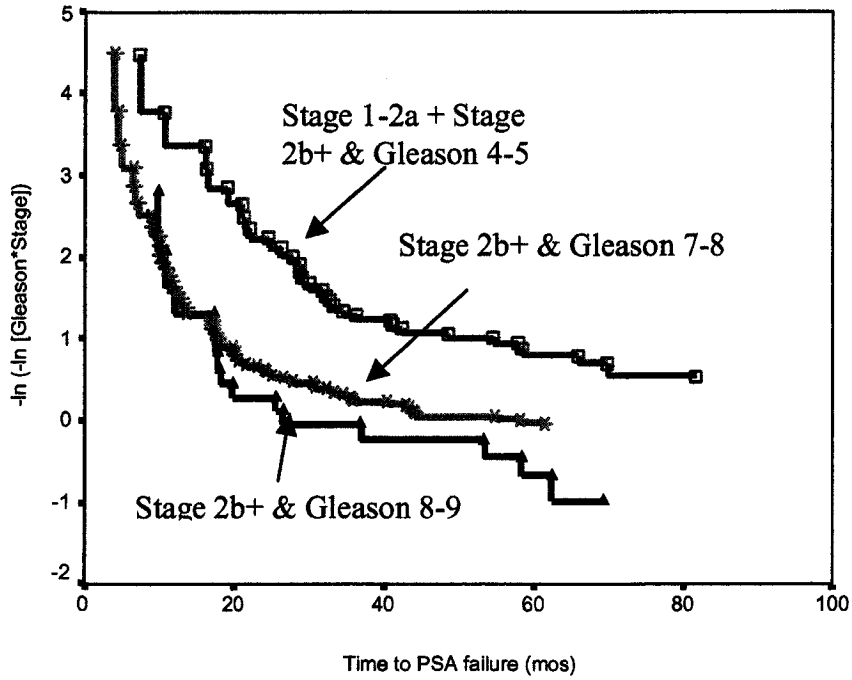


Figure D-7: Verification of PH assumption for ADT in the equation predicting for biochemical progression: Univariate evaluation of -log log plot

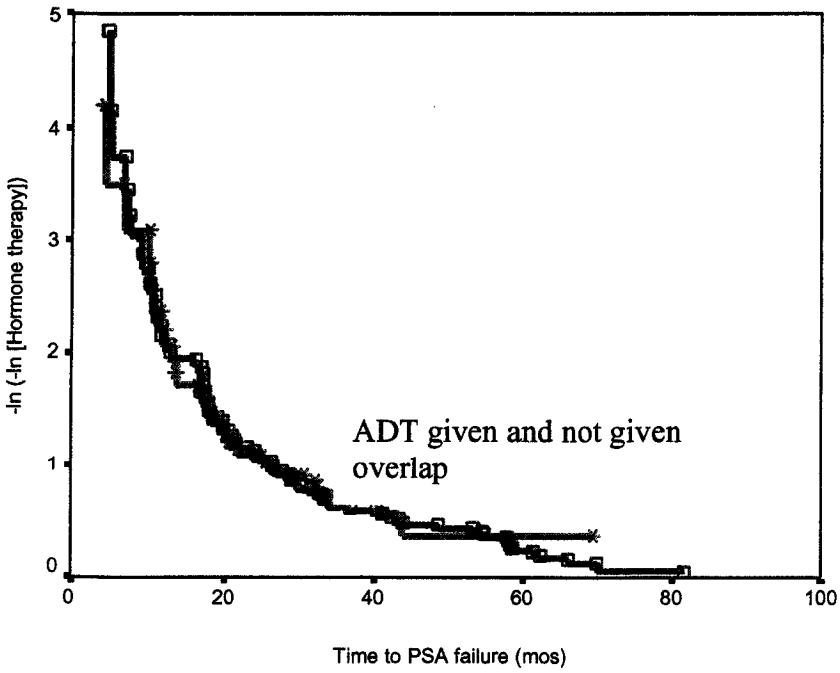


Figure D-8: Verification of PH assumption for MVD (%) in the equation predicting for biochemical progression: Univariate evaluation of -log log plot

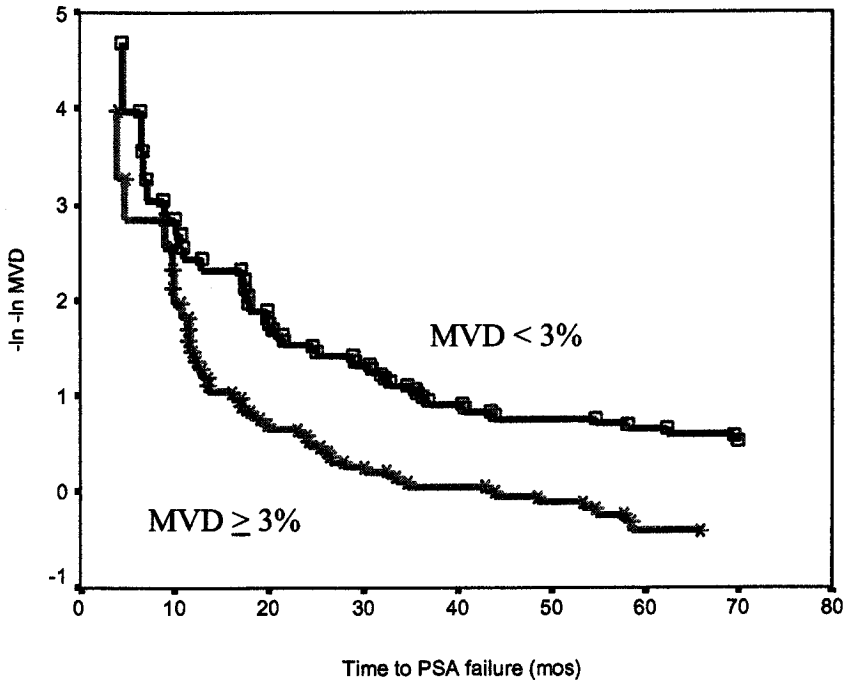


Table D-14: Verification of PH assumption for variables in the equations predicting for biochemical progression: Extended Cox model

Interaction term	Wald statistics
Ln (time) * radiotherapy fraction size	0.084
Ln (time) * Prior hormone therapy	0.12
Ln (time) * ln PSA (ng/ml)	0.083
Ln (time) * Stage 2b+ & Gleason 6-7	**0.021**
Ln (time) * Stage 2b+ & Gleason 8-9	0.64
Ln (time) * MVD (3% cut off)	0.32

Results of the extended Cox model in which a product term built between a time dependent variable (ln time) and, in turn, each variable is incorporated in the model. The test is used to evaluate deviation from the PH assumption. A significant product term suggests that the effect of the variable changes over time and the PH assumption is not met. All variables except the combination term for Stage and Gleason meet the PH assumption. The PH assumption for the traditional factors was investigated in the traditional factor model and for MVD in the biomarker model.

Table D-15: Interpretation of time dependent variable component in the traditional factor model

	Beta of the Stage*Gleason	Beta of G0*Stage*Gleason interaction	Total Beta	Hazard ratio
Stage*Gleason reference	0	0	0	1.000
Stage2b+*Gleason 6-7 <40 months	2.01		2.01	7.46
Stage2b+*Gleason 6-7 >40 months	2.01	-2.85	-0.84	0.43
Stage2b+*Gleason 8-9	1.26		1.26	3.52

Table D-16: Interpretation of time dependent variable component in the biomarker model

	Beta of the Stage*Gleason	Beta of G0*Stage*Gleason interaction	Total Beta	Hazard ratio
Stage*Gleason reference	0	0	0	1.000
Stage2b+*Gleason 6-7 <40 months	2.13		2.13	8.41
Stage2b+*Gleason 6-7 >40 months	2.13	-2.92	-0.79	0.46
Stage2b+*Gleason 8-9	1.55		1.55	4.69

Table D-17: Final biochemical progression traditional and biomarker models

Variable Category	ln PSA ng/ml	XRT 1.8Gy	Stage (stg)* Gleason (Gls) Stg ≥2b&Gls 6-7 <40months	Stg ≥2b&Gls 6-7 >40months	Stg ≥2b &Gls 8-9	ADT given	MVD
Beta of model							
Traditional	+ 0.5677	+0.563	+ 2.010	− 0.836	+ 1.258	− 0.598	NA[1]
Biomarker	+ 0.5256	+0.491	+ 2.130	− 0.785	+ 1.545	− 0.598	+ 0.549

[1] NA = Not applicable

Table D-18: Summary information of hazard ratios derived from the 2 models predicting for biochemical progression

	All TF	MVD
Mean	15.98	20.32
Median	3.49	3.97
Range		
Minimum	0.32	105.17
Maximum	0.59	143.69
Percentile		
10	1.21	1.22
20	1.52	1.94
30	2.42	2.47
40	3.11	2.89
50	3.49	3.97
60	5.31	5.85
70	15.22	20.95
80	29.15	33.61
90	53.58	67.83

Table D-19: Hazard ratios derived from equations predicting for biochemical progression categorized into quartiles

Quartile # and figure label	All TF	Bax model
First quartile = ♦	0.59-1.86	0.59-2.13
Second quartile = ▲	2.07-3.49	2.13-3.20
Third quartile = ▼	3.51-21.40	3.27-27.35
Fourth quartile = ■	21.82-105.17	29.29-143.69

Patients were grouped into quartiles based on their hazard ratio to evaluate the spread provided by the models. The table lists the hazard ratio intervals falling within each quartile.

Figure D-9: Kaplan Meier curves of the 4 strata derived from the traditional factor model in all patients

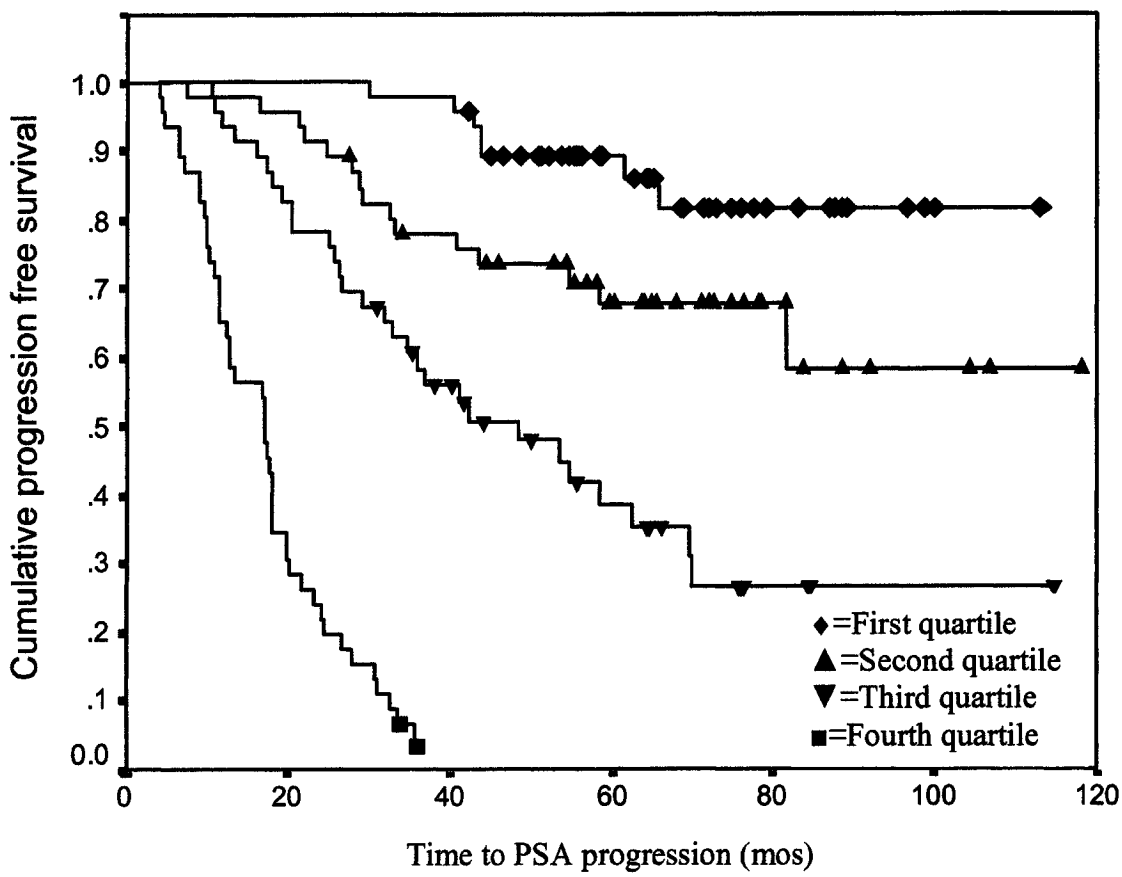
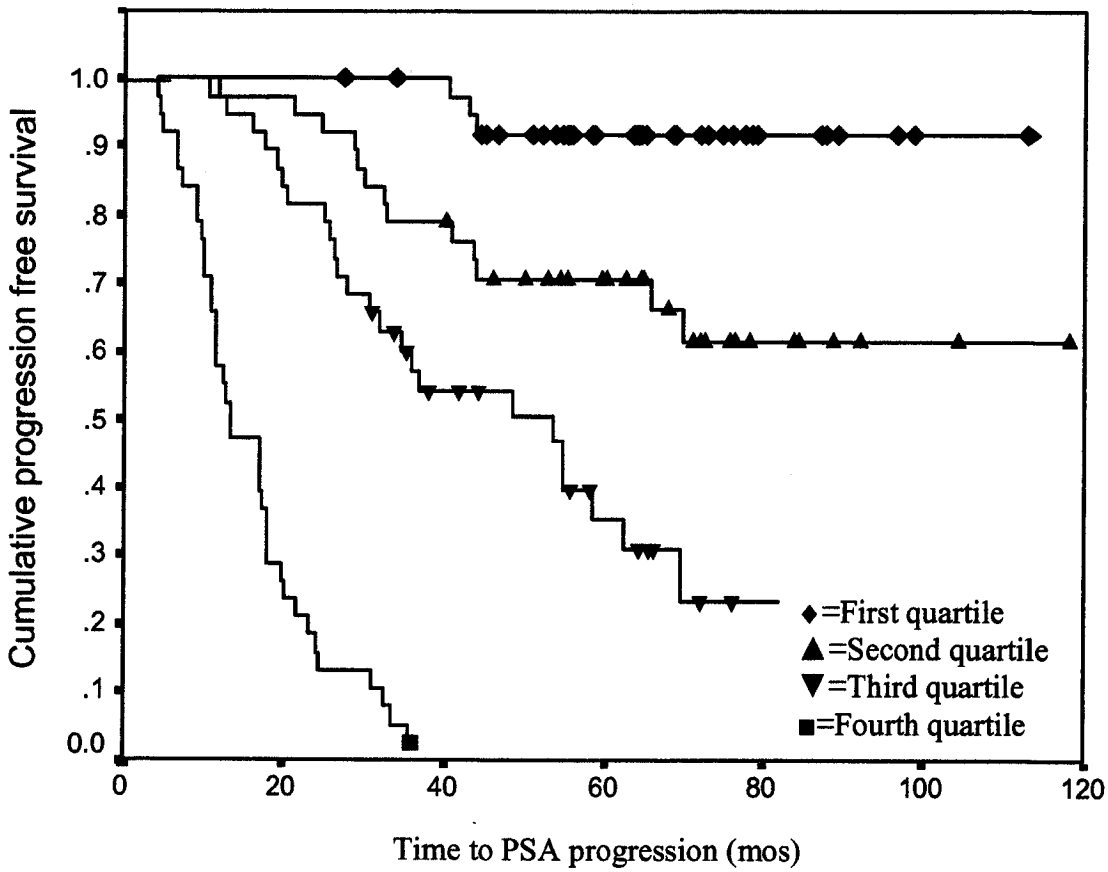


Figure D-10: Kaplan Meier curves of the 4 strata derived from the biomarker model



together, they engage epistemological questions about the formal quality of true narratives, as well as about the historiographic process in relation to the public circulation of facts.[1] To begin, the first preface sets a clear trajectory for the formal and aesthetic considerations that organize Carey's collection of "interesting occurrences" into a historical narrative whose purpose it would be to reflect on the epidemic, and to educate the local community about the inner workings of a public health disaster still fresh in its memory.[2] In this context, Carey draws a clear distinction between the factual content of his narrative and its formal stylistic features. His claim to authenticity is founded on an address to the reader in which he explains that: "I have not attempted any embellishment or ornament of stile; but have merely aimed at telling plain facts in plain language. I have taken every precaution to arrive at the truth" (v). Although Carey defines ornament and style

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[1] The first three editions appeared on November 13, 23, and 30, 1793. A fourth edition appeared shortly afterward, and was followed by the "improved" edition on January 16, 1794.

[2] Benjamin Rush first identified the epidemic as "bilious remitting yellow fever" on August 19, and believed that its cause lay in a cargo of rotting coffee that had been left to putrefy on Ball's wharf (Powell 12). The yellow fever epidemic was very short and extremely deadly. The disease appeared in Philadelphia in mid-August, and began to abate in late October. The vector of transmission, for yellow fever is the Aëdes aegypti – a mosquito that could not survive the fall frost. More than four thousand people died during the epidemic, with a death rate that exceeded one hundred persons per day during its early October peak. Philadelphians had no knowledge of the modes of transmission of yellow fever (whether, for example, it was contagious or not), and spent considerable energy debating whether it was of local or foreign origin.

through their absence from the text ("I have not attempted any"), his assertion is tentative, as if the attempt were not entirely successful.

While the self-conscious effort to avoid artifice suggests an uneasy relation between truth and style, as if the presence of one detracted from the other, the attention that Carey draws to this relation implies that a true narrative of the epidemic could be told: with precaution and with a careful attention to language, the event could be understood as a composite aggregation of facts that were in turn susceptible to historicizing narratives. In this manner, even as the preface points to the text's potential formal and stylistic flaws, it does so in order to posit an internal coherence to the epidemic. The argument is, of course, tautological: if the first edition does not yet reflect the coherence that it suggests is central to the epidemic, this posited coherence would assuredly appear in future editions. Indeed, Carey does not hide the fact that his work is marred by formal problems, or that these problems could be corrected with additional effort. Far from it, he argues that such problems are integral to the text, but that they are a product of external pressures induced by the necessity of sending a text to market and circulating it in public:

> For the desultory plan of some part of the pamphlet, I have to offer the following apology; many of the circumstances and reflections towards the conclusion, which would have come with more propriety in the beginning, did not occur, until some of the first half-sheets were not only written, but printed. I had no choice, therefore, but either to omit them, or place them somewhat out of order. I preferred the latter. (v)

The scene of Carey composing his pamphlet while proof sheets returned from the printer highlights the art of bringing a work to the public. In the case of his *Short Account*, the mechanics of publication undermine what he claims would otherwise have been a well-ordered narrative. But they also stand in as a guarantor of the truth that the text purports to reveal: the rush to publish implies that he did not have the time to refine his work, so that the form of this narrative is guided not by the nature of the epidemic, but by the medium through which its history circulates. Despite Carey's earlier claim to the contrary, the distinction he draws between plain language and the ornament of style is one that he willingly subordinates to the constraints of print technology. Although he does not use "form" and "style" interchangeably, together these terms are imagined as elements external to truthful historical accounts, and their absence from Carey's text – indeed the promise of their future absence (or in the case of form, its future transparence) – calls attention to its factual nature.

This interplay between composition and publication locates the text's formal problems not as antithetical to truth, but as temporary placeholders that point to its further engagement with a reading public in order to assume its proper coherence. Carey follows up on the provisional nature of his earlier statements with a conditional offer to amend and perfect the pamphlet if market forces would allow him to do so:

> Desirous of having this account correct and complete, I have printed off but a small number of copies of the present

> edition: and shall esteem myself most particularly obliged to any person who will be so kind to point out errors, to be corrected in, or suggest facts, to be added to, a new edition, which I propose to put to press very soon, and which will, I hope be found more ample than the present one. (vi)

By printing a small initial run with the intention of adding subsequent editions to correct any errors, Carey inserts economic considerations into the truth-form dyad that he sets up at the outset and creates a temporal framework for the circulation of knowledge in which futurity holds the promise of truth, and is accessible through the distribution of multiple editions. The urgency with which the several editions made it to the public suggests an ontological ambiguity that appropriates its claim to truth via its circulation in print: truth is available through successive revisions, and through repeated negotiations with a reading public.

As the pamphlet attains its aura of authenticity, formal concerns presumably recede – or become transparent; the fourth preface makes no mention of form or style, having apparently disengaged those questions. This disengagement is, of course, illusory, because Carey's show of moving beyond formal and stylistic problems in the fourth preface is a well-contrived misdirection founded in the inclusion of the distinct prefaces. When brought together in the fourth improved edition, they offer a progression that draws attention to the pamphlet's formal history as evidence of its authenticity: in point of fact, the prefaces not only highlight the fact that Carey is deeply concerned with form, but reveal the artifice of his stance to the contrary. Where the pamphlet was once tentative, and had a

"desultory plan," the fourth preface implies (by its silence on the matter) that the text was now well ordered. In this manner, the evolution of prefaces simultaneously foregrounds Carey's formal and stylistic interests, and acts as a testimonial to the pamphlet's factual nature. Where Carey had first claimed that style could hinder the presentation of facts, and that his ill-formed narrative was due to temporal constraints, the text now suggested that a transparent form was a sign of felicity, and presumably offered itself as a model for factual narratives. Ironically, the claim to authenticity is all based on artifice.[3]

The injunction for readers to correct Carey's errors and misrepresentations implies an important role for the economics of print circulation in the production of knowledge. Although he may have been unable to articulate this notion concretely

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[3] This progression is apparent in the two middle prefaces as well. In the second preface, Carey announced that despite his intention to enlarge the pamphlet, and "to have new-modelled it, so as to preserve a connexion between its several parts, in which it is extremely deficient...its speedy sale, and the demand for more copies, render it impossible for me to do more, at present, than make such corrections as the kindness of a few friends had led them to point out" (vi). This statement displaces any formal or factual issue that might be raised with respect to the second edition onto the economic network that prevented Carey from completing the work in the manner he would have liked to. He writes that the third edition "comes before the public...in some measure, in a new form. I have reduced it to as methodical a state, as in my power, but not as much so as I could wish, nor, I fear, as the reader may expect" (vii). While this progression describes the evolution of the text explicitly, and explains that its was still coming into its proper form, Carey goes on to say (still in the third preface), that temporal constraints continued to plague its overall shape, although he is unwilling to let this problem disrupt his project: "I beg to inform the reader, that this day ends one month, since the writing of the pamphlet commenced. I know that the shortness of the time employed, is no justification of a bad performance; but it may somewhat extenuate the defects of a middling one" (vii).

in his first edition, he was quite clear about it in the fourth. No longer aimed at a local audience, this fourth edition drops its affect of self-conscious formal critique. Rather than drawing attention to style, the fourth preface is expressly addressed to a community of readers beyond Philadelphia's borders, and recommends itself to this wider public as a repository of information for resurrecting lost economic networks. Always with an eye toward educating the public, Carey articulates the stakes behind his work on a scale that shifts the central medical preoccupation of the epidemic toward individual and familial structures of ownership and inheritance. Noting that a number of the epidemic's victims were not native Philadelphians, Carey contends that among these strangers, "were probably some, by whose death, estates have fallen to heirs at a distance" (viii). This assertion leads to the central innovation of the fourth edition: a necrology that claims to correct the inaccuracies of official morbidity statistics, and that was largely underwritten by Carey himself, who says that he "employed suitable persons to go thro' the city and liberties, and make enquiry at every house, without exception, for the names and occupations of the dead" (viii).[4]

While bills of mortality traditionally group victims according to broad demographic criteria (age, sex, race, geographic location), this necrology painstakingly lists the more than four thousand victims of the epidemic alphabetically, adding, where possible, kinship ties among the dead. In doing so, it

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[4] An earlier copy of the necrology appeared in the third edition, but the preface does not include a reference to it (Powell 281).

creates a compelling portrait of the epidemic that is remarkable in its simplicity: few inferences can be drawn about the progression of yellow fever in the fall of 1793 (addresses and dates of death, for example, are not included), but this snapshot gave Philadelphians an accounting of their losses as well as the means by which to re-imagine their much transformed community. Despite the impressiveness of this private census, Carey acknowledged that the list itself was neither complete, nor wholly accurate. If, as he claimed, the purpose behind the necrology was to identify foreign victims, one is left to puzzle the anonymous reference to "seven French strangers," which could in no way help their next-of-kin identify them. Nevertheless, Carey asks his audience to ignore these shortcomings, and quickly points out that the necrology is a crucial artifact of the epidemic:

> Imperfect as the list still remains, I hope it will be found useful in removing anxious doubts, and conveying to persons in different countries, the melancholy information of the decease of relatives, which, but for such a channel of communication, would, in many cases, be difficult, if not impossible to acquire for years to come. (viii)

Although his stated reasons for publishing the necrology seem unlikely, the drive to identify each of the victims and to circulate the list as a testimonial to the epidemic's effects doubtless has value as a public service: the focus on improving channels of communication, for example, offers an economic framework for post-epidemic markets in which information exchange becomes an increasingly important product to be packaged and traded. In doing so, the *Short Account* creates value for itself first by identifying communication as a market, and then by

advertising its utility in such markets. In this sense, regardless of how far the pamphlet was disseminated beyond Philadelphia's borders, it served as a model for open public discourse – first according to the interplay between audience and author (as revealed by the formal evolution of the text), and second, in its engagement with local, national, and international markets.

Formally, the necrology stands on its own as an addendum to the *Short Account* with little commentary or context instructing its audience about how it should be read. Because it seems to speak for itself, it is the exemplary model for Carey's goal of using plain language to speak plain facts unfettered by stylistic and formal considerations. On the surface, the necrology's form is transparent: an alphabetical list of victims suggests little in the way of ideological apparatus, unless it is a democratic ideology in which no victim is more important than an other, or is given preference over fellow citizens. And yet the illusory nature of such transparence is the hallmark of modern epidemiological analysis. The necrology is certainly not intended to explain how the epidemic developed, but it organizes its effects into two easily recognizable categories: familial and professional.[5] That

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[5] In truth, the necrology is not simply drawn along familial and professional lines. There are occasional references to nationality and, in the case of African-Americans, to race. In fact, the necrology's very first entry is for "Abigail, a Negress." Abigail's race stands in the place of family name and occupation, framing race in a socio-economic arena. This figures much more prominently in the debate over the role of African-Americans during the epidemic. The belief that they were immune (or had a heightened immunity) to yellow fever led Benjamin Rush to call on the black community to attend to the sick. Absalom Jones, Richard Allen and William Gray of the African Society organized these efforts, although

these categories would appear obvious or transparent ways to represent the effects of yellow fever on Philadelphia underscores the socio-economic axes along which Carey organizes his history of the epidemic, and thus reveals the taxonomy of the market that he exploits. Of course, the clearest beneficiary of this market was Carey, because the necrology was the primary motivating force for publishing the fourth edition of his pamphlet. Furthermore, by asserting itself as a model channel of communication, the necrology offers a guideline for modes of information exchange in the wake of local and national disasters: it represents a prototypical narrative that submits plain facts in plain language, and therefore helps to anchor the rest of the pamphlet by positioning itself as the text's logical and chronological end.

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the reward for their efforts was, at times, surprising (Powell 95). Carey singled out the three men for praise, but he railed against what he called "the vilest of the blacks [who] extorted two, three, four, and even five dollars a night for such attendance, as would have been well paid by a single dollar. Some of them were even detected in plundering the houses of the sick" (63). In response to this attack, Jones and Allen published *A Narrative of the Proceedings of the Black People, During the Late Awful Calamity in Philadelphia*. In it, they take Carey to task for singling them out for praise. Despite what appear to be good intentions, they wrote that "by naming us, he leaves those others in the hazardous state of being classed with those who are called the 'vilest'" (13). Finally, and perhaps most damning, Jones and Allen expose the economic impetus behind Carey's *Short Account*, turning his charges of extortion back against him: "he was wrong in giving so partial and injurious an account of the coloured nurses; if they have taken advantage of the public distress, is it any more than he hath done of its desire for information? We believe he has made more money by the sale of his "Scraps" than a dozen of the greatest extortioners among the coloured nurses" (8).

In this vein, the most intriguing chapters of the *Short Account* are the so-called "scraps" or fragments that Carey presents without attempting to create a coherent narrative. Not all of these scraps pertain directly to the immediate causes or remedies of the epidemic, but they provide a wider glimpse of its social and cultural effects on the community.[6] The absence of any obvious narrative frame for the scraps implies a certain journalistic detachment, as if the information could have been gathered by Carey's census takers while they were compiling the necrology. Among these scraps is a startling passage that provides insight into early Republican anxieties about the exchange of information. Taking the notion of circulation literally, the fragment dramatizes fears of infection on local and national scales through the hidden dangers posed by postal systems:

> Some of the postmasters, in the different states, used the precaution to dip Philadelphia letters into vinegar with a pair of tongs, before they handled them. Several of the subscribers for Philadelphia papers, made their servants sprinkle them with vinegar, and dry them at the fire, before they would venture to touch them. (79)

This fragment superimposes fears of contagion onto correspondence networks by suggesting that the postal system could act as a vector for the transmission of

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[6] One such entry speaks directly to the way that epidemics can frame temporal spaces in a community, albeit in an unexpected, if not uncanny, manner. Carey makes note of an anecdotal curiosity relating to the fact that among the refugees to leave Philadelphia at the height of the epidemic were a great many watchmakers. Since few people were left to pay "attention to how time passed," the watchmen who remained literally lost track of time, and began to call out the hours incorrectly (78).

yellow fever. While the example pertains specifically to the 1793 epidemic, the wider implication is clear: circulation of correspondence is a potential physical danger to individuals. This danger is figured on two scales: locally, through the public distribution of newspapers, and nationally, through the circulation of private correspondence. Although Carey doesn't spell it out, the step from distributing pathogenic agents to disseminating dangerous and seditious information is an easy, if not obvious one to make. Because private correspondence is, by its very nature, a hidden discourse, postal networks both maintain open channels of communication and pose a grave danger to the body politic through the incipient and potentially contagious threat of national rebellion.[7]

As is so often the case during public health crises, the anxiety about the dangers of correspondence plays on the tension between individual and communal bodies. Certainly, when the mechanism of transmission for a disease is unknown, the body politic – like the citizen's body – is threatened with dissolution unless it can isolate and treat pathogenic agents. And where a public document like Carey's *Short Account* is intended to alleviate the fear of spreading dangerous information,

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[7] This is all the more apparent because the nation was so young. Carey opens his *Short Account* with a commentary about the recent political stability of the United States, which he traces to its open markets and its "manufactures, trade and commerce" (9). Nevertheless, he makes it clear that this stability – even if promising – is but a recent development in America, and by virtue of the fact that the chance was so rapid, it could just as easily reverse: "a few revolving years exhibited the interesting spectacle of a young country, with a new form of government, emerging from a state which approached very near to anarchy, and acquiring all the stability and nerve of the best-toned and oldest nations" (9).

private correspondence does not. By the late fall of 1793, at the tail end of the epidemic in Philadelphia, the anxiety over yellow fever could be overshadowed by an anxiety in which correspondence might threaten not just the citizens of one city, but of an entire nation. Although the physical threat that yellow fever presented to the rest of the Union was not great, the threat of dissolution literally came to a head when the United States government was forced to evacuate Philadelphia during the epidemic, nearly causing a constitutional crisis over where and when the congress and cabinet could meet, and who had the authority to reconvene them (Powell 261).

The political and medial crises of the epidemic found their confluence in the most likely source of the infection. While Carey does not seem overly concerned with a fear of foreign contagion, he is convinced that it was "unquestionably… imported from the West Indies" (67). As evidence, he provides a list of vessels that arrived from San Domingo just prior to the outbreak, and also includes "extracts" from English papers describing the prevalence of the disease in the West Indies (68). These facts clearly suggest a correlation between yellow fever and emigration from the Caribbean. This correlation is further strengthened by the fact that earlier in the summer there had been a vast influx of French refugees from San Domingo who sought to escape the slave revolution; many refugees headed to Philadelphia after evacuating Cap François, and arrived "just a few weeks" before the epidemic erupted, and very likely brought the fever with them (11). Although these refugees were very likely the source of the epidemic, what is remarkably left unsaid in

Carey's account is the fear that a slave revolt could spread to the United States from San Domingo, threatening the nation even more than the yellow fever. These political anxieties about revolt were heightened by the widely held belief that African-Americans were immune to the disease. Their immunity, when considered in conjunction with white susceptibility to yellow fever could presumably lead to a great military advantage, as the slaves of San Domingo discovered over the next ten years. While slaves would presumably not communicate to each other via official postal networks, the contagion of revolution could potentially spread through the United States secretly, aided by hidden channels, until it erupted into public view and destroyed the body politic.

II. Epidemiology and Fiction

Charles Brockden Brown's 1799 novel *Ormond; or, the Secret Witness* opens with a similar set of formal and epistemological concerns to the ones expressed by Carey in the first preface to his *Short Account*. But rather than including an author's advertisement or a note to the reader, as Brown often does, the task of introducing the novel falls on its fictional narrator, Sophia Courtland. Sophia frames the story in a letter to her German correspondent, I.E. Rosenberg, explaining the nature of her tale, and justifying its formal qualities as follows:

> My narrative will have little of that merit which flows from unity of design. You are desirous of hearing an authentic, and not a fictitious, tale. It will, therefore, be my duty to relate events in no artificial or elaborate order, and without that harmonious congruity and luminous amplification which might justly be displayed in a tale flowing merely from invention. It will be little more than a biographical sketch, in which the facts are distributed and amplified, not as a poetical taste would prescribe, but as the materials afforded me, sometimes abundant and sometimes scanty, would permit. (37)

Unlike Carey's first edition, *Ormond* is a tale "flowing merely from invention," so that Brown's decision to ventriloquize this set piece through a fictional character calls attention to itself as a rhetorical device, and immediately raises questions about fact, fiction, and form in a novel that is concerned with forgery and conspiracy. This means that *Ormond*'s formal and stylistic flaws – such as they might be – are counterfeit; that they serve to enhance the novel's central conceit in which Brown writes himself out of the text. This manipulation gives notice that truth – both in the story and in the novel – will be a deeply contested category: where Carey insisted that truth, properly told, could be made free of stylistic embellishments and formal flaws, Sophia complicates this notion by pointing to the supposed formal unity of fictional tales. Much as in Carey's preface, Sophia explains that her narrative has been shaped by the availability of information, and that the novel's form therefore stands as evidence of its factual nature. Furthermore, she echoes Carey's claim with respect to the ornament of style and "luminous amplifications," but because these echoes exist in a fictional narrative,

they undermine the truth-claims that she makes: if the novel has the same formal qualities as a true narrative, Brown blurs the boundaries of fact and fiction, and suggests that the question of form is much more complicated than either Sophia or Carey would admit. Ultimately, Sophia's letter displaces anxieties about the text's formal structure onto epistemological grounds only to bring those grounds into question themselves.

These anxieties are readily apparent in the novel's opening chapters, when Craig Mansfield's crime against the Dudley family is finally revealed after five years of apparently faithful service. Craig first appeared at Stephen Dudley's pharmacy under the name of Wakefield, as a model of Franklinian youth. He claimed to have left England because he "conceived it his duty to provide for himself...[and came] to America, in search of the means of independent subsistence" (41). Raising himself in Dudley's opinions through hard work, and building a reputation for "stability and integrity" (42), Craig advances from apprentice to partner until Dudley offers to take his (fictional) younger brother as an apprentice as well. All of this advancement is built upon an elaborate masquerade. The fact that Craig could reinvent himself and present a false front so convincingly and without raising suspicions is the ultimate nightmare of self-representation gone bad. Even more so than his counterfeit English background, Craig's true history – with his humble beginnings in Portsmouth, New Hampshire – implies that he has a deep affinity for exploiting social and economic opportunities

for advancement in America. But in Craig's case, such opportunities are merely the means toward more lucrative fraudulent schemes. Craig therefore dramatizes the fear that American markets – if they are structured to encourage opportunities for self-advancement – are nonetheless highly susceptible to fraud by virtue of the fact that they provide for nearly seamless, and potentially unlimited advancement, and that this advancement can be manipulated through misrepresentations of the self.

The problem with self-representation as Craig exploits it in the novel lies not only in the danger that it poses to public markets, but to domestic spaces as well. At the same time that he insinuates himself into Stephen Dudley's professional realm, Craig infiltrates the Dudley household, and destroys it despite his apparent affections for the family. In doing so, he reveals how permeable the boundary between family and profession is in early Republican America. If Craig's actions mimic a form of secret contagion, it is no accident that the Dudley family's tribulations begin with his crime and reach their climax during the Philadelphia yellow fever epidemic. The dissolution of the Dudley family – including their removal from New York to Philadelphia, Stephen's unemployment and eventual blindness, his wife's death, and the fact that their young daughter Constantia (aged 17 at the time of the epidemic) must support the family – adds a third, familial body (besides individual and national) to those that can be threatened by contagion. Indeed, while domestic spaces were not an explicit concern in the

debates about the epidemics discussed thus far, both Brown's representation of the Dudley family, and Carey's insistence on listing familial bonds in his necrology demonstrate a shift in the public discourse surrounding disease.

And as in Carey's narrative, the figure of contagion in the early part of the novel is split between the yellow fever and Craig's manipulation of correspondence. The fact that he not only invents his history, but that he forges the corroborating evidence for it – including a series of letter between he and his fictional family – demonstrates the way in which private correspondence can be used for nefarious ends. In addition to this, Craig intercepts Stephen Dudley's letters, imitates his handwriting, and alters it to serve his confidence scheme. Ironically, Craig's lie is eventually brought to light by a letter from his real mother that arrives when he is out of town. If postal services can be exploited for fraud, then so too can they regulate criminal behavior by virtue of the fact that anyone – including Craig's illiterate mother – can use them as channels of communication. But for Brown's purposes, the fact that private correspondence can so easily undermine familial and economic bonds becomes an important issue with regard to the text, which is, after all framed as a quasi-epistolary narrative. The novel therefore enacts the tensions that it describes while trying to use those enactments to anchor its formal and epistemological structure. The book provides a model – much like Carey's *Short Account* does – for the proper circulation of information; it is a secret history made public. In this manner, the epistemological questions that

Brown raises at the outset of the novel serve to educate the reader about the dangers of taking information at face value. Indeed Sophia says as much when, at the end of her opening epistle, she tells Rosenberg: "Society and manners constitute your favourite study; and I am willing to believe that my relation will supply you with knowledge, on these heads, not to be otherwise obtained. If these details be, in that respect, unsatisfactory, all that I can add is my counsel to go and examine for yourself" (38).

While the first part of *Ormond* explores the dangers that private correspondence can pose to familial order, this danger is literalized with the appearance of yellow fever in the novel. It is no small coincidence that in the same moment that the fever is first mentioned, it is transformed in Constantia Dudley's consciousness from an existence "chiefly in the form of rumour" (64) to an event in fact; the fictive appearance of yellow fever in the novel mirrors its passing from narrative to reality in the story, and this transformation occurs at the intersection of Brown's model of circulation.[8] As soon as Constantia discovers that her education in the sciences and the classics leaves her unqualified to teach female students and

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[8] A second place where illness and narrative intersect occurs in the character of Whiston, the Dudley's hysterical neighbor, who acts as a vector for rumors until he flees the city: "the rumours and conjectures industriously collected [by Whiston] during the day were, in the evening, copiously detailed to his neighbours, and his own mind appeared to be disburdened of its cares in proportion as he filled others with terror and inquietude" (70). Ironically, after he abandons his sister and flees to the country, Whiston dies alone and is "suffered to decay piecemeal" (74). It appears that the model for his death may have been taken from Carey's account of a poor man who died while villagers refused to help him for fear of being infected with the yellow fever (84).

therefore unable to earn extra income with which to support her father, she devises a plan to better her chances of employment by teaching herself Italian and French. In order to do so, however, she must purchase a second-hand grammar book from her former book and paper seller – Mr. Watson. As Constantia makes her way to Watson's shop, however, she discovers that he has died of the yellow fever in the night prior to her arrival.

The fact that Watson was a book and paper seller only reinforces anxieties about correspondence being an agent of infection. Spatially, Watson's shop is the nexus where readers and writers would congregate to begin the process of circulating books and letters. It is therefore a natural focal point for the eruption of epidemic if correspondence too is to be considered a vector of contagion. The fact that his is the first case mentioned in the novel only underscores the relation between contagion and narrative all the more explicitly. His death is therefore doubly charged, because it represents the advent of disease as well as a further danger of uncontrollable epidemic in the community: when four thousand people can die in just over ten weeks, the prospects for the young Republic's health seem tenuous indeed, especially given the revolutionary fervor in France and San Domingo.

Ultimately, Charles Brockden Brown was not a contagionist, subscribing instead to Benjamin Rush's ideas about putrefaction. But his novel plays both theories off of each other in order to dramatize the interconnectedness of illness and

narrative. And because the mystery regarding the source and modes of transmission of yellow fever is one that seemed to baffle eighteenth-century physicians, writers such as Carey and Brown were left to chronicle the epidemic by resorting to social, political, and economic frameworks. Their attention to the formal difficulties of their texts is not simply an aesthetic consideration. Instead, the self-conscious engagement of form in their respective prefatory remarks suggests a serious epistemological problem: the ontological ambiguities that Carey and Brown reveal, reflects uncertainty about what disease is, and how it functions. For these writers, this uncertainty has its analogue in the very nature of representation, so that the confluence of illness and narrative in this history, and in this novel is not unexpected. It is, however, their expression of this confluence as a formal (rather than simply thematic) quality that demonstrates the particular elegance of their epidemiological modes of inquiry. And it is this same expression that is deeply bound to early American history, American rhetoric, and finally, to American narrative.

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