Barts and The London
Queen Mary's School of Medicine and Dentistry

EXPERIMENTAL PATHOLOGY
BSc PROJECT

TITLE: "TISSUE MICROARRAY ANALYSIS OF PROSTATE CANCER"

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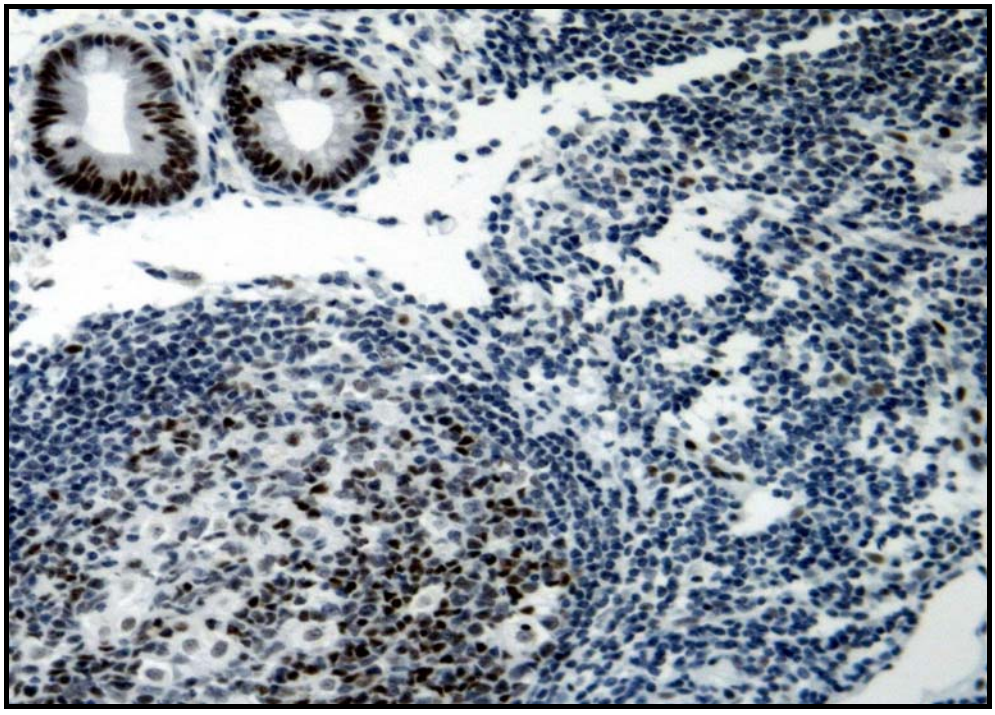
2005-2006



ACKNOWLEDGEMENTS

I would like to acknowledge the contribution of everyone that helped me with this project.

First and foremost, I am extremely grateful and indebted to Professor Steven Greenwald and Dr. Cathy Baker who nominated me for the Wolfson Intercalated Award and to Lord Turnberg MD FRCP (Chair), Professor Abraham Guz MD FRCP (London), and Professor Sue Malcolm PhD FRCPath (London) of the Royal College of Physicians who honoured me with the Wolfson Intercalated Award (£5,000).

Moreover, I would like to thank my project supervisor Dr. Daniel Berney for his invaluable support throughout the year.

In addition, I would like to thank the statisticians Dr. Gabrielle Fisher and Ms. Laurence Ambroisine of the Wolfson Institute of Preventive Medicine and Cancer Research UK, who performed the statistical analysis of the data in this project.

Finally, I would like to thank Ms. Susanne Jordan, senior laboratory technician at St. Bartholomew's Hospital, and Ms. Sak Kudahetti, PhD student, for teaching me the technique of immunohistochemistry.

DEDICATIONS

I dedicate this project to:

My father Professor Ioannis Garas MD PhD-my inspiration, my mother Emily Garas MSc, and my brothers Ioannis-Alexios Garas MEng, and Aris Garas.

Mrs. Marianna Latsis, and Mrs. Eutichia Papakonstadinou for their tremendous support all these years. There is no way that I could possibly convey how obliged I am to both of them, and this is the least I can do to express my eternal gratitude.

Dr. Nicholas Perry FRCS FRCR, an excellent physician, Professor Konstantinos Grammenos OBE DSc, a pioneer in his field, and Mr. Petros Vassilaras, a great teacher, all of whom are very dear family friends.

ABBREVIATIONS

-BPH: benign prostatic hyperplasia

-DAB: diaminobenzidine

-Gleason score: histological grading system for prostatic carcinoma based on histological pattern of growth and infiltration

-IHC: immunohistochemistry

-PBS: phosphate buffer solution

-PCa: prostate cancer

-PIN: prostatic intraepithelial neoplasia

-PSA: prostate specific antigen

-Rb: retinoblastoma

-TMA: tissue microarray

-TRUS: transrectal ultrasound

-TURP: transurethral resection of the prostate

-WW: watchful waiting

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ABSTRACT

Background: Prostate cancer (PCa) is the commonest non-cutaneous malignancy in males and the second leading cause of male cancer mortality. However, the natural history of PCa is highly variable, and therefore difficult to predict. Consequently, urologists are unable to decide with confidence whether a patient would benefit from early aggressive treatment, or, if conservative management with regular follow-up (watchful waiting) would suffice. As a result, many patients are "overtreated". This "overtreatment" carries with it significant morbidity and mortality. Currently, the best prognosticators for clinically localised PCa are the Gleason score and PSA level.

Purpose: To assess whether any of the cell cycle proteins p53, Rb, MDM-2, p21, and Ki-67 could be used as new independent prognostic biomarkers for clinically localised PCa.

Methods: The tumour specimens used in this project come from patients that were conservatively managed (watchful waiting), to enable an assessment of the natural history of their tumours. Follow-up was for a mean period of ten years. To our knowledge, this represents the first such study in the available international literature. Tissue microarray (TMA) technology was used to increase the efficiency of the analysis.

Results: A significant correlation between Gleason score, in 3 or 5 levels, and the percentage of stained cells for p21, p53, Ki67 and MDM-2 was found. Moreover, p21 expression had a significant association with the risk of death from PCa when using the univariate Cox proportional hazard model. However, after including the PSA level and Gleason score in the multivariate Cox model, p21 expression had no longer a significant association with the risk of death from prostate cancer.

Conclusions: p21 is an important independent prognostic biomarker in clinically localised PCa as its overexpression signifies a worse prognosis, necessitating early aggressive treatment. Moreover, p21, p53, Ki67 and MDM-2 phenotypes are useful in the assessment of the grade of clinically localised PCa, due to their significant associations with the Gleason score.

INTRODUCTION

Prostate cancer epidemiology, importance, and clinical problems

In the western world, prostate cancer (PCa) is the commonest non-cutaneous malignancy in males, and is second, only to lung cancer, as a cause of cancer-related mortality in males.[1-4] In 2000, 513,000 new PCa cases were diagnosed, while in the same year, 250,000 men died of prostate cancer throughout the world.[5] Consequently, PCa constitutes a significant public health problem.[2]

The incidence and mortality of PCa increases more rapidly with age compared to any other cancer (Figure 1).[6] Autopsy studies have revealed that in men over 50 years of age, the incidence exceeds 30%, this number rising to virtually 100% by 80 years.[4;7]

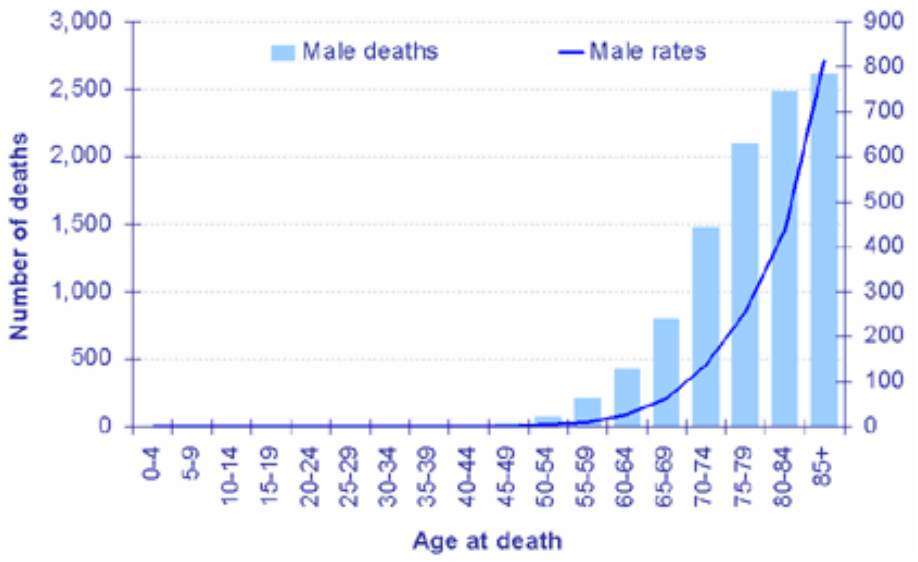


Figure 1 Number of deaths and age-specific mortality rates for prostate cancer, UK 2004.[8]

Over the past decade, the incidence of PCa has been increasing (Figure 2).[9;10] This has been partly attributed to the introduction of prostate-specific antigen (PSA) and digital rectal examination (DRE) screening in the USA.[9] In other countries, such as the UK, although no PSA screening programme is endorsed by the NHS, it is available to men on request.[11] The increased awareness of the problem among the general population has resulted in greater numbers of men seeking to be screened for PCa. In opposition to the increasing incidence, the mortality from PCa was shown to subsequently decrease.[9] This decline, it is argued by some to be due to the intense screening (USA)

leading to early radical treatment of clinically localised PCa.[12] This view however is not widely accepted, especially outside the USA.[9]

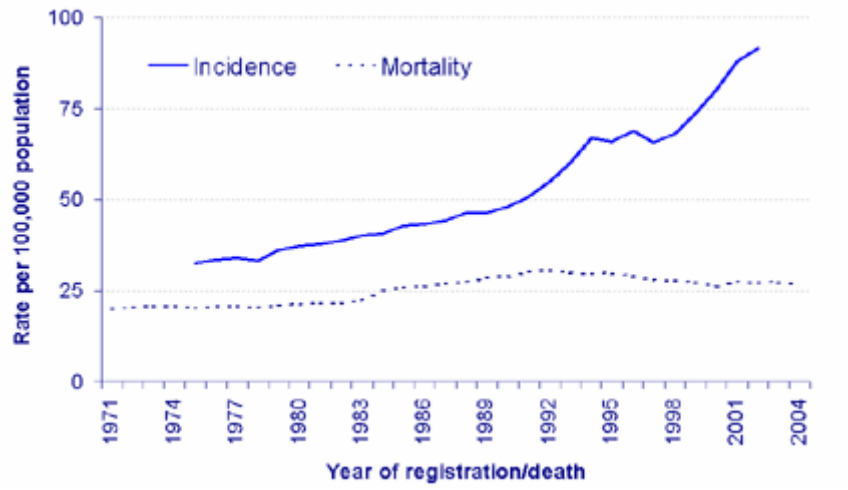


Figure 2 Age-standardised incidence and mortality rates for prostate cancer, Great Britain, 1971-2004.[8]

A very important characteristic of PCa is that its aggressiveness varies significantly.[6] Many of these tumours have a long doubling time and remain latent for many years, hence being of little clinical interest, particularly if the patient is elderly. On the other hand, in younger patients (with a longer life expectancy), PCa may be a more aggressive disease leading to the patient's death.[9]

On the whole, "most men die with rather than from PCa". Despite that, it is imperative to distinguish between the more aggressive and potentially lethal tumour from the more latent and relatively indolent one.[13] Consequently, it is crucial to define the true utility of the currently used classical markers for PCa and to evaluate the role of newer potentially independent prognostic biomarkers obtained by immunohistochemistry (IHC).[14]

Difficulties with PCa are that the natural history of the disease varies extensively and hence is very difficult to predict, while the age of the patient at diagnosis ranges from 40 to over 80 years.[15] The later further complicates decision-making in the management of clinically localised PCa.

Aims of this project

Currently, the best prognostic biomarkers for clinically localised PCa are the Gleason score and the baseline PSA level. These, although strong and independent, can only provide good prognostic information for the highest and lowest 20% 10-year mortality subgroups. However, for the intermediate patient group, which constitutes the majority of PCa patients (60%), improved prognostic biomarkers, that would add information independently to the ones currently in use, need to be identified. If such new independent prognostic factors are identified then so many men will be spared of unnecessary treatment associated with serious morbidity (urinary incontinence, impotence, risks of major surgery) and hence serious consequences on the quality of life,[11] while for those with more aggressive PCa needing early intervention (radical prostatectomy, radiotherapy), the benefits of the treatment will outweigh the risks. Moreover, conservative treatment (watchful waiting) of indolent tumours should prove to reduce the anxiety of patients and urologists.

The aim of this project is to assess whether any of the cell cycle proteins Rb, p21, p53, Ki-67, and MDM-2 (see appendix 1) could be used as new independent prognostic biomarkers that would allow a more accurate prediction of the natural history of clinically localised PCa. This has been done through the use of tissue microarray (TMA) technology (Figure 3). If such additional biomarkers are identified, that independently add information to the current prognosticators[11] they will be of great clinical value in the decision making of how to treat clinically localised PCa on an individual patient basis.[16]

The tumour specimens used in this project were clinically localised ones managed by watchful waiting. They were obtained either by TRUS-guided biopsy or by TURP chips. This project forms part of the Transatlantic Prostate Cancer Group Study (USA & UK) and follow-up data for a mean period of ten years were provided for the statistical analysis.[11]

To our knowledge, this study is the first of its kind in the available international literature, as it assesses clinically localised prostate cancers managed by watchful waiting. In this way, the natural history of these tumours was assessed, as no

intervention was performed which would have interfered with the natural evolution of the disease. This is in opposition to studies that have been published to date, where the prostate specimens examined were either radical prostatectomy specimens or had undergone radiotherapy, and hence the natural history could no more be assessed with certainty.

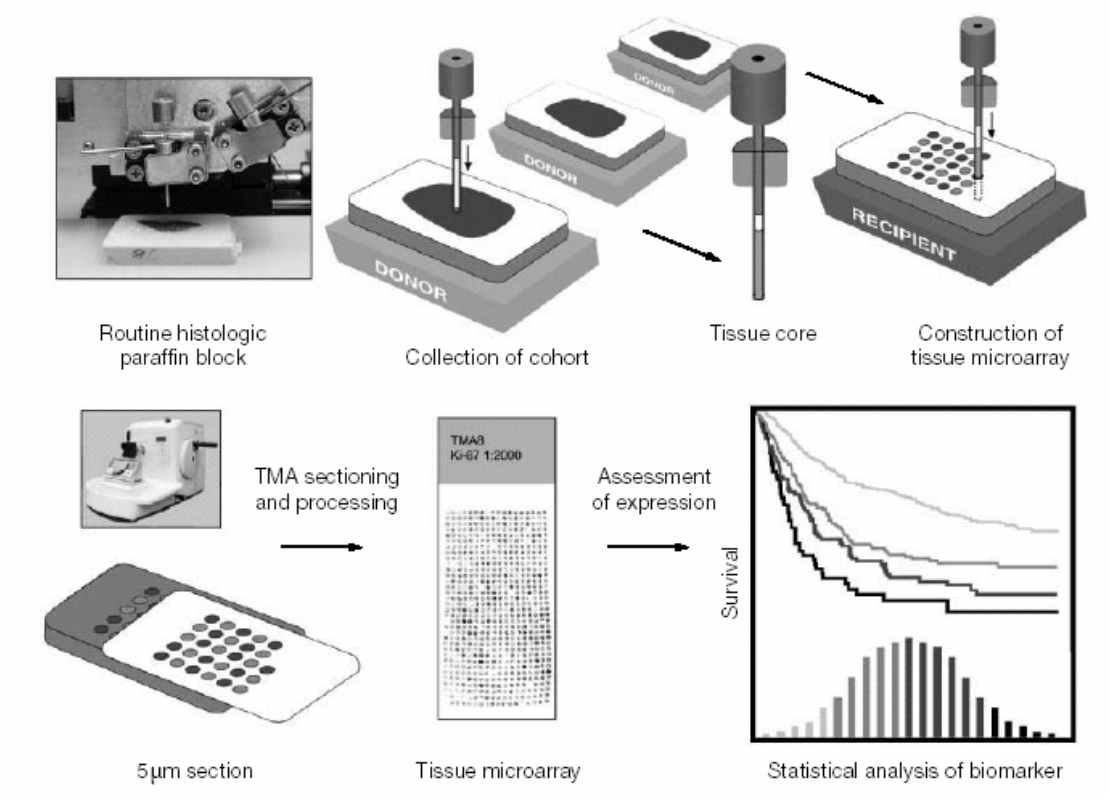


Figure 3 Illustration of TMA construction and its use in biomarker evaluation.[17]

Tissue Microarray Technology

Tissue microarray (TMA) technology, chosen for the analysis of PCa in this project involves taking small tissue cores from the original tumour, and placing them on a slide. In this study, each core had a diameter of 0.6mm while the distance between two adjacent cores was 0.8mm.[17;18] Consequently, each slide consisted of about 100 cores on average (Figure 4). Once the TMA slides have been prepared, they can be treated in a variety of ways (IHC for protein detection, fluorescence in situ hybridization (FISH) for DNA detection, or mRNA-ISH for mRNA detection).[17] In this study, TMA slides were analysed by IHC as protein over/under expression was looked for and then this was related to the clinical outcome of the corresponding PCa patient in order to try and identify new independent prognostic biomarkers for clinically localised PCa.

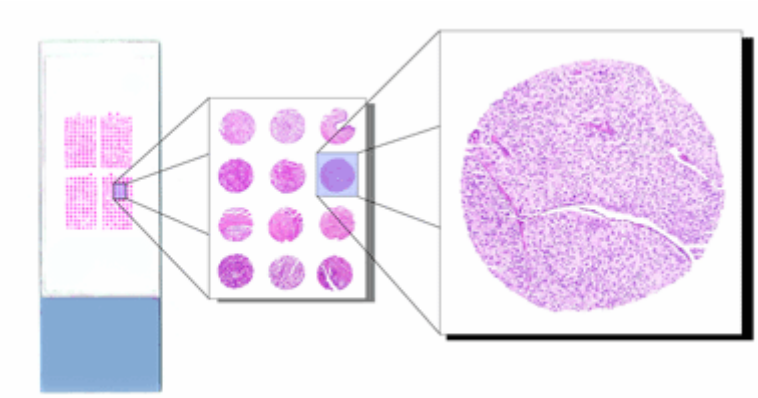


Figure 4 A typical TMA slide, and how a TMA core looks under the microscope.[19]

TMAs were first described in their current form by Kononen *et al* in 1998. A year later, TMAs were introduced in urology by Budendorf *et al* in a project where gene amplifications in PCa was studied through the use of FISH.[20-22]

TMA technology constitutes a powerful research tool that has made it popular in cancer research worldwide. More and more studies are using TMAs (Figure 5). This is because TMA technology possesses multiple advantages in relation to the traditional laboratory techniques such as conventional IHC or FISH.[17;18;21]

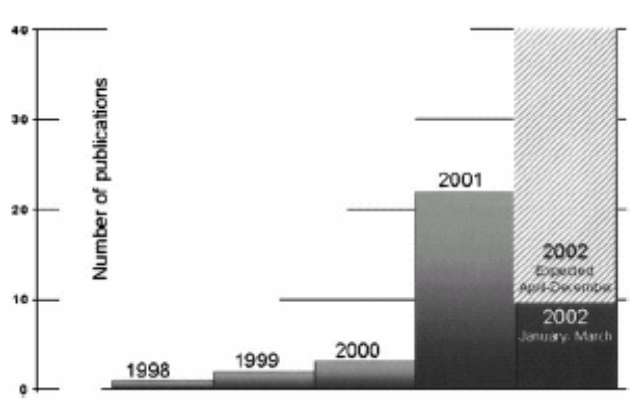


Figure 5 Data from Medline demonstrate an increased in the popularity of TMAs in studies.[21]

TMA technology provides a "high throughput" method for the identification of new, independent prognostic biomarkers for PCa, as IHC can be performed to hundreds of tumour cores simultaneously.[23] With TMA technology, the number of tissue specimens that can be analysed in a single IHC session increases by an order of magnitude, while the time needed for this is reduced by an order of magnitude. Also, the cost is heavily reduced as the amount of antibody needed is much less.[16-18;24] Moreover, TMA results are at least as good as the ones obtained from conventional IHC or FISH, as several trials have demonstrated.[17;22;25] In addition, intra-observer variability is minimised as all the cores can be examined on the same day.[21] Hence, it can be concluded that TMA comprises an ideal tool for the rapid analysis and characterisation of hundreds of potential biomarkers in a more convenient way than standard IHC would allow.[24;26]

Moreover, the minute size of TMA cores minimizes destruction (tissue loss) of the original block.[16;24] Hence, most of the original block is preserved, so that it can be used for diagnosis and/or stored for future research.[18] This, also assists in the management of pathology archives as TMA slides are much easier to handle, store and retrieve than entire tumour blocks.[17] TMA also assures that all cores are treated under exactly the same conditions during the experiment, for exactly the same amount of time with exactly the same reagents. This ensures "experimental uniformity" as well as increasing efficiency, since for every one hundred (or more) conventional slides, only one TMA slide needs to be stained.[18;20;27]

For all of the above reasons, and also due to the fact that TMAs can be digitalised and be put on electronic databases in the World Wide Web (Figure 6), TMAs greatly assist cancer research by easing collaborations between institutions throughout the world, as is the case with this study (St. Bartholomew's Hospital, London, UK, Royal Marsden Hospital, London, UK and Memorial Sloan-Kettering Cancer Centre, NY, USA).[24;27]

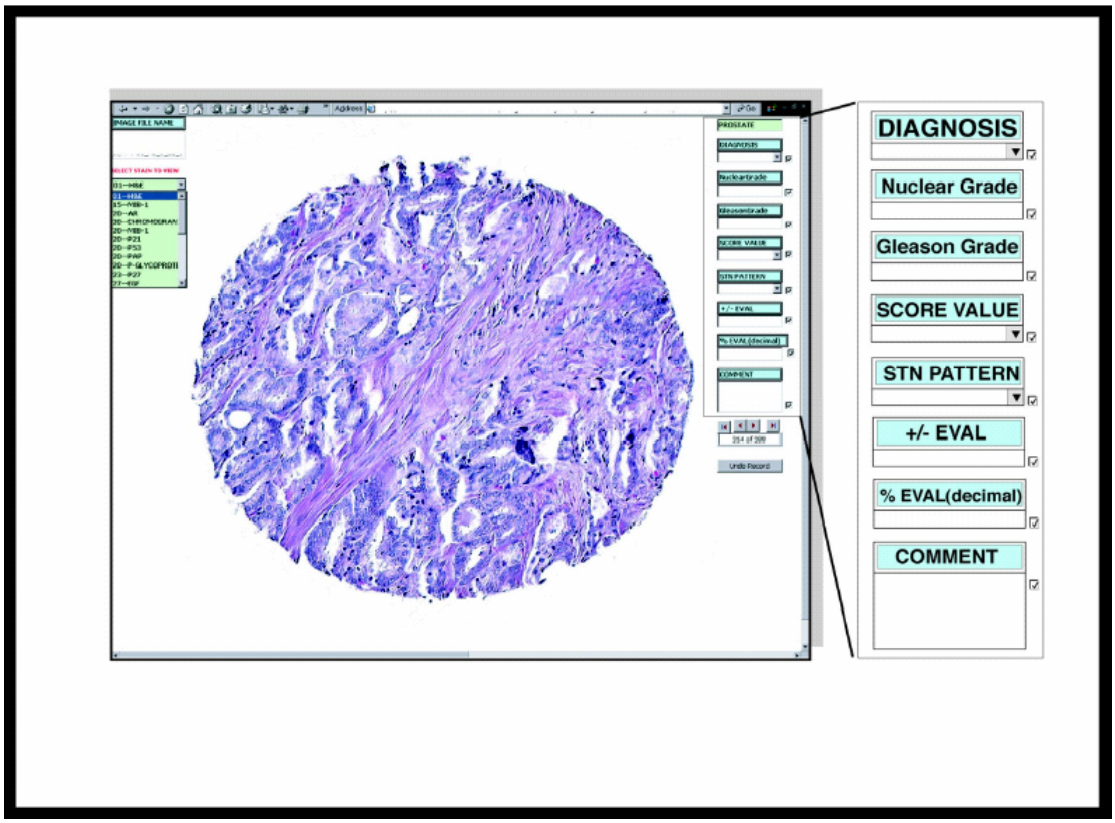


Figure 6 Digital analysis of TMA using special software. Data are then entered in a central Internet database.[27]

However, TMA technology has been criticised that it does not take into account intratumour heterogeneity.[24;28] It has been suggested that a single 0.6mm core (Figure 7) cannot be fully representative of the entire tumour it originates from.[20;21] This is particularly true for PCa, which represents an "extremely heterogeneous epithelial tumour".[16] If only one 0.6mm core per PCa tumour is used, then about 20% of alterations will be missed.[21;24;28] To deal with this problem, in this project, each PCa tumour was represented by 3-4 cores on each TMA slide. This particular number was chosen because several trials have shown that 3-4 is the optimal number of cores (per

tumour) essential to allow an accurate prediction of prognosis post-prostatectomy.[16;20] As Rubin *et al* have demonstrated less than 3 cores may not be adequately representative, but more than 4 cores "will not add significant information".[16;23] Moreover, Kononen *et al* said, "the results demonstrate that minute tissue samples in an array format can be sufficiently representative of their donor tumours to establish associations between molecular alterations and clinical endpoints" and "that, contrary to expectations, tissue heterogeneity did not negatively influence the predictive power of the TMA results".[29] Even if some of the cores are not representative, this effect will be "statistically diluted" by the large number of data that TMA allows to be analysed.[17] Here comes the important role of the pathologist. For a core to be representative it has to be sampled from the appropriate area (particularly relevant in heterogeneous tumours such as PCa).[16] Unfortunately, this represents a weakness of TMA technology, as there is natural human variation between which area will be selected by different pathologists (or even by the same pathologist on different occasions) to be cored for the TMA.[17] This means that the selection of the regions to be cored for the TMA is to an extent, subjective. However, best efforts are done to reduce subjectivity to a minimum, by standardising the sampling strategy. This is done by randomly sampling the largest or highest-grade tumour nodule.[16]

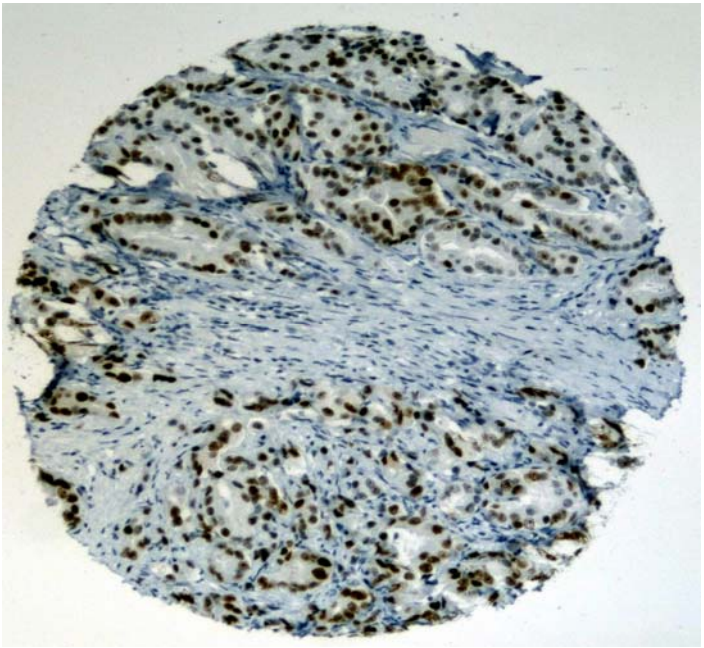


Figure 7 TMA core from a prostatic adenocarcinoma showing p53 staining.

Despite being a powerful research tool, the use of TMAs in the hospital setting seems to be limited.[20;21] This is because even a 95% concordance between core and entire tumour (although excellent for research analysis of prognostic biomarkers) may not be acceptable on an individual patient basis.[21]

The disadvantages of TMAs are in general the same as those of any wax-embedded material.[21] However, a particular problem of TMAs is that there is an inevitable loss of about 10-15% of cores during the process of slide preparation.[20;21] Also, antigen loss has been reported to occur very soon after tumour sectioning (most probably due to oxidation). This necessitates that IHC of the TMA slides is performed as soon as possible after tumour sectioning.[18]

Moreover, TMA analysis is a highly complex issue. Familiarity by the pathologist analysing the TMA is essential to prevent serious errors.[27] In addition, TMAs are prone to observer error and subjectivity.[30] Furthermore, TMAs can generate colossal amounts of data in a relatively short time. Analysing them can be a laborious, time-consuming, and error-prone process. To overcome all these problems, and make TMA analysis more efficient and objective, computerised TMA has been developed.[30] This makes use of software, different ones being developed and available on the market (AQUA, BLISS, Biogenex etc.), which allow "rapid, automated, continuous and quantitative analysis of TMAs",[31] as well as TMA digital database creation.[18;27]

MATERIALS AND METHODS

Patient Selection

The patients selected for this study form part of the Transatlantic Prostate Cancer Group Study (USA & UK), which represents collaboration between St. Bartholomew's Hospital, London, UK, Royal Marsden Hospital, London, UK and Memorial Sloan-Kettering Cancer Centre, NY, USA. They were all patients that were diagnosed with clinically localised PCa, either incidentally by TURP for BPH, or by TRUS-guided biopsy. All of these patients were managed by watchful waiting and follow-up data for a mean period of ten years was available.

All patients taking part in this study were under the age of 76 years at the time at which they were diagnosed with PCa. A baseline PSA measurement was obtained at the time of diagnosis. Exclusion criteria involved if from the time of diagnosis until 6 months later, the patient had a PSA measurement that exceeded 100 $ngml^{-1}$, had undergone radical prostatectomy or radiotherapy, had developed metastases, or had died from any cause.[11]

Ethical Approval

Ethical approval for this project was given by COREC as part of the Transatlantic Prostate Cancer Group Study (USA & UK). The lead investigator for this study is Professor Jack Cuzick.

Data

The diagnoses for the TMA cores, as well as the percent stain, and intensity for each of the cell cycle proteins in each TMA core are available as Microsoft Excel files in appendix 3.

However, due to the policy of the Wolfson Institute of Preventive Medicine, London, UK, the follow-up data could not be given to anyone until they are published as part of the larger Transatlantic Prostate Cancer Group Study. Instead, they performed the statistical analysis for this project. The statisticians that analysed the data were Dr. Gabrielle Fisher and Ms. Laurence Ambroisine of the Wolfson Institute of Preventive Medicine and Cancer Research UK.

Tissue used in the construction of TMAs and the evaluation of the TMA cores

As the patients involved in this study were conservatively managed, no radical prostatectomy specimens were available for tissue sampling and subsequent TMA core formation.

Instead, the tissue used to construct the TMA cores was obtained in one of two ways:

It was either obtained from TURP chips, TURP being performed to the patient as a treatment for symptomatic BPH, at which time, the presence of malignant prostatic tissue was an incidental finding.

Alternatively, malignant prostatic tissue was obtained from TRUS-guided biopsy material. TRUS was performed as part of the investigations for symptoms of prostatism and needle biopsies were taken to exclude (or confirm) the presence of neoplasia, in order to assist decision making for the future management of these patients.

Immunohistochemical evaluation (assessment of percentage staining and staining intensity) was performed by Dr. Daniel Berney and the author.

Tissue Microarray Construction

The tissue microarrays (TMAs) were sent to St. Bartholomew's Hospital, London, UK from Memorial Sloan-Kettering Cancer Centre, NY, USA. The TMA slides were constructed at the Royal Marsden Hospital, London, UK.

The first step of TMA construction involved the assembling of the haematoxylin and eosin-stained slides of the original (donor) tumour specimens to be used to construct the TMA slides. Then on each slide, the relevant (cancer) areas to be used for the TMA cores were marked by a pathologist. From each slide, 3-4 cores were taken to account for intratumour heterogeneity, a particular feature of PCa.[16;20;23]

Once the specific areas to be sampled from each donor slide had been carefully selected, the sampling process was started. This was done through the use of an arraying instrument, the tissue microarrayer (Figure 8), which allowed great accuracy. The tissue microarrayer consisted of an immobile platform onto which rested an empty (recipient) wax block, and a turret that could be freely moved in the X-Y plane. The turret position was controlled by two precision micrometer screws, each one possessing a digital dial. In this way any point on the X-Y plane could be mapped to a 1.0μm accuracy.[21]

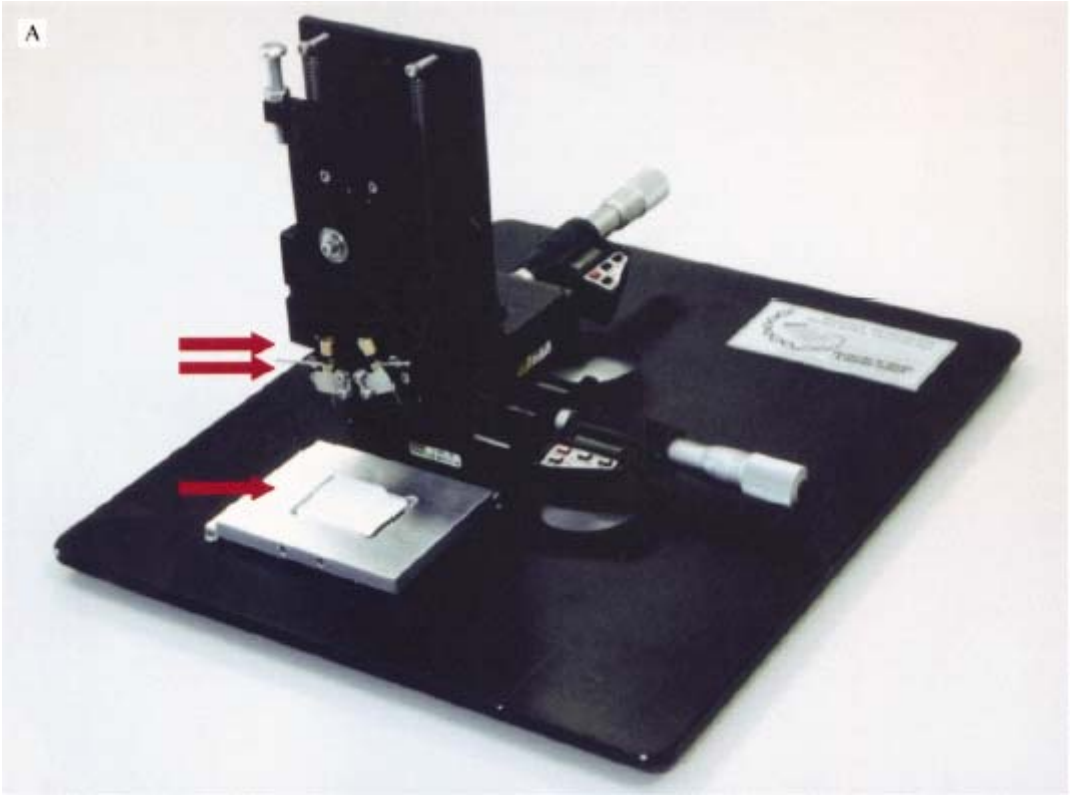


Figure 8 Commercial tissue microarrayer. Single arrow indicates the empty recipient wax block on the immobile stage. Double arrow indicates the mobile turret with two hollow stainless steel core punches.[21]

Apart from locating exactly the X-Y coordinates in the two-dimensional (2-D) plane, to exactly match a point in space (3-D), and hence exactly transfer the core from the donor to the recipient block, the depth at which the turret stopped when pushed downwards was adjusted. This was achieved by setting it at 0.5mm higher than the block holder.[32]

The turret had two hollow stainless-steel needles (punches). The right-hand punch was used to sample tissue from the donor block (internal diameter of 0.6mm), and the left-hand punch was used for creating a hole to the recipient wax block (internal diameter slightly less than 0.6mm) into which the piece of tissue obtained from the donor block was inserted to form a TMA core. The two-micrometer screws with their digital displays, allowed these two core punches to be moved on the X-Y plane with extreme (1μm) accuracy. These are illustrated in Figure 9.

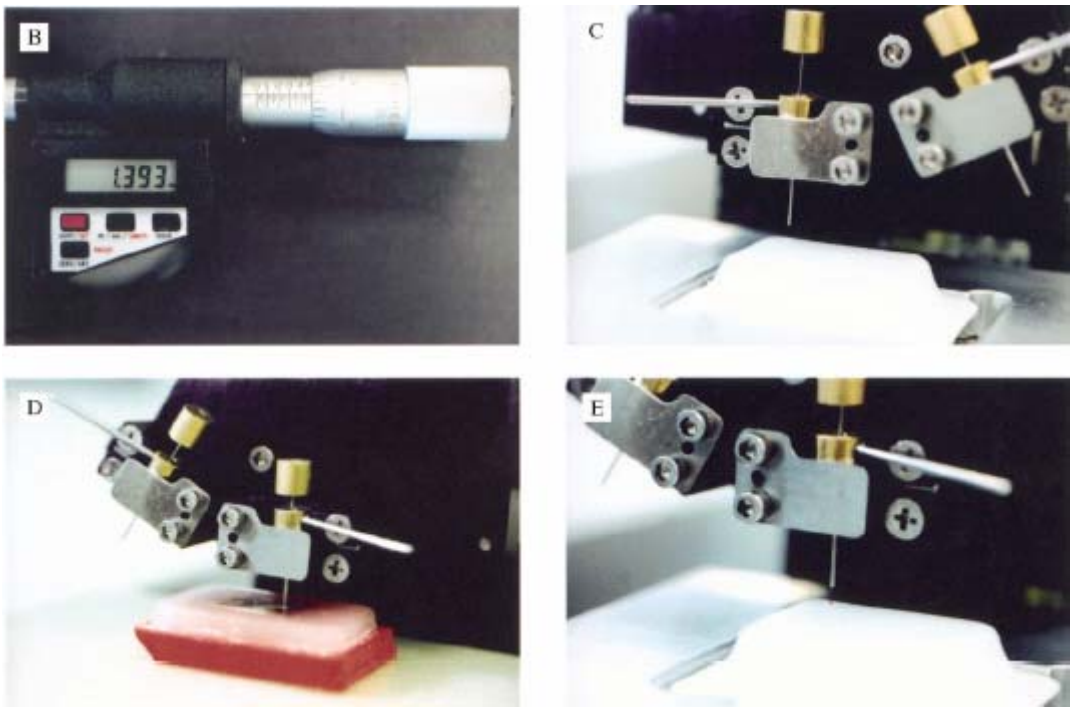


Figure 9 B. Micrometer screws possessing a digital dial. C. Left-hand punch used to make a hole into the empty recipient wax block. D. Right-hand punch brought in exactly the same position, on top of the area to be sampled in the donor block. E. The donor block, resting on a small bridge, is positioned above the platform onto which rests immobilised the recipient wax block. Hence, the core that has being obtained is inserted into the hole in the recipient TMA wax block.[21]

Once a hole had been created with the left-hand punch, the right-hand punch was brought exactly on top of the area to be sampled (marked by the pathologist). The two-micrometer dials were used to ensure absolute accuracy during the sampling process. Then, the donor block, resting on a small bridge, was positioned above the platform onto which rested immobilised the recipient wax block. By using the two-micrometer dials, it was ensured that the X- and Y-coordinates of the "punch position" where the sampled core was contained and the corresponding area in the recipient block where the core would be inserted, exactly matched. Then, once this was the case, the core was inserted into the corresponding hole in the recipient wax block. The needle contents (i.e. the core) were transferred from the needle into the recipient hole by pushing the stylet (a stainless steel wire closely fit into the needle) carefully downwards.[20;21;24;32]

This cyclical process of sampling cores from donor tissue blocks and re-implanting them in a pre-created hole on a recipient block was repeated continuously until all the

required cores were on the recipient block. At that point, the required TMA block was complete. Each TMA block contained approximately one hundred (0.6mm diameter) cores. (Figure 10) Every prostate tissue was represented by 3-4 cores on each slide to account for intratumour heterogeneity.[16;20;23]

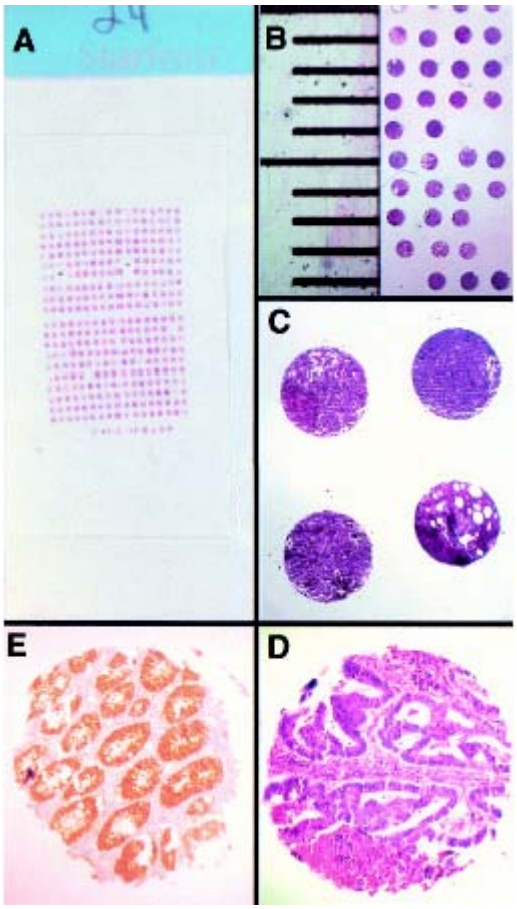


Figure 10 A. Typical TMA slide with hundreds of cores on it. At its top part, slide is labelled with pencil. B.C.D.E. Progressive magnification of the TMA cores seen under the microscope.[33]

Once each TMA block construction was completed, sections through each TMA block were cut with the microtome. The thickness at which sections were cut was 8μm. However, before the TMA block was actually cut, it was gently heated to 37°C for about 10-15min. This was done to make the wax a bit softer and hence easier to cut. Subsequently, the side of the block that contained the cores was compressed evenly by a flat surface (glass slide) in order to bring all the cores at the same horizontal level, which was the level of the surface of the block.[21;24;32]

An advantage of heating the TMA blocks was that it ensures strong adherence of the cores to the walls of the cylindrical holes in which they have been placed.[32]

The next step involved sectioning of the TMA blocks. However, before this was done, an adhesive-coated tape was applied on the surface of the block that the cores faced. After the tape was applied, the microtome knife was used to cut just below this surface. The thin tissue section that was produced by cutting the TMA block with the microtome, was adherent to the tape. Subsequently, the tape was lifted, and with it so were the core sections which were adherent to it. Once lifted, the tape with the core sections was transferred on to the surface of a microscope slide, onto which the core sections (still adherent to the tape) were rolled with a roller. The microscope slide, which was coated with PSA adhesive, was then exposed to UV light for a period of 45sec. After that, it was submersed in TPC solvent. At that point, the tape was rapidly removed by using forceps. Then, the slide was placed in xylene for 1min and then left to dry in air. Once air-dried, the slide was rapidly dipped in melted (60°C) paraffin once. After, it was allowed to cool and then placed in a slide box for storage, ready to undergo immunohistochemistry. The TMA slide can be safely stored in the slide box for a long period of time. It is at that stage that the TMA slides reached St. Bartholomew's Hospital, London, UK.[21;24;32]

Immunohistochemistry

The method that was followed for the immunohistochemistry (IHC) was in accordance to the IHC protocol used in St. Bartholomew's Hospital.

In terms of the actual IHC procedure, the TMA slides were treated identically to any formalin-fixed, paraffin-embedded histological section would have been.

Briefly, the procedure involved dewaxing the TMA slides (which had been pre-labelled with pencil), and blocking the endogenous peroxidase activity in 2% H_2O_2 in methanol. Immersion into alcohol, rehydration, and washing followed.

The next step involved pressure-cooking. Once pressure-cooking was completed, the slides were rehydrated, and then prevented from drying by applying on them tween buffer solution.

After the slides were wiped, the primary antibody was added (excepting negative controls), and the slides were incubated in a metallic box at room temperature for 40 min. For every antibody, positive and negative controls were used. After 40 min, the slides were washed with tween buffer solution, wiped, and the secondary antibody was added. Subsequently, they were incubated with it for 30 min. When the 30 min had passed, the slides were washed with tween buffer solution, wiped, and the tertiary antibody was added. They were then incubated with it for 20 min. When the 20 min had passed, the slides were washed with tween buffer solution, wiped, and then activated diaminobenzidine (DAB) was added on them. They were incubated with DAB for 10 min. After 10 min, the slides were put back on the slide holder and then briefly washed in cold tap water. Subsequently, the nuclei were stained in haematoxylin for 5 min, differentiated in acid alcohol for 2 sec, and blued in tap water for 5 min. Finally, the slides were dehydrated, cleared, and mounted.

A detailed description of the entire IHC procedure, including the preparation of the antigen unmasking solution, the blocking solution, the pressure-cooking technique, which specific antibodies and controls were used, and how DAB was formed and activated is available in appendix 2.

Statistical Analysis

A total of 193 prostate specimens were analysed. The total number of cores was 955, as 3-4 TMA cores were taken from each prostate specimen to account for intratumour heterogeneity.[16;20;23] Of all cores, the cancerous ones (458) were used for the statistical analysis.

All patients contributing to this project form part of the Transatlantic Prostate Cancer Group Study (USA & UK) and constitute patients with localised prostate cancers (diagnosed by TURP or needle biopsy), which were managed conservatively (watchful-waiting).

Follow-up data for all patients existed for a mean period of ten years. These included patient status at fixed times after the diagnosis (alive with no evidence of progression, alive but with disease recurrence, death from PCa, death from other causes). Follow-up times started six months after the diagnosis.

The clinical variables recorded were the PSA level and the Gleason score. The pathological variables recorded were the percentage of cores that stained for each particular cell cycle protein, the percentage of cells that stained in each core (only nuclear staining was considered), and the intensity of staining in the cells that stained (categorised into weak, medium, and strong intensity). For Ki67, no staining intensity was recorded as for Ki67, staining was considered to be either present or absent.

Initially a descriptive analysis of the data was performed. This was presented as histograms that demonstrated the distribution of the percentage staining for each of the cell cycle proteins.

Subsequently, bar charts were constructed to demonstrate the distribution of the staining intensity for each cell cycle protein. This was done for every cell cycle protein apart from Ki67 for the reasons explained above.

The relationship of percentage staining for each cell cycle protein with revised Gleason score was studied by using a series of one-way analyses of variance. The results were demonstrated on boxplots.

Finally, all factors were evaluated as dichotomous variables to study their prognostic significance on death from PCa. These were coded as follows: Rb (0, =0% *vs* 1, >0%), p21 (0, =0% *vs* 1, >0%), p53 (0, =0% *vs* 1, >0%), Ki67 (0, ≤5% *vs* 1, >5%) and MDM-2 (0, ≤85% *vs* 1, >85%). This was initially done by using univariate Cox proportional hazard models. After including the PSA levels and Gleason score in the multivariate Cox model, a multivariate analysis was also performed to determine any possible association between the expression (or loss of expression) of each cell cycle protein and death from PCa.

Identical analyses were performed to analyse the association between protein expression (or loss of expression) and death from any cause.

Finally, all significant findings were presented on Kaplan Meier survival curves.

The software used for the statistical analysis was Stata 8.2 for Windows.

RESULTS

A total of 955 cores, corresponding to 193 patients, were available: 458 cores were diagnosed as cancerous, 61 as mixed, 359 as normal, 10 as PIN, and 62 were "undetermined".

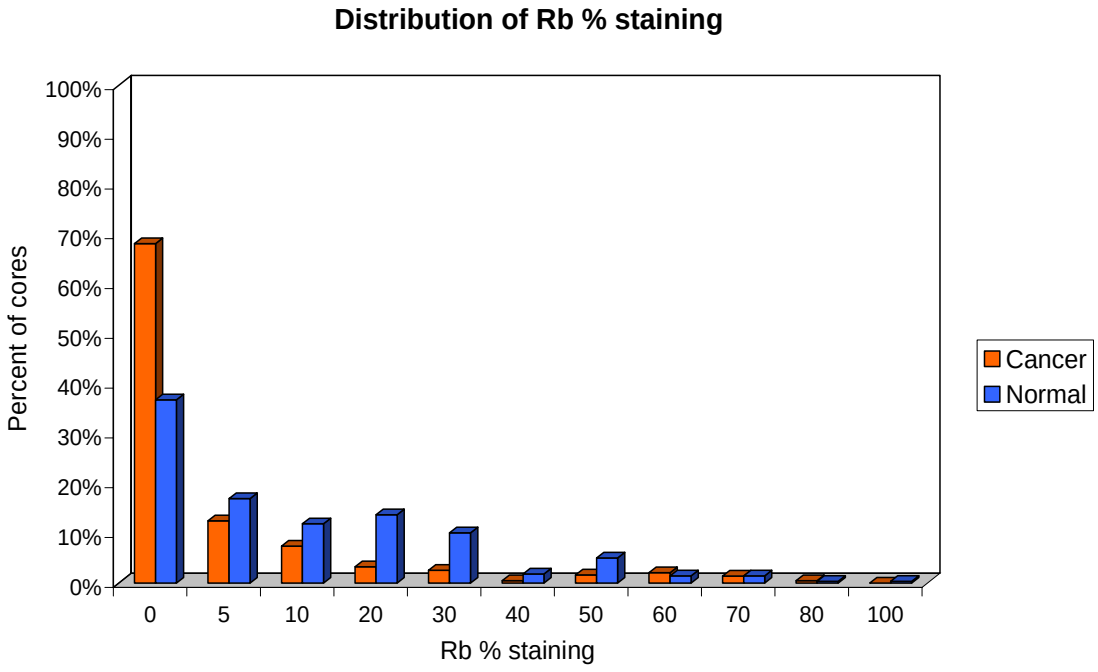
The analysis was carried out, by taking into account the 458 cores with cancer.

STATISTICAL ANALYSIS OF THE 458 CANCEROUS CORES

Descriptive analysis

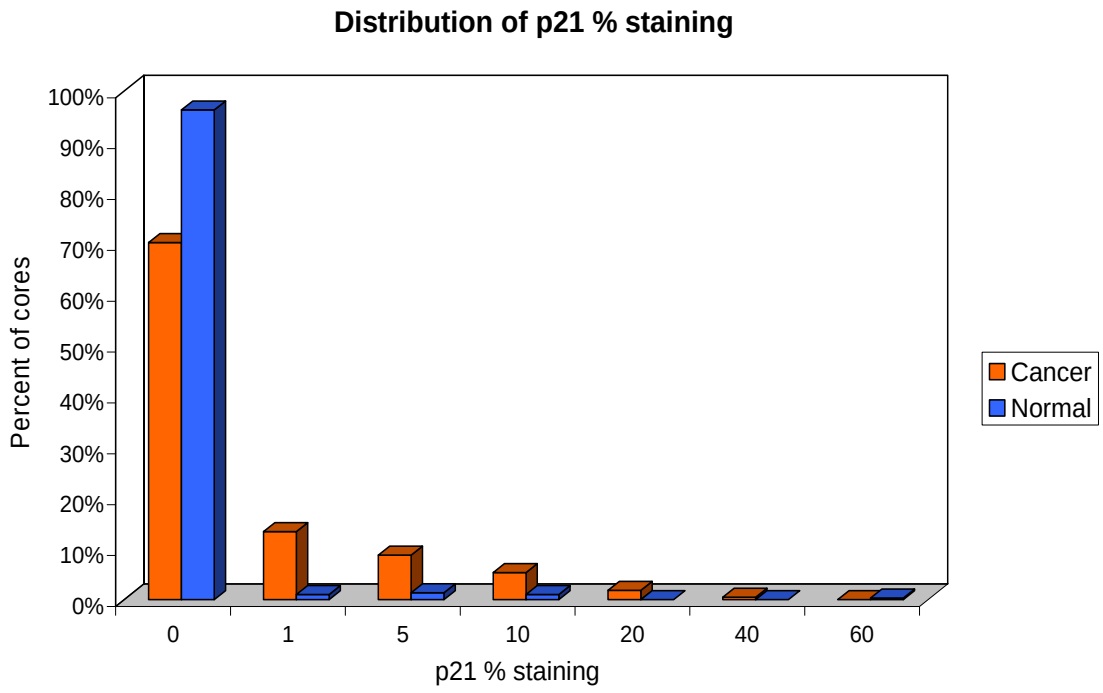
Four hundred and fifty eight cores were collected from 166 patients.

Information on Rb percentage of stained cells was not available for 27 cancer cores. The following histogram presents the distributions of percentage of stained cells for Rb in normal cores and cancer cores.



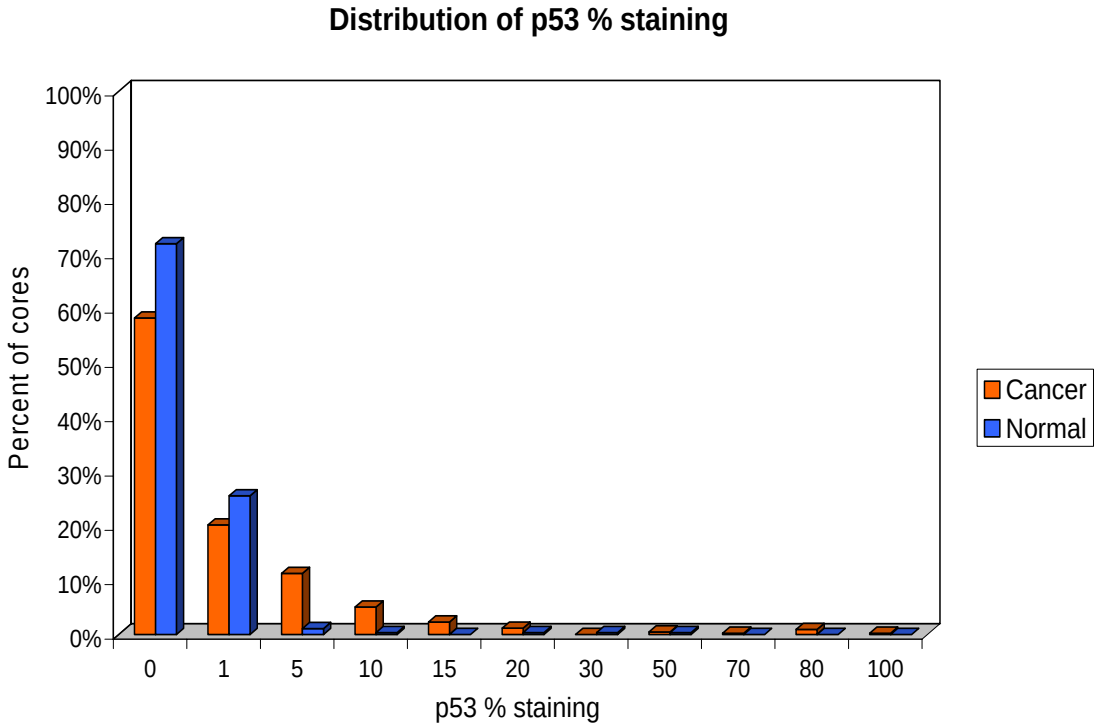
Histogram 1 The majority of cancer cores (70%) did not stain at all for Rb.

Information on p21 percentage of stained cells was not available for 25 cancer cores. The following histogram presents the distributions of percentage of stained cells for p21 in normal cores and cancer cores.



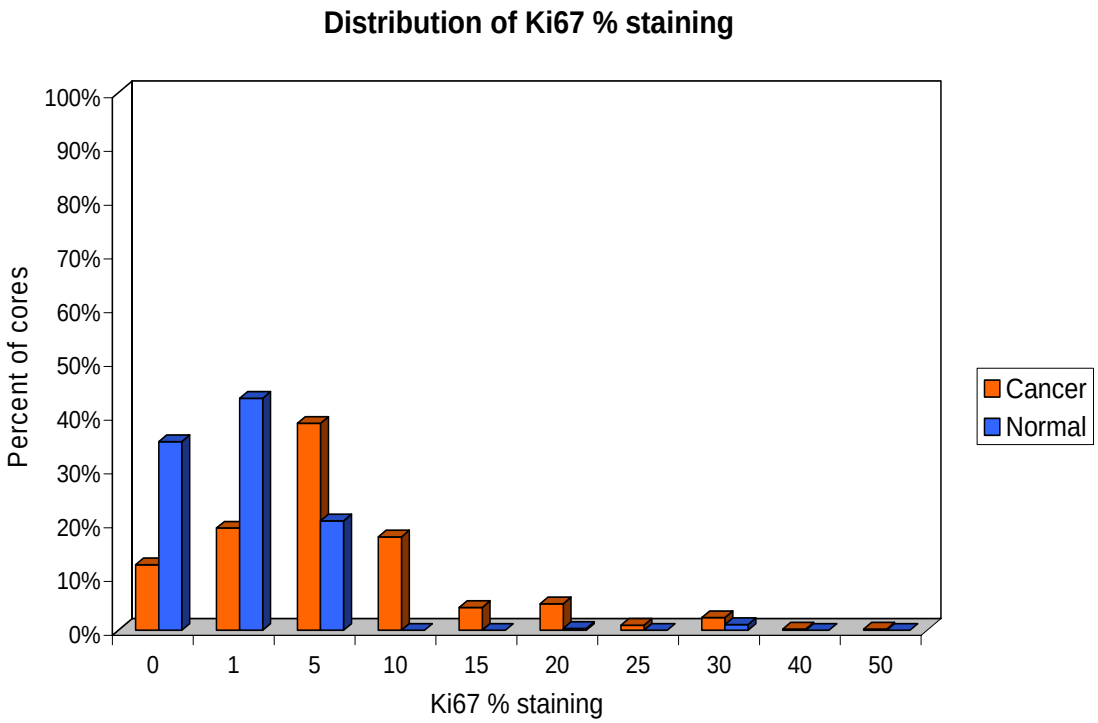
Histogram 2 The vast majority of normal cores (almost 100%) did not stain for p21.

Information on p53 percentage of stained cells was not available for 22 cancer cores. The following histogram presents the distributions of percentage of stained cells for p53 in normal cores and cancer cores.



Histogram 3 The majority of normal cores (74%) did not stain at all for p53**.**

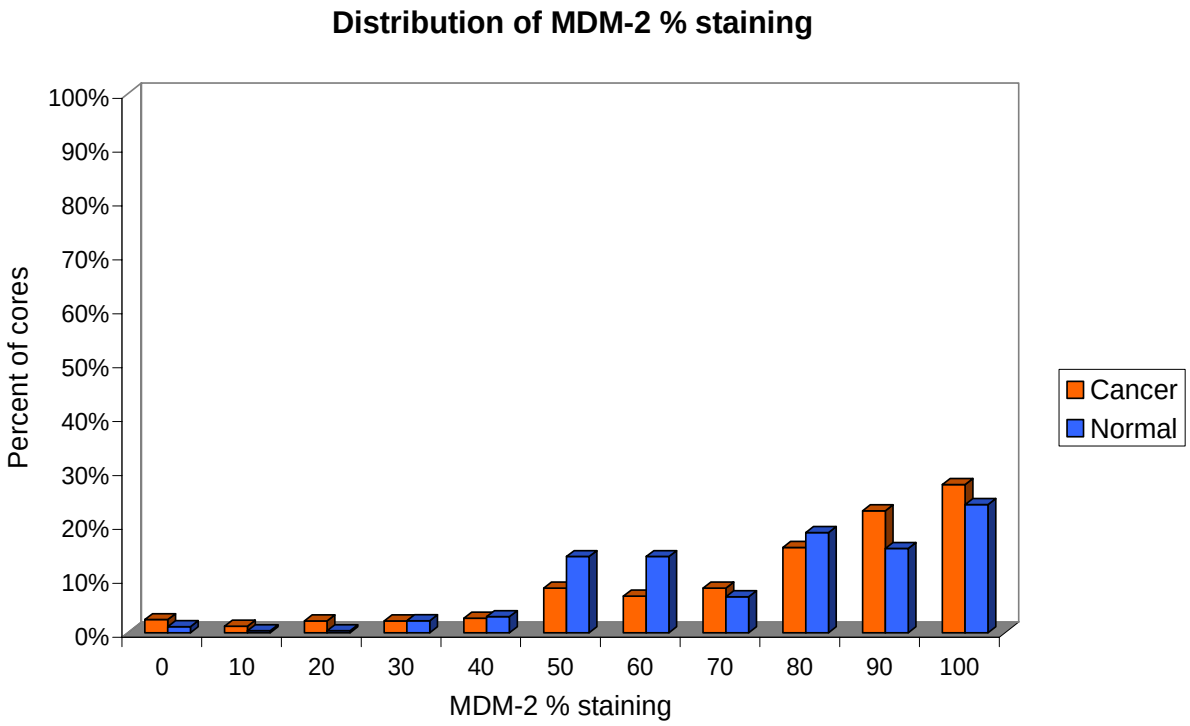
Information on Ki67 percentage of stained cells was not available for 32 cancer cores. The following histogram presents the distributions of percentage of stained cells for Ki67 in normal cores and cancer cores.



Histogram 4 The relative proportion of cancerous cores that stained for Ki67 was significantly greater than the corresponding proportion of normal cores (excepting 1% staining category)**.**

Information on MDM-2 percentage of stained cells was not available for 47 cancer cores.

The following histogram presents the distributions of percentage of stained cells for MDM-2 in normal cores and cancer cores.



Histogram 5 As the percent staining increased, so did the percentage of cores that stained for MDM-2.

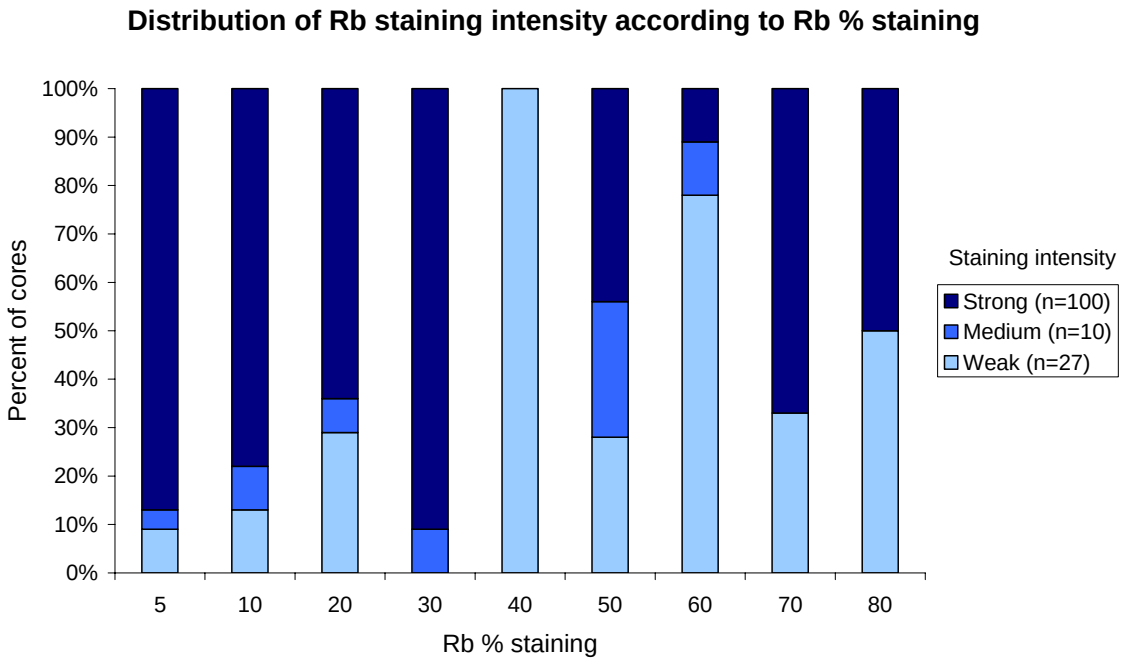
Analysis of staining intensity

Staining intensity was only available for positively staining cells, which corresponded to 137 cores for Rb, 129 cores for p21, 182 cores for p53 and 401 cores for MDM-2. Staining intensity was not evaluated for Ki67, as for Ki67, staining was considered to be either present or absent.

The following table and bar chart show the relationship between Rb staining intensity and Rb percentage of stained cells (Fisher's exact test, $p < 0.0001$).

Table 1

Rb % stain	Rb intensity		
	Weak n=27	**Medium** n=10	**Strong** n=100
5	9%	4%	87%
10	13%	9%	78%
20	29%	7%	64%
30	0%	9%	91%
40	100%	0%	0%
50	28%	28%	44%
60	78%	11%	11%
70	33%	0%	67%
80	50%	0%	50%

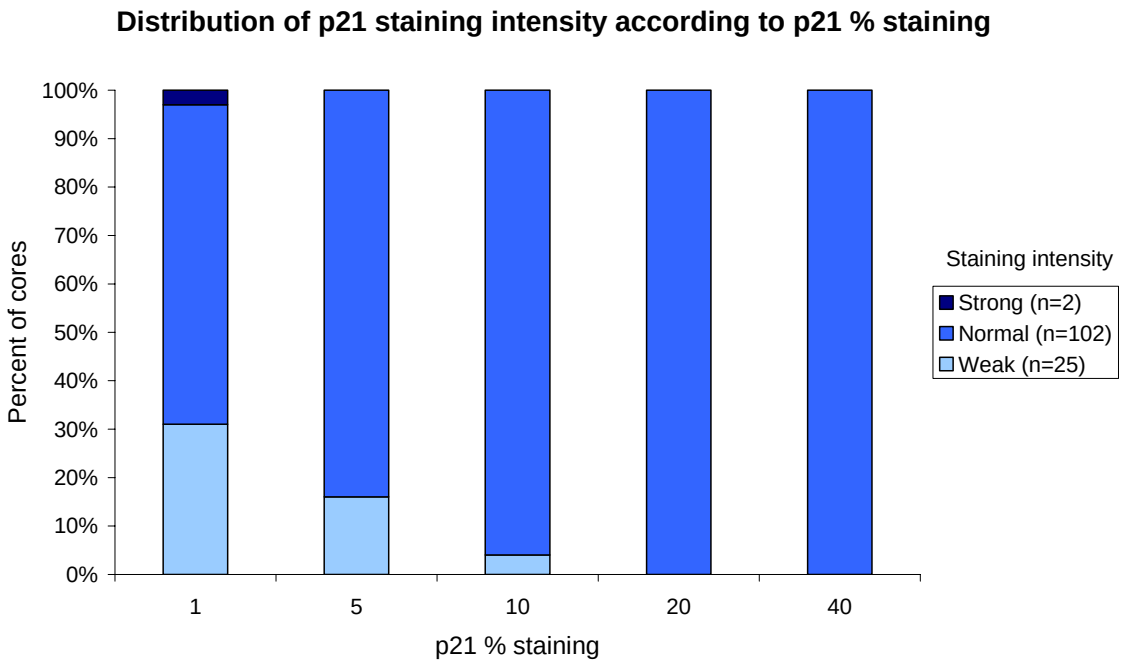


Bar Chart 1 The greatest proportions of strong staining were seen in cores where the percentage of staining cells was small (5-30%).

The following table and bar chart show the relationship between p21 staining intensity and p21 percentage of stained cells (Fisher's exact test, p = 0.046).

Table 2

p21 % stain	p21 intensity		
	Weak n=25	**Medium** n=102	**Strong** n=2
1	31%	66%	3%
5	16%	84%	0%
10	4%	96%	0%
20	0%	100%	0%
40	0%	100%	0%

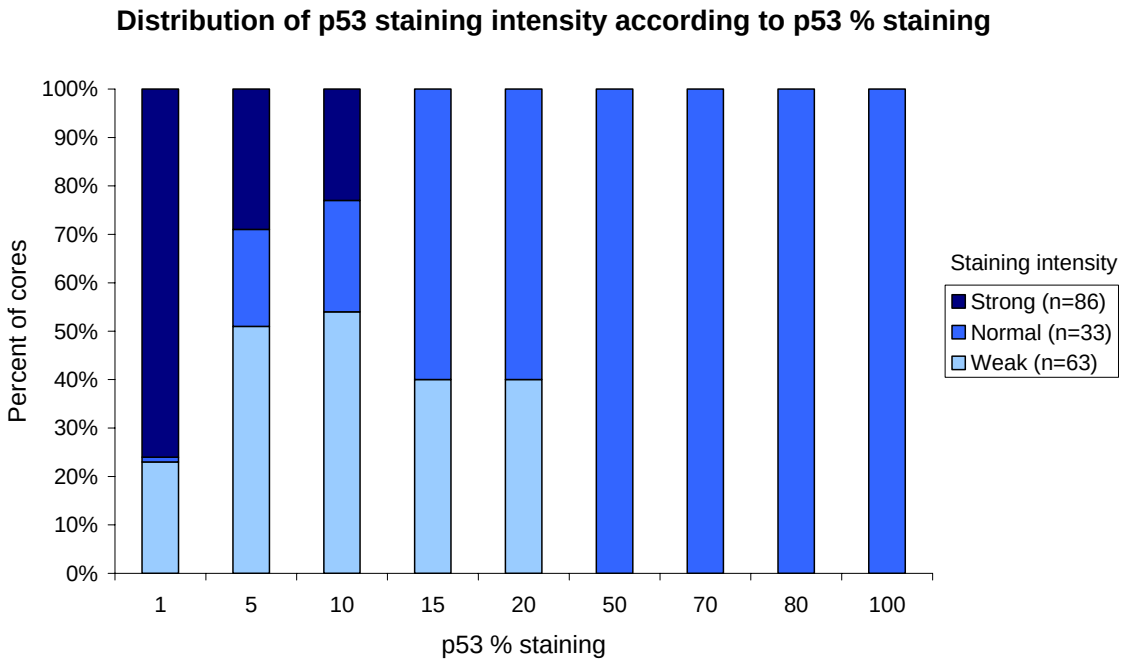


Bar Chart 2 At all percentage staining for p21, the staining was predominantly of normal intensity.

The following table and bar chart show the relationship between p53 staining intensity and p53 percentage of stained cells (Fisher's exact test, $p < 0.0001$).

Table 3

p53 % stain	p53 intensity		
	Weak n=63	**Medium** n=33	**Strong** n=86
1	23%	1%	76%
5	51%	20%	29%
10	54%	23%	23%
15	40%	60%	0%
20	40%	60%	0%
50	0%	100%	0%
70	0%	100%	0%
80	0%	100%	0%
100	0%	100%	0%

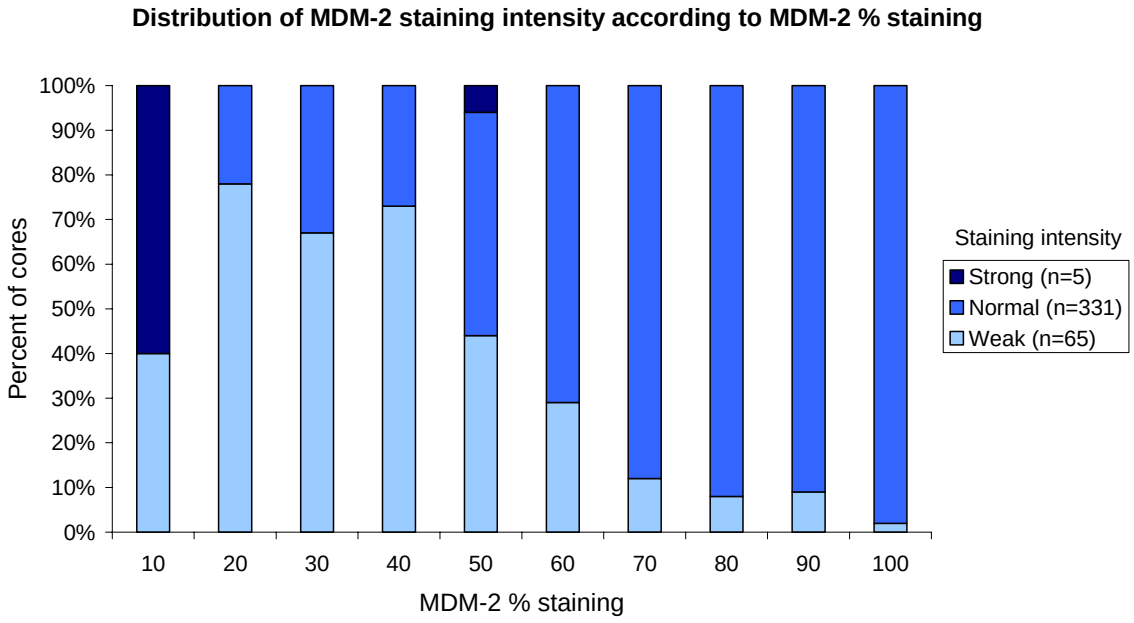


Bar Chart 3 The staining was predominantly of normal intensity.

The following table and bar chart show the relationship between MDM-2 staining intensity and MDM-2 percentage of stained cells (Fisher's exact test, $p < 0.0001$).

Table 4

	MDM-2 intensity		
MDM-2 % stain	**Weak** n=65	**Medium** n=331	**Strong** n=5
10	40%	0%	60%
20	78%	22%	0%
30	67%	33%	0%
40	73%	27%	0%
50	44%	50%	6%
60	29%	71%	0%
70	12%	88%	0%
80	8%	92%	0%
90	9%	91%	0%
100	2%	98%	0%



Bar Chart 4 Weak intensity predominated up to the 50% staining category, but from then on, the relative proportion of normal intensity increased with the percent of cores.

Percentage staining analysis and its relationship to revised Gleason score

To study the relationship between expression of each protein and Gleason score, the development of metastases, death from prostate cancer and death from any cause, the average percentage of stained cells by patient was calculated for each protein.

The following table presents the summary statistics for the different protein percentages of stained cells:

Table 5

	Rb % stain	p21 % stain	p53 % stain	Ki67 % stain	MDM-2 % stain
Mean	6.4	1.9	3.5	6.6	77.8
Standard deviation	13.5	5.1	11.5	6.8	22.0
Minimum	0	0	0	0	0
25% percentile	0	0	0	2.5	70
Median	0	0	0.3	5	85
75% percentile	5	1.7	2	7.5	92.5
Maximum	70	40	100	50	100

Relationship of percentage of staining with revised Gleason score

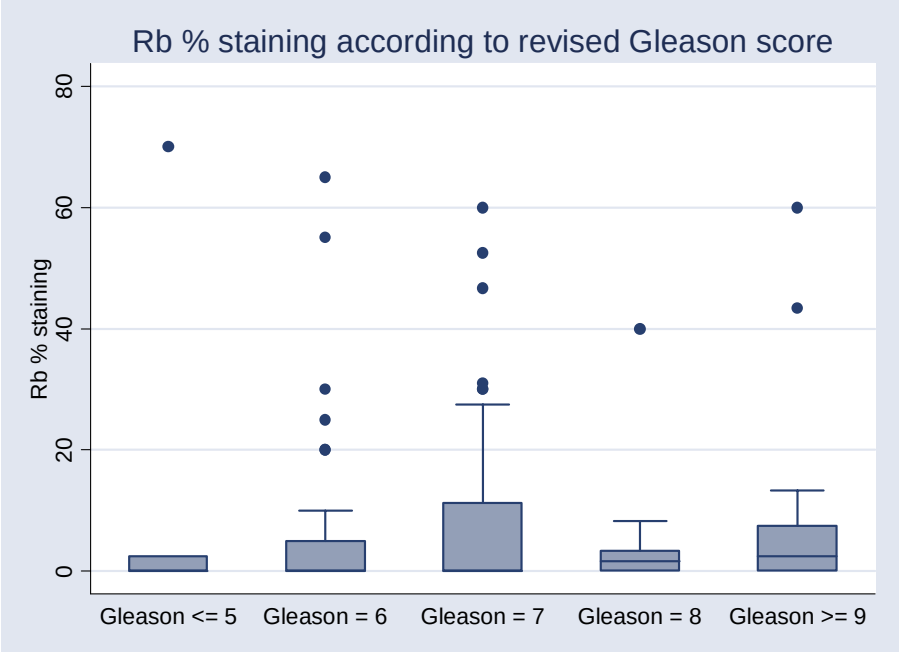
The relationship between each protein percentage of stained cells and Gleason score (categorised in 3 and in 5 levels) was studied using a series of one-way analyses of variance.

To summarise, there is a significant correlation between Gleason score, in 3 or 5 levels, and the percentage of stained cells for p21, p53, Ki67 and MDM-2.

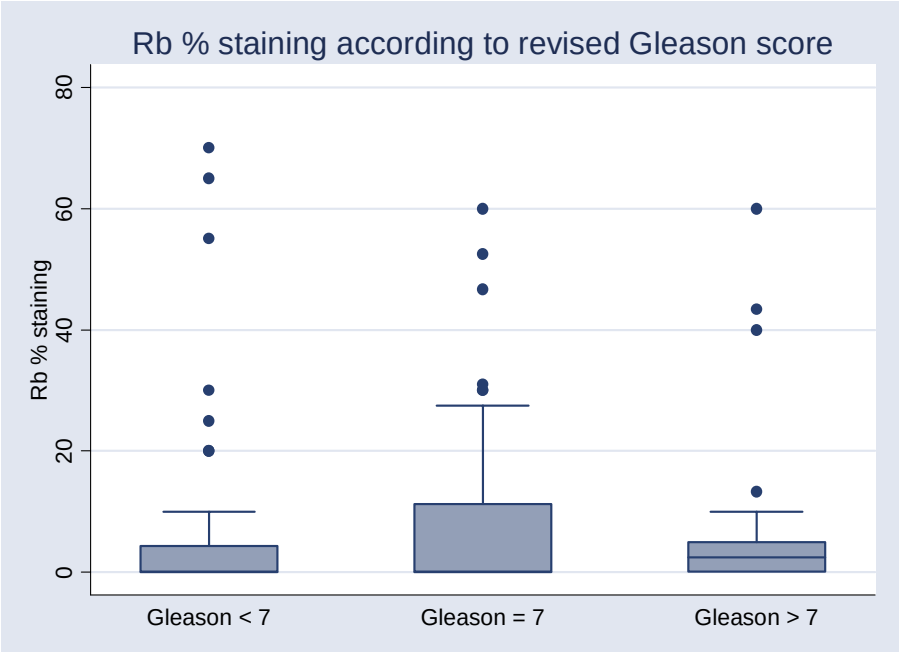
The results are detailed and illustrated in the following tables and box-plots.

Table 6

		Rb % stain	p-value
Gleason score (5 levels)	Gleason score <= 5 (n=7)	10.4±26.3	0.4334
	Gleason score = 6 (n=63)	4.9±11.7	
	Gleason score = 7 (n=47)	8.7±14.7	
	Gleason score = 8 (n=21)	3.7±8.6	
	Gleason score >= 9 (n=25)	7.6±14.3	
Gleason score (3 levels)	Gleason score < 7 (n=70)	5.4±13.6	0.3927
	Gleason score = 7 (n=47)	8.7±14.7	
	Gleason score > 7 (n=46)	5.8±12.0	



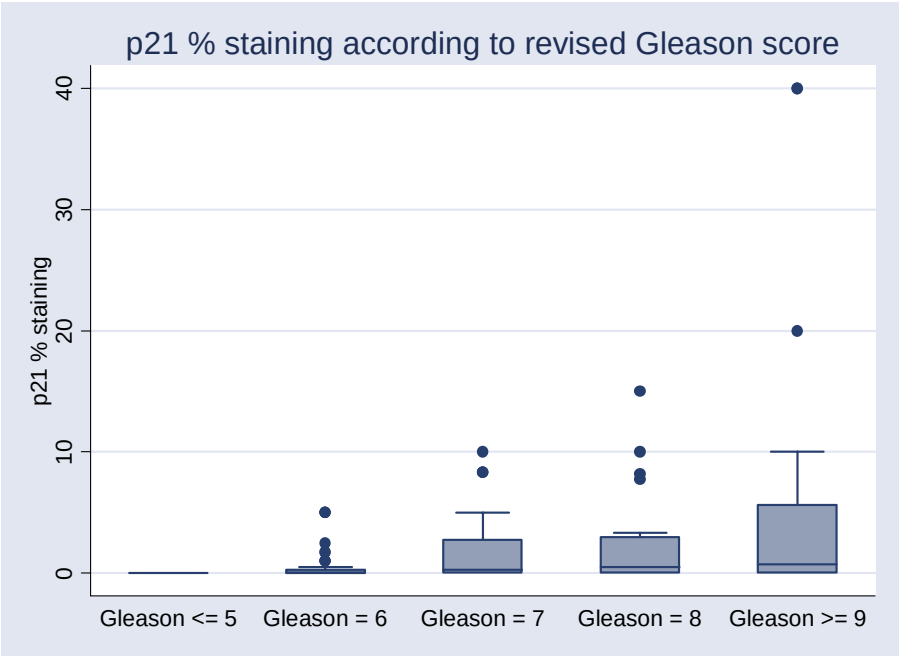
Boxplot 1 For the lowest (<5) and highest (>8) Gleason scores, the Rb % staining is low and similar while in the middle (Gleason score 7), there is higher Rb % staining.



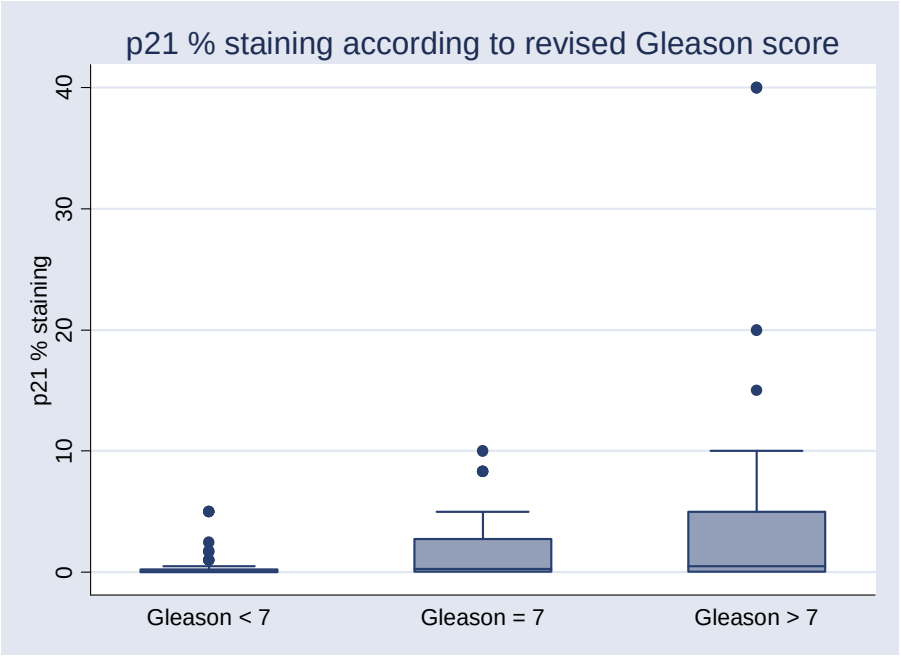
Boxplot 2 For the lowest (<7) and highest (>7) Gleason scores, the Rb % staining is low and similar while in the middle (Gleason score 7), there is higher Rb % staining.

Table 7

		p21 % stain	p-value
Gleason score (5 levels)	Gleason score <= 5 (n=7)	0±0	0.0001
	Gleason score = 6 (n=61)	0.4±1.1	
	Gleason score = 7 (n=48)	1.8±2.7	
	Gleason score = 8 (n=21)	2.7±4.1	
	Gleason score >= 9 (n=25)	6.1±11.5	
Gleason score (3 levels)	Gleason score < 7 (n=68)	0.4±1.1	0.0001
	Gleason score = 7 (n=48)	1.8±2.7	
	Gleason score > 7 (n=46)	4.5±8.9	



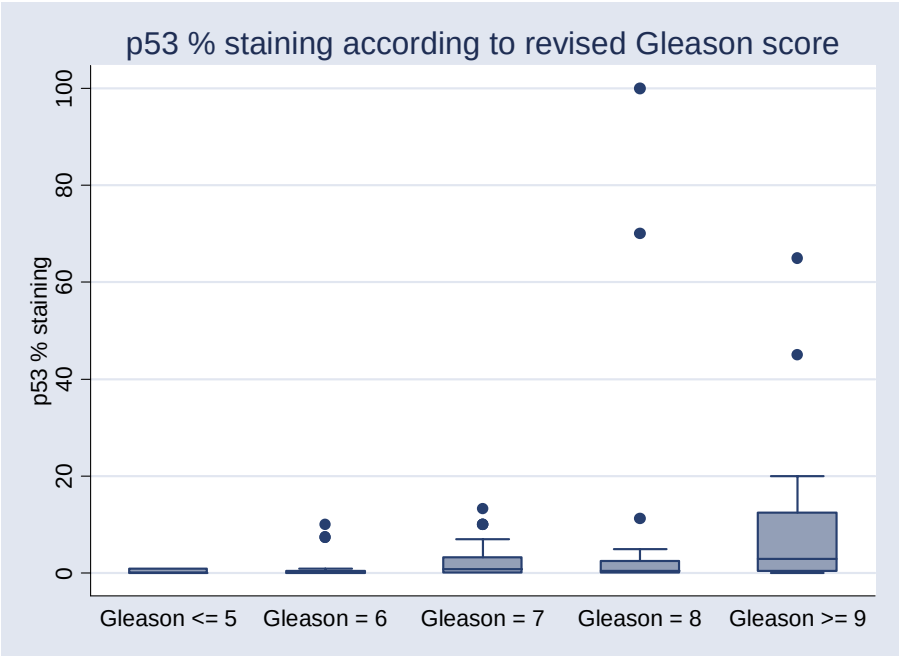
Boxplot 3 As the p21 percent stain increased, so did the revised Gleason score.



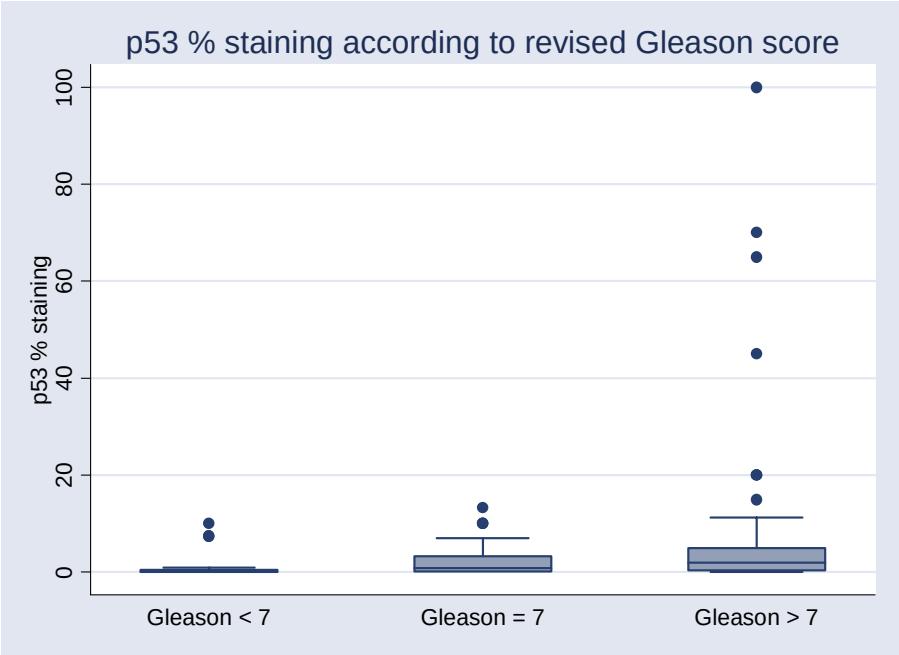
Boxplot 4 As the p21 percent stain increased, so did the revised Gleason score.

Table 8

		p53 % stain	p-value
Gleason score (5 levels)	Gleason score <= 5 (n=7)	0.3±0.5	0.0009
	Gleason score = 6 (n=63)	0.6±1.8	
	Gleason score = 7 (n=48)	2.2±3.2	
	Gleason score = 8 (n=21)	9.6±25.7	
	Gleason score >= 9 (n=25)	9.6±15.8	
Gleason score (3 levels)	Gleason score < 7 (n=70)	0.6±1.7	0.0001
	Gleason score = 7 (n=48)	2.2±3.2	
	Gleason score > 7 (n=46)	9.6±20.7	



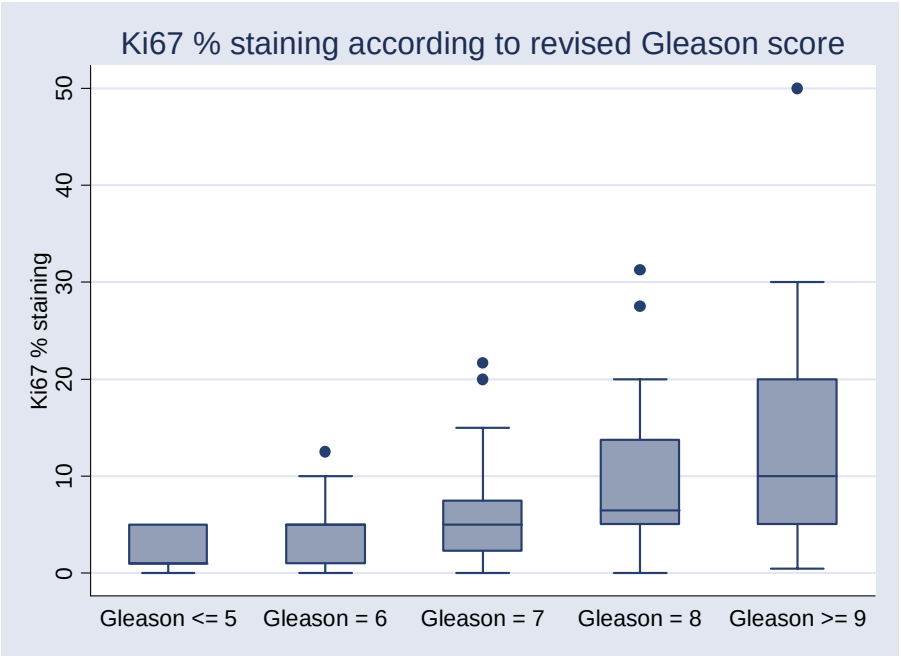
Boxplot 5 As the p53 percent stain increased, so did the revised Gleason score**.**



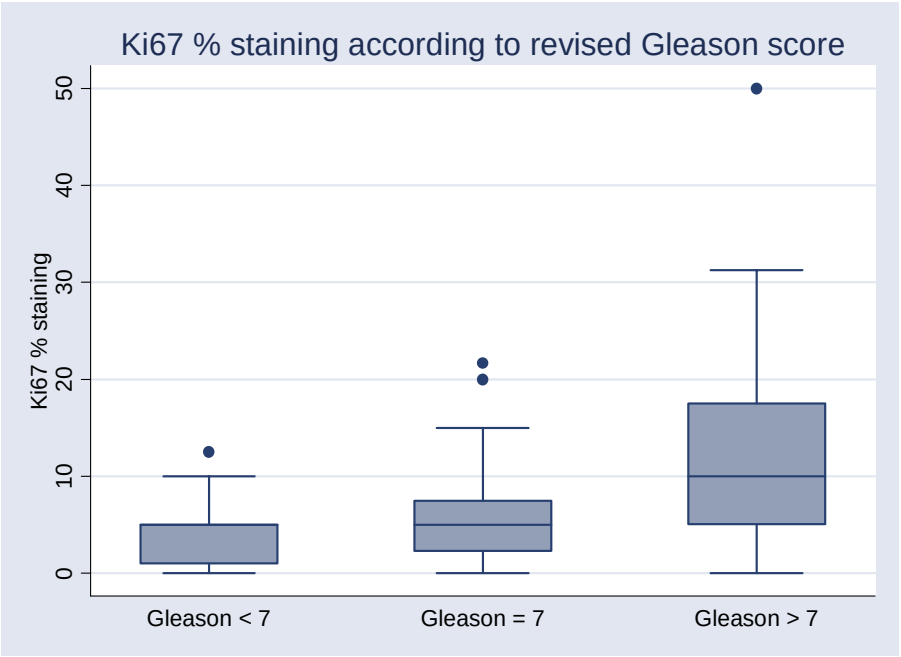
Boxplot 6 As the p53 percent stain increased, so did the revised Gleason score**.**

Table 9

		Ki67 % stain	p-value
Gleason score (5 levels)	Gleason score <= 5 (n=7)	2.6±2.3	<0.0001
	Gleason score = 6 (n=61)	4.0±2.8	
	Gleason score = 7 (n=48)	5.7±4.8	
	Gleason score = 8 (n=21)	10.3±8.3	
	Gleason score >= 9 (n=25)	13.1±10.7	
Gleason score (3 levels)	Gleason score < 7 (n=68)	3.9±2.8	<0.0001
	Gleason score = 7 (n=48)	5.7±4.8	
	Gleason score > 7 (n=46)	11.8±9.6	



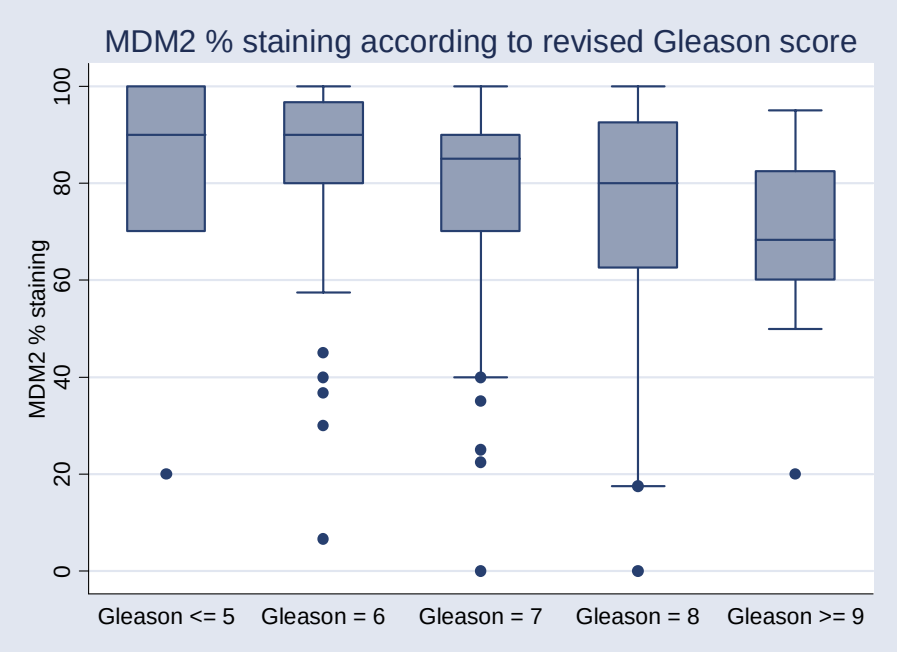
Boxplot 7 As the Ki67 percent stain increased, so did the revised Gleason score**.**



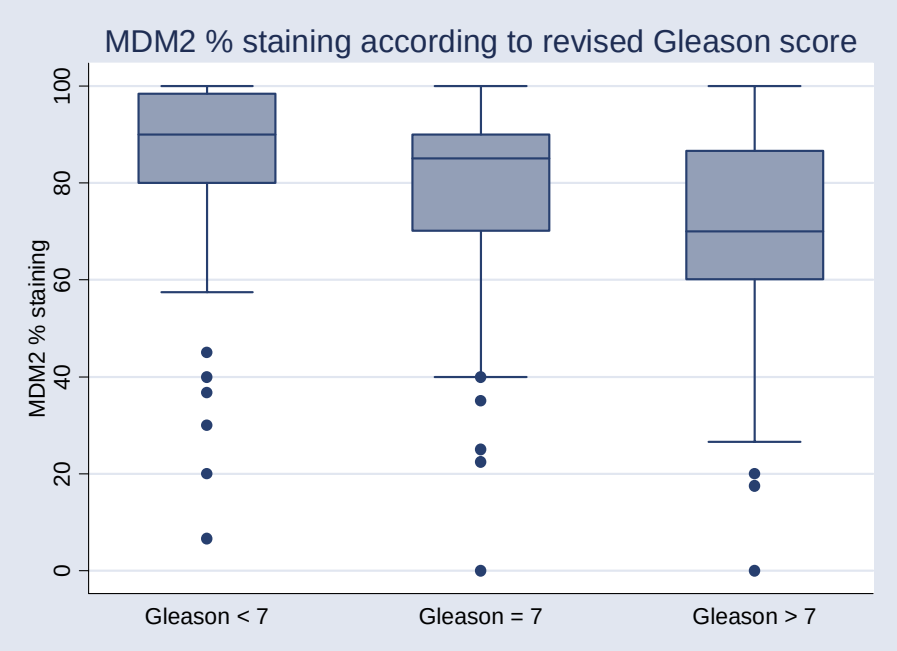
Boxplot 8 As the Ki67 percent stain increased, so did the revised Gleason score**.**

Table 10

		MDM-2 % stain	p-value
Gleason score (5 levels)	Gleason score <= 5 (n=7)	81.4±29.1	0.0392
	Gleason score = 6 (n=58)	83.4±19.1	
	Gleason score = 7 (n=45)	77.0±22.3	
	Gleason score = 8 (n=21)	71.9±28.4	
	Gleason score >= 9 (n=25)	68.4±16.6	
Gleason score (3 levels)	Gleason score < 7 (n=65)	83.1±20.1	0.0075
	Gleason score = 7 (n=45)	77.0±22.3	
	Gleason score > 7 (n=46)	70.0±22.7	



Boxplot 9 As the MDM-2 percent stain decreased, the revised Gleason score increased**.**



Boxplot 10 As the MDM-2 percent stain decreased, the revised Gleason score increased**.**

The following table summarises the correlations between each cell cycle protein and the revised Gleason score.

Table 11

Cell Cycle Protein Expression	Correlation with Gleason Score (3 levels)	Statistical significance of correlation (p-value)
Retinoblastoma (Rb)	No correlation	Insignificant (0.3927)
p21	Positive	Significant (0.0001)
p53	Positive	Significant (0.0001)
Ki67	Positive	Significant (<0.0001)
MDM-2	Negative	Significant (0.0075)

Prognostic significance on death from prostate cancer

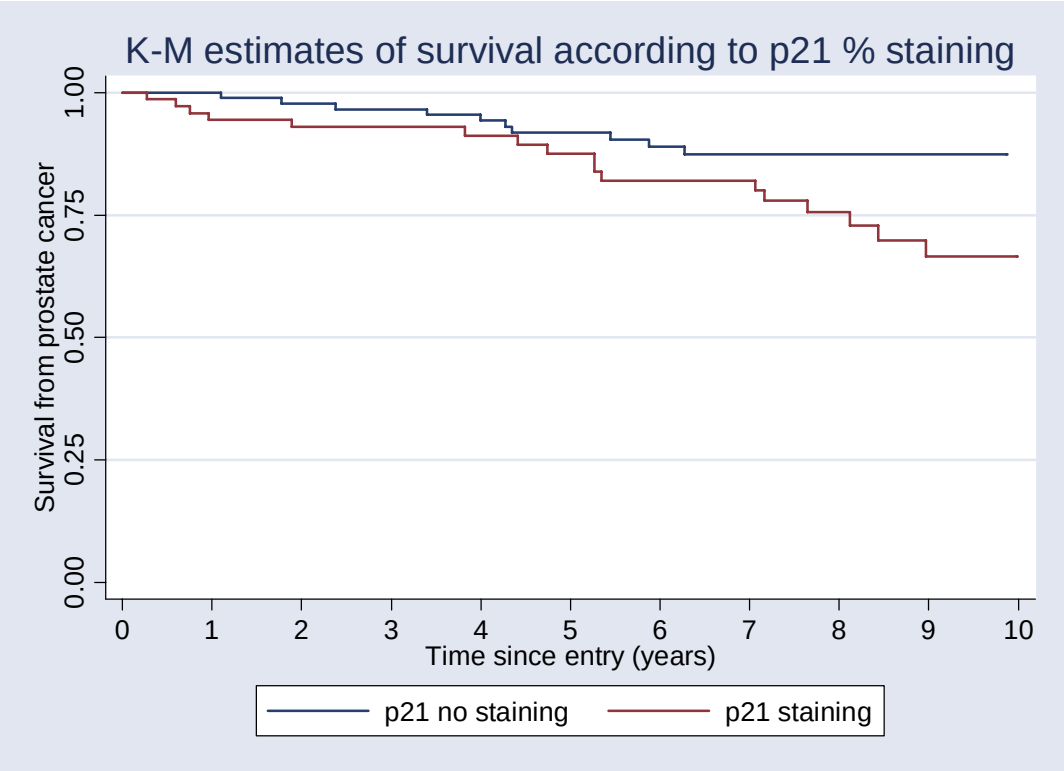
Univariate analyses

All factors were evaluated as dichotomous variables to study their prognostic significance on death from prostate cancer, using univariate Cox proportional hazard models. These were coded as follows: Rb (0, =0% *vs* 1, >0%), p21 (0, =0% *vs* 1, >0%), p53 (0, =0% *vs* 1, >0%), Ki67 (0, ≤5% *vs* 1, >5%) and MDM-2 (0, ≤85% *vs* 1, >85%).

The univariate analyses showed that:

- Rb expression had no significant association with the risk of death from prostate cancer (P=0.0856).
- p21 expression had a significant association with the risk of death from prostate cancer (Hazard Ratio, HR=2.53, 95% CI = [1.18-5.46], P=0.0142).
- p53 expression had no significant association with the risk of death from prostate cancer (P=0.1308).
- Ki67 expression had no significant association with the risk of death from prostate cancer (P=0.0512).
- MDM-2 expression had no significant association with the risk of death from prostate cancer (P=0.3148).

The following graph presents the Kaplan Meier curve corresponding to the significant association between p21 expression and risk of death from prostate cancer.



Kaplan Meier curve 1 p21 expression is associated with a significantly higher risk of death from prostate cancer.

Multivariate Analysis

p21 expression had no longer a significant association with risk of death from prostate cancer after including the PSA level and Gleason score in the multivariate Cox model.

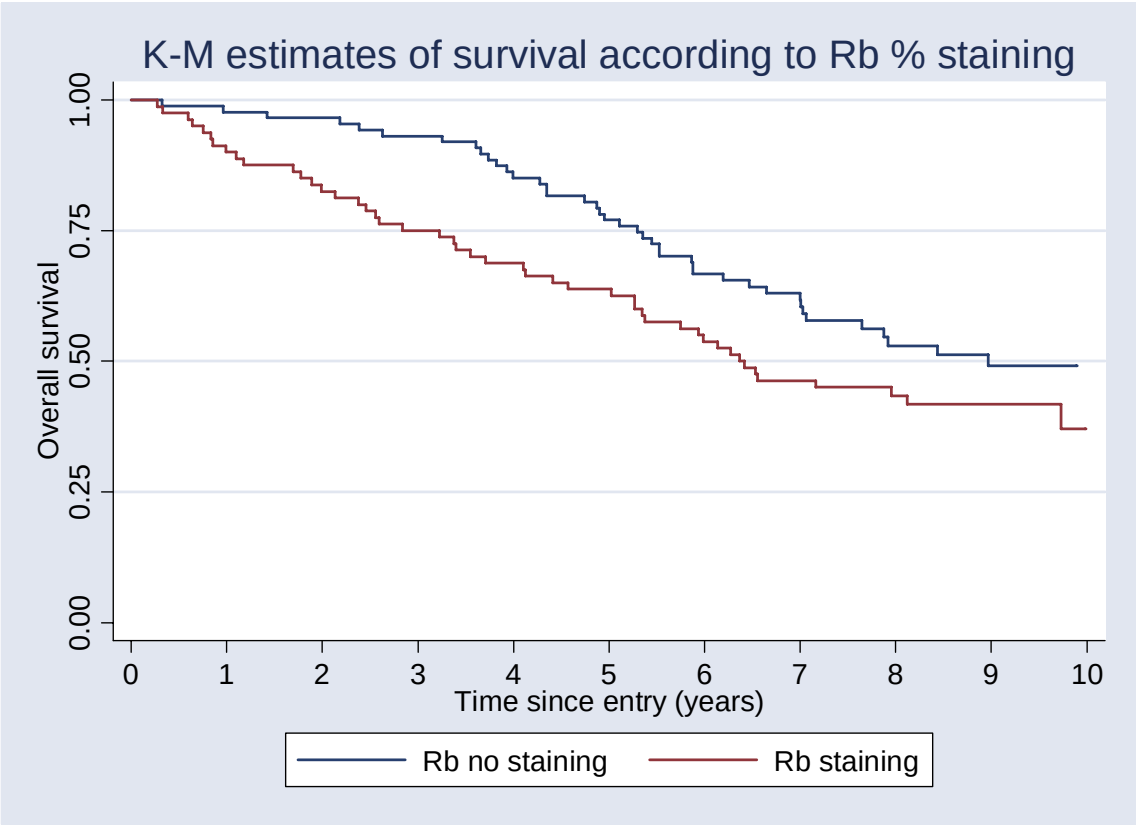
Prognostic significance on death from any cause

Univariate analyses

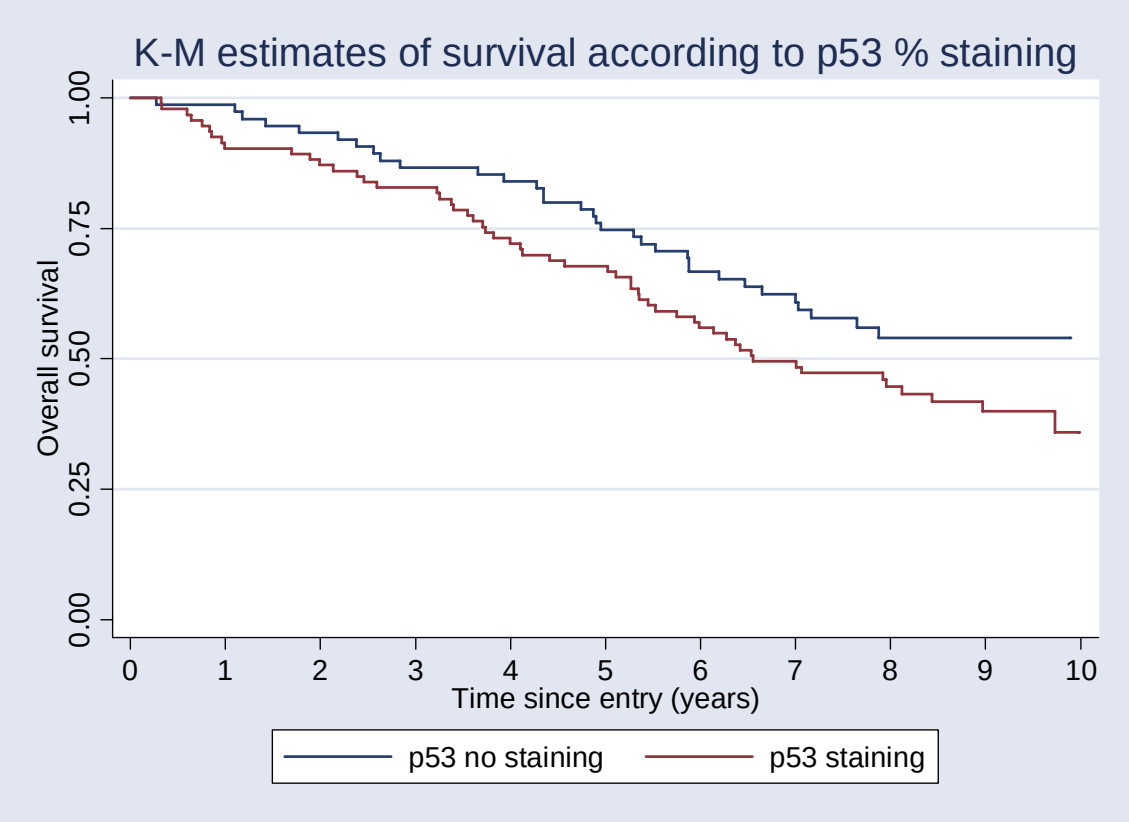
The univariate analyses showed that:

- Rb expression had a significant association with the risk of death from any cause (Hazard Ratio, HR=1.50, 95% CI = [1.00-2.27], P=0.0493).
- p21 expression had no significant association with overall survival (P=0.1066).
- p53 expression had a significant association with the risk of death from any cause (Hazard Ratio, HR=1.58, 95% CI = [1.03-2.42], P=0.0318).
- Ki67 expression had a significant association with the risk of death from any cause (Hazard Ratio, HR=2.27, 95% CI = [1.51-3.43], P=0.0001).
- MDM-2 expression had no significant association with overall survival (P=0.2058).

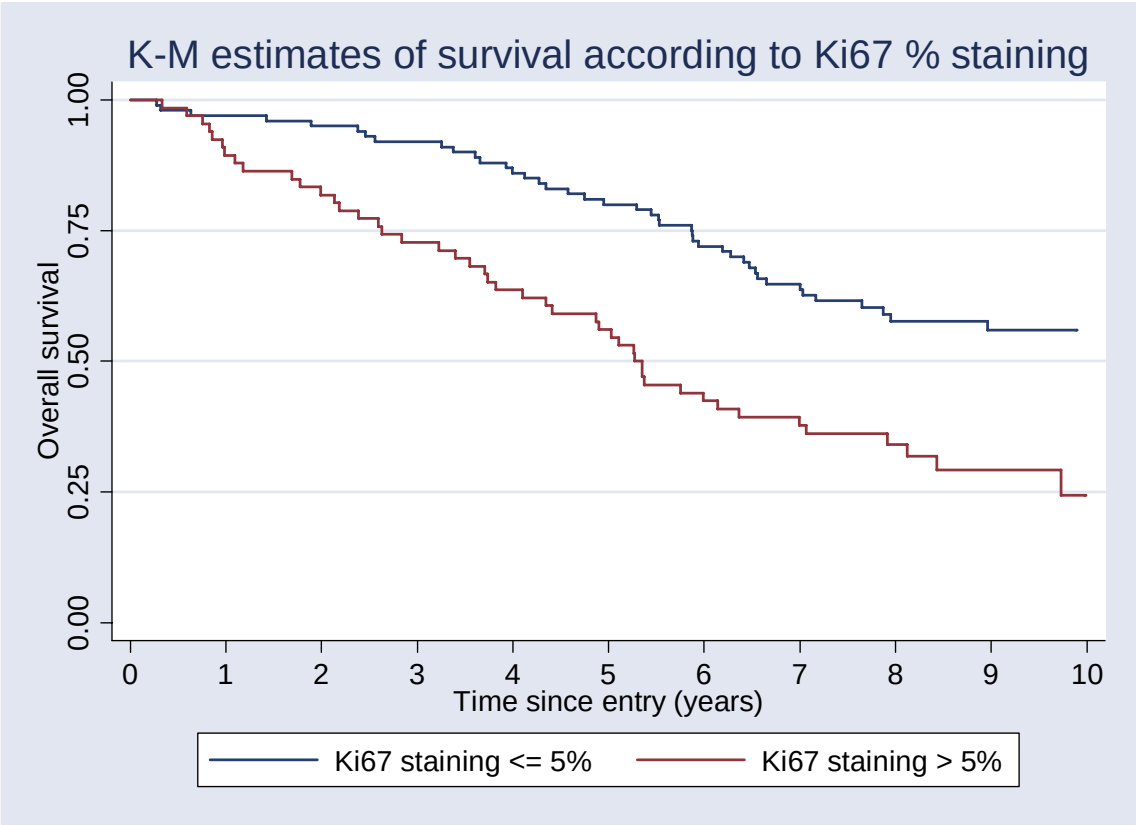
The three following graphs present the Kaplan Meier curves corresponding to the significant associations for Rb, p53 and Ki67 expression and risk of death from any cause.



Kaplan Meier curve 2 Rb expression had a significantly higher risk of death from any cause.



Kaplan Meier curve 3 p53 expression had a significantly higher risk of death from any cause.



Kaplan Meier curve 4 Ki67 expression had a significantly higher risk of death from any cause.

Multivariate analyses

- After including the PSA and the Gleason score in the Cox model, Rb expression was no longer significantly associated with reduced overall survival.

- After including the PSA and the Gleason score in the Cox model, p53 expression was no longer significantly associated with reduced overall survival.

- After including the PSA (in 4 levels) and the Gleason score (in 3 levels) in the Cox model, Ki67 expression was still significantly associated with reduced overall survival (Hazard Ratio, HR=1.95, 95% CI = [1.23-3.10], P=0.0002).

DISCUSSION

Retinoblastoma (Rb) protein

As is evident from histogram 1, the majority of cancer cores (70%) did not stain at all for Rb. This is in contradiction to the normal cores, of which only 38% did not stain at all for Rb. As the percentage of the cells that stained for Rb increased, the proportion of both cancerous and normal cores decreased. Virtually no cancer or normal core had 100% of its cells staining for Rb. These findings are in contradiction with other studies that have suggested that loss of Rb protein expression is not a common finding in PCa.[34]

In relation to the staining intensity (bar chart 1), the greatest proportions of strong staining were seen in cores where the percentage of staining cells was small (5-30%) (Figures 11, 12). All cells in the cores where there was 40% Rb staining, stained weakly. From then on, the proportion of cells staining weakly for Rb increased with increasing Rb % staining.

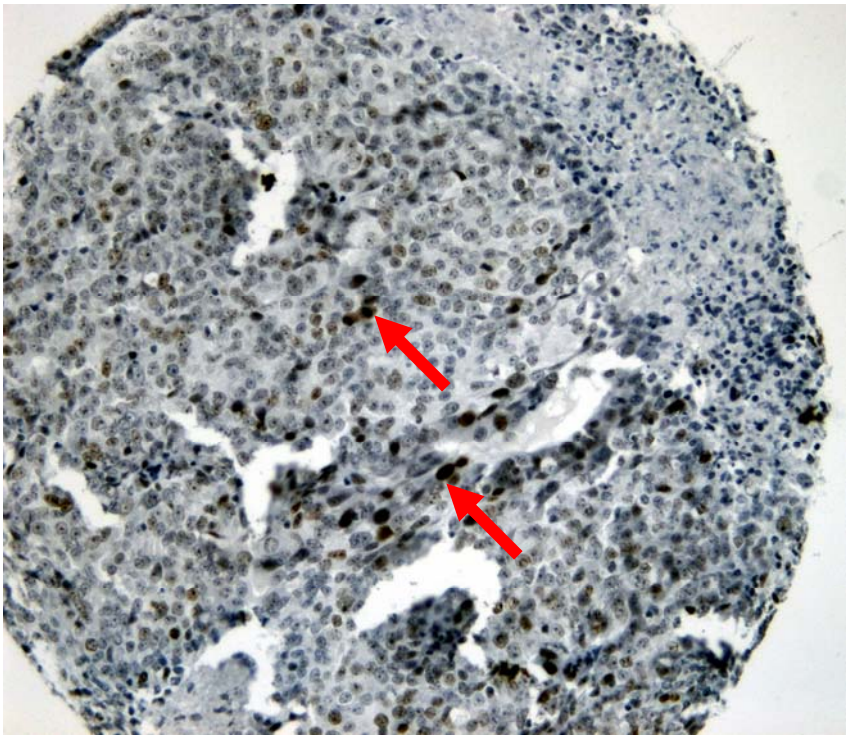


Figure 11 Photomicrograph showing cells staining strongly for Rb (red arrows).

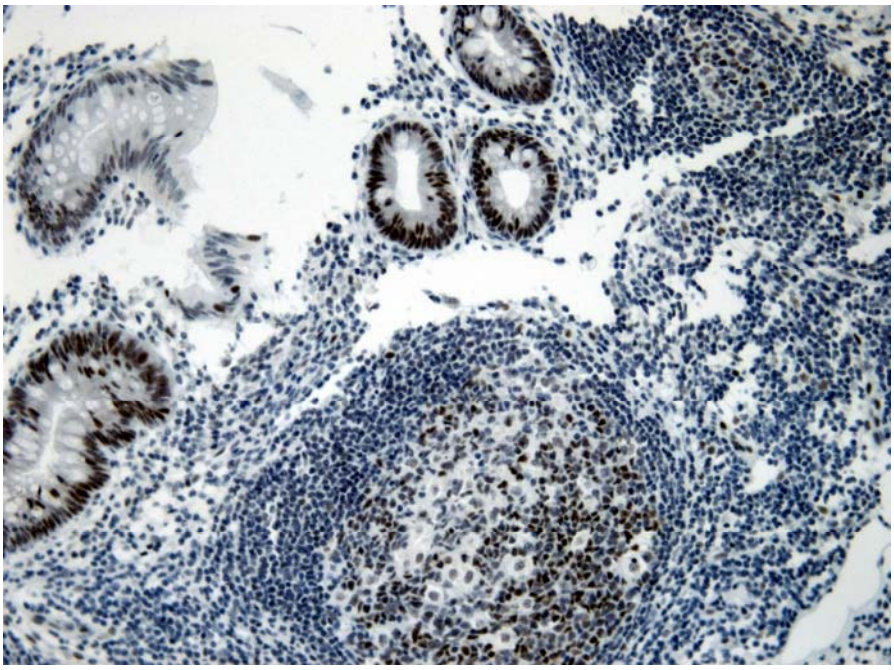


Figure 12 Retinoblastoma positive control (tonsil).

The pattern of the association between Rb % staining and revised Gleason score follows that of a normal (Gaussian) distribution. As can be seen from boxplots 1 and 2, for the lowest (<7) and highest (>7) Gleason scores, the Rb % staining is low and similar while in the middle (Gleason score 7), there is higher Rb % staining. Hence, no significant correlation was found between Gleason score, in 3 or 5 levels, and the percentage of stained cells for Rb. This comes into disagreement with other studies that have demonstrated a significant association between Gleason score and Rb loss.[34]

Through the use of univariate Cox proportional hazard models, no significant association was found between Rb expression and the risk of death from prostate cancer (P=0.0856). This result has also been shown in other trials that have concluded that decrease or loss of Rb expression has no effect on the PCa outcome.[35] Furthermore, a study by Dunsmuir *et al* concluded that Rb expression was of limited prognostic value in clinically localised PCa.[34] In a review article, Ross *et al* came to the conclusion that Rb is altered in only a small subset of PCa cases.[6] Another study also proved the small significance of Rb as a prognostic marker for clinically localised PCa, as Rb loss, which closely correlates to Rb mutations,[36-39] occurs with comparable frequencies in both localised, low-grade cancers as in more advanced ones.[4] On the other hand, other research groups have arrived to the opposite

conclusions, as they have associated the loss of Rb with disease progression and PCa-related mortality.[15]

Hence, the prognostic value of Rb in PCa is debatable according to the current literature, and no confident conclusion on its importance can be drawn at present.[36;40]

In this particular project, no significant association was found between Rb expression and the risk of death from prostate cancer (univariate Cox proportional hazard models, P=0.0856). However, Rb expression had a significantly higher risk of death from any cause (Hazard Ratio, HR=1.50, 95% CI = [1.00-2.27], P=0.0493), as shown in Kaplan Meier curve 2. In the multivariate analysis, after including PSA levels and the Gleason score in the Cox model, Rb expression was no longer significantly associated with reduced overall survival.

p21

As is evident from histogram 2, the vast majority of normal cores (almost 100%) did not stain for p21. This finding has been confirmed in other studies.[41;42] The majority of cancerous cores (72%) also did not stain for p21. However, of the cores that had cells that stained, especially in cores where 1, 5, 10, and 20% of the cells were staining, the proportion of the cancerous cores with staining cells was significantly higher to the one of the normal cores.

In relation to the staining intensity (bar chart 2), at all percentage staining for p21, the staining was predominantly of normal intensity. In the 20 and 40% staining cores, all cells stained at a normal intensity. (Figures 13, 14)

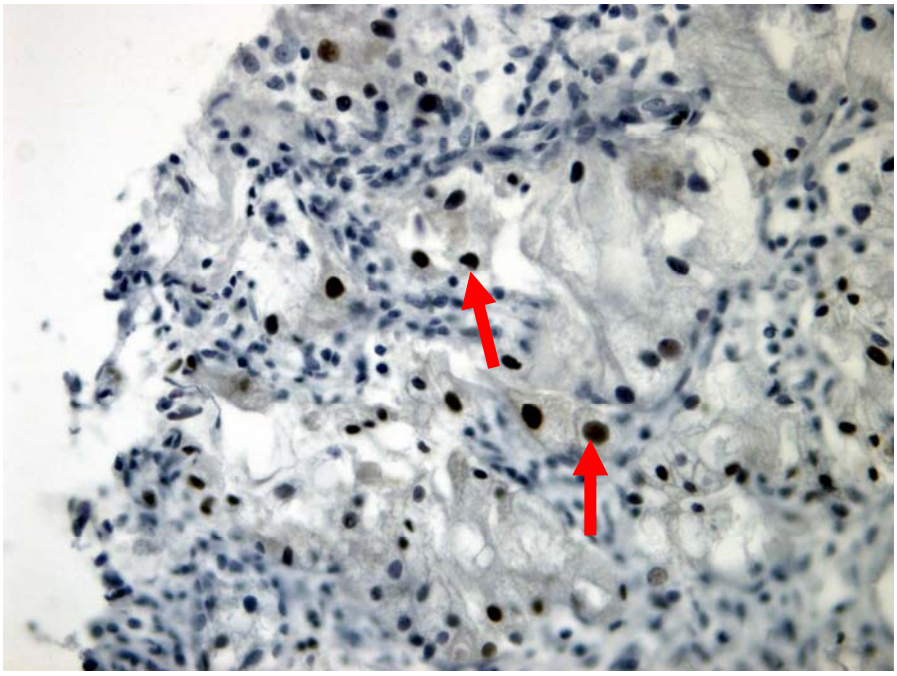


Figure 13 Photomicrograph showing a small proportion of cells staining for p21 (red arrows).

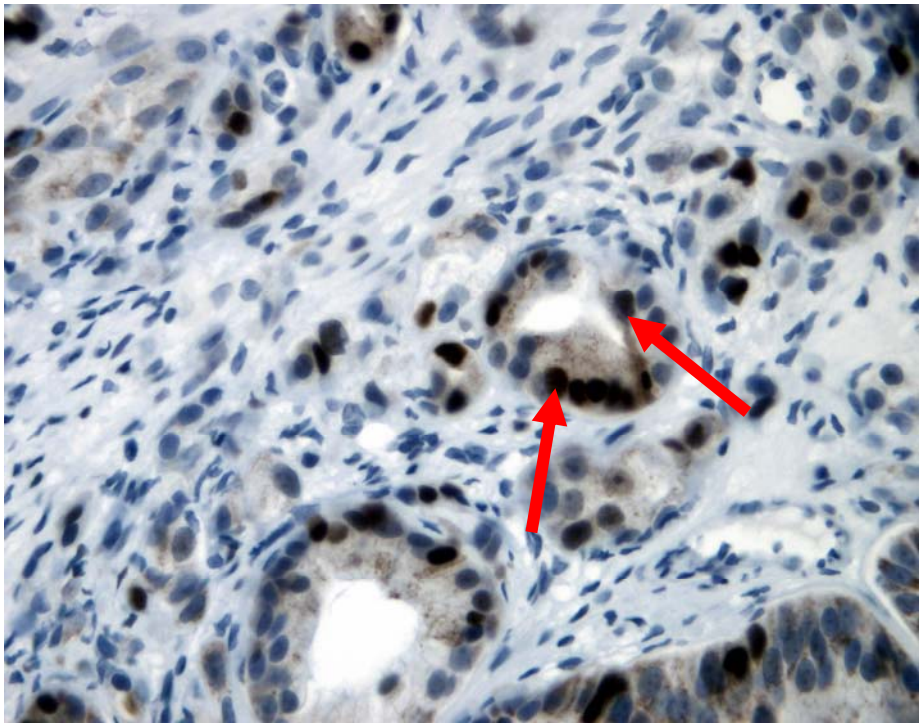


Figure 14 Photomicrograph showing staining for p21 of normal intensity (red arrows).

The pattern of p21 % staining shows an important correlation with revised Gleason score. As the p21 percent stain increased, so did the revised Gleason score. Hence, there is a significant correlation between Gleason score, in 3 or 5 levels, and the percentage of stained cells for p21 as can be seen from boxplots 3 and 4. This trend between p21 overexpression and higher Gleason score has also been shown in other papers.[10;43] However, other studies have found opposite results.[9;44;45]

Through the use of univariate Cox proportional hazard models, and as can be seen from Kaplan Meier curve 1, p21 expression is associated with a significantly higher risk of death from prostate cancer (Hazard Ratio, HR=2.53, 95% CI = [1.18-5.46], P=0.0142). This is a very important finding as it signifies the importance of p21 as an independent prognostic biomarker for clinically localised PCa. Other studies have also confirmed this conclusion. Such studies, have demonstrated that of the patients who had undergone radical prostatectomy as a monotherapy, those that had p21-positive tumours had a significantly higher risk of PSA failure than those who had p21-negative tumours.[36;41] Also, Doganavsargil *et al* reported that a higher p21 expression is an adverse prognostic factor.[42] The prognostic value of p21 in localised PCa is independent to the p53 status.[36] Further evidence on the prognostic value of

p21 comes from studies from Baretton *et al* and Sarkar *et al* that demonstrated that patients belonging to the p21 expression group, had more rapidly progressing tumours, greater rates of recurrence, and higher PCa-related mortality.[10;43;46] p21 expression, has also been shown to be "a strong and independent poor prognostic factor in patients with advanced stage PCa treated by androgen ablation".[47]

The association between p21 overexpression and poor prognosis discussed above, contradicts with the current belief that p21 is a tumour suppressor protein.[42;43] It would be expected that more aggressive, less well-differentiated tumours would actually lack p21. The p21 that is seen to have accumulated in the proliferating cells of the more aggressive tumours has obviously failed to arrest growth in tumour cells.[10] The evidence about whether it is over- or under-expression of p21 that should serve as a poor, independent prognostic biomarker, is conflicting.[42;44;48;49] In opposition to the findings of this study and of other studies mentioned above, other studies have found that it is the lack of expression of p21 that is associated with a poorer prognosis. However, in this poor prognosis group, the lack of p21 staining was associated with an overexpression of p53 and also some of these studies have been criticised for their weak statistical power.[9;41;42;44] Similarly, a study by Cheng *et al* preservation of p21 immunoreactivity is associated with a longer disease-free survival.[6;48]

Contrary to all of the above in relation to p21, some other studies have concluded that p21 expression plays no role in PCa prognosis.[45;50] However, these studies have been criticised due to the small number of mixed stage PCa tumours.[41]

p53

As is evident from histogram 3, the majority of normal cores (74%) did not stain at all for p53. The majority of cancerous cores (59%) also did not stain for p53. In other papers, the percentages of cancerous cores staining for p53 differ, varying from 6 to 20%. These findings suggest that most probably, p53 mutations are not a common feature in the pathogenesis of early PCa.[4;51] As the percentage of the cells that stain for p53 increased, the proportion of both cancerous and normal cores decreased. Virtually no cancer or normal core had 100% of its cells staining for p53. Of the cores that contained cells that stained for p53, the relative proportion of cancerous cores significantly exceeded the corresponding proportion of normal cores in the 5, 10, and 15% staining categories. The detection of p53 through IHC is usually associated with mutated p53. However, this is not always the rule, as, if the mutated p53 is not stable, it will not be detected by IHC.[15;43;52;53] In the 1% staining category and in the no-staining category, this pattern was reversed.

The staining was predominantly of normal intensity (bar chart 3), particularly in the 50% staining cores and onwards, where all the cells stained normally (Figures 15, 16, 17).

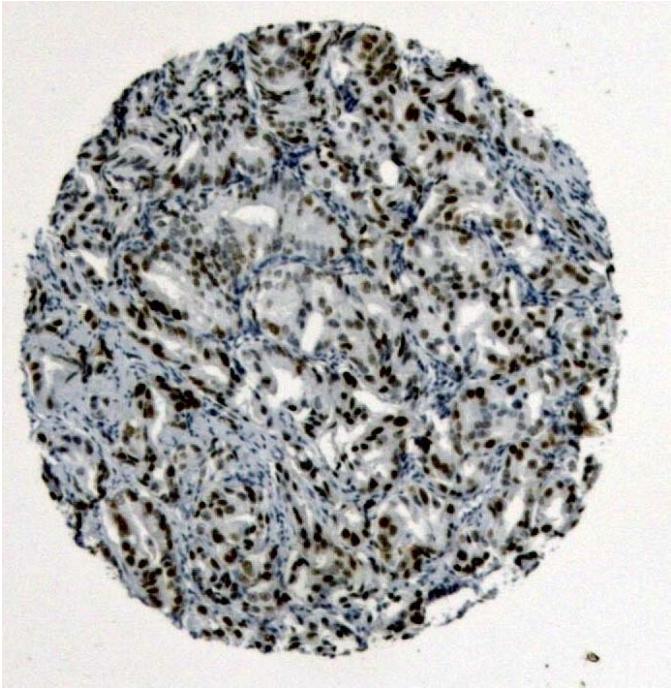


Figure 15 Positive control for p53 (colorectal carcinoma).

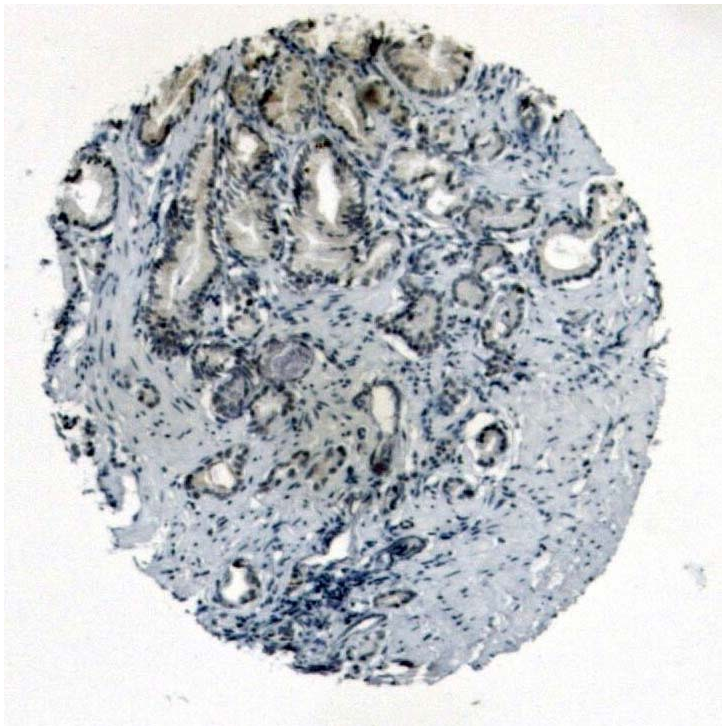


Figure 16 Photomicrograph showing minimal p53 staining.

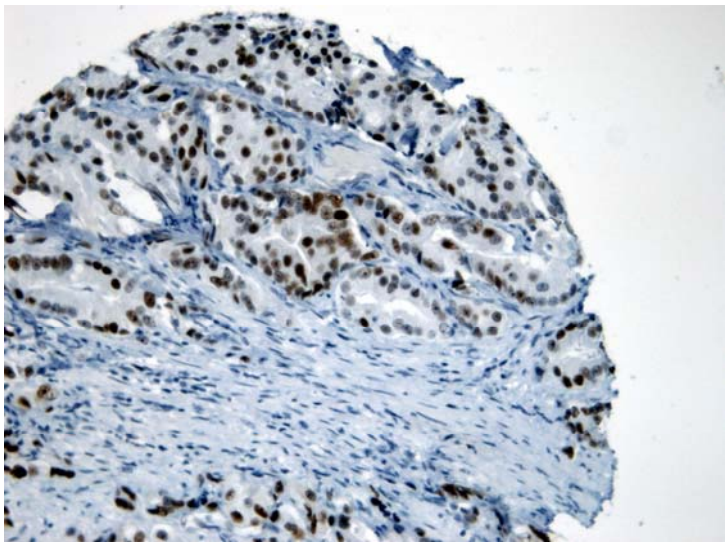


Figure 17 Photomicrograph showing cells staining for p53.

The pattern of p53 % staining shows an important correlation with revised Gleason score. As the p53 percent stain increased, so did the revised Gleason score. Hence, there is a significant correlation between Gleason score, in 3 or 5 levels, and the percentage of stained cells for p53 as can be seen from boxplots 5 and 6. This association has also been shown in other trials.[9;44;54] However, in a further trial, no significant correlation was identified between the two variables.[34]

Through the use of univariate Cox proportional hazard models, no significant association was found between p53 expression and the risk of death from prostate cancer (P=0.1308). Multiple trials have come to the same conclusion.[4;15;34;55;56] On the other hand, other papers have found a positive association between p53 over-expression and PCa-related mortality.[6;57-60] Also, p53 over-expression has been shown to be predictive of biochemical relapse post-prostatectomy or radiotherapy, when used as monotherapy.[43;61-65] In a similar way, p53 nuclear accumulation has been linked to a higher grade and more advanced stage of the disease.[47;66-68]

Consequently, as can be concluded by the above studies, "the role of p53 and its prognostic value in human PCa remains unclear",[13] and its value as a prognostic biomarker in localised PCa has been strongly debated.[15;36]

However, p53 expression had a significantly higher risk of death from any cause (Hazard Ratio, HR=1.58, 95% CI = [1.03-2.42], P=0.0318), as shown in Kaplan Meier curve 3. In the multivariate analysis, after including PSA levels and the Gleason score in the Cox model, p53 expression was no longer significantly associated with reduced overall survival.

Ki67

As is evident from histogram 4, with the exception of the 1% staining category, the relative proportion of cancerous cores that stained for Ki67 was significantly greater than the corresponding proportion of normal cores. As expected, the opposite pattern was seen in the cores that showed no staining, where the normal core percentage (37%) was significantly higher than the cancerous core percentage (12%). From the 5% staining category and onwards, as the % staining decreased, the % of cores that stained also decreased.

Staining for Ki67 was considered to be either present or not, and hence no distinctions in the intensity of staining for Ki67 were made (Figures 18, 19).

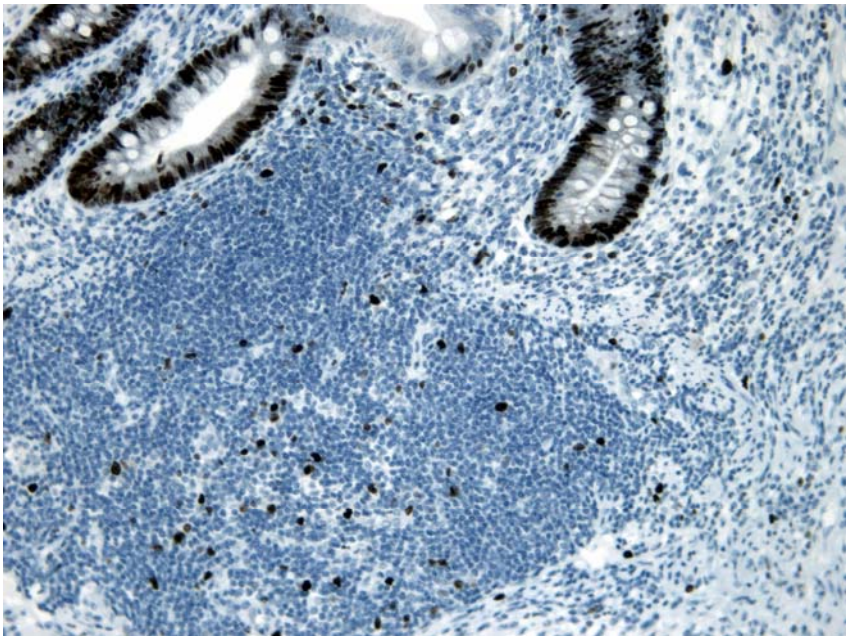


Figure 18 Ki67 positive control (tonsil).

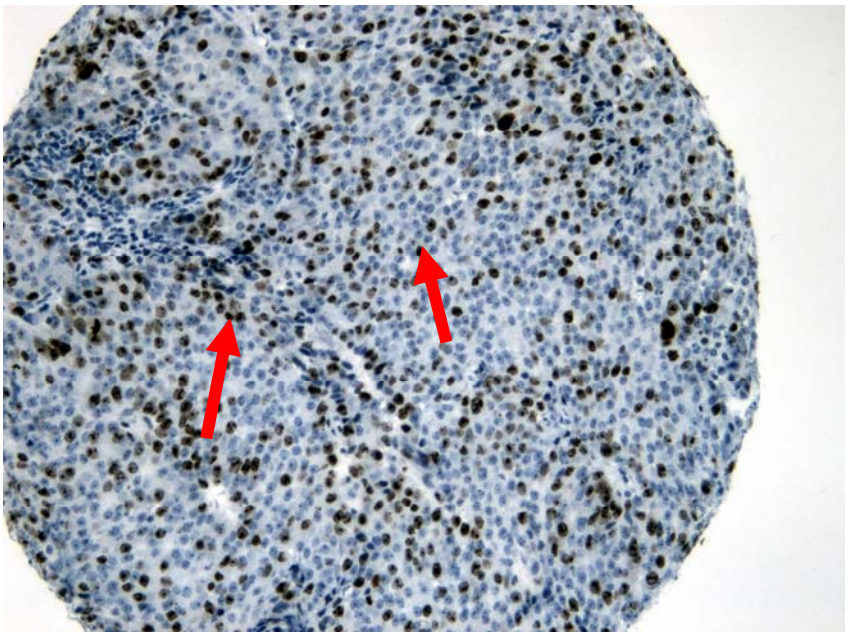


Figure 19 TMA core with cells staining for Ki67 (red arrows).

The pattern of Ki67 % staining shows an important correlation with revised Gleason score. As the Ki67 percent stain increased, so did the revised Gleason score. Hence, there is a significant correlation between Gleason score, in 3 or 5 levels, and the percentage of stained cells for Ki67 as can be seen from boxplots 7 and 8. Other studies have also shown a statistically significant correlation between MIB-1 (a monoclonal antibody that binds to parts of the Ki67 antigen) and Gleason score.[34] Furthermore, associations between the proliferative fraction of localised prostate tumours (measured indirectly by using MIB-1) and advancing tumour grade and stage have been found in other studies.[4;36;69-71] The Ki67 indices of prostate tumours that have recurred have been demonstrated to be about double the Ki67 indices of the corresponding primary tumours.[36;72] However, on the whole there are conflicting results in the literature on the association between Ki67 and tumour grade and stage. Although certain studies (such as the ones mentioned above) have shown an association between Ki67 expression and PCa stage, grade, and related prognosis, other studies have not.[73] Tumour heterogeneity is a particularly important factor that contributes to this conflict by posing problems in the interpretation of results.[13] Despite this, low Ki67 labelling index has been independently linked to an improved prognosis.[4;73;74]

Through the use of univariate Cox proportional hazard models, no significant association was found between Ki67 expression and the risk of death from prostate cancer (P=0.0512). Other reports have found Ki67 (through MIB-1) to be an independent predictor of the development of metastases and of survival in advanced PCa. This does not necessarily come into opposition with the findings of this project, as this project is concerned with PCa tumours that were managed by watchful waiting, therefore being more likely to be localised, rather than advanced tumours.[34;70;71;73] Different studies have demonstrated that Ki67 can be used as an independent marker of prognosis in patients that have undergone radical prostatectomy,[36;75] radiotherapy,[36;76] or watchful waiting[36;72] as a treatment for clinically localised PCa.

Ki67 expression had a significantly higher risk of death from any cause (Hazard Ratio, HR=2.27, 95% CI = [1.51-3.43], P=0.0001), as shown in Kaplan Meier curve 4. In the multivariate analysis, after including PSA levels (in 4 levels) and the Gleason score (in 3 levels) in the Cox model, Ki67 expression was still significantly associated with reduced overall survival (Hazard Ratio, HR=1.95, 95% CI = [1.23-3.10], P=0.0002).

MDM-2

As is evident from histogram 5, as the percent staining increased, so did the percentage of cores that stained for MDM-2. In 30% of the cancerous cores, all cells stained for MDM-2. Other studies have shown very similar results (32.5% of PCa cases overexpressing MDM-2).[61] In the 50, 60, and 80% staining categories, the relative proportions of normal cores that stained for MDM-2 was significantly higher than for the cancerous cores. However, the reverse pattern was present for the 70, 90, and 100% staining categories. Hence, there was no pattern in terms of the expression of MDM-2 between normal and cancerous cores. MDM-2 mutations are a rare event in clinically localised PCa.[77]

In relation to the staining intensity (bar chart 4), weak intensity predominated up to the 50% staining category, but from then on, the relative proportion of normal intensity increased with the percent of cores. At 100% staining, almost all cores stained normally for MDM-2 (Figures 20, 21).

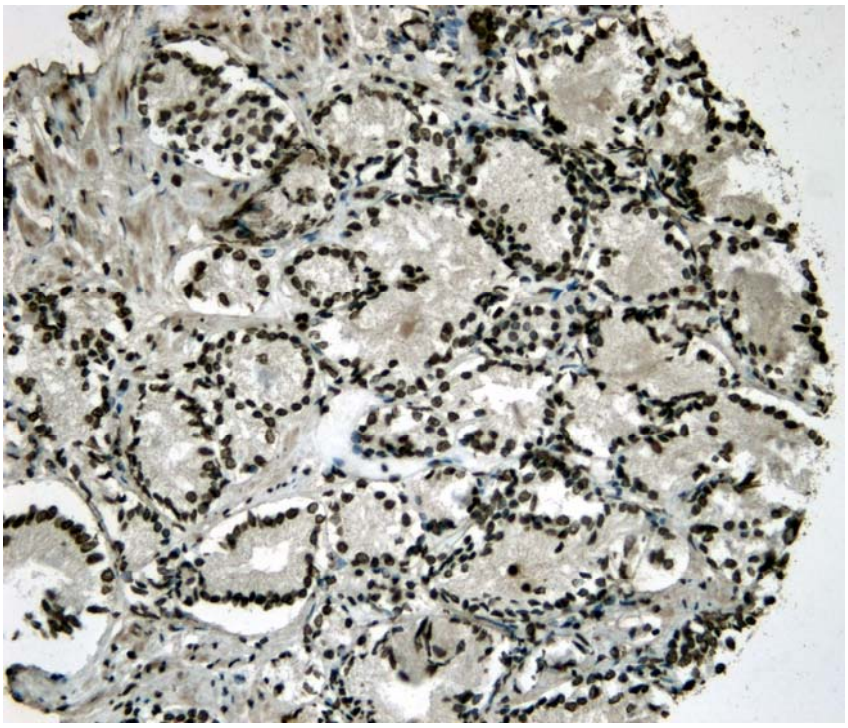


Figure 20 Photomicrograph showing strong positivity for MDM-2.

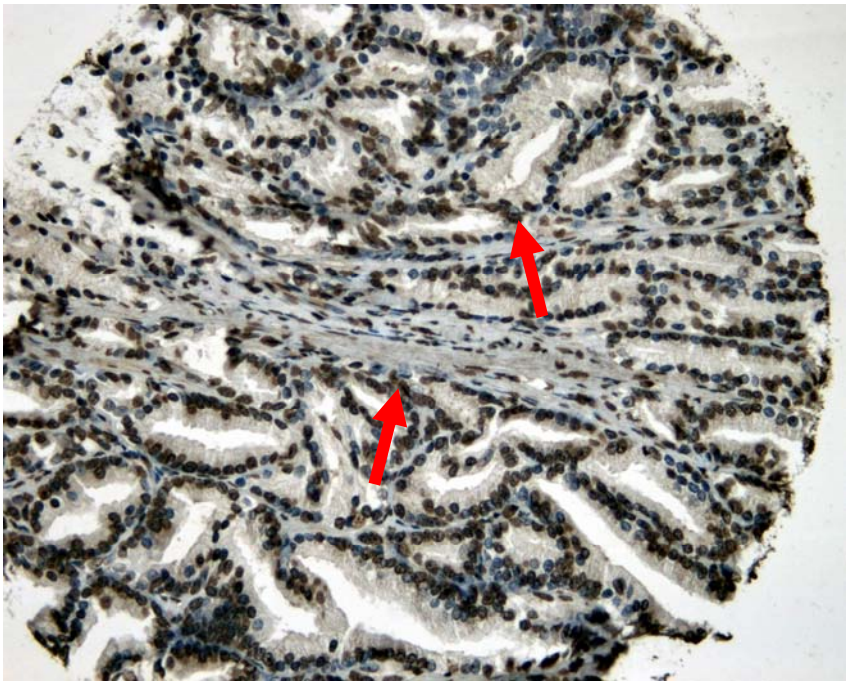


Figure 21 Photomicrograph showing prominent nuclear MDM-2 staining of normal intensity (red arrows).

The pattern of MDM-2 % staining shows an important correlation with revised Gleason score. As the MDM-2 percent stain decreased, the revised Gleason score increased. This relationship has also been shown in other studies, where a lower MDM-2 staining was present in prostate tumours with a higher Gleason score.[77] A significant correlation exists between Gleason score, in 3 or 5 levels, and the percentage of stained cells for MDM-2 as can be seen from boxplots 9 and 10. Consequently, MDM-2 phenotype can serve as a useful marker that will assist the pathologist in the differentiation between early and advanced prostate tumours. This has been shown in multiple studies.[61;77;78]

Through the use of univariate Cox proportional hazard models, no significant association was found between MDM-2 expression and the risk of death from prostate cancer (P=0.3148). This is confirmed by other studies.[61] In addition, univariate analysis showed that MDM-2 expression had no significant association with overall survival (P=0.2058).

Limitations of this study

The cohort used in this study consisted of 458 cancerous cores. Obviously, the larger the cohort, the more representative the results will be of the broader prostate tumour population. Hence, the relatively small cohort size could be considered one of the limitations of this study. Future studies could use larger numbers of tumour cores to generate more accurate data.

Another limitation in this study could be the fact that only the cancerous cores were included in the statistical analysis, while all other cores (mixed, normal, PIN, and "undetermined") were excluded. This could represent a form of selection bias. However, the reason why this was done was to ensure that the results would reveal correlations between protein expression and clinicopathological parameters (Gleason score, survival), specifically relating to prostatic adenocarcinoma. It was thought that if other prostatic pathologies were included in the statistical analysis, the quality of the results might be potentially affected.

Moreover, a limitation involves the use of IHC. The full procedure, although described clearly and in detail in appendix 2, thoroughly followed, and widely available, it constitutes an error prone process. This is because it is time-consuming and needs particular attention to the finest detail in every step, if the results are to be accurate and reproducible. Practice is the only way to master the technique. Also, the lack of standardisation of protocols and antibodies between different laboratories makes the results less comparable to each other. It is important that future studies follow the current IHC protocol as closely as possible, to ensure precision and comparability between their data and the current ones.

The TMA cores were evaluated for the percentage staining and staining intensity by Dr. Daniel Berney and the author. This represents a weakness in the study, as it makes the analysis subject to observer bias. To minimise this bias, the evaluation was performed blind, as the follow-up data were not known to the observers at that point. In addition, by involving the same two people evaluating together all TMA cores, inter-observer variation was prevented. Only cells with nuclear staining were counted, cells with cytoplasmic staining were excluded. All these measures ensured the

consistency of the results. To further reduce subjectivity, future studies could use specialised software programmes in the analysis of percentage staining and staining intensity in the TMA cores.

An important factor that could affect the reliability of the results of this study is the intratumour heterogeneity, which is particularly present in PCa tumours.[16] It could be argued that a single TMA core cannot possibly be representative of the entire tumour from which it has been derived. The issue of heterogeneity and how it was dealt with is described in the introduction.

CONCLUSION

To our knowledge, this study is the first of its kind in the available international literature, as it assesses clinically localised prostate cancers managed by watchful waiting. In this way, it has become possible to assess the natural history of these tumours, as no intervention was performed that could potentially interfere and alter the progression of the disease. This is in opposition to studies that have been published to date, where at least part of the prostate specimens examined were either radical prostatectomy specimens or had undergone radiotherapy, and hence the natural history could no more be assessed with certainty.

The most important finding of this study is the identification of the significant association between p21 overexpression and the increased risk of death from prostate cancer. Hence, patients overexpressing p21 would possibly benefit from early aggressive treatment, such as radical prostatectomy or radiotherapy. However, further studies will be needed to confirm this finding. Such studies should involve a larger cohort of patients, longer follow-up period, and also standardisation of the immunohistochemical protocols (including specific antibodies). Further studies need to be conducted to determine the role of the other cell cycle proteins, as important associations with disease-specific survival may be revealed.

Other important results from this study include the significant correlations that were identified between p21, p53, Ki67, and MDM-2 phenotypes and the Gleason score. Once more, further larger studies will be needed to support or decline this evidence. The role of Rb expression in relation to the Gleason score also needs further assessment.

Future projects could try to confirm the above findings by using larger patient cohorts in their studies. In addition they could make use of specialised software, which should theoretically be more objective and efficient in the analysis of TMAs, eliminating observer error and speeding up this process. Finally, to determine whether such software would be useful in the clinical setting, the results of the manual data analysis could be compared with those of the digital analysis.

APPENDIX 1

The molecular pathology of prostate cancer

For the human organism to develop and function normally, strict control over gene expression is essential. This is performed by transcription factors. As their name implies, transcription factors are proteins that regulate the transcription (i.e. the conversion of DNA into RNA) of particular genes. Their role is vital so that gene expression (i.e. protein production) occurs in the appropriate organs or tissues and at particular times (eg. as a response to specific stimuli). However, strict control can also be exerted post transcription.[79]

Certain diseases occur (partly or entirely) as a result of errors in the control over gene expression (often the result of mutations in certain transcription factors), one of the most common being cancer. In numerous cases, cancer results from the inappropriate activation of oncogenes, a specific type of genes that normally stimulate cellular growth (proliferation) through their protein products. This can occur as a result of a chromosomal translocation. On the other hand, cancer can also result from the inactivation of antioncogenes (also known as tumour suppressor genes), a different type of genes that inhibit cellular growth through their protein products. Such an inactivation can be the result of a mutation or deletion. All these events interfere with the strict control normally exerted over the cell cycle (Figure 22), eventually resulting in carcinogenesis.[79]

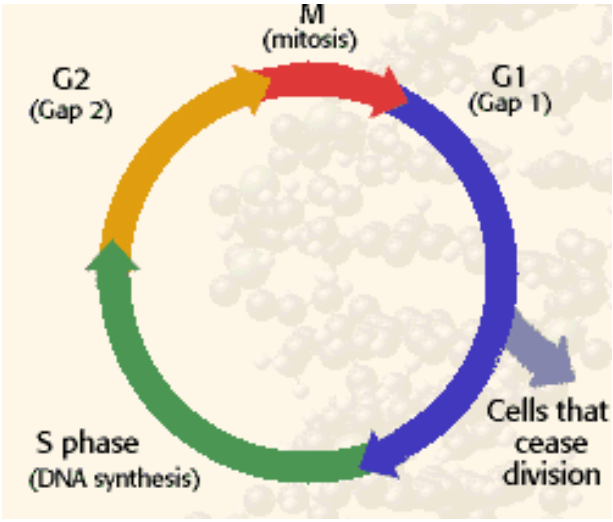


Figure 22 The stages of the cell cycle.[80]

There are numerous examples of such genes. p53 (Figures 23, 24) is a tumour suppressor gene[81] as is retinoblastoma (Rb)[82] (Figure 25) while MDM-2 is an oncogene.[83] p53 also known as the "guardian of the genome",[79] arrests the cell cycle at the G1 checkpoint if DNA is damaged. This is achieved by stimulating the expression of p21, which in turn arrests the cell cycle both directly by inhibiting DNA replication in cells that are in the S phase and indirectly by inhibiting Cdk's (cyclin-dependent kinases).[82] In addition, p53 induces the expression of the *bax* gene, which through its protein product, makes cells who's DNA is irreparably damaged to undergo apoptosis.[79]

Figure 23 3-D structure of p53 bound to DNA.[84]

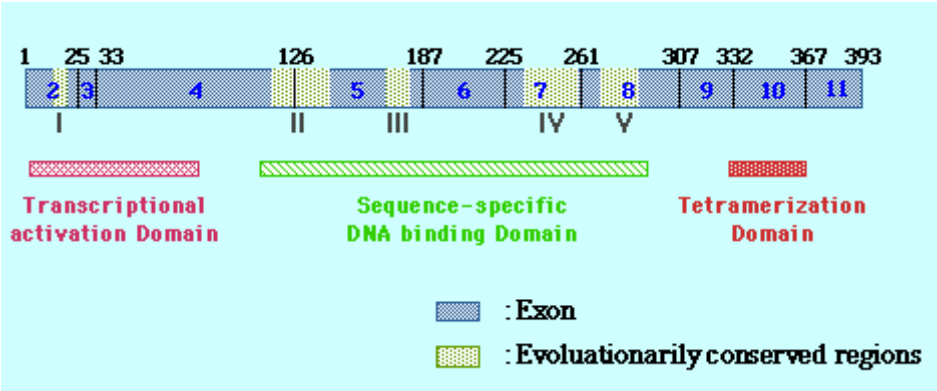


Figure 24 The structural organisation of p53 protein.[85]

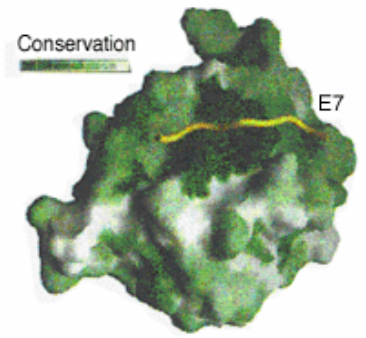


Figure 25 Complex of Rb protein with E7 viral oncoprotein.[86]

Consequently, p53, through its above functions, serves in maintaining the integrity of the genome. Abnormal (or non-) function of p53 for any reason will result in genomic instability (through numerous mutations in the DNA of cells), which can eventually result in tumour formation.[81] Interestingly, sporadic somatic p53 mutations have been identified in numerous human carcinomas,[79] including the most common ones such as those of the prostate, breast, lung and colon.[81] The most common p53 mutation involves the formation of an abnormal p53 which (as opposed to normal p53) does not have the ability to bind to the DNA and hence cannot arrest the cell cycle nor induce apoptosis in the (DNA) damaged cells. On top of this, the mutant p53 interacts with the normal one to prevent it from binding to the DNA. Due to this, even if an individual only has a single somatic mutant copy of p53, that individual is still at a much higher risk of developing a malignancy than if he/she had both p53 copies unaffected.[79]

The p53 protein may be inactivated in one of two ways. Either through mutation in the p53 gene itself or by being inactivated by MDM-2 (through its protein products which stimulate ubiquitination and proteosomal degradation of p53 by binding to it)[79;83] (Figure 26). An example of the latter would be that of an individual with two unaffected copies of p53, but high MDM-2 protein levels (due to MDM-2 gene amplification), who develops a soft-tissue sarcoma due to the imbalance between oncogene and tumour suppressor gene concentrations.[79]

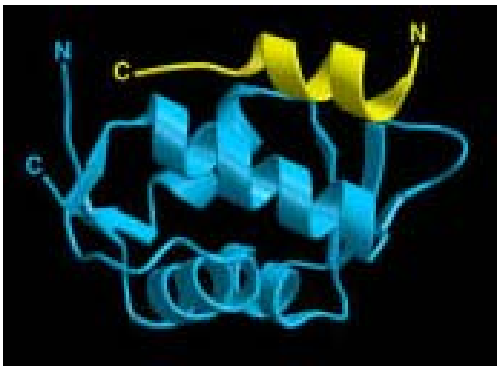


Figure 26 MDM-2 bound to the transactivation domain of p53.[87]

Inappropriate overexpression of MDM-2 has been shown to be present in 5-10% of all human tumours.[88] Apart from MDM-2 affecting p53, the opposite may also occur, as p53 can cause transcription and translation of the MDM-2 gene. Hence, p53 and MDM-2 are both equally important parts of the same loop (Figure 27). It is important to understand that MDM-2 can inhibit p53 functions both directly but also indirectly by binding straight to p21 to induce p53 proteasome-mediated degradation. Furthermore, MDM-2 can exert its functions in a p53-independent manner by various ways, one of which includes binding to retinoblastoma gene product.[83]

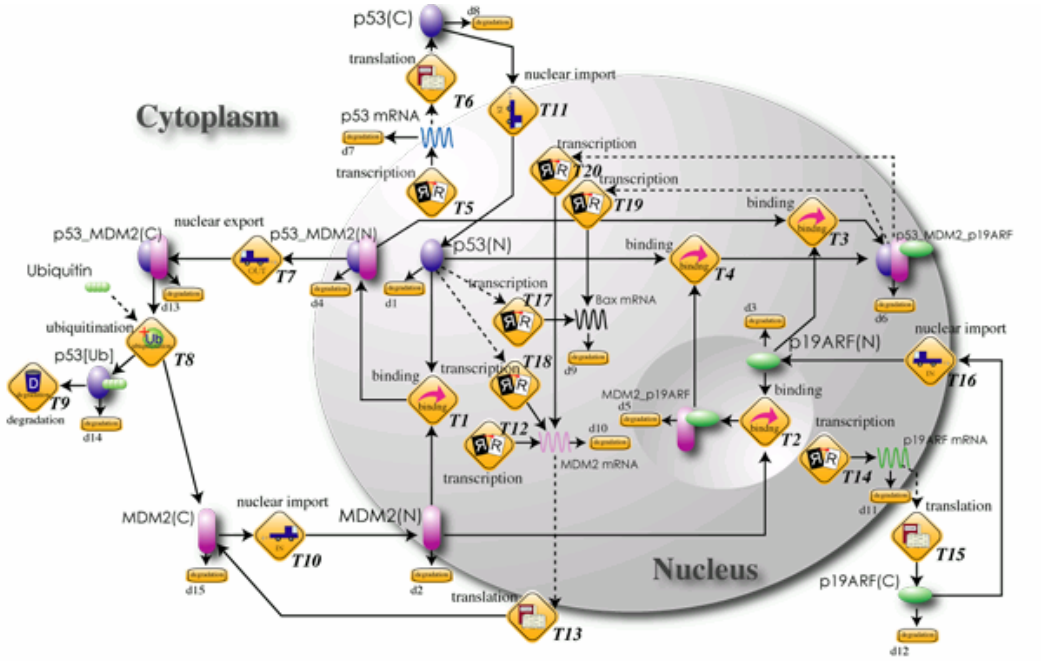


Figure 27 MDM-2 and p53 molecular interactions.[89]

It can therefore be concluded that MDM-2 is a very important oncogene when considering the pathogenesis of many malignant disorders in humans (commonly overexpressed in sarcomas, as well as in haematological and solid tumours). This is because it can affect the growth and development of cells as well as suppress apoptosis in (DNA) damaged cells in numerous ways.[83]

Both p53 and MDM-2 have been specifically implicated in the pathogenesis of prostate cancer (PCa).[81;83] p53 has been mostly associated with advanced PCa, since p53 abnormalities are rarely present in clinically localised PCa,[81] while they are commonly seen in association with advanced disease, dedifferentiation, and the emergence of hormone (androgen) resistance.[90]

In a similar way, the expression of MDM-2 is also associated with an advanced stage; something that further suggests that MDM-2 overexpression inhibits p53 to allow PCa to progress.[83]

On the other hand, the same is not valid for Rb. It has been shown that loss of Rb1 presents with a similar frequency in clinically localised as well as advanced PCa.[91]

Moreover, Ki-67 (another cell-cycle-regulated protein) can be considered as a proliferation indicator. This is because Ki-67 is consistently overexpressed in all tumour cells, but not in normal matched tissues, where its expression is low.[91]
The above lead to the conclusion that immunohistochemically obtained p53 and MDM-2 status of a PCa tumour allows an accurate prediction of how advanced the tumour is and of its chance of recurrence. However, under- and overexpression of Rb and Ki-67 respectively, only allow distinction between benign and malignant prostate tissue.[81]

Several studies have demonstrated that the degree of expression of p53 closely correlates to higher tumour grade and the presence of metastases.[14] Overexpression of p53 is considered to be an aggressive feature of cancer. Of interest is the fact that certain PCa cells that show nuclear p53 accumulation have been shown to possess hormone (androgen) independence.[92]

In addition, inactivation of the Rb gene has been linked to PCa progression. Both p53 and Rb have been shown to be predictors of disease-specific survival in post-prostatectomy patients.[14]

With regards to Ki-67, multiple studies have demonstrated its usefulness as an independent prognostic marker for PCa.[92]

Furthermore, MDM-2 expression is associated with more advanced malignant disease.[83]

By using p53 and MDM-2 as molecular biomarkers[81] it may therefore be possible to predict how aggressively an individual clinically localised PCa will behave at the time it has been histologically diagnosed.

If achieved, this will be a revolutionary step forwards in the management of clinically localised PCa, as it will be possible to categorise PCa patients in terms of the aggressiveness potential of their tumour in a much more accurate and reliable way than the prognostic criteria currently used (Gleason score, TNM stage, surgical margin status and pre- and post-treatment PSA levels). Thus it will be possible to

tailor subsequent management accordingly.[93] Furthermore, based on this information, new treatments for prostate cancer may be developed. These will be exerting their effects at the molecular level by using for example MDM-2 as a potential target for gene therapy.[83]

APPENDIX 2

Immunohistochemistry

The method that was followed for the immunohistochemistry (IHC) and that is described here, was in accordance to the IHC protocol used in St. Bartholomew's Hospital, developed by Ms. Susanne Jordan.

Immunohistochemistry method

Initially, the TMA slides, after been placed on the slide holder, were dewaxed by placing them into xylene. They were placed in two different xylene glass containers, in each being left for 5min.

They were then placed in absolute alcohol for 2min.

Subsequently, they were placed in blocking solution (2% H_2O_2 in methanol) for 10min (two different glass containers, in each one for 5min).

The TMA slides were then immersed in absolute alcohol for 10min (two different glass containers, in each one for 5min).

Following that, slides were taken to cold tap water for a period of at least 2min.

The TMA slides were then placed into boiling water, inside the pressure cooker.

Once pressure-cooking finished, and the lid was removed, cold tap water was poured onto the slides in the cooker for 5min. Subsequently, the slides were removed from the cooker with forceps and immediately placed into tap water, to prevent them from drying out.

Following that, slides were placed in phosphate buffer solution (PBS) until they were positioned in the metal box.

Once in the metal box, tween buffer solution was applied on the slides to prevent them from drying.

At that point, one by one, the slides were taken out, wiped carefully without touching the cores, and with a green marker a rectangle was drawn around the cores. Immediately after, the slide was placed back to the metal box, and tween buffer solution was applied to it to prevent drying.

Once this was done for all TMA slides, the slides were placed on an inclined plane inside the metal box. Once most of the tween buffer solution had drained due to gravity (except tween buffer solution covering the cores as it was trapped in the green rectangle drawn around them), the slides were rewiped. At that point, the tween buffer solution was also removed from inside the green rectangle by absorbing it with a piece of cloth without touching the cores.

The primary antibody was then added.

The primary antibody was not added to the negative controls.

Specific positive controls were used for each cell cycle protein (stated below).

The metallic box was then closed and the tissues were incubated with the primary antibody for 40min.

After 40min, the slides were washed with tween buffer solution, wiped, and the secondary antibody was added.

The metallic box was then closed and the tissues were incubated with the secondary antibody for 30min.

After 30min, the slides were washed with tween buffer solution, wiped, and the tertiary antibody was added.

The metallic box was then closed and the tissues were incubated with the tertiary antibody for 20min.

After 20min, the slides were washed with tween buffer solution, wiped, and then activated diaminobenzidine (DAB) was added on them.

The metallic box was then closed and the tissues were incubated with activated DAB for 10min.

After 10min, the slides were put back on the slide holder and then briefly washed in cold tap water.

The nuclei were then stained in haematoxylin for 5min.

Subsequently, they were rinsed in tap water, dipped for 2sec in acid alcohol, and then blued in tap water for 5min.

After that, the TMA slides were immersed in alcohol xylene for 2min.

The TMA slides were then immersed in alcohol for 2min.

Subsequently, the TMA slides were left in alcohol water for 2min.

After, the TMA slides were placed in xylene for 10min (two different glass containers containing xylene, in each one for 5min).

Finally, the TMA slides were mounted using a special apparatus.

Additional information

-Preparation of antigen unmasking solution:

50ml of antigen unmasking (retrieval) solution were added to 5l of distilled water (to mark of container). Once added, the two were mixed by shaking. The pH of the 0.01M solution was subsequently checked by a pHmeter. It was always assured that the pH of the antigen unmasking solution was in the range 6.0-6.2.

-Preparation of blocking solution:

To block endogenous peroxidase activity:

400ml of industrial methylated spirits (IMS) were mixed with 8ml of hydrogen peroxide (H_2O_2). If smaller quantities were required then 50ml of IMS were mixed with 750μl of hydrogen peroxide (H_2O_2).

-Pressure cooker technique:

3l of vector antigen unmasking solution were placed into a Delicio pressure cooker, the lid was closed, and the pressure cooker was placed on a hotplate, which was already been set to full heat (dial turned to 450). The solution was left on the hotplate until it started boiling. Once the solution had started boiling, it was taken off the hotplate, the lid was removed, and by using forceps; the slides were placed into the solution ensuring that they were fully covered. The lid was then placed back into position and locked, the pressure selector was set to 2 and the pressure cooker was placed back on the hotplate. The solution was then allowed to reheat until a steady steam flow came out from the outlet valve (due to high pressure). At that point, the timer was set to 10min. After 10min, the heater was turned to zero and the pressure cooker was taken off the hotplate and placed in the sink. Cold tap water was poured on the pressure cooker. Meanwhile, the pressure selector was turned gradually to the steam symbol to release the pressure from inside the pressure cooker. Once the pressure indicator dropped, the lid was unlocked and removed. Cold tap water was

allowed to pour directly on the slides into the solution for 5min, and after that, the slides were taken out by using the forceps and immediately placed in tap water to prevent them from drying out. Subsequently, the slides were placed in PBS and then in the metallic box.

-Primary antibodies:

Rb: Mouse anti-Human Monoclonal Antibody
Novacastra
Clone 1F6
1:50 dilution
Positive Control: Tonsil

p21: Mouse anti-Human Monoclonal Antibody
DakoCytomation
Clone SX118
1:1000 dilution
Positive Control: Breast Cancer

Ki67: Rabbit anti-Human Monoclonal Antibody
DakoCytomation
Clone A0047
1:1000 dilution
Positive Control: Tonsil

P53: Mouse anti-Human Monoclonal Antibody
DakoCytomation
Clone DO7
1:1000 dilution
Positive Control: Colorectal Carcinoma

MDM-2: Mouse anti-Human Monoclonal Antibody
Novacastra
Clone 1B10
1:100 dilution
Positive Control: Glioma

All primary monoclonal antibodies were diluted with 1% BSA with azide.

-Secondary antibody:

The secondary antibody used was **Biotinylated universal antibody** code BA-1400. It was used at 1:200 dilution and 1% BSA with azide was used as the diluent.

-Tertiary antibody:

For the formation of the tertiary antibody, the **Vector Elite ABC kit** PK6100 was used.

The following were mixed:

-20μl from bottle A
-20μl from bottle B
-1000μl phosphate buffer solution (PBS)

Once mixed, a 30min period was allowed before it was used.

- DAB formation:

By using plastic forceps, one Kemtec DAB tablet was dissolved in 10ml of distilled water. It was then left for 15min.

-DAB activation:

DAB was activated by adding 10μl of 3% hydrogen peroxide (H_2O_2) per 1ml of DAB solution.

APPENDIX 3

TMA Data

-TMA 19-03

-TMA 31-03

-TMA 34-03

-TMA 45-03

-TMA 51-02

Transatlantic Prostate Cancer Tissue Microarray Data 19-03 BSc Project 2006										
ID	Designation (C/N)	Rb % stain	Rb intensity	p21 % stain	p21 intensity	Ki67 % stain	p53 % stain	p53 intensity	MDM-2 % stain	MDM-2 intensity
A1	m	0		1	w	10	x		100	s
B1	n	0		0		x	0		100	s
C1										
D1										
E1										
F1										
G1										
H1										
I1										
J1										
K1										
L1										
M1										
A2	n	x		x		x	x		x	
B2	c	60	m	5	s	10	1	w	80	s
C2		x		x		x	x		x	
D2	n	100	m	0		1	1	w	90	s
E2	n	50	w	0		0	1	w	80	s
F2										
G2										
H2										
I2										
J2										
K2										
L2										
M2										
A3	c	0		1	s	5	x		90	s
B3	c	0		1	s	5	1	w	100	s
C3	c	0		10	s	1	0		90	s
D3	c	0		5	s	10	10	w	80	s
E3	x	0		0		x	x		100	s
F3	c	x		x		10	0		x	
G3	c	10	w	0		5	0		100	s

H3	c	0		0		1	0		100	s
I3	x	x		x		x	x		x	
J3	x	x		x		x	x		x	
K3	c	0		1	s	5	0		100	s
L3	n	30	w	0		1	1	w	100	s
M3	c	0		0		0	0		100	s
A4	x	x		x		x	x		x	
B4	n	0		0		x	x		80	s
C4	n	10	w	0		1	1	w	70	s
D4	c	30	w	0		5	1	w	90	s
E4	c	30	w	0		1	1	w	100	s
F4	c	30	w	0		1	1	w	100	s
G4	n	30	w	0		5	1	w	60	s
H4	m	10	w	0		1	1	w	90	s
I4	n	30	w	0		1	1	w	80	s
J4	n	20	w	0		1	1	w	80	s
K4	c	0		0		1	0		90	s
L4	c	10	w	0		5	1	w	80	s
M4	c	40	m	0		5	1	w	x	
A5	c	60	m	0		1	1	w	90	s
B5	n	0		0		1	0		70	s
C5	c	0		0		10	1	w	80	s
D5	x	x		x		x	x		30	m
E5	x	0		0		x	x		60	s
F5	n	0		0		x	x		x	
G5	n	60	m	0		5	1	w	80	s
H5	c	30	w	5	s	10	5	w	90	s
I5	c	50	m	1	m	5	1	w	90	s
J5	c	60	m	0		1	0		100	s
K5	m	0		0		0	0		x	
L5	n	0		0		1	1	w	100	s
M5	x	x		x		x	x		100	s
A6	x	30	m	5	m	0	0		80	s
B6	n	40	w	5	m	0	0		80	s
C6	n	40	m	0		5	1	w	60	s
D6	c	0		0		20	0		90	s

E6	c	0		0		15	1	w	90	s
F6	c	0		0		5	5	w	80	s
G6	x	x		x		x	x		x	
H6	n	0		0		5	0		100	s
I6	n	0		0		5	0		90	s
J6	c	20	w	0		10	1	w	100	s
K6	c	10	w	5	s	10	5	w	100	s
L6	c	10	w	20	s	10	10	w	90	s
M6	n	30	w	0		1	1	w	80	s
A7	n	5	w	0		1	0		70	s
B7	n	50	w	0		1	0		70	s
C7	c	10	w	5	m	1	0		60	s
D7	n	70	w	0		1	1	w	x	
E7	n	50	w	0		1	x		50	s
F7	n	x		x		x	x		x	
G7	x	x		0		x	x		60	s
H7	c	70	w	5	m	1	10	w	100	s
I7	c	60	w	0		1	5	w	100	s
J7	n	20	w	0		1	0		90	s
K7	n	20	w	0		0	0		90	s
L7	c	x		x		x	x		x	
M7	c	10	w	0		5	1	w	70	s
A8	c	50	w	0		5	0		80	s
B8	n	50	w	0		1	1	w	80	s
C8	n	50	w	0		1	1	w	80	s
D8	c	0		0		1	1	w	80	s
E8	c	0		0		1	0		60	s
F8	n	0		0		1	1	w	90	s
G8	c	0		0		1	0		80	s
H8	n	0		0		0	0		50	s
I8	n	0		0		0	0		50	s
J8	n	x		x		x	x		x	
K8	n	x		x		x	x		x	
L8	m	50	m	0		5	1	w	90	s
M8	m	50	m	0		0	0		90	s
A9	c	70	w	0		1	1	w	90	s

B9	n	10	w	0		1	1	w	90	s
C9	n	40	w	0		1	5	m	80	s
D9	n	30	s	0		30	50	s	10	s
E9	n	x		x		x	x		60	s
F9	n	x		0		x	x		60	s
G9	x	0		0		x	x		70	s
H9	c	0		x		x	x		30	s
I9	c	0		0		0	0		50	s
J9	c	0		1	m	10	5	m	100	s
K9	c	0		1	m	5	5	w	100	s
L9	n	5	w	0		20	0		80	s
M9	n	0		0		0	1	w	x	
A10	c	x		x		0	1	m	x	
B10	c	10	m	0		1	0		50	s
C10	c	60	m	0		5	0		60	s
D10	c	60	m	0		5	1	w	70	s
E10	c	x		0		x	x		80	s
F10	m	10	w	0		5	1	w	70	s
G10	n	x		x		x	x		x	
H10	n	0		0		5	1	w	60	s
I10	c	0		0		10	0		90	s
J10	c	0		0		5	0		80	s
K10	c	0		0		5	0		90	s
L10	n	30	w	0		5	1	w	90	s
M10	n	20	w	0		5	1	w	100	s
A11	c	70	m	1	s	1	0		100	s
B11	c	70	m	0		10	1	m	100	s
C11	c	10	w	0		10	1	m	100	s
D11	c	0		0		5	0		100	s
E11	n	0		0		x	x		100	s
F11	c	0		0		10	5	s	100	s
G11	x	x		x		x	x		x	
H11	c	0		x		5	0		x	
I11	x	0		0		x	x		100	s
J11	c	0		5	m	5	0		90	s
K11	n	10	w	0		1	0		70	s

L11	n	20	w	0		1	0		80	s
M11	n	10	w	0		5	0		60	s
A12	n	10	w	0		0	1	w	80	s
B12	n	20	w	0		1	1	w	90	s
C12	n	20	w	0		1	0		80	s
D12	n	10	w	0		0	0		90	s
E12	n	10	w	0		0	0		80	s
F12	c	10	m	10	s	30	15	s	60	m
G12	c	10	m	10	s	30	15	s	50	m
H12	n	10	w	0		30	5	m	50	s
I12	n	0		x		30	x		x	
J12	c	0		0		5	0		100	s
K12	c	0		0		x	0		100	s
L12	m	0		0		1	0		100	s
M12	c	0		0		1	0		100	s
A13	n	x		0		0	0		60	s
B13	n	10	w	1	m	5	0		70	s
C13	c	5	w	1	m	5	0		70	s
D13	n	x		x		x	x		50	s
E13	n	5	w	0		0	0		60	s
F13	n	5	w	0		1	0		60	s
G13	c	0		0		1	0		100	s
H13	c	0		0		1	0		100	s
I13	n	30	w	0		1	1	w	90	s
J13	n	20	w	0		0	1	w	90	s
K13	m	10	w	0		1	5	m	80	s
L13	c	0		0		5	0		80	s
M13	c	0		0		1	0		70	s
A14	c	0		0		5	0		90	s
B14	n	5	w	0		5	0		80	s
C14	n	5	w	0		0	0		80	s
D14	c	0		0		5	0		90	s
E14	c	0		0		5	0		90	s
F14	c	0		0		5	0		90	s
G14	c	0		0		x	x		90	s
H14	n	5	w	0		0	0		100	s

I14	n	40	w	0		0	0		100	s
J14	c	0		0		10	10	s	90	s
K14	c	0		0		5	20	s	90	s
L14	c	5	w	0		10	70	s	80	s
M14	c	10	s	1	s	15	80	s	100	s
A15	n	30	w	0		x	x		50	s
B15	n	30	w	0		0	0		50	s
C15	n	20	w	0		0	0		60	s
D15	c	20	w	20	s	5	1	m	90	s
E15	n	0		0		0	0		100	s
F15	x	x		0		x	x		90	s
G15	c	0		0		5	0		80	s
H15	x	x		x		x	x		x	
I15	n	40	w	0		5	1	w	100	s
J15	n	50	w	0		5	0		80	s
K15	c	5	m	0		5	0		100	s
L15	c	0		0		5	0		100	s
M15	n	5	w	0		1	0		100	s
A16	n	0		0		1	0		90	s
B16	x	x		x		x	x		x	
C16	n	10	w	0		1	0		50	s
D16	pin	10	w	0		5	0		60	s
E16	pin	10	w	0		5	1	w	60	s
F16	x	x		x		x	x		x	
G16	x	x		0		x	x		100	s
H16	n	0		0		0	0		90	s
I16	n	10	w	0		5	1	w	80	s
J16	c	5	w	1	m	10	5	m	90	s
K16	m	0		0		10	1	w	100	s
L16	n	5	w	0		0	0		100	s
M16	n	10	w	0		5	20	s	100	s
A17	n	x		x		x	x		x	
B17	n	x		x		x	x		x	
C17	c	0		0		x	x		90	s
D17	n	20	w	0		5	0		60	s
E17	n	30	w	0		1	0		70	s

F17	x	x		x		x	x		x	
G17	x	x		x		x	x		x	
H17	x	0		0		x	x		90	s
I17	x	0		0		x	x		60	s
J17	n	0		0		0	1	w	50	s
K17	n	5	w	0		1	0		80	s
L17	c	0		0		5	0		90	s
M17	n	5	w	0		1	0		80	s
A18	n	70	m	0		1	0		80	s
B18	n	50	m	0		0	1	w	50	s
C18	c	0		1	s	5	0		50	s
D18	c	0		1	s	5	0		80	s
E18	c	0		5	s	5	0		80	s
F18	n	x		x		x	x		x	
G18	n	0		0		5	1	w	100	s
H18	c	10	w	10	s	5	5	m	90	s
I18	c	0		5	s	5	0		100	s
J18	c	0		0		10	0		90	s
K18	c	0		0		5	0		90	s
L18	n	0		0		1	0		90	s
M18	n	5	w	0		5	0		90	s
A19	c	10	w	0		5	1	w	90	s
B19	c	10	w	0		5	0		80	s
C19	n	10	w	0		x	x		40	s
D19	n	0		x		x	x		40	s
E19	c	0		0		0	1	w	70	s
F19	n	x		0		x	x		60	s
G19	n			x		x	x		x	

Transatlantic Prostate Cancer Tissue Microarray Data 31-03 BSc Project 2006										
ID	Designation (C/N)	Rb % stain	Rb intensity	p21 % stain	p21 intensity	Ki67 % stain	p53 % stain	p53 intensity	MDM-2 % stain	MDM-2 intensity
A1	c	0		0		0	0		70	s
B1	c	0		0		1	1	w	70	s
C1										
D1										
E1										
F1										
G1										
H1										
I1										
J1										
K1										
L1										
M1										
A2	c	0		0		5	1	w	100	s
B2	n	0		0		0	x		90	s
C2		x		x		x	x		x	
D2	n	0		0		1	0		60	s
E2	c	5	w	0		1	1	w	90	s
F2		x		x		x	x		x	
G2	m	x		x		0	1	w	100	s
H2	n	5	w	x		1	1	w	100	s
I2		x		x		x	x		x	
J2	c	0		0		15	100	s	50	s
K2	c	0		0		20	x		50	s
L2						x	x		x	
M2						x	x		x	
A3	m	5	w	1	m	5	1	w	70	s
B3	m	0		0		1	x		80	s
C3	c	0		1	m	5	1	w	90	s
D3	c	0		0		1	0		60	s
E3	n	20	w	0		1	1	w	100	s
F3	c	80	m	0		5	5	w	80	m
G3	c	70	w	1	w	5	5	w	90	m
H3	c	50	w	1	m	10	10	w	90	m
I3	c	10	w	5	s	10	5	w	80	m

J3	n	60	m	0		1	1	w	60	s
K3	n	0		0		1	1	w	100	s
L3	c	0		0		5	0		100	s
M3	c	10	w	1	s	5	0		100	s
A4	n	x		x		x	x		x	
B4	c	0		0		5	0		100	s
C4	c	5	w	0		5	0		70	s
D4	c	0		0		5	0		100	s
E4	c	0		0		5	0		80	s
F4	n	x		x		x	0		x	
G4	n	0		0		5	0		x	
H4	m	0		10	s	5	1	w	90	s
I4	c	x		x		x	x		x	
J4	c	0		0		5	1	w	90	s
K4	c	0		0		5	1	w	60	m
L4	n	50	w	0		0	1	w	90	s
M4	n	10	w	0		1	1	w	90	s
A5	c	x		x		x	x		x	
B5	m	0		0		1	1	w	60	s
C5	c	60	m	1	m	5	1	w	80	s
D5	c	50	m	0		1	1	w	90	s
E5	c	0		0		5	1	w	90	s
F5	m	0		1	m	5	1	w	x	
G5	c	x		0		x	1	w	x	
H5	c	5	w	10	s	5	5	w	70	s
I5	n	5	w	0		5	x		70	s
J5	c	0		0		5	0		50	s
K5	c	0		0		5	0		100	s
L5	c	0		0		5	0		100	s
M5	c	0		0		1	0		100	s
A6	n	30	m	0		5	1	w	60	s
B6	n	x		0		x	x		x	
C6	c	0		0		1	0		100	s
D6	x	x		x		x	x		x	
E6	n	50	w	0		5	1	w	100	s
F6	c	5	w	0		5	0		100	s
G6	c	5	w	0		5	0		90	s

H6	c	0		x		x	0		x	
I6	c	0		0		x	0		x	
J6	n	5	w	0		5	0		100	s
K6	c	20	w	0		5	1	w	100	s
L6	c	0		x		x	0		x	
M6	c	0		x		x	x		x	
A7	n	10	w	x		x	1	w	x	
B7	n	x		0		1	x		50	s
C7	c	0		0		10	1	w	90	m
D7	x	x		x		x	x		100	s
E7	c	x		0		x	x		x	
F7	c	x		0		x	x		60	s
G7	c	20	m	0		10	0		90	s
H7	c	20	m	0		10	x		50	s
I7	c	x		0		x	x		x	
J7	c	0		0		x	0		60	s
K7	m	0		0		1	0		100	s
L7	m	0		0		0	0		100	s
M7	n	0		x		x	0		x	
A8	n	x		x		x	x		x	
B8	c	0		10	s	30	5	m	90	m
C8	c	0		10	s	25	5	m	90	m
D8	c	0		0		15	1	w	10	w
E8	c	0		0		10	1	w	20	m
F8	n	x		x		x	1	w	x	
G8	n	80	m	0		5	1	w	90	s
H8	m	30	w	1	w	1	1	w	70	s
I8	c	20	w	0		5	0		90	s
J8	n	30	s	10	w	1	1	w	90	s
K8	n	x		0		x	x		90	s
L8	c	0		x		x	0		x	
M8	c	0		0		1	1	w	x	
A9	n	x		0		0	x		80	s
B9	n	20	w	0		1	1	w	90	s
C9	m	0		0		5	0		100	s
D9	c	0		0		5	1	w	100	s
E9	n	0		0		5	0		90	s

F9	c	5	w	x		5	0		x	
G9	n	x		x		x	x		x	
H9	c	x		x		5	0		x	
I9	n	20	m	0		1	1	w	100	s
J9	x	x		x		x	x		x	
K9	x	x		x		x	x		x	
L9	x	x		x		x	x		x	
M9	n	5	w	0		0	x		100	s
A10	m	0		x		x	0		x	
B10	m	x		x		x	0		x	
C10	x	x		x		x	0		x	
D10	n	30	w	0		5	1	w	x	
E10	c	60	m	1	s	1	5	w	80	s
F10	c	20	m	0		0	0		90	s
G10	c	0		0		1	0		100	s
H10	c	0		0		1	0		100	s
I10				x		x	x			
J10						x	x			
K10						x	x			
L10						x	x			
M10						x	x			
A11										
B11										
C11										
D11										
E11										
F11										
G11										
H11										
I11										
J11										
K11										
L11										
M11										
A12	c	10	w	0		1	0		90	s
B12	c	20	w	0		1	1	w	70	m
C12	c	0		0		1	0		100	s

D12	c	0		0		1	0		100	s
E12	n	x		x		x	x		x	
F12	n	0		x		x	0		x	
G12	c	5	m	1	w	15	15	s	20	m
H12	c	20	m	10	s	15	10	s	30	m
I12	c	0		0		15	5	s	30	m
J12	c	20	s	5	s	15	10	s	20	m
K12	m	30	w	0		10	1	w	90	m
L12	m	5	w	0		5	1	w	100	s
M12	c	0		1	m	5	0		80	s
A13	c	0		1	s	5	0		50	s
B13	c	x		x		x	x		x	
C13	c	5	w	40	s	25	x		50	s
D13	c	x		x		15	10	m	x	
E13	x	x		x		x	x		x	
F13	n	x		x		x	0		x	
G13	n	x		x		x	1	w	x	
H13	x	x		0		5	x		40	s
I13	c	30	w	0		10	0		50	s
J13	c	0		0		5	0		50	s
K13	c	0		0		10	0		90	s
L13	n	x		x		x	x		x	
M13	n	30	m	1	w	5	0		60	s
A14	m	5	w	10	s	15	5	s	90	s
B14	c	5	w	5	s	10	5	s	60	s
C14	c	5	w	1	s	10	0		50	s
D14	c	5	w	1	s	x	5	s	80	s
E14	n	30	w	0		1	1	s	40	s
F14	n	30	w	0		5	0		50	s
G14	c	x		x		x	x		x	
H14	c	5	w	0		x	1	w	x	
I14	c	5	w	0		10	0		70	s
J14	c	0		0		10	0		80	s
K14	n	0		0		x	1	w	x	
L14	n	0		0		1	0		90	s
M14	c	10	w	0		1	0		90	s
A15	c	x		0		1	0		x	

B15	n	x		x		1	x		x	
C15	n	x		x		x	x		x	
D15	n	30	w	0		1	0		100	s
E15	n	x		x		x	x		x	
F15	m	10	w	0		5	10	s	100	s
G15	m	30	w	1	s	10	15	s	60	s
H15	c	30	w	0		5	10	s	40	m
I15	c	5	w	0		1	10	s	30	m
J15	n	20	w	0		1	0		50	s
K15	n	20	w	0		1	0		40	s
L15	c	0		0		5	0		50	m
M15	c	0		x		x	0		x	
A16	c	0		0		1	0		70	s
B16	c	0		0		15	0		80	s
C16	n	30	w	0		1	1	w	50	s
D16	n	30	w	0		1	0		40	s
E16	c	10	w	0		5	0		80	s
F16	n	10	w	0		1	0		100	s
G16	n	30	w	0		1	0		80	s
H16	c	50	s	10	s	10	15	m	80	s
I16	m	0		0		25	10	m	x	
J16	c	5	w	10	s	25	15	m	100	s
K16	c	10	m	5	s	30	10	m	90	s
L16	n	20	w	0		5	0		80	s
M16	n	0		0		1	0		90	s
A17	pin	x		0		10	0		90	s
B17	pin	0		0		1	0		80	s
C17	pin	10	m	1	m	50	0		80	s
D17	c	5	w	0		15	0		70	s
E17	n	30	m	0		1	1	w	100	s
F17	c	x		x		x	0		x	
G17	c	0		0		5	0		100	s
H17	m	20	w	0		5	1	w	100	s
I17	c	5	w	0		5	1	w	100	s
J17	c	0		x		x	0		x	
K17	n	10	w	0		1	0		100	s
L17	n	x		0		1	x		100	s

M17	x		x		x		x		x	
A18			x		x		x		x	
B18			x		x		x		x	
C18			0		0		5	0		50	s
D18			x		x		x		x	
E18			0		x		x	1	w	x	

Transatlantic Prostate Cancer Tissue Microarray Data 34-03 BSc Project 2006										
ID	Designation (C/N)	Rb % stain	Rb intensity	p21 % stain	p21 intensity	Ki67 % stain	p53 % stain	p53 intensity	MDM-2 % stain	MDM-2 intensity
A1	c	0		0		5	0		100	s
B1	c	0		1	s	5	1	w	90	s
C1										
D1										
E1										
F1										
G1										
H1										
I1										
J1										
K1										
L1										
M1										
A2	c	0		0		1	0		100	s
B2	n	10	w	0		1	1	w	100	s
C2		x		x		x	x		x	
D2	n	5	m	0		5	0		50	s
E2	n	5	w	0		1	0		100	s
F2		x		x		x	x		x	
G2	m	5	w	5	s	5	0		50	s
H2	n	5	w	x		5	0		50	s
I2										
J2										
K2										
L2										
M2										
A3	n	20	w	0		0	0		x	
B3	n	30	w	0		1	0		100	s
C3	c	x		10	s	20	5	m	50	m
D3	c	40	m	10	s	30	10	m	80	m
E3	c	30	s	10	s	30	15	s	60	s
F3	c	50	s	10	s	30	15	s	60	s
G3	n	x		0		0	x		50	s
H3	n	0		0		1	0		100	s
I3	c	0		0		1	0		100	m

J3	c	0		0		0	0		100	m
K3	c	0		0		1	0		100	s
L3	c	0		0		5	0		100	s
M3	c	0		0		10	x		100	s
A4	c	0		0		x	0		x	
B4	n	0		0		1	0		100	s
C4	n	0		0		1	0		100	s
D4	c	20	w	1	s	10	5	m	50	w
E4	c	20	w	1	s	20	10	m	70	s
F4	c	30	w	0		10	5	w	60	m
G4	c	30	w	0		10	10	w	50	w
H4	n	x		0		x	x		x	
I4	n	x		0		x	x		x	
J4	c	20	w	0		5	1	w	100	s
K4	c	20	w	0		0	0		100	s
L4	n	20	w	0		0	0		60	s
M4	n	0		0		5	x		100	s
A5	c	10	w	1	s	5	0		x	
B5	n	30	w	0		0	0		100	s
C5	n	30	w	0		1	0		100	s
D5	c	0		0		1	0		90	s
E5	c	0		0		1	0		80	s
F5	c	0		0		1	0		100	s
G5	c	0		0		5	0		100	s
H5	n	20	w	60	s	1	1	w	50	m
I5	n	x		0		x	x		x	
J5	c	5	w	0		10	1	m	80	m
K5	c	0		0		10	0		50	m
L5	n	10	w	0		5	x		x	
M5	n	0		0		5	0		50	m
A6	c	0		0		20	20	m	x	
B6	c	5	w	0		20	20	m	80	s
C6	m	0		5	s	10	15	s	90	s
D6	n	5	w	0		1	5	s	50	s
E6	n	20	m	x		5	x		x	
F6	n	10	w	0		1	0		100	s
G6	c	5	w	5	s	20	5	m	90	m

H6	c	5	w	5	s	20	1	m	90	s
I6	c	0		5	s	10	x		80	s
J6	c	10	w	10	s	30	1	m	80	s
K6	n	50	w	0		5	1	w	70	s
L6	n	60	w	10	s	0	0		100	s
M6	c	0		0		1	0		100	s
A7	c	0		0		1	0		x	
B7	c	0		0		1	0		100	s
C7	c	0		1	s	1	0		100	s
D7	n	x		x		x	x		x	
E7	n	0		0		0	0		100	s
F7	c	0		0		0	0		x	
G7	m	0		0		0	0		50	s
H7	n	0		0		5	0		50	w
I7	n	0		0		0	0		100	s
J7	c	x		0		0	0		x	
K7	m	0		1	s	5	1	w	100	m
L7	n	20	w	1	s	1	0		80	m
M7	n	5	w	0		5	1	w	60	s
A8	c	5	m	5	s	10	5	s	x	
B8	c	5	s	10	s	5	5	s	90	s
C8	x	x		x		x	x		x	
D8	c	0		10	s	5	1	m	100	s
E8	n	0		0		1	0		100	s
F8	c	0		5	s	5	0		100	s
G8	n	10	w	0		1	1	s	70	s
H8	n	0		0		1	0		80	m
I8	c	0		5	s	10	10	m	90	m
J8	c	10	w	5	s	5	10	m	80	s
K8	n	x		x		x	x		x	
L8	c	0		0		5	1	w	90	s
M8	c	0		x		5	0		50	s
A9	c	0		0		5	0		x	
B9	c	0		0		x	0		70	s
C9	c	0		0		5	1	m	80	s
D9	c	0		0		5	0		90	s
E9	c	x		0		5	0		90	s

F9	c	0		5	s	10	0		100	s
G9	n	x		x		x	0		x	
H9	n	20	w	0		0	0		60	s
I9	c	5	w	20	s	1	0		80	s
J9	c	0		10	s	5	5	m	90	m
K9	c	0		10	s	10	5	m	100	s
L9	c	0		20	s	10	0		100	s
M9	n	5	w	0		1	0		50	s
A10	n	5	w	0		5	1	w	x	
B10	c	0		0		15	0		70	s
C10	c	0		0		10	0		100	s
D10	c	0		0		1	0		100	s
E10	c	0		0		0	0		100	s
F10	n	0		0		0	0		100	s
G10	n	0		0		1	0		90	s
H10	c	x		0		10	x		x	
I10	c	0		0		5	1	m	50	s
J10	c	0		5	s	15	15	m	80	s
K10	c	5	m	5	s	15	10	m	60	s
L10	n	5	w	0		1	0		60	s
M10	n	x		x		x	x		x	
A11	n	20	w	x		1	0		x	
B11	n	20	w	0		5	0		100	s
C11	c	0		0		1	0		80	s
D11	n	x		0		0	x		50	s
E11	n	x		x		x	x		x	
F11	m	10	w	0		5	0		100	s
G11	m	5	w	0		1	0		100	s
H11	n	5	w	0		5	0		90	s
I11	n	5	w	0		5	0		80	s
J11	pin	0		0		10	5	m	80	m
K11	pin	10	w	0		10	5	m	50	m
L11	n	x		x		x	10	m	x	
M11	c	10	w	5	s	10	5	m	60	s
A12	n	x		x		x	x		x	
B12	c	70	w	1	m	10	5	m	100	s
C12	c	80	w	5	m	10	5	w	90	s

D12	c	0		0		5	10	m	90	s
E12	c	5	w	0		10	10	m	80	s
F12	m	10	w	0		1	15	m	80	s
G12	c	0		0		10	5	s	90	s
H12	n	x		x		x	x		x	
I12	x	x		x		x	0		x	
J12	n	0		0		0	0		100	s
K12	c	x		x		0	0		x	
L12	m	5	w	1	s	10	0		100	s
M12	n	x		x		x	x		x	
A13	c	0		0		1	0		100	s
B13	n	5	w	0		0	0		60	s
C13	n	5	w	0		0	0		80	s
D13	pin	0		0		1	0		50	s
E13	c	0		5	m	1	1	w	40	m
F13	n	0		0		1	0		50	s
G13	n	0		0		1	0		100	s
H13	n	0		0		1	0		50	s
I13	n	0		5	m	0	0		80	s
J13	c	x		0		1	0		60	s
K13	c	0		0		1	1	m	90	s
L13	c	30	w	0		5	5	m	60	s
M13	c	10	w	0		5	0		70	s
A14	n	x		0		1	0		70	s
B14	c	0		0		1	0		90	s
C14	c	0		20	s	5	0		80	s
D14	m	0		10	s	5	0		90	s
E14	x	x		x		x	x		x	
F14	n	0		0		0	0		100	s
G14	c	0		5	s	10	5	m	100	s
H14	c	0		5	s	10	0		100	s
I14	c	0		1	s	1	1	w	100	s
J14	c	0		0		1	5	w	100	s
K14	n	0		0		0	0		60	m
L14	n	0		0		0	0		80	m
M14	c	0		0		5	0		100	s
A15	c	0		0		5	0		100	s

B15	c	0		1	m	5	0		100	s
C15	n	0		0		5	0		50	m
D15	n	0		0		1	0		90	s
E15	m	0		0		5	5	m	100	s
F15	c	0		1	s	5	5	m	100	s
G15	c	0		0		20	1	m	70	s
H15	m	0		0		10	0		70	s
I15	n	10	w	0		1	0		80	s
J15	n	x		0		0	0		60	s
K15	n	0		0		0	0		90	s
L15	n	0		0		5	0		100	s
M15	n	5	w	0		5	1	w	70	s
A16	m	0		0		15	5	w	90	s
B16	c	0		0		20	1	w	80	s
C16	n	x		0		0	x		x	
D16	n	0		0		1	0		60	s
E16	n	0		0		0	0		60	s
F16	c	0		1	s	5	0		90	s
G16	c	0		0		5	0		50	s
H16	c	0		0		10	0		80	s
I16	c	0		0		10	0		60	s
J16	n	x		0		0	0		100	s
K16	n	0		0		0	0		100	s
L16	c	0		0		10	1	w	80	s
M16	c	0		0		5	1	w	80	m
A17	c	0		0		15	5	m	70	s
B17	c	0		1	s	20	5	m	60	m
C17	c	5	w	1	s	0	5	m	50	m
D17	c	0		0		5	10	m	50	m
E17	c	0		1	m	1	0		70	s
F17	c	0		0		5	1	w	40	m
G17	n	5	w	0		0	0		80	s
H17	n	x		0		0	0		70	s
I17									x	

Transatlantic Prostate Cancer Tissue Microarray Data 45-03 BSc Project 2006										
ID	Designation (C/N)	Rb%stain	Rb intensity	p21%stain	p21 intensity	Ki67%stain	p53 % stain	p53 intensity	MDM-2 % stain	MDM-2 intensity
A1	c	0		0		0	1	w	40	m
B1	c	50	w	1	m	0	1	w	50	m
C1						x				
D1						x				
E1										
F1										
G1										
H1										
I1										
J1										
K1										
L1										
M1										
A2										
B2										
C2										
D2										
E2										
F2										
G2										
H2										
I2										
J2										
K2										
L2										
M2										
A3	c	30	w	0		0	1	w	50	m
B3	c	0		0		5	0		100	s
C3		x		x		x	x		x	
D3										
E3										
F3										
G3										
H3										
I3										
J3										
K3										
L3										
M3										
A4	c	0		1	m	5	0		100	s

B4	n	30	w	0		0	1	w	100	s
C4	n	20	w	0		0	1	w	60	s
D4	c	0		0		5	1	w	100	s
E4	x	x		x		x	x		x	
F4	c	0		0		5	0		70	s
G4	c	0		0		5	0		80	s
H4	n	10	w	5	s	1	1	w	80	s
I4	n	x		x		x	x		x	
J4	c	0		0		5	0		90	s
K4	c	0		0		5	0		100	s
L4	n	0		5	s	1	0		60	s
M4	n	5	w	x		1	0		40	m
A5	c	x		0		x	x		x	
B5										
C5										
D5										
E5										
F5										
G5										
H5										
I5										
J5										
K5										
L5										
M5										
A6	c	5	w	0		0	0		70	s
B6	c	0		0		0	0		80	s
C6	c	0		0		0	0		100	s
D6	n	0		0		0	0		100	s
E6	n	5	w	0		0	0		70	s
F6	c	0		5	s	0	1	m	60	m
G6	c	0		1	s	1	0		60	m
H6	c	0		0		1	0		60	s
I6	c	0		0		0	0		50	s
J6	n	5	w	0		0	1	m	30	s
K6	n	5	w	0		0	1	w	x	
L6	c	x		0		x	0		x	
M6	c	0		x		5	0		20	m
A7	x	x		x		x	x		x	
B7	n	x		x		x	x		x	
C7	c	0		0		1	0		80	s
D7	m	0		0		1	0		60	s

E7	n	5	w	0		0	0		50	s
F7	n	5	w	0		0	0		50	s
G7	c	5	w	x		5	0		40	s
H7	c	0		1	s	5	0		70	s
I7	c	5	w	0		5	0		70	s
J7	c	5	w	0		5	1	w	80	s
K7	n	5	w	10	m	1	1	w	x	
L7	n	0		0		1	1	w	50	s
M7	c	0		0		0	0		0	
A8	x	x		x		x	x		0	
B8	c	0		0		0	0		0	
C8	c	0		0		0	0		0	
D8	c	5	w	1	s	5	0		x	
E8	c	0		1	s	5	0		20	m
F8	n	10	w	0		0	0		0	
G8	n	5	w	0		0	0		x	
H8	m	0		0		0	0		50	s
I8	c	0		1	m	0	5	w	60	s
J8	c	0		0		20	0		80	s
K8	m	0		0		20	1	w	50	s
L8	c	0		0		1	0		60	s
M8	c	0		x		0	0		80	s
A9	n	x		x		0	0		80	s
B9	n	0		0		0	0		80	s
C9	c	0		0		1	0		50	m
D9	c	0		0		0	0		40	m
E9	n	0		0		1	0		30	s
F9	n	0		0		0	0		40	s
G9	c	0		0		5	0		80	s
H9	c	5	w	0		5	1	w	90	s
I9	n	5	w	0		0	x		100	s
J9	n	x		x		x	x		100	s
K9	c	0		0		5	0		100	s
L9	c	0		0		0	0		100	s
M9	c	0		0		1	0		100	s
A10	c	0		0		10	1	w	100	s
B10	n	10	w	0		5	1	w	80	s
C10	n	x		x		x	0		60	s
D10	c	10	w	0		5	15	s	90	s
E10	c	0		0		1	5	s	80	s
F10	m	0		0		1	5	m	100	s
G10	n	20	w	0		0	1	w	x	

H10	n	10	w	0		1	1	w	100	s
I10	n	5	w	0		5	0		60	s
J10	c	0		0		10	0		100	s
K10	c	0		0		20	0		100	s
L10	c	0		0		15	0		x	
M10	c	0		0		10	0		90	s
A11	n	0		0		1	1	w	80	s
B11	n	x		x		x	x		x	
C11	n	0		0		1	0		x	
D11	n	0		0		0	x		50	s
E11	n	5	w	0		0	0		50	s
F11	n	0		0		0	0		50	s
G11	m	0		5	s	30	0		80	s
H11	m	0		5	s	30	0		80	s
I11	m	0		5	s	30	0		90	s
J11	n	50	w	0		1	0		60	s
K11	n	20	w	0		1	1	w	50	s
L11	c	0		5	s	5	80	s	100	s
M11	n	0		0		1	30	s	x	
A12	m	5	w	5	s	10	100	s	100	s
B12	c	5	w	1	s	5	50	s	90	s
C12	n	x		x		x	0		100	s
D12	n	60	w	0		1	0		100	s
E12	c	0		0		0	0		0	
F12	c	0		0		0	0		0	
G12	c	0		0		0	0		0	
H12	c	0		0		0	0		0	
I12	n	0		0		0	0		x	
J12	n	0		0		0	0		x	
K12	pin	0		0		10	0		50	m
L12	pin	0		0		10	0		50	m
M12	n	0		0		x	x		x	
A13	x	x		0		x	x		x	
B13	n	x		x		x	1	w	60	s
C13	n	0		0		0	0		x	
D13	n	20	w	0		1	0		100	s
E13	c	0		0		1	0		90	s
F13	n	0		0		1	0		x	
G13	n	5	w	0		1	1	w	90	s
H13	c	0		0		1	1	w	100	s
I13	c	5	w	0		5	1	w	100	s
J13	n	20	w	0		0	0		100	s

K13	n	20	m	0		0	0		100	s
L13	c	0		0		0	0		0	
M13	c	0		0		1	0		x	
A14	c	0		0		0	0		20	s
B14	c	0		0		0	0		0	
C14	n	0		0		0	0		0	
D14	n	0		0		0	0		0	
E14	c	0		0		1	0		20	m
F14	c	0		0		0	0		30	m
G14	c	0		0		0	0		10	m
H14	c	0		0		1	0		30	m
I14	n	0		0		0	0		x	
J14	c	0		0		0	0		x	
K14	c	0		0		0	0		20	m
L14	c	0		0		5	0		40	m
M14		x		x		x	x		x	
A15	x	x		x		x	x		x	
B15	n	0		0		0	0		x	
C15		x		x		x	x		x	
D15	n	0		0		0	0		50	m
E15	c	x		x		x	0		10	w
F15	c	5	w	1	m	0	0		0	
G15	c	0		5	m	0	0		50	m
H15	c	0		1	m	0	0		10	w
I15	n	0		0		0	0		30	m
J15	n	5	w	0		0	0		50	m
K15	x	x		x		x	x		x	
L15	c	0		1	s	1	0		70	s
M15	c	0		0		5	1	m	80	s
A16	c	0		0		1	0		100	s
B16	n	0		0		0	0		80	s
C16	n	x		0		0	0		100	s
D16	c	0		10	s	5	1	w	x	
E16	n	0		0		1	0		x	
F16	x	x		0		x	x		x	
G16	c	0		0		10	5	m	x	
H16	n	x		0		x	x		x	
I16	c	5	w	0		0	0		50	m
J16	c	0		0		1	0		50	m
K16	c	0		0		0	5	m	80	s
L16	c	0		10	m	1	15	m	60	m
M16	n	0		0		0	0		30	s

A17	n	0		0		0	0		x	
B17	c	0		0		5	0		80	s
C17	c	0		0		0	0		80	s
D17	c	0		1	s	0	0		70	m
E17	c	0		0		0	1	m	70	m
F17	n	x		0		0	x		x	
G17	n	0		0		0	0		x	
H17	c	0		0		0	0		x	
I17	c	0		0		0	0		20	s
J17	c	0		0		0	0		30	s
K17	c	0		0		0	0		60	s
L17	n	0		0		0	x		40	s
M17	n	0		0		0	0		30	s
A18	m	0		0		0	0		80	s
B18	n	0		0		0	0		50	s
C18	c	0		0		0	0		x	
D18	m	0		0		0	0		x	
E18	n	0		0		0	0		30	m
F18	n	0		0		0	0		20	m

Transatlantic Prostate Cancer Tissue Microarray Data 51-02 BSc Project 2006										
ID	Designation (C/N)	Rb%stain	Rb intensity	p21%stain	p21 intensity	Ki67%stain	p53 % stain	p53 intensity	MDM-2 % stain	MDM-2 intensity
A1	c	0		0		x	0		90	s
B1	m	10	w	0		x	1	m	60	s
C1										
D1										
E1										
F1										
G1										
H1										
I1										
J1										
K1										
L1	n			0		1	0		70	s
M1	c			0		5	0		90	s
A2		x		x		x	x		x	
B2		x		x		x	x		x	
C2	c	0		0		5	0		90	s
D2	c	0		0		1	0		100	s
E2		x		x		x	x		x	
F2	c	0		0		5	0		100	s
G2	n	30	m	0		5	0		60	s
H2	n	x		0		1	0		60	s
I2	m	0		0		1	0		50	s
J2	m	0		0		1	0		70	s
K2	n	10	w	0		5	0		80	s
L2		x		x		x	x		x	
M2		x		x		x	x		x	
A3	n	20	w	x		0	0		80	s
B3	c	0		1	s	5	0		80	s
C3	c	0		0		5	0		90	s
D3	c	0		0		5	0		90	s
E3	m	0		0		5	0		90	s
F3	n	5	w	0		5	0		90	s
G3	n	0		0		1	0		60	s
H3										
I3										
J3										
K3										
L3										
M3										
A4	c	0		0		1	0		100	s

B4	c	0		0		1	0		100	s
C4	n	0		0		1	0		100	s
D4	n	5	w	0		1	0		50	s
E4	c	5	w	0		10	1	w	90	s
F4	c	5	w	0		5	1	w	100	s
G4	n	x		0		x	x		100	s
H4	n	5	w	0		1	0		90	s
I4	n	0		0		1	0		80	s
J4	c	0		0		5	0		90	s
K4	n	0		0		0	1	w	80	s
L4	n	20	w	0		1	0		90	s
M4	m	10	w	1	w	5	0		80	s
A5	c	0		0		5	0		90	s
B5	c	0		0		10	0		90	s
C5	n	10	w	0		1	0		70	s
D5	n	0		0		1	0		70	s
E5	n	0		0		1	0		80	s
F5	c	0		0		10	0		90	s
G5	c	10	w	0		5	0		90	s
H5	n	20	w	0		1	0		80	s
I5	n	10	w	0		5	0		60	s
J5	x	x		x		x	x		x	
K5	c	5	w	5	s	5	1	w	90	s
L5	c	0		0		5	1	w	80	s
M5	c	0		0		5	0		90	s
A6	n	10	w	x		x	0		x	
B6	n	20	w	0		1	0		90	s
C6	x	x		x		x	x		x	
D6	c	5	s	0		20	0		10	m
E6	c	10	s	1	m	20	0		30	m
F6	c	10	s	5	s	20	1	w	40	m
G6	n	x		x		x	x		x	
H6	n	x		x		x	x		x	
I6	x	x		x		x	x		x	
J6	x	x		x		x	x		x	
K6	c	0		20	s	20	20	s	70	s
L6	x	x		x		x	x		x	
M6	x	x		x		x	x		x	
A7	c	0		0		10	0		100	s
B7	n	x		x		x	x		x	
C7	n	x		0		1	0		100	s
D7	c	0		1	s	5	0		100	s

E7	c	0		0		5	0		100	s
F7	c	10	w	1	s	5	0		80	s
G7	c	5	w	5	s	5	0		40	s
H7	x	x		x		x	x		x	
I7	n	70	m	0		1	0		80	s
J7	c	0		5	s	5	0		100	s
K7	x	x		x		x	x		x	
L7	c	0		1	s	10	1	m	100	s
M7	c	5	w	5	s	5	1	s	100	s
A8	n	10	w	0		0	x		80	s
B8	n	x		0		0	x		90	s
C8	x	x		x		x	x		x	
D8	n	x		0		1	x		90	s
E8	c	0		0		5	5	s	70	s
F8	c	0		0		5	5	m	60	m
G8	n	20	w	0		1	0		80	s
H8	n	50	m	0		1	0		90	s
I8	c	0		0		1	0		90	s
J8	n	0		0		5	0		90	s
K8	n	0		0		5	0		90	s
L8	c	60	s	40	s	50	20	s	90	s
M8	x	60	s	30	s	50	20	s	90	s
A9	n	5	w	0		5	0		60	s
B9	n	x		x		x	x		x	
C9	n	x		x		x	x		x	
D9	n	x		x		x	x		x	
E9	m	x		0		5	0		80	s
F9	n	x		0		x	x		x	
G9	n	x		0		x	x		x	
H9	c	5	m	0		10	1	m	90	s
I9	c	0		0		5	1	m	90	s
J9	c	5	w	0		5	5	m	90	s
K9	c	0		0		5	1	m	80	s
L9	n	0		0		1	0		70	s
M9	c	0		0		15	0		100	s
A10	c	0		0		10	0		100	s
B10	x	x		0		5	x		x	
C10	c	0		0		10	0		100	s
D10	n	30	w	0		1	0		80	s
E10	n	70	w	0		0	0		90	s
F10	c	5	w	0		30	1	w	80	s
G10	c	5	w	1	s	25	1	w	80	s

H10	c	5	w	20	s	40	0		90	s
I10	c	0		10	s	30	1	w	90	s
J10	n	50	m	x		x	0		x	
K10	x	x		x		x	x		x	
L10	n	x		x		x	x		x	
M10	n	50	w	0		1	0		60	s
A11	n	20	w	0		5	0		60	s
B11	n	10	w	0		5	0		90	s
C11	c	5	w	0		10	0		80	s
D11	c	0		0		5	0		70	s
E11	c	0		0		5	0		30	s
F11	n	0		0		x	0		x	
G11	n	0		0		0	0		50	s
H11	c	0		0		10	10	m	50	m
I11	c	0		0		5	10	m	60	s
J11	c	0		1	s	5	0		50	m
K11	c	0		0		5	0		50	s
L11	c	0		1	s	5	1	w	100	s
M11	c	0		1	s	5	1	w	90	s
A12	c	0		0		5	0		90	s
B12	c	0		0		5	0		100	s
C12	m	0		0		0	0		x	
D12	x	x		x		x	x		x	
E12	c	5	w	0		10	1	m	80	s
F12	x	x		x		x	x		x	
G12	c	0		0		5	0		80	s
H12	n	x		x		x	x		x	
I12	c	0		0		1	0		100	s
J12	c	0		0		1	0		100	s
K12	n	10	w	0		5	0		80	s
L12	n	20	w	0		1	0		90	s
M12	c	0		0		15	80	s	100	s
A13	x	x		x		x	x		x	
B13	c	10	w	0		10	50	s	70	s
C13	c	x		0		x	80	s	x	
D13	n	x		0		1	x		x	
E13	n	x		0		5	0		x	
F13	c	0		0		5	0		40	s
G13	x	x		x		x	x		x	
H13	x	x		0		1	x		60	s
I13	c	0		5	s	20	5	m	90	s
J13	c	0		1	s	20	5	m	40	m

K13	c	0		20	s	20	5	s	50	m
L13	c	0		5	s	20	1	w	70	m
M13	m	20	w	0		1	0		90	s
A14	c	0		10	s	10	5	m	70	s
B14	m	10	w	0		5	0		70	s
C14	c	0		0		10	0		90	s
D14	m	10	w	0		1	1	w	60	s
E14	m	10	w	0		10	1	w	60	s
F14	n	30	w	0		1	0		100	s
G14	n	20	w	0		0	0		100	s
H14	c	10	w	0		10	0		60	s
I14	c	0		0		10	x		70	s
J14	n	x		x		0	x		x	
K14	n	x		x		x	x		x	
L14	x	x		x		x	x		x	
M14	x	x		0		10	x		x	
A15	n	0		0		1	0		x	
B15	n	x		x		x	x		x	
C15	x			x		x	x		x	
D15	x			x		x	x		x	

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