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Abstract: Background: External beam radiotherapy is delivered using a uniform fractionation schedule for localised prostate tumours, individualising fractionation according to tumour biology could improve outcomes. Additionally recurrence rates following radiotherapy vary considerably, better prognostic markers could improve treatment stratification. This study assessed if the cellular proliferation marker Ki67 provides prognostic information and predicts response to radiotherapy fractionation in patients participating in \*\*\*\*, a randomised trial of three radiotherapy fractionation schedules (74Gy/37f vs 60Gy/20f vs 57Gy/19f).

Methods: A matched case:control study design was used, patients with biochemical/clinical failure >2 years after radiotherapy (BCR) were matched 1:1 to patients without recurrence using established prognostic factors (Gleason score, PSA, tumour-stage) and fractionation schedule. Immunohistochemistry was used to stain diagnostic biopsy specimens for Ki67, which were scored using the unweighted global method. Conditional logistic regression models estimated the prognostic value of mean and maximum Ki67 scores on BCR risk. Biomarker-fractionation interaction terms determined whether Ki67 was predictive of BCR by fractionation.

Results: Using 173 matched pairs, the median for mean and maximum Ki67 scores were 6.6% (IQR:3.9-9.8) and 11.0% (IQR:7.0-15.0) respectively. Both scores were significant predictors of BCR in models adjusted for established prognostic factors. Conditioning on matching variables and age, the odds of BCR was estimated to increase by 9% per 1% increase in mean Ki67 score (OR=1.09, 95%CI:1.04-1.15,p=0.001). Interaction terms between Ki67 and fractionation schedules were not statistically significant.

Conclusions: Diagnostic Ki67 did not predict BCR according to fractionation schedule in \*\*\*, however it was a strong independent prognostic factor for BCR.

Suggested Reviewers:

Opposed Reviewers:

Professor Anthony Zietman  
Editor-in-Chief  
IJROBP

Dear Professor Zietman

*Re: Ki67 is an independent predictor of recurrence in the largest randomised trial of 3 radiation fractionation schedules in localised prostate cancer*

We are very grateful for your consideration of the above manuscript for publication in *the IJROBP*. This manuscript evaluates the well-established proliferation marker Ki67 in localised prostate cancer, for the first time using methodology that has been internationally validated and accounts for spatial intra-tumoural heterogeneity. The manuscript assesses both the association of Ki67 with overall risk of recurrence after radiotherapy, and risk of recurrence according to radiotherapy fractionation schedule.

The CHHiP trial is the largest randomised trial in localised prostate cancer to compare different radiation schedules reported to date. It therefore represents a unique opportunity to research biomarkers related to personalised fractionation. Our results offer important reassurance that shorter, more convenient hypofractionated schedules are not detrimental in tumours with relatively high proliferation.

In addition, Ki67 predicted recurrence independently of established prognostic factors including Gleason score. This routinely available and affordable test could therefore aid treatment stratification in patients with localised prostate cancer, for example by intensification of androgen deprivation treatment such as with abiraterone or docetaxel, especially as highly proliferative tumours tend to respond better to cytotoxic chemotherapy.

Thank you very much for your kind consideration of this manuscript that we believe has important translational relevance and will be of interest to your readership.

Kind regards

Dr Navita Somaiah

Professor Anthony Zietman  
Editor-in-Chief  
IJROBP

Dear Professor Zietman

*Re: Ki67 is an independent predictor of recurrence in the largest randomised trial of 3 radiation fractionation schedules in localised prostate cancer*

Thank you for your email dated 24.12.2017. We have uploaded the missing tables and apologise for the omission.

As no changes have been made to the actual blinded manuscript, we have uploaded two copies without tracked changes (as tracked and clean versions required by the system).

Kind regards

Dr Navita Somaiah

Title page

**Ki67 is an independent predictor of recurrence in the largest randomised trial of 3 radiation fractionation schedules in localised prostate cancer**

**Running title:** Ki67 to predict recurrence and fraction sensitivity

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**Conflicts of interest:** A declaration from each author has been completed

## **Contributors**

ACW – study design, acquisition of data, scoring, analysis and interpretation of data; drafting and revising manuscript; final approval of submitted version

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CC - study design, interpretation of data; final approval of submitted version

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## Summary

Radiotherapy is delivered using uniform fractionation for localised prostate tumours despite varying recurrence rates. Biomarkers to guide treatment stratification and predict fraction size sensitivity are needed. This study evaluated Ki67 in localised prostate cancer, for the first time using an internationally validated methodology accounting for intra-tumoural heterogeneity. Ki67 did not predict recurrence according to fractionation, providing reassurance that hypofractionated schedules can be safely administered in highly proliferative tumours. Ki67 predicted biochemical/clinical recurrence independently of established prognostic factors including Gleason score.



## **Ki67 is an independent predictor of recurrence in the largest randomised trial of 3 radiation fractionation schedules in localised prostate cancer**

### **Abstract**

Background: External beam radiotherapy is delivered using a uniform fractionation schedule for localised prostate tumours, individualising fractionation according to tumour biology could improve outcomes. Additionally recurrence rates following radiotherapy vary considerably, better prognostic markers could improve treatment stratification. This study assessed if the cellular proliferation marker Ki67 provides prognostic information and predicts response to radiotherapy fractionation in patients participating in " ", a randomised trial of three radiotherapy fractionation schedules (74Gy/37f vs 60Gy/20f vs 57Gy/19f).

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Conditioning on matching variables and age, the odds of BCR was estimated to increase by 9% per 1% increase in mean Ki67 score (OR=1.09, 95%CI:1.04–1.15, $p=0.001$ ). Interaction terms between Ki67 and fractionation schedules were not statistically significant.

Conclusions: Diagnostic Ki67 did not predict BCR according to fractionation schedule in ", however it was a strong independent prognostic factor for BCR.

**Keywords:** radiation fractionation, Ki67, prediction of recurrence, prostate cancer

## Introduction

Prostate cancer (PCa) is the second most common cancer worldwide for males, more than 1.11 million new cases were diagnosed in 2012 [1]. In the developed world, increased PSA testing means that most patients are diagnosed with localised disease, for which external beam radiotherapy (EBRT), brachytherapy and prostatectomy are important radical treatment options.

Recurrence rates following EBRT for localised PCa vary considerably from approximately 10% to 40-50% [2,3]. Recurrences are inadequately predicted using current prognostic algorithms that incorporate Gleason grade, T-stage and presenting PSA. Identification of prognostic biomarkers to aid treatment stratification would therefore be clinically useful.

In addition, EBRT is delivered using a uniform fractionation schedule for all localised PCa i.e. a “one size fits all approach”. This is despite a wide variation in the biology of localised PCa [4], including proliferation rate [5]. A personalised approach to fractionation therefore offers considerable potential to improve therapeutic outcomes. Biomarkers predicting sensitivity to RT fraction size have recently been identified as a key area for radiobiological research [6].

There is a tight inverse association between the proliferative indices of normal tissues and fractionation sensitivity. Tissues with high proliferation indices such as gastro-intestinal mucosa and epidermis, are insensitive to fraction size. In contrast late-reacting normal tissues, such as kidney, have low proliferative indices and are very sensitive to fraction size [7,8]. This study tests the hypothesis that the same association between proliferative indices and fractionation sensitivity in normal tissues extends to localised PCa.

The “ trial (”) randomly assigned 3216 men to conventional fractionation (74 Gy in 37 fractions over 7.4 weeks) or one of two hypofractionated schedules (60 Gy in 20 fractions over 4 weeks or 57 Gy in 19 fractions over 3.8 weeks) [3]. Trans-“ is the main translational sub-study within ”, tissue blocks from over 2000 patients have been collected. It provides an excellent opportunity to test the above hypothesis. The expectation is that highly proliferative cancers will show insensitivity to fraction size and be more likely to relapse after the reduced total dose in hypofractionated (>2Gy) schedules. In contrast slowly proliferating tumours are expected to be sensitive to fraction size hence more likely to relapse after conventional fractionation (2Gy) schedules [7].

## **Materials and Methods**

### *Study design*

A matched case:control methodology was used to select study participants. The study was approved by the London Multi-centre Research Ethics Committee (04/MRE02/10) and the local ethics committees of all participating centres. Patients experiencing recurrence (cases) were matched 1:1 to patients without recurrence (controls). Matching criteria included fractionation schedule (74Gy/37f, 60Gy/20f or 57Gy/19f) and established prognostic factors including PSA (<10/10-20/>20ng/ml), Gleason grade (3+3/3+4/4+3/≥4+4) and T-stage (T1/T2/T3). All tissue samples were centrally reviewed by a specialist uropathologist (CMC), including assignment of Gleason grade according to recent ISUP and WHO recommendations [9,10]. The centrally assigned Gleason grade was used for matching.

### *Immunohistochemistry staining and scoring*

Full-face sections from the diagnostic biopsy blocks were used for immunohistochemistry staining. This decision followed a pilot study that demonstrated construction of tissue microarray, using the checkerboard technique [11], resulted in inadequate tumour cellularity (tables S1 and S2). Immunohistochemistry staining methods are outlined in the supplementary appendix. All slides were scored using bright field microscopy by two independent investigators blinded to recurrence status and fractionation schedule. The CK5/6 basal marker distinguished pre-invasive from invasive disease. Prostatic intra-epithelial neoplasia and intra-ductal carcinoma were not scored. A minimum of 100 tumour cells were required to score each case.

The unweighted global assessment of Ki67 developed by the International Ki67 Working Group was used to score all prostate biopsies [12,13]. This includes assessment of intra-tumoural spatial heterogeneity, which is well-recognised in localised PCa [14]. The global assessment has met pre-specified criteria for scoring reproducibility in an international phase III study using core biopsies of breast tumours [13]. It involves counting 100 tumour cells in up to 4 high power fields to derive a mean Ki67 score (figure 1). Fields are chosen following an assessment of overall heterogeneity in staining. The final mean Ki67 score consisted of the average of the two scoring investigator's mean Ki67 scores for each case. Maximum Ki67 was assessed by one investigator and consisted of the highest scoring individual field (figure 1). This was included because the highest proliferative tumour area may be important for radiotherapy response.

All cases with a discrepancy in initial mean Ki67 score >10% were re-scored [15]. Further rescores were carried out if the discrepancy remained >10%.

### *Study endpoints*

Mean and maximum Ki67 scores were evaluated. Recurrence was defined as patients with biochemical [16] or clinical failure after radiotherapy (BCR). Patients experiencing BCR within two years of radiotherapy commencement were excluded because they are more likely to have developed distant metastases than local recurrence due to radiotherapy failure [2]. All data pertaining to recurrence was taken from a “ data snapshot (11/09/2015) where median follow up was 62.4 months (IQR: 53.9-77.0). Non-recurrence was defined in patients with no evidence of BCR alive at the data snapshot.

### *Statistical analysis*

Agreement in Ki67 scores between the two scoring investigators was assessed using Bland-Altman plots to measure the difference between the scores versus the mean of the mean Ki67 scores [17]. The concordance correlation coefficient was used to quantify agreement. The difference in the mean Ki67 scores (mean and maximum) between the matched cases and controls by fractionation schedule was compared using paired t-tests.

Both Ki67 endpoints were analysed as continuous variables to maximise statistical power [18]. Multivariable conditional logistic regression models were fitted to estimate the prognostic value of Ki67 on the risk of BCR, using the entire Trans-“ case-control study cohort. To determine whether Ki67 predicted BCR by fractionation, a biomarker-fractionation interaction term was included. Three comparisons were undertaken to avoid confounding by different recurrence rates across trial arms (74Gy/37f versus 60Gy/20f, 74y/37f versus 57Gy/19f and 60Gy/20f versus 57Gy/19f). Based on an alpha of 0.017, we estimated a power of 75.5%,

74.8% and 70.0% to detect an interaction between each fractionation schedule and Ki67 respectively (table S4).

All statistical analysis was conducted using STATAv13.0 and R (version:i3863.3.3).

## **Results**

### *Patient characteristics*

437 cases were assessed by both scoring investigators. Ki67 scores were provided by both investigators in 400 cases, in 37 cases there was insufficient tumour present. The final matched dataset comprised 173 patients with BCR after start of radiotherapy (cases) and 173 patients without recurrence (controls). Matching was achieved to 100% of relevant criteria in all cases analysed. 54 patients were excluded as they did not have an appropriate match, these were usually controls with no available matching case. Table 1 shows the distribution of the matching variables and age for the controls and cases.

### *Agreement in Ki67 scores*

Of the total of 400 cases scored by both investigators, in 12 (3.0%) cases the difference in mean Ki67 between the two scoring investigators was  $\geq 10\%$  (IQR:11.8-15.1%). These were re-scored by both scoring investigators. All re-scores were within the required  $<10\%$  discrepancy.

Scatter plots comparing each scoring investigator's final scores, and Bland-Altman plots comparing the difference in final score versus the mean Ki67 are shown in figure 2 (original scores figure S1). The Bland-Altman plots indicate that the difference in score tended to increase as the mean Ki67 score increased. The overall

agreement was considered to be good with a concordance correlation coefficient of 0.74 (95% CI: 0.70-0.78,  $p<0.001$ ) for the final scores. For the original scores prior to rescore, the concordance correlation coefficient was 0.63 (95% CI: 0.58-0.68,  $p<0.001$ ).

#### *Prediction of biochemical/clinical recurrence*

Multivariable conditional logistic regression models using the entire Trans-“ case-control study sample showed that both mean Ki67 and maximum Ki67 were statistically significant predictors of BCR (tables 2 and S3). For each unit increase in mean Ki67 the odds of BCR is estimated to increase by 9% (OR=1.09, 95% CI: 1.04-1.15,  $p=0.001$ ) having adjusted for matching variables and age. It is clinically relevant that the prediction of recurrence by mean Ki67 is independent of Gleason grade. The lack of correlation between mean Ki67 and Gleason grade is also displayed in the box and whisker plot (figure 3). For the maximum Ki67, the odds of BCR were estimated to increase by 5% for each unit increase in the maximum Ki67 score (OR=1.05, 95% CI: 1.01-1.09,  $p=0.006$ ) in the multivariable model.

#### *Prediction of fraction sensitivity*

The interaction tests between either mean or maximum Ki67 and fractionation schedule was not statistically significant for all comparisons (table 3 and S3). The distribution of mean and maximum Ki67 scores according to fractionation schedule and recurrence status, including a statistical comparison of the difference between cases and controls within fractionation arms is shown in figure S2.

## **Discussion**



This study measures Ki67 staining indices in localised PCa treated with different radiotherapy fractionation schedules. It indicates that the global unweighted method for scoring Ki67 can be used with good agreement between independent scoring investigators without prior experience of this method. To our knowledge this is the first report using the global unweighted method in PCa. However it is an established method to aid treatment stratification in breast cancer [19] where Ki67 is used clinically to distinguish between low proliferation luminal A and higher proliferative luminal B breast cancer subtypes [20].

The statistically significant association between mean Ki67 and prediction of BCR has potential clinical application. Our results require external validation in additional patient cohorts, with particular attention to the spectrum of Ki67 expression in different risk groups and a rigorous assessment of scoring concordance in prostate biopsies across different centres. Patients with high mean Ki67 but otherwise lower risk factors could be recommended longer or more intensive androgen deprivation (ADT), with possible addition of Docetaxel or abiraterone [21]. Patients with low mean Ki67 could be reassured that they are likely to have a good prognosis and might be candidates for studies of reduced ADT. This study suggests that Ki67 is of maximal predictive benefit when used as a continuous variable, this method of stratification is used effectively in the clinic for Ki67, and other expression profiling-based algorithms [19,22].

Our exclusion of patients with BCR less than two years after radiotherapy means the estimates of the predictive value of Ki67 are likely to be conservative. Maximum Ki67 was also a statistically significant predictor of BCR which is worthy of further study as a single field assessment of 100 cells is quicker than 4 fields for mean Ki67.

The apparent lack of interaction between Ki67 and fractionation schedule also has clinical implications. The range of proliferative indices seen indicates that, the predominantly intermediate risk, PCa included in the trial are usually slowly proliferative. However when including those cancers showing relatively high proliferation rates, there was no suggestion of a detriment using hypofractionated radiotherapy schedules giving 3Gy/fraction. Other tumour types encompass wider ranges in proliferation and show higher average proliferation [7]. Our results should not be interpreted as demonstrating a general lack of association between proliferation and fraction sensitivity. An important confounding factor may be the complex interplay between fraction sensitivity and overall treatment time [23]. Additionally we acknowledge that the statistical power of tests of interaction are low, and that a relatively small proportion of high risk PCa were included in ”.

3112 of 3216 (96.7%) of patients recruited to “ were treated with ADT from just after their diagnostic biopsy until completion of radiotherapy. ADT may modulate fraction sensitivity as it can markedly reduce proliferation and affect repair of double stranded DNA breaks (dsDNA) [24]. ADT can also inhibit the cell cycle at the G1/S checkpoint as part of induction of senescence [25,26]. This inhibition could restrict use of dsDNA repair pathway homologous recombination, which operates exclusively in S and G2 and is thought to mediate resistance to fraction sensitivity [27,28]. Cells would instead rely on error prone non-homologous end joining which operates throughout the cell cycle and is important for fraction sensitivity [27,28]. In the PROFIT trial of radiotherapy fractionation, men did not receive ADT and outcomes were similar to “ [29]. This suggests that ADT does not have a major impact on average fraction sensitivity in PCa, however ADT may have confounded

the interaction between proliferation and BCR according to fractionation schedule in our study.

Our results are supported by a recent report by Pollack et al [30]. In this, a single cut-point (11.3%) was used to score Ki67, fractionation schedules differed to our study and there were fewer failure events. However Ki67 demonstrated independent prediction of prognosis and did not predict fraction sensitivity. It is relevant that Ki67 immunohistochemistry is routinely available and affordable for most pathology laboratories, and automated scoring algorithms are showing potential clinical applicability [31].

This study assessing Ki67 in patients treated with different radiotherapy fractionation schedules reaches two conclusions. Firstly, it does not suggest that there is a detriment to using hypofractionated radiotherapy schedules in PCa showing relatively high proliferation. Secondly, Ki67 is a highly statistically significant biomarker predicting recurrence, independent of established prognostic factors. As localised PCa shows diverse clinical outcomes, Ki67 has a potential clinical application to guide treatment stratification.

## **Titles and legends to figures**

**Figure 1:** Scoring Ki67 using the global unweighted method<sup>a</sup>.

<sup>a</sup>This case contained 50% high proliferation tumour and 50% low proliferation tumour therefore high power fields were selected for 2 highly proliferative areas and 2 low proliferative areas. In this case the number of positive staining cells were: field 1 39/100, field 2 33/100, field 3 5/100 and field 4 5/100. This gives a mean Ki67 score of 20.5% and a maximum Ki67 score of 39%.

**Figure 2:** A: Scatterplot showing concordance in final mean Ki67 between independent scoring investigators, B: Brand-Altman plot showing difference in final scores between investigator 1 and investigator 2 versus means scores.

**Figure 3:** Box and whisker plot showing relationship between Mean Ki67 and Gleason grade group.

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## **Ki67 is an independent predictor of recurrence in the largest randomised trial of 3 radiation fractionation schedules in localised prostate cancer**

### **Abstract**

Background: External beam radiotherapy is delivered using a uniform fractionation schedule for localised prostate tumours, individualising fractionation according to tumour biology could improve outcomes. Additionally recurrence rates following radiotherapy vary considerably, better prognostic markers could improve treatment stratification. This study assessed if the cellular proliferation marker Ki67 provides prognostic information and predicts response to radiotherapy fractionation in patients participating in " ", a randomised trial of three radiotherapy fractionation schedules (74Gy/37f vs 60Gy/20f vs 57Gy/19f).

Methods: A matched case:control study design was used, patients with biochemical/clinical failure >2 years after radiotherapy (BCR) were matched 1:1 to patients without recurrence using established prognostic factors (Gleason score, PSA, tumour-stage) and fractionation schedule. Immunohistochemistry was used to stain diagnostic biopsy specimens for Ki67, which were scored using the unweighted global method. Conditional logistic regression models estimated the prognostic value of mean and maximum Ki67 scores on BCR risk. Biomarker-fractionation interaction terms determined whether Ki67 was predictive of BCR by fractionation.

Results: Using 173 matched pairs, the median for mean and maximum Ki67 scores were 6.6% (IQR:3.9-9.8) and 11.0% (IQR:7.0-15.0) respectively. Both scores were significant predictors of BCR in models adjusted for established prognostic factors.



Conditioning on matching variables and age, the odds of BCR was estimated to increase by 9% per 1% increase in mean Ki67 score (OR=1.09, 95%CI:1.04–1.15, $p=0.001$ ). Interaction terms between Ki67 and fractionation schedules were not statistically significant.

Conclusions: Diagnostic Ki67 did not predict BCR according to fractionation schedule in ", however it was a strong independent prognostic factor for BCR.

**Keywords:** radiation fractionation, Ki67, prediction of recurrence, prostate cancer

## Introduction

Prostate cancer (PCa) is the second most common cancer worldwide for males, more than 1.11 million new cases were diagnosed in 2012 [1]. In the developed world, increased PSA testing means that most patients are diagnosed with localised disease, for which external beam radiotherapy (EBRT), brachytherapy and prostatectomy are important radical treatment options.

Recurrence rates following EBRT for localised PCa vary considerably from approximately 10% to 40-50% [2,3]. Recurrences are inadequately predicted using current prognostic algorithms that incorporate Gleason grade, T-stage and presenting PSA. Identification of prognostic biomarkers to aid treatment stratification would therefore be clinically useful.

In addition, EBRT is delivered using a uniform fractionation schedule for all localised PCa i.e. a “one size fits all approach”. This is despite a wide variation in the biology of localised PCa [4], including proliferation rate [5]. A personalised approach to fractionation therefore offers considerable potential to improve therapeutic outcomes. Biomarkers predicting sensitivity to RT fraction size have recently been identified as a key area for radiobiological research [6].

There is a tight inverse association between the proliferative indices of normal tissues and fractionation sensitivity. Tissues with high proliferation indices such as gastro-intestinal mucosa and epidermis, are insensitive to fraction size. In contrast late-reacting normal tissues, such as kidney, have low proliferative indices and are very sensitive to fraction size [7,8]. This study tests the hypothesis that the same association between proliferative indices and fractionation sensitivity in normal tissues extends to localised PCa.

The “ trial (”) randomly assigned 3216 men to conventional fractionation (74 Gy in 37 fractions over 7.4 weeks) or one of two hypofractionated schedules (60 Gy in 20 fractions over 4 weeks or 57 Gy in 19 fractions over 3.8 weeks) [3]. Trans-“ is the main translational sub-study within ”, tissue blocks from over 2000 patients have been collected. It provides an excellent opportunity to test the above hypothesis. The expectation is that highly proliferative cancers will show insensitivity to fraction size and be more likely to relapse after the reduced total dose in hypofractionated (>2Gy) schedules. In contrast slowly proliferating tumours are expected to be sensitive to fraction size hence more likely to relapse after conventional fractionation (2Gy) schedules [7].

## **Materials and Methods**

### *Study design*

A matched case:control methodology was used to select study participants. The study was approved by the London Multi-centre Research Ethics Committee (04/MRE02/10) and the local ethics committees of all participating centres. Patients experiencing recurrence (cases) were matched 1:1 to patients without recurrence (controls). Matching criteria included fractionation schedule (74Gy/37f, 60Gy/20f or 57Gy/19f) and established prognostic factors including PSA (<10/10-20/>20ng/ml), Gleason grade (3+3/3+4/4+3/≥4+4) and T-stage (T1/T2/T3). All tissue samples were centrally reviewed by a specialist uropathologist (CMC), including assignment of Gleason grade according to recent ISUP and WHO recommendations [9,10]. The centrally assigned Gleason grade was used for matching.

### *Immunohistochemistry staining and scoring*

Full-face sections from the diagnostic biopsy blocks were used for immunohistochemistry staining. This decision followed a pilot study that demonstrated construction of tissue microarray, using the checkerboard technique [11], resulted in inadequate tumour cellularity (tables S1 and S2). Immunohistochemistry staining methods are outlined in the supplementary appendix. All slides were scored using bright field microscopy by two independent investigators blinded to recurrence status and fractionation schedule. The CK5/6 basal marker distinguished pre-invasive from invasive disease. Prostatic intra-epithelial neoplasia and intra-ductal carcinoma were not scored. A minimum of 100 tumour cells were required to score each case.

The unweighted global assessment of Ki67 developed by the International Ki67 Working Group was used to score all prostate biopsies [12,13]. This includes assessment of intra-tumoural spatial heterogeneity, which is well-recognised in localised PCa [14]. The global assessment has met pre-specified criteria for scoring reproducibility in an international phase III study using core biopsies of breast tumours [13]. It involves counting 100 tumour cells in up to 4 high power fields to derive a mean Ki67 score (figure 1). Fields are chosen following an assessment of overall heterogeneity in staining. The final mean Ki67 score consisted of the average of the two scoring investigator's mean Ki67 scores for each case. Maximum Ki67 was assessed by one investigator and consisted of the highest scoring individual field (figure 1). This was included because the highest proliferative tumour area may be important for radiotherapy response.

All cases with a discrepancy in initial mean Ki67 score >10% were re-scored [15]. Further rescores were carried out if the discrepancy remained >10%.

### *Study endpoints*

Mean and maximum Ki67 scores were evaluated. Recurrence was defined as patients with biochemical [16] or clinical failure after radiotherapy (BCR). Patients experiencing BCR within two years of radiotherapy commencement were excluded because they are more likely to have developed distant metastases than local recurrence due to radiotherapy failure [2]. All data pertaining to recurrence was taken from a “ data snapshot (11/09/2015) where median follow up was 62.4 months (IQR: 53.9-77.0). Non-recurrence was defined in patients with no evidence of BCR alive at the data snapshot.

### *Statistical analysis*

Agreement in Ki67 scores between the two scoring investigators was assessed using Bland-Altman plots to measure the difference between the scores versus the mean of the mean Ki67 scores [17]. The concordance correlation coefficient was used to quantify agreement. The difference in the mean Ki67 scores (mean and maximum) between the matched cases and controls by fractionation schedule was compared using paired t-tests.

Both Ki67 endpoints were analysed as continuous variables to maximise statistical power [18]. Multivariable conditional logistic regression models were fitted to estimate the prognostic value of Ki67 on the risk of BCR, using the entire Trans-“ case-control study cohort. To determine whether Ki67 predicted BCR by fractionation, a biomarker-fractionation interaction term was included. Three comparisons were undertaken to avoid confounding by different recurrence rates across trial arms (74Gy/37f versus 60Gy/20f, 74y/37f versus 57Gy/19f and 60Gy/20f versus 57Gy/19f). Based on an alpha of 0.017, we estimated a power of 75.5%,

74.8% and 70.0% to detect an interaction between each fractionation schedule and Ki67 respectively (table S4).

All statistical analysis was conducted using STATAv13.0 and R (version:i3863.3.3).

## **Results**

### *Patient characteristics*

437 cases were assessed by both scoring investigators. Ki67 scores were provided by both investigators in 400 cases, in 37 cases there was insufficient tumour present. The final matched dataset comprised 173 patients with BCR after start of radiotherapy (cases) and 173 patients without recurrence (controls). Matching was achieved to 100% of relevant criteria in all cases analysed. 54 patients were excluded as they did not have an appropriate match, these were usually controls with no available matching case. Table 1 shows the distribution of the matching variables and age for the controls and cases.

### *Agreement in Ki67 scores*

Of the total of 400 cases scored by both investigators, in 12 (3.0%) cases the difference in mean Ki67 between the two scoring investigators was  $\geq 10\%$  (IQR:11.8-15.1%). These were re-scored by both scoring investigators. All re-scores were within the required  $<10\%$  discrepancy.

Scatter plots comparing each scoring investigator's final scores, and Bland-Altman plots comparing the difference in final score versus the mean Ki67 are shown in figure 2 (original scores figure S1). The Bland-Altman plots indicate that the difference in score tended to increase as the mean Ki67 score increased. The overall

agreement was considered to be good with a concordance correlation coefficient of 0.74 (95% CI: 0.70-0.78,  $p<0.001$ ) for the final scores. For the original scores prior to rescore, the concordance correlation coefficient was 0.63 (95% CI: 0.58-0.68,  $p<0.001$ ).

#### *Prediction of biochemical/clinical recurrence*

Multivariable conditional logistic regression models using the entire Trans-“ case-control study sample showed that both mean Ki67 and maximum Ki67 were statistically significant predictors of BCR (tables 2 and S3). For each unit increase in mean Ki67 the odds of BCR is estimated to increase by 9% (OR=1.09, 95% CI: 1.04-1.15,  $p=0.001$ ) having adjusted for matching variables and age. It is clinically relevant that the prediction of recurrence by mean Ki67 is independent of Gleason grade. The lack of correlation between mean Ki67 and Gleason grade is also displayed in the box and whisker plot (figure 3). For the maximum Ki67, the odds of BCR were estimated to increase by 5% for each unit increase in the maximum Ki67 score (OR=1.05, 95% CI: 1.01-1.09,  $p=0.006$ ) in the multivariable model.

#### *Prediction of fraction sensitivity*

The interaction tests between either mean or maximum Ki67 and fractionation schedule was not statistically significant for all comparisons (table 3 and S3). The distribution of mean and maximum Ki67 scores according to fractionation schedule and recurrence status, including a statistical comparison of the difference between cases and controls within fractionation arms is shown in figure S2.

## **Discussion**

This study measures Ki67 staining indices in localised PCa treated with different radiotherapy fractionation schedules. It indicates that the global unweighted method for scoring Ki67 can be used with good agreement between independent scoring investigators without prior experience of this method. To our knowledge this is the first report using the global unweighted method in PCa. However it is an established method to aid treatment stratification in breast cancer [19] where Ki67 is used clinically to distinguish between low proliferation luminal A and higher proliferative luminal B breast cancer subtypes [20].

The statistically significant association between mean Ki67 and prediction of BCR has potential clinical application. Our results require external validation in additional patient cohorts, with particular attention to the spectrum of Ki67 expression in different risk groups and a rigorous assessment of scoring concordance in prostate biopsies across different centres. Patients with high mean Ki67 but otherwise lower risk factors could be recommended longer or more intensive androgen deprivation (ADT), with possible addition of Docetaxel or abiraterone [21]. Patients with low mean Ki67 could be reassured that they are likely to have a good prognosis and might be candidates for studies of reduced ADT. This study suggests that Ki67 is of maximal predictive benefit when used as a continuous variable, this method of stratification is used effectively in the clinic for Ki67, and other expression profiling-based algorithms [19,22].

Our exclusion of patients with BCR less than two years after radiotherapy means the estimates of the predictive value of Ki67 are likely to be conservative. Maximum Ki67 was also a statistically significant predictor of BCR which is worthy of further study as a single field assessment of 100 cells is quicker than 4 fields for mean Ki67.



The apparent lack of interaction between Ki67 and fractionation schedule also has clinical implications. The range of proliferative indices seen indicates that, the predominantly intermediate risk, PCa included in the trial are usually slowly proliferative. However when including those cancers showing relatively high proliferation rates, there was no suggestion of a detriment using hypofractionated radiotherapy schedules giving 3Gy/fraction. Other tumour types encompass wider ranges in proliferation and show higher average proliferation [7]. Our results should not be interpreted as demonstrating a general lack of association between proliferation and fraction sensitivity. An important confounding factor may be the complex interplay between fraction sensitivity and overall treatment time [23]. Additionally we acknowledge that the statistical power of tests of interaction are low, and that a relatively small proportion of high risk PCa were included in ”.

3112 of 3216 (96.7%) of patients recruited to “ were treated with ADT from just after their diagnostic biopsy until completion of radiotherapy. ADT may modulate fraction sensitivity as it can markedly reduce proliferation and affect repair of double stranded DNA breaks (dsDNA) [24]. ADT can also inhibit the cell cycle at the G1/S checkpoint as part of induction of senescence [25,26]. This inhibition could restrict use of dsDNA repair pathway homologous recombination, which operates exclusively in S and G2 and is thought to mediate resistance to fraction sensitivity [27,28]. Cells would instead rely on error prone non-homologous end joining which operates throughout the cell cycle and is important for fraction sensitivity [27,28]. In the PROFIT trial of radiotherapy fractionation, men did not receive ADT and outcomes were similar to “ [29]. This suggests that ADT does not have a major impact on average fraction sensitivity in PCa, however ADT may have confounded

the interaction between proliferation and BCR according to fractionation schedule in our study.

Our results are supported by a recent report by Pollack et al [30]. In this, a single cut-point (11.3%) was used to score Ki67, fractionation schedules differed to our study and there were fewer failure events. However Ki67 demonstrated independent prediction of prognosis and did not predict fraction sensitivity. It is relevant that Ki67 immunohistochemistry is routinely available and affordable for most pathology laboratories, and automated scoring algorithms are showing potential clinical applicability [31].

This study assessing Ki67 in patients treated with different radiotherapy fractionation schedules reaches two conclusions. Firstly, it does not suggest that there is a detriment to using hypofractionated radiotherapy schedules in PCa showing relatively high proliferation. Secondly, Ki67 is a highly statistically significant biomarker predicting recurrence, independent of established prognostic factors. As localised PCa shows diverse clinical outcomes, Ki67 has a potential clinical application to guide treatment stratification.

## **Titles and legends to figures**

**Figure 1:** Scoring Ki67 using the global unweighted method<sup>a</sup>.

<sup>a</sup>This case contained 50% high proliferation tumour and 50% low proliferation tumour therefore high power fields were selected for 2 highly proliferative areas and 2 low proliferative areas. In this case the number of positive staining cells were: field 1 39/100, field 2 33/100, field 3 5/100 and field 4 5/100. This gives a mean Ki67 score of 20.5% and a maximum Ki67 score of 39%.

**Figure 2:** A: Scatterplot showing concordance in final mean Ki67 between independent scoring investigators, B: Brand-Altman plot showing difference in final scores between investigator 1 and investigator 2 versus means scores.

**Figure 3:** Box and whisker plot showing relationship between Mean Ki67 and Gleason grade group.

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Table 1. Distribution of the matching variables and age by fractionation schedules.

	74Gy (N = 116, 33.5 %)			60Gy (N = 104, 30.1 %)			57Gy (N = 126, 36.4 %)		
	Controls (N = 58)	Cases (N = 58)		Controls (N = 52)	Cases (N = 52)		Controls (N = 63)	Cases (N = 63)	
	N	N	%	N	N	%	N	N	%
<b>PSA</b>									
<10	17	17	29.3	20	20	38.5	33	33	52.4
10- & <20	37	37	63.8	27	27	51.9	26	26	41.3
20-	4	4	6.9	5	5	9.6	4	4	6.3
<b>Tumour stage</b>									
T1	11	11	19.0	8	8	15.4	20	20	31.7
T2	42	42	72.4	38	38	73.1	35	35	55.6
T3	5	5	8.6	6	6	11.5	8	8	12.7
<b>Gleason Score</b>									
≤ 6	6	6	10.3	5	5	9.6	8	8	12.7
3+4	31	31	53.4	30	30	57.7	30	30	47.6
4+3	16	16	27.6	10	10	19.2	15	15	23.8
≥ 8	5	5	8.6	7	7	13.5	10	10	15.9
<b>Age at randomisation (years)</b>	<b>Mean (SD)</b>	<b>Mean (SD)</b>		<b>Mean (SD)</b>	<b>Mean (SD)</b>		<b>Mean (SD)</b>	<b>Mean (SD)</b>	
	69.4 (6.3)	69.8 (6.6)		68.9 (5.4)	68.0 (5.8)		70.1 (6.1)	68.4 (6.2)	
<b>PSA</b>	<b>Median (IQR)</b>	<b>Median (IQR)</b>		<b>Median (IQR)</b>	<b>Median (IQR)</b>		<b>Median (IQR)</b>	<b>Median (IQR)</b>	
	12 (8.9-15.8)	11.9 (9.1-16.2)		12.2 (8.6-15.1)	11.6 (8.6-18.1)		9.6 (7.3-13.0)	9.7 (7.2-15.0)	

**Table 2: Odds ratio for BCR estimated by multivariable conditional logistic regression models (n=346) using Ki67 as a continuous variable, for mean and maximum Ki67.**

<b>Ki67</b>			
<b>Biomarker</b>	<b>OR*</b>	<b>95 % CI</b>	<b>P value</b>
<b>mean Ki67 scores</b>	1.09	1.04 - 1.15	0.001
<b>max Ki67 scores</b>	1.05	1.01 - 1.09	0.006

\*Odds ratios (OR) are adjusted for the matching variables and age at randomisation.



**Table 3: Odds ratio for BCR estimated from multivariable conditional logistic regression models without and with interaction terms between the mean Ki67 scores and fractionation schedules.**

Schedules	Variable	OR	95 % CI (OR)	P value (OR)	P value for interaction*
<b>74 Gy &amp; 60 Gy</b>	mean Ki67	1.09	1.02 – 1.17	0.007	0.26
<b>74 Gy &amp; 57 Gy</b>	mean Ki67	1.07	1.01 – 1.14	0.03	0.59
<b>60 Gy &amp; 57 Gy</b>	mean Ki67	1.11	1.04 – 1.19	0.001	0.59

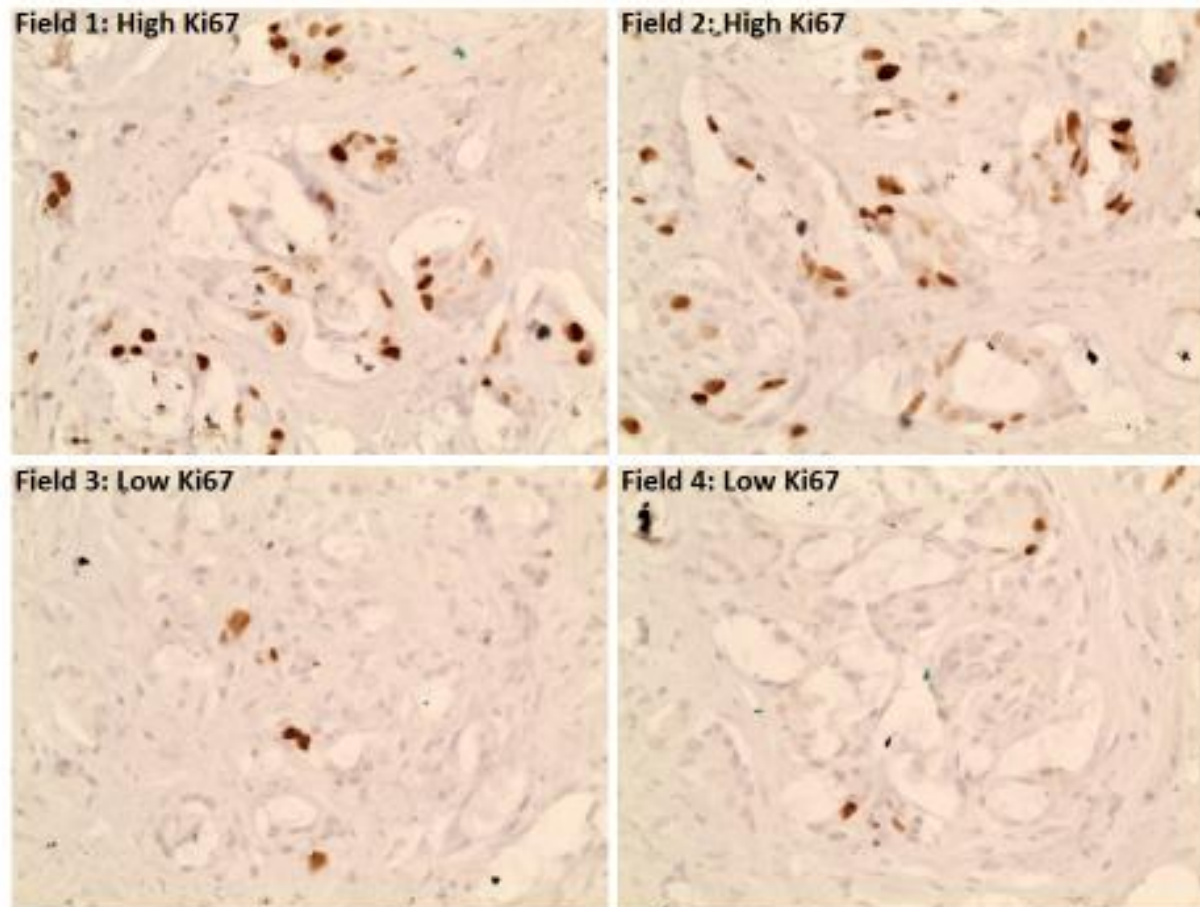
OR's are adjusted for matching variables and age at randomisation. \*P value for the interaction between the mean Ki67 scores and fractionation schedules.

## Figure 1

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### Figure 1: Scoring Ki67 using the global unweighted method<sup>a</sup>.

<sup>a</sup>This case contained 50% high proliferation tumour and 50% low proliferation tumour therefore high power fields were selected for 2 highly proliferative areas and 2 low proliferative areas. In this case the number of positive staining cells were: field 1 39/100, field 2 33/100, field 3 5/100 and field 4 5/100. This gives a mean Ki67 score of 20.5% and a maximum Ki67 score of 39%.

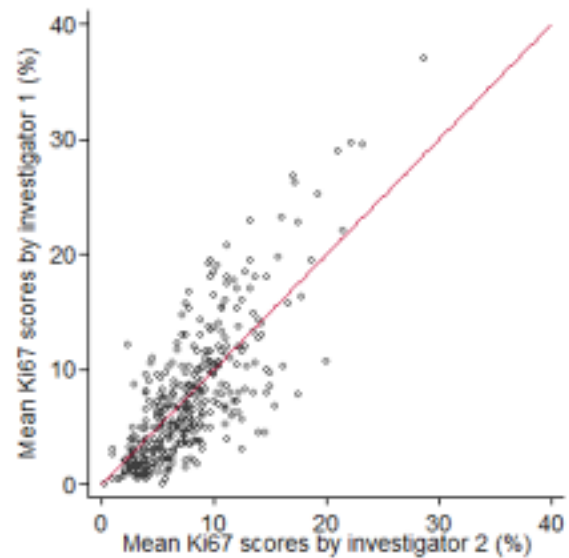


## Figure 2

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Figure 2: A: Scatterplot showing concordance in final mean Ki67 between independent scoring investigators, B: Brand-Altman plot showing difference in final scores between investigator 1 and investigator 2 versus means scores.

**A**



**B**

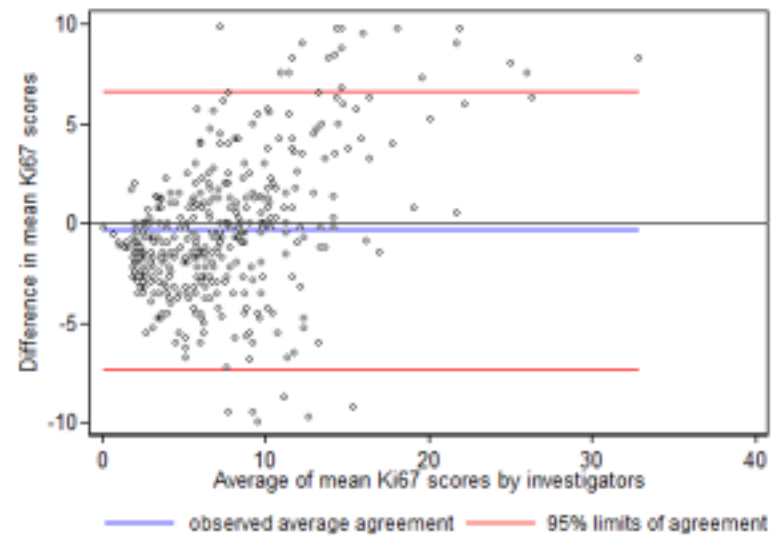
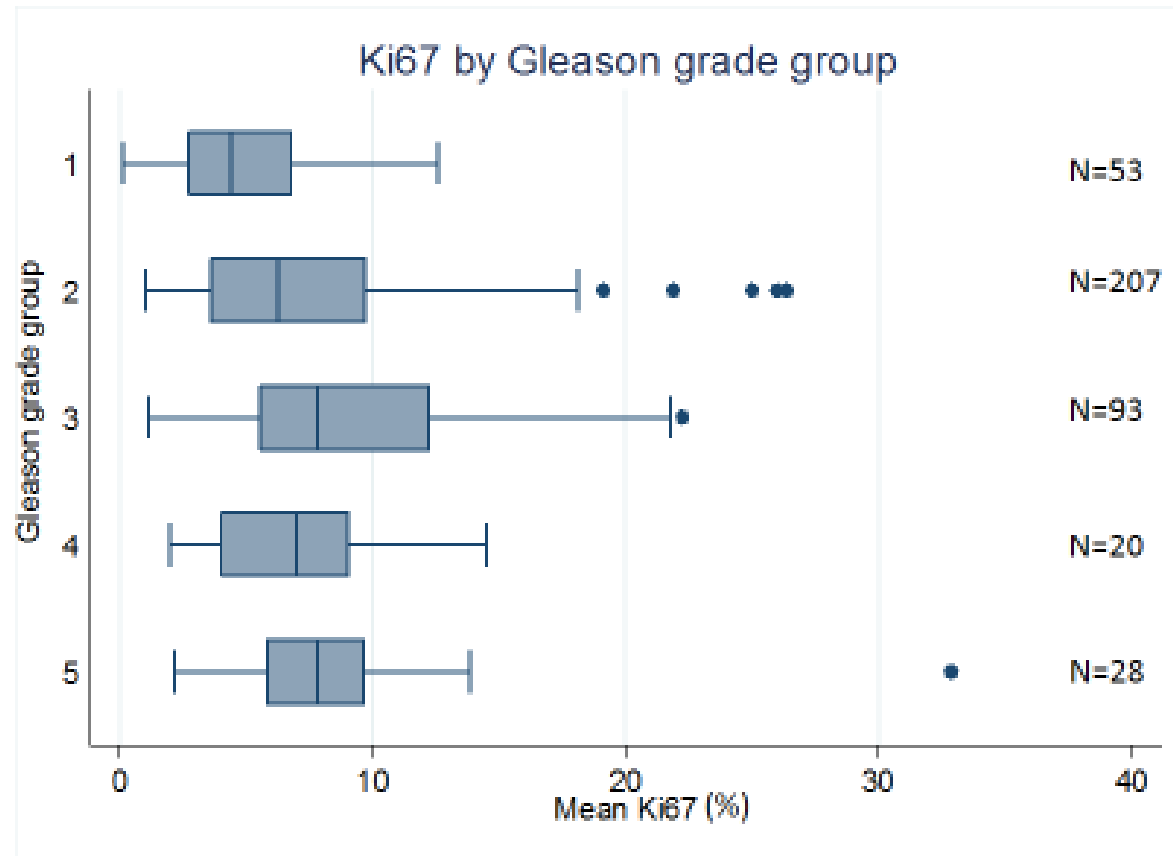


Figure 3: Box and whisker plot showing relationship between Mean Ki67 and Gleason grade group.



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