The Effect of VEGF-Targeted Therapy on Biomarker Expression in Sequential Tissue from Patients with Metastatic Clear Cell Renal Cancer

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Abstract

**Purpose:** To investigate how biologically relevant markers change in response to antiangiogenic therapy in metastatic clear cell renal cancer (mRCC) and correlate these changes with outcome.

**Experimental Design:** The study used sequential tumor tissue and functional imaging (taken at baseline and 12–16 weeks) obtained from three similar phase II studies. All three studies investigated the role of VEGF tyrosine kinase inhibitors (TKI) before planned nephrectomy in untreated mRCC (n = 85). The effect of targeted therapy on ten biomarkers was measured from sequential tissue. Comparative genomic hybridization (CGH) array and DNA methylation profiling (MethylCap-seq) was performed in matched frozen pairs. Biomarker expression was correlated with early progression (progression as best response) and delayed progression (between 12–16 weeks).

**Results:** VEGF TKI treatment caused a significant reduction in vessel density (CD31), phospho-S6K expression, PDL-1 expression, and FOXP3 expression (P < 0.05 for each). It also caused a significant increase in cytoplasmic FGF-2, MET receptor expression in vessels, Fuhrman tumor grade, and Ki-67 (P < 0.05 for each). Higher levels of Ki-67 and CD31 were associated with delayed progression (P < 0.05). Multiple samples (n = 5) from the same tumor showed marked heterogeneity of tumor grade, which increased significantly with treatment. Array CGH showed extensive intrapatient variability, which did not occur in DNA methylation analysis.

**Conclusion:** TKI treatment is associated with dynamic changes in relevant biomarkers, despite significant heterogeneity in chromosomal and protein, but not epigenetic expression. Changes to Ki-67 expression and tumor grade indicate that treatment is associated with an increase in the aggressive phenotype of the tumor.

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Introduction

Antiangiogenic tyrosine kinase inhibitors (TKI) are established as first-line therapy for metastatic clear cell renal cancer (mRCC; ref. 1, 2). However, a marked variability of response to therapy occurs. A minority of tumors are inherently resistant to therapy (early progression), whereas a larger proportion are initially sensitive and subsequently develop resistance to therapy (delayed progression; ref. 3). There are currently no clinically validated tumor biomarkers for renal cancer that predict the onset of treatment resistance (4). Most studies to date have focused on tumor samples taken before therapy; however, preclinical data show that treatment with antiangiogenic agents induces dynamic changes in tumor biology (5, 6). We hypothesize that identification of these dynamic changes is required to elucidate the mechanisms of resistance to antiangiogenic agents.

Here, we prospectively collected sequential tissue from three trials that were all designed to investigate the role of antiangiogenic TKIs before planned nephrectomy in treatment-naïve patients with mRCC. The expression of relevant biomarkers before and after 12 to 16 weeks of TKI therapy was measured from the primary renal tumor. The absolute levels and dynamic changes to biomarkers were assessed...
Translational Relevance
The mechanisms of acquired resistance to VEGF-targeted therapy have not been fully elucidated. By investigating renal tumor tissue taken before and after treatment, this study demonstrates that consistent changes to tumor biomarkers occur with therapy. These dynamic changes occur in clinically relevant biomarkers such as PD-1, MET, and FGF-2. Candidate drugs targeting these three proteins are currently under investigation in renal cancer. Our data show that CD31 (a vascular marker) decreases with treatment. Failure to achieve a sustained reduction in CD31 expression is associated with poor prognosis, suggesting that continued vascular suppression is important to maximize treatment response. Treatment is associated with an increase in tumor grade and Ki-67 expression, which suggests the development of a more aggressive tumor phenotype.

and correlated with outcome in 85 patients. Patients with early or delayed disease progression were studied separately and compared with nonprogressors. The markers examined were selected because they fulfilled one or more of the following criteria: (i) markers linked mechanistically with response and/or resistance to antiangiogenic therapy, (ii) markers of pathways that are currently under investigation as a therapeutic option in RCC, and (iii) markers of immunologic significance. Markers examined included: vessel density (CD31), pericyte coverage, Ki-67, FGF-2, MET receptor, PDL-1, phosphorylated S6K, CD45, CD3, or FOXP3 (7–15). Multiple sampling of the same tumor was performed to further explore the importance of heterogeneity in RCC. This included histologic, chromosomal, and DNA methylation analysis.

The hypothesis was that VEGF therapy resulted in dynamic changes to specific biomarkers that correlated with outcome despite the presence of tumor heterogeneity. Our biomarker work was performed on tissue from the renal cancer and not metastatic sites, which are responsible for mortality. Therefore, the final part of our work examined the relationship between the primary renal tumor and metastatic sites. This was performed by comparing metabolic response to VEGF therapy in these sites using $^{18}$Ffluoro-2-deoxy-D-glucose–positron emission tomography (FDG-PET).

Patients and Methods
Clinical trials
Patients included in this analysis originated from three independent prospective single-arm phase II studies, two using sunitinib and one pazopanib (16–18). All three studies investigated VEGF TKI therapy before planned nephrectomy in biopsy-proven untreated mRCC. The trials were institutional review board–approved [EudraCT 2006-004511-21, 2006-006491-38, and 2009-016675-29]. The analysis of biomarkers from the sequential tissue taken was a secondary endpoint of all of these prospective studies. Patients signed written informed consent for this research.

The three studies had very similar inclusion criteria: two of the studies investigated sunitinib (50 mg: 4/2 weeks) for 12 to 16 weeks before nephrectomy, whereas the final study investigated pazopanib (800 mg once daily) for 12 to 14 weeks before planned nephrectomy. Both sunitinib studies are complete and published, whereas the pazopanib study has reached interim analysis which is published. The three studies all included a translational component in which key proteins were measured from excess tissue from the renal biopsy and nephrectomy. Paraffin-embedded samples were treated in an identical manner. Sequential fresh-frozen tissue was also available from one study ($n = 15$; 2006-006491-38). VEGF TKI therapy was stopped 2 to 14 days (median 3 days) before the nephrectomy. All patients restarted the same VEGF TKI therapy after the nephrectomy irrespective of progression during the treatment break.

Not all patients had tissue available. Tissue was available in 56 patients at baseline and in 61 patients after 12 to 16 weeks of therapy. The commonest reason for tissue not being available at baseline was inadequate amounts of excess material being present after histologic analysis. The commonest reason for sequential tissue not being available was treatment cessation before the 12- to 16-week time point.

Patients were separated into three groups, namely, (i) early progression, those with progression of disease as their best response to therapy; (ii) delayed progression, those patients with progression directly after the subsequent tissue collection at 12 to 18 weeks (after initial stable disease); and (iii) nonprogression, the remaining population. Characteristics of these patients are shown in Table 1. Patients with early and delayed progression have a significantly worse outcome than the nonprogressors, justifying the investigation of the three groups.

Histologic, CGH array, and DNA methylation analysis
Sequential samples were assessed for Fuhrman grade, Ki-67 expression, and presence of necrosis by 2 independent pathologists. Subsequent histologic analysis investigated five separate samples from the same tumor for heterogeneity in study NCT 2006-006491-38 ($n = 23$). Patients were separated into low grade (Fuhrman 1 and 2), high grade (Fuhrman 3 and 4), or mixed grade (presence of both low and high grade). Those patients with mixed grade were defined as having histologic heterogeneity. An indirect comparison was made with multiple biopsies taken from untreated nephrectomy samples (sourced from treatment-naïve patients with mRCC from the same research group; $n = 22$). Discrepancies between histopathologists were resolved by central review where required. Five different areas were selected from each tumor and one slide per area was assessed.

Array CGH and DNA methylation analysis (MethylCap-seq) was performed on the same sequential frozen pairs ($n = 14$). Analysis was performed once from the biopsy and
in triplicate from the nephrectomy sample for each patient. Genomic DNA extraction was conducted using the Qiagen DNeasy Blood and Tissue (Qiagen) kit as per the manufacturer’s instructions.

For array CGH, DNA was labeled with Cy3 from NimbleGen Dual-Color Labeling Kit according to the manufacturer’s instructions. Similarly, pooled DNA from lymphoblastoid cell lines were labeled with Cy5 and used as a reference. The sex of the cell lines was matched to that of the patient sample. Once labeled 20 μg of test and reference sample were combined and added to a NimbleGen 12 × 135 array and hybridized, following which, slides were scanned (NimbleGen MS 200 Microarray Scanner). Array CGH data were preprocessed and analyzed using an in-house R script (BACE.R) in R version 2.13.0, as previously described (19, 20). Categorical array comparative

<table>
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<th>Table 1. Patient characteristics before study entry</th>
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Abbreviations: CR, complete remission; PD, progression of disease; MSKCC, Memorial Sloan-Kettering Cancer Center; SD, stable disease.

*Two patients were not included because of early cessation of treatment.

Significantly longer overall survival compared with patients with early and late progression (P < 0.05).
genomic hybridization (aCGH) states (i.e., gains, losses, and amplifications) were used for clustering, using Ward clustering algorithm based upon Euclidean distance.

MethylCap-seq involves the in vitro capture of methylated DNA and subsequent analysis of enriched fragments by massively parallel sequencing (21). Unsupervised hierarchical clustering analysis was performed. Data analysis was performed with R 2.15.3.

Protein analysis
A tissue microrarray (TMA) was constructed from biopsy and nephrectomy tissue samples. For protein analysis, sections were pretreated using heat-mediated antigen retrieval with sodium citrate buffer. The following antibodies were used to assess protein expression; CD31 (1:600; AstraZeneca), FGF-2 (1:100; Peprotech), MET receptor (1:200; Invitrogen), p-S6K (1:200; Cell Signaling Technology), CD3 (1:100; Abcam), CD45 (1:100; Dako), FOXP3 (1:200; Abcam), and PDL-1 (1:200; Abcam). Appropriate isotype controls were used in all cases. Results were analyzed centrally by 2 histopathologists who were blinded to patient outcome data (R. Doshi and D. Berney). Vessel density (CD31), CD45, CD3, or FOXP3-positive immune cells, were quantified using a computerized image analysis system (ARIOL Applied Imaging, Genetix) using visually-trained parameters. To determine pericyte coverage, TMAs underwent triple staining for CD31, α-smooth muscle actin (α-SMA), and 4’,6-diamidino-2-phenylindole (DAPI) and the percentage of α-SMA-coated vessels was scored by 2 observers.

Radiologic and FDG-PET–CT assessment
All patients were assessed according to RECIST v1.1. Computed tomography (CT) was performed at base line, before nephrectomy (week 12–14) and after nephrectomy (week 16–18). Patients then followed up with regular imaging until progression. Radiologic analysis took place centrally.

FDG-PET–CT scans were performed at baseline and before nephrectomy (48 hours after cessation of VEGF TKI therapy) in 34 patients. Changes in standard uptake variable (SUV) to positive lesions (SUV > 2.5) with therapy were measured. A correlation between the primary renal lesion and the metastatic sites was examined. The correlation between changes in SUV and outcome in this population had been previously published (22).

Statistical analysis
Analysis of the data took place centrally in November 2012. Descriptive statistics were used to compare groups. Univariable and multivariable analysis was performed. Further analysis was performed to investigate if dynamic changes to biomarkers correlated with outcome. Student t tests were used to compare biomarker levels in progressors and nonprogressors. A P value of less than 0.05 was considered to be significant. Outcome was estimated using the Kaplan–Meier method. A Fisher exact test was used to compare groups expressing different histopathologic features.

Results

Patient characteristics
The CONSORT diagram in Fig. 1 shows the disposition of patients at baseline and their subsequent course on study. All 85 patients were diagnosed with untreated mRCC. All patients started VEGF-targeted therapy with the intention of having subsequent tissue taken. Table 1 compares patients’ characteristics according to their best response to therapy. Seventeen patients had early disease progression, whereas 24 patients had delayed progression and 44 were in the nonprogressor group. Survival was significantly different in these three groups with early progressors having the worst outcome and those without progression having the best outcome (see Table 1). The survival for patients treated with pazopanib and sunitinib was similar [HR for pazopanib, 1.16 (95% confidence interval (CI), 0.64–2.1)]. A number of patients did not have a nephrectomy (n = 19), prior progression of disease/cessation of therapy (n = 17) being the most common reason for this.

Biomarker expression and outcome from baseline tissue
To investigate biomarkers associated with early or delayed progression, baseline untreated tissue was analyzed (Fig. 2). None of the ten biomarkers correlated with progression.

The effect of treatment on tumor pathology and biomarkers
Paired tumor tissue showed a significant increase in Fuhrman grade (increased = 61% of patients, vs. reduction = 29%; P < 0.05), Ki-67 (increased = 63%, vs. reduction = 23%; P < 0.05), and necrosis (increased = 58%, vs. reduction = 2%; P < 0.05) with therapy. The proportion of samples scoring Fuhrman grade ≥ 3 increased with treatment from 31% to 78% (P < 0.05).

Immunohistochemistry (IHC) demonstrated that treatment had a significant effect on several tumor markers including CD31 (tumor vessel density; median change –56%; P < 0.05), cytoplasmic FGF-2 (+90%; P < 0.05), and MET receptor expression in blood vessels (P < 0.05). A significant change in p-S6K (–50%; P < 0.05) and changes in immune cell markers PDL-1 (–34%; P < 0.05) and FOXP3 (P < 0.05) were also observed (Fig. 3A). No significant changes occurred in other biomarkers examined. The comparative effect of sunitinib and pazopanib on biomarker expression was investigated. There was no difference in the effects of the two drugs on the expression of the majority of biomarkers in the paired samples. However, sunitinib, but not pazopanib, resulted in reduced expression of the immune cell markers CD45 and CD3 [median change –84% vs. +13% (P < 0.05); median change –38% vs. +117% (P < 0.05), respectively].

Biomarker expression and outcome from treated tissue
Subsequently, we examined if any of the biomarkers which significantly changed with therapy (Fig. 3A) correlated with delayed progression of disease. Ki-67 and CD31...
expression was significantly higher in tissue taken from patients with delayed progression compared with nonprogressors ($P < 0.05$ for both; Fig. 3B).

An increase in CD31 with therapy also correlated with reduced overall survival ($P < 0.05$). The only other biomarker in which a change was associated with a poor outcome was Fuhrman grade, in which an increase was associated with a poor outcome ($P < 0.05$).

The relationship between biomarker expression and overall survival

In multivariable analysis, significant baseline prognostic factors included Heng prognostic score [HR, 4.61 (1.30–16.31)], number of metastatic sites [3.37 (HR, 1.16–9.78)], tumor grade [HR, 6.62 (2.02–21.63)] and high pericyte coverage at baseline [HR, 4.20 (1.48–11.9; $P < 0.05$)]. The prognostic significance of a change in the biomarkers with therapy (above and below the median change) was also examined. Multivariable analysis revealed that a reduction in pericyte coverage was the only factor associated with a poor outcome [HR, 3.65 (1.36–9.80); $P = 0.01$].

Tumor heterogeneity in treated and untreated samples

Five samples were taken from treated tumors ($n = 23$) to examine for heterogeneity of tumor grade and biomarker expression ($n = 5$). The majority of treated tumors exhibited both low- and high-grade histologic features (mixed features). Mixed histologic grade was significantly higher in the treated group compared with the untreated group (65% vs. 20%; $P < 0.05$; Fig. 4A). Variability in biomarker expression between different regions of the same tumor was also observed (Fig. 4B). CGH array analysis showed marked intrapatient variability in multiple samples ($n = 3$) taken from the same patient. Only 2 of 15 patients’ tumors clustered (Fig. 4C). Further analysis showed no increase in DNA instability in the treated samples compared with untreated samples. There were no consistent chromosomal changes associated with sunitinib therapy. Analysis of the DNA methylation data showed consistent clustering of...
individual patient’s tumors irrespective of exposure to sunitinib treatment (Fig. 4D).

The relationship between the primary renal tumor and the metastatic sites
Sequential FDG-PET–CT scans were performed at baseline and after three cycles of sunitinib (i.e., at the time of the tissue collection) in 34 patients. The relationship between the metabolic activity in the primary renal tumor and metastatic sites was examined. There was a positive correlation between baseline metabolic activity (SUV) in the primary tumor and the metastatic sites for individual patients (r = 0.72; P < 0.01; Fig. 5A). There was also a significant correlation in the metabolic response (ΔSUV) between response in the primary tumor and metastatic sites (r = 0.46; P < 0.01; Fig. 5B).

Discussion
There is currently a lack of tissue biomarkers to predict response to VEGF receptor-targeted TKIs. In this work, sequential tissue taken before and during TKI treatment was used to explore the hypothesis that dynamic changes to biomarker expression occur in resistance. We focused on the correlation of biomarker expression with progression (initial and delayed) rather than response. This is because initial response does not seem to correlate with outcome (unlike progression) and usually occurs early in the treatment.
process before acquired resistance appears (23). Correlation between radiologic response (RECIST v1.1) and biomarker was performed but did not identify prognostic changes (data not shown).

Efficacy of VEGF receptor–targeted TKIs is thought to stem primarily from suppression of tumor angiogenesis. Here, TKI treatment significantly reduced tumor vessel density, supporting recent findings obtained in sequential tissue from patients with mRCC (12). Importantly, we show also that failure to achieve a reduction in vessel density is associated with progression of disease. Continued suppression of the vasculature is therefore important for continued clinical response, supporting the results of a recent randomized phase III trial (24). The inhibitory effect of TKIs on the phosphoinositide 3-kinase (PI3K) pathway (pS6K) demonstrated the broad effect of these agents and suggests possible overlapping mechanism of action with the mTOR inhibitors that also target the PI3K pathway. This may be a potential explanation for cross resistance that is speculated to occur between these two classes of agents in mRCC.

Figure 3. Molecular markers before and after 12 to 16 weeks of VEGF TKI therapy in matched pairs. A, the effect of treatment on biomarker expression. Analysis of biomarkers occurred only where paired samples (untreated and treated in the left and right columns, respectively) were available. Pretreatment samples were taken before the onset of therapy and posttreatment samples were taken 12 to 16 weeks after the treatment onset. A proportion of patients did not have post treatment samples taken ($n = 17$). The untreated samples act as a control to identify the effects of treatment on tissue. B, the association of delayed progression with those biomarkers that changed significantly with treatment. Tissue was taken after 12 to 16 weeks of therapy. Patients were separated into two groups (those with delayed progression and those without progression). Patients with delayed progression have higher levels of Ki-67 and CD31 (a Student t test was used and $P$ values < 0.05 were considered significant).
Preclinical studies suggest that VEGF-targeted therapy may increase tumor aggressiveness, including accelerated tumor growth (25), increased tumor invasion (26), and increased metastasis (25, 26). If manifested in patients, these effects would limit therapeutic efficacy; however, there is little or no compelling clinical evidence that VEGF-targeted therapy leads to increased tumor aggressiveness (27). Here, we show that tumor grade and tumor cell proliferation were both increased in patients with mRCC after treatment with VEGF TKIs. Also, increased proliferation (Ki-67) was associated with a poor prognosis. Therefore, we present the first evidence for a phenotype of increased tumor aggressiveness in response to VEGF-targeted therapy in human mRCC. It is possible that some of these effects, especially on tumor grade, are related to the treatment stress of VEGF therapy. This effect occurs in prostate cancer, in which luteinizing hormone-releasing hormone (LHRH) agonist results in a more aggressive phenotype due to treatment-related factors (28).

FGF-2 may mediate resistance to VEGF-targeted therapy by providing an alternative proangiogenic signal for endothelial cells (6, 9). Increased circulating concentrations of FGF-2 have been reported in patients progressing on VEGF-targeted therapy (29). However, the relevance of this circulating pool is not clear, because FGF-2 exerts a paracrine effect to promote angiogenesis and tumor cell proliferation (30). Here we confirm, for the first time, that circulating FGF-2 is increased as a result of TKI treatment. Combined, these data support a strategy to target both VEGF and FGF receptor signaling to achieve sustained suppression of mRCC tumor growth (9). Dovitinib targets both VEGF and FGF receptor signaling and is currently under investigation in the TKI refractory setting.

Preclinical studies show that VEGF-targeted therapy in upregulated expression of MET receptor in tumor cells, facilitating increased tumor invasion and metastasis (31, 32). Here, we found no evidence for upregulated MET receptor in the tumor cell compartment. Instead, we found...
significant upregulation of MET receptor in the vascular compartment in response to TKI. Of interest, the MET receptor ligand, hepatocyte growth factor, may also mediate resistance to VEGF-targeted therapy by providing an alternative proangiogenic signal for endothelial cells (8). Conceivably, upregulated expression of MET in the vascular compartment could help facilitate this mechanism of acquired resistance.

Pericyte coverage of vessels in human tumors is variable, with conflicting reports about the prognostic significance (32, 33). Our results show that high baseline levels of pericyte coverage correlate with a poor outcome, supporting previous work in RCC (33). Conflicting data also exist about the effects of antiangiogenic therapy on pericyte coverage (32, 34, 35). Here, we demonstrate that therapy can exert opposing effects on pericyte coverage (increase in 13%, decrease in 26%, and no change in 61%). We also observed that a reduction in pericyte coverage adversely affected survival in multivariable analysis. This may be related to the fact that pericyte loss can compromise tumor vessel integrity, promoting metastasis (32, 35). Because loss of pericytes was not associated with progression in our study, but did strongly associate with overall survival, there may be a tumor growth–independent effect of pericyte loss on outcome.

Immune therapy has been used in renal cancer and is currently under evaluation in the form of PD1/PDL-1 inhibitors (36). Our work supports in vivo experiments that show that VEGF TKIs have specific effects on immune parameters, including regulatory T-cell expression. The...
suppression of PDL-1 required particular attention as the biomarker is thought to predict response to PD-1 inhibitors (10). This alteration questions the use of archived untreated tissue to estimate VEGF-resistant biomarker expression.

Recent exome analysis revealed the existence of significant regional heterogeneity from 1 patient with mRCC (37). Our histology, protein, and chromosomal analysis shows marked intratumoral heterogeneity across a cohort of patients. However, DNA methylation results showed all samples from individual patients clustered. This suggests the variability seen in protein expression and histology results more from genetic rather than epigenetic instability. Consistent chromosomal changes were not seen with sunitinib treatment. This may in part be due to the marked heterogeneity.

The presence of heterogeneity is challenging as it may mask the detection of biologically significant changes due to sampling bias. However, despite this heterogeneity, we still observed significant changes in several key biomarkers, including CD31, Ki-67, S6K, PDL-1, MET receptor, and FGF-2. In our opinion, the ability to detect a significant change in these biomarkers across a sample that is intrinsically heterogeneous only acts to further reinforce the biologic significance of these changes.

Finally, a positive correlation between a metabolic response in the primary tumor and the metastatic sites occurred, suggesting that primary and metastatic sites may respond in a similar fashion to therapy. However, it does not necessarily mean that dynamic changes to biomarkers in the metastatic sites match those seen in the primary tumor. Collection of sequential tissue from specific metastatic sites is extremely challenging. Therefore, further exploration of this relationship may not be possible.

There are several shortcomings in this work. Specifically, not all patients were able to have sequential tissue taken due to lack of excess tissue for sampling and patients coming off study. Also, the tissue originated from three rather than one study (although they were almost identical in design). Despite being the largest series to report, in this setting the number of samples remains modest. Moreover, multiple testing occurred because of the number of biomarkers selected. In addition, our analysis of sequential biomarker expression may be influenced by the natural course of disease rather than treatment. Finally, this work was a post hoc analysis of tissue from three studies and was exploratory in nature. Therefore these results are hypothesis generating and require further exploration in clinical and laboratory-based models. Although potential biomarkers from sequential RCC tissue (such as CD31) appear feasible, there are major challenges before this becomes clinical practice. The two major hurdles are the feasibility of taking sequential tissue in large randomized trials and the chromosomal and protein variability effecting biomarker expression.

Disclosure of Potential Conflicts of Interest

C.G. Stewart has speaker honorarium. A. Bex has honoraria from Pfizer and is a consultant/advisory board member of Novartis. W. Van Criekinge is employed by MDxHealth. T. Powles has honoraria, received a commercial research grant, and is a consultant/advisory board member of Pfizer and GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Sharpe, G.D. Stewart, I. Mackay, C. Van Neste, C. Rofe, I. Kayani, E. Wan, F.C. O’Mahony, S. Chowdhury, M. Gerlinger, A. Sahdev, T. De Meyer, W. Van Criekinge, A.R. Reynolds, T. Powles

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Study supervision: N. Smith, B. Davies, T. Powles

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References


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