ABSTRACT

Biomarkers for more precise patient care are needed in metastatic prostate cancer. We have reported a phase II trial (TOPARP-A) of the PARP inhibitor olaparib in metastatic prostate cancer, demonstrating antitumor activity associating with homologous recombination DNA repair defects. We now report targeted and whole-exome sequencing of serial circulating free DNA (cfDNA) samples collected during this trial. Decreases in cfDNA concentration independently associated with outcome in multivariable analyses (HR for overall survival at week 8: 0.19; 95% CI, 0.06–0.56; \( P = 0.003 \)). All tumor tissue somatic DNA repair mutations were detectable in cfDNA; allele frequency of somatic mutations decreased selectively in responding patients (\( \chi^2 \) \( P < 0.001 \)). At disease progression, following response to olaparib, multiple subclonal aberrations reverting germline and somatic DNA repair mutations (\( BRCA2, PALB2 \)) back in frame emerged as mechanisms of resistance. These data support the role of liquid biopsies as a predictive, prognostic, response, and resistance biomarker in metastatic prostate cancer.

SIGNIFICANCE: We report prospectively planned, serial, circulating free DNA (cfDNA) analyses from patients with metastatic prostate cancer treated on an investigator-initiated phase II trial of olaparib. These analyses provide predictive, prognostic, response, and resistance data with "second hit" mutations first detectable at disease progression, suggesting clonal evolution from treatment-selective pressure and platinum resistance. Cancer Discov; 7(9); 1–12. ©2017 AACR.
Metastatic prostate cancer is a major cause of cancer mortality in males globally. Studies indicate substantial inter- and intrapatient genomic heterogeneity, although treatment to date has not incorporated molecular stratification (1–7). Clinical qualification of predictive biomarkers is a major need for these invariably lethal tumors. We previously reported that 20% to 30% of lethal prostate cancers have deleterious aberrations in genes involved in DNA repair by homologous recombination [homologous recombination deficiency (HRD)], including BRCA2, ATM, BRCA1, PALB2, FANCA, CHEK2, and CDK12 (4). Studies indicate that HRD-associated mutations associate with a worse prognosis, with these aberrations being inherited in approximately 10% to 12% of men with lethal prostate cancer (8, 9).

We also recently reported an investigator-initiated phase II clinical trial (TOPARP-A) of the PARP inhibitor olaparib (Lynparza) in patients with metastatic prostate cancer, describing antitumor activity associating with HRD (10). These data led to olaparib being given “Breakthrough Designation” by the FDA for advanced prostate cancer associated with BRCA2/ATM defects, and registration trials of different PARP inhibitors being currently pursued (11–13). A major regulatory approval challenge for these drug development efforts remains the lack of proven surrogates of survival benefit. We hypothesized that circulating biomarkers can enhance drug development and patient care, informing on treatment response and resistance. Response biomarkers are crucial to improving the care of advanced prostate cancer, which is often characterized by metastatic disease only to bone, which is not easily evaluable (14). Circulating free DNA (cfDNA) in blood, acquired from plasma by a simple blood test, provides repeated serial access to tumor DNA as a minimally invasive “liquid biopsy” (15–19).

We report here preplanned cfDNA analyses from serial blood samples from the TOPARP-A trial. We had hypothesized that cfDNA analyses can enhance drug development and patient care and that: (i) changes in cfDNA concentrations in response to treatment would be prognostic;
(ii) next-generation sequencing of cfDNA would detect the predictive HRD-associated mutations found in tumor biopsies; (iii) changes in cfDNA somatic mutation allele frequency on therapy would associate with response; and (iv) serial cfDNA analyses could detect the evolution of resistant subclones at disease progression.

RESULTS
Patient Characteristics and Blood Sample Disposition

Fifty patients were treated in the TOPARP-A trial; 49 were evaluable for response, with 16 patients responding to treatment. Overall, 16 of these 49 patients had prostate cancers with a deleterious aberration in homologous recombination DNA repair genes, with 14 of these patients responding to treatment. Serial samples for cfDNA studies were available for 46 of 49 patients (94%). We now report on these analyses utilizing updated outcome data based on a data snapshot taken on May 24, 2016. One patient with a BRCA2 homozygous deletion remained on therapy and free of progression at the data cutoff, after 22 months of therapy. Overall, the median cfDNA baseline concentration across the trial population was 31.6 ng/mL [interquartile range (IQR), 19.4–57.1]. Next-generation targeted sequencing of cfDNA was successful for 43 of 46 patients (93%; Supplementary Fig. S1).

Prognostic Relevance of Changes in cfDNA Concentration Following PARP Inhibition

Changes in cfDNA concentration were evaluated in patients responding (n = 16) or not responding (n = 30) to olaparib. After 4 weeks of therapy, there was a median −51.4% change in responders (IQR, −76.5% to −20.4%), differing significantly from nonresponders (IQR, −72.6% to −29.5%) and a median −33.4% change in nonresponders (IQR, −52.3% to −25.8%; P = 0.006). After 8 weeks of therapy, responders continued to experience sustained declines (median −49.6% change; IQR, −76.5% to −20.4%), differing significantly from nonresponders (median +2.1% increase; IQR, −43.6% to +57.8%; P = 0.006; Supplementary Fig. S2).

Next, we explored how declines in cfDNA concentrations correlated with patient outcome based on radiologic progression-free survival (rPFS) and overall survival (OS). cfDNA concentration falls robustly correlated with rPFS as early as after 4 weeks of therapy [HR, 1.70; 95% confidence interval (CI), 1.13–2.55; P = 0.01 for cfDNA log fold change; HR, 0.41; 95% CI, 0.21–0.80; P = 0.009 for absence/presence of ≥50% fall from baseline of cfDNA concentration]. cfDNA concentration falls after 8 weeks of olaparib correlated with both prolonged rPFS and OS (Fig. 1).

In multivariable analyses including established prognostic factors, such as LDH and circulating tumor cell (CTC) count conversions, a ≥50% decline in cfDNA concentration after 4 weeks of olaparib was independently associated with longer rPFS. These changes at 4 weeks were not statistically significant for OS (HR, 0.47; 95% CI, 0.18–1.07; P = 0.07). Nevertheless, a ≥50% fall in cfDNA concentration after 8 weeks of therapy was independently associated with longer OS (HR for ≥50% cfDNA decline and OS in multivariable analyses: 0.19; 95% CI, 0.06–0.56; P = 0.003; Table 1).

Changes in Allele Frequency of Somatic Mutations

We then performed next-generation targeted sequencing of serial cfDNA samples to assess allele frequency of somatic mutations during PARP inhibitor therapy as an indirect estimate of tumor burden. A total of 254 plasma samples were analyzed. We detected somatic mutations in cfDNA of 33 of 43 baseline samples. Olaparib treatment led to sustained decreases in cfDNA mutation allele frequencies in responding patients; sustained (≥28 weeks) falls in cfDNA somatic mutation allele frequencies were not observed in nonresponding patients, although 3 of 29 detected somatic events in nonresponding patients decreased transiently after 4 weeks of olaparib (χ² P < 0.001; Fig. 2A).

Somatic HRD-Associated Mutations in cfDNA

Overall, 6 subjects in the TOPARP-A trial had tumors with somatic mutations likely to be associated with HRD (3 in ATM, 2 in BRCA2, 1 in PALB2; 5/6 responded to olaparib); all 6 mutations were detected in baseline cfDNA. In all 5 responding patients, these somatic mutation allele frequencies all decreased to <5% following olaparib treatment (Fig. 2B). In the nonresponding patient, the somatic mutation allele frequency remained unchanged at 4% throughout therapy.

In addition, in one nonresponding patient we detected two ATM frameshift mutations in cfDNA not previously detected in the tumor biopsy. Retrospective inspection of tumor data detected both mutations in 3% of sequencing reads. We obtained a further metastatic biopsy from a different anatomic location after treatment; both of these ATM mutations were present in 18% and 29% of reads, respectively. We also evaluated this patient’s primary prostate tumor biopsy taken at diagnosis; one of these mutations was not present and the other was visualized in 1% of reads. Overall, these data indicate that these were probably subclonal mutations.

Loss of Heterozygosity in Germline Mutation Carriers

Five patients with pathogenic BRCA2 or ATM germline mutations responded to olaparib in the trial. In 4 of these, we had previously documented loss of heterozygosity (LOH) for these pathogenic mutations in tumor biopsies. In these 4 patients, falls in the cfDNA allele frequencies of these germline mutations toward 50% were observed as they responded to olaparib, suggesting elimination of the tumor clone with LOH. For the last patient with an ATM mutation, but no LOH in his tumor biopsy, allele frequencies remained between 45% and 55% in the cfDNA at all time points in concordance with the tumor tissue findings (Fig. 2C).

Mechanisms of PARP Inhibitor Resistance Detected in cfDNA

Ten of 16 patients having an initial tumor response to olaparib had cfDNA samples acquired at the time of resistance and disease progression. In addition to targeted sequencing, we successfully performed whole-exome sequencing (WES) from paired plasma samples collected before olaparib treatment and at disease progression for 6 patients to study mechanisms of secondary resistance to PARP inhibition. In a seventh case, we performed WES in the progression sample...
Response and Resistance to PARP Inhibition in cfDNA

and targeted sequencing in the baseline cfDNA sample, due to low DNA yield.

In both of the patients with a germline BRCA2 frameshift mutation, we identified at the time of tumor progression additional somatic BRCA2 mutations in cfDNA restoring the normal open reading frame (Fig. 3).

One of these patients had a germline BRCA2 p.-1056fs mutation and LOH in the pretrial tumor biopsy. At the time of disease progression, a further somatic deletion of four base pairs emerged, resulting in an overall in-frame change (p.Q1145*). These aberrations again indicate possible divergence of this new event independently by targeted next-generation sequencing (MiSeq). In addition, we also detected the emergence at progression of a somatic ARID1A mutation (p.K1057_Q1063delinsTEQA) was detected; these data suggest these other emerging subclones may have originated in different metastases.

A second germline BRCA2 mutation carrier (p.E1514fs*15) developed an additional deletion of 28 bp at progression, reverting BRCA2 back in frame. We confirmed the emergence of this new event independently by targeted next-generation sequencing (MiSeq). In addition, we also detected the emergence at progression of a somatic ARID1A mutation (p.Q1145*). These aberrations again indicate possible divergent clonal evolutionary resistance mechanisms as a result of PARP inhibition–generated selective pressures.

In nongermline mutation carriers, we also identified new emerging genomic events at progression leading to the reversion of a somatic BRCA2 mutation (Fig. 5). A somatic 2-bp frameshift deletion was detected in WES of the original tumor biopsy and in targeted sequencing of the baseline plasma sample (p.Y2154fs*21). Because of DNA yield, we could not obtain WES of the baseline cfDNA sample in this patient. At progression, WES revealed two alternative deletions resulting in an in-frame deletion but restoring the open

Figure 1. Kaplan–Meier plots showing differences in rPFS and OS based on the presence or absence of a ≥50% fall in total cfDNA concentration after 4 weeks and after 8 weeks of therapy with olaparib.
In addition, secondary genomic events causing reversion back to normal reading frame of a somatic PALB2 mutation were also detected for one patient. The pretreatment sample contained a 2-bp frameshift deletion (p.-253fs*3) with LOH. At the time of progression, after 9 months of treatment, two different new mutations restoring the PALB2 reading frame were identified (Fig. 5). Both events coexisted with the original clone, again indicating divergent resistant subclones.

We examined the nucleotide sequences flanking the BRCA2 and PALB2 original frameshift deletions in these cases with

**Table 1.** Univariate and multivariable Cox regression analyses of the association cfDNA plasma concentrations (ng/mL) with patient outcome including established prognostic factors.

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| 50% cfDNA [c] decline | 0.41 (0.21–0.80) | 0.44 (0.09–0.66) |
| ≥30% CTC decline      | 0.33 (0.15–0.74) | 0.38 (0.19–0.74) |
| ≥30% cfDNA [c] decline | 0.61 (0.33–1.14) | 0.61 (0.31–1.19) |
| ≥50% cfDNA [c] decline | 0.41 (0.21–0.80) | 0.24 (0.11–0.52) |

Abbreviations: ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase.

**Figure 2.** A, Waterfall plot summarizing change in allele frequency (AF; defined as allele frequency on treatment subtracted by the allele frequency at baseline *100) after 4- and 8 weeks of therapy. Stars, patients considered responders to olaparib in the TOPARP-A trial in the predefined primary endpoint. An absolute decrease of ≥10% in allele frequency was observed in 18 of 27 somatic mutations detected in responding patients as compared with 3 of 29 somatic events monitored in nonresponding patients (*χ2* *P* < 0.001). None of the three allele frequency falls in nonresponding patients were maintained after 8 weeks of therapy. B, Four examples of how AF of somatic HRD-associated mutations decrease in response to therapy, in parallel with decreases in total cfDNA concentrations. A patient with an ATM p.-2288fs mutation had intermittent increases and decreases in AF in parallel to drug interruptions due to hematologic toxicity. C, Changes in cfDNA mutation allele frequency over time in germline deleterious mutation carriers (BRCA2 and ATM) in 5 patients from the TOPARP-A trial. Those patients with LOH at baseline have the cfDNA mutation allele frequency trending toward 50% in response to therapy, probably due to elimination of the tumor clone. This is not seen in the serial cfDNA samples from the patient whose tumor did not have LOH.

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Response and Resistance to PARP Inhibition in cfDNA

A

Absolute change in allele frequency

B

Allele frequency vs. Total cfDNA ng/mL

C

Allele frequency for germline mutations in cfDNA

- ATM p.N2875S
- BRCA2 p.-2154fs
- P015 (BRCA2)
- P016 (BRCA2)
- P036 (ATM)
- P005 (ATM)
- PALB2 p.-253fs
- PTEN p.-19fs
- ERCC2 intronic
- TSC intronic
- TRIM24
- P016 (BRCA2)
- P025 (BRCA2)
- P030 (BRCA2)
- P036 (ATM)
- P005 (ATM)

Weeks of therapy

Weeks of therapy
Figure 3. Visual representation of emerging de novo mutations at progression that likely result in acquired drug resistance in two patients with germline BRCA2 mutations. Top, a patient with a germline deleterious BRCA2 frameshift insertion that was present in both tumor and cfDNA at baseline presents at disease progression with a new frameshift deletion that restores the BRCA2 reading frame; bottom, a second patient with a germline deleterious BRCA2 mutation is depicted; at progression cfDNA WES identified multiple clones with different previously undetected mutations all resulting in reversion of the BRCA2 reading frame to normal.
secondary gene mutations. In some of these multiple resistant subclones emerging in parallel, we observed short regions of nucleotide sequence identity flanking the new deletions. This association with microhomology regions suggests that these might have evolved as a consequence of defective homologous recombination and the utilization of alternative error-prone DNA repair mechanisms.

Finally, in one patient with HDAC2 biallelic somatic loss in the tumor biopsy, WES demonstrated subclonal divergent evolution and new mutations emerging at progression in TP53 and TSC2 (GAP domain), which have been associated with drug resistance (Supplementary Table S1; refs. 20, 21).

Among the remaining patients with plasma cfDNA evaluated at secondary resistance, we did not detect any other such events, although 2 of these patients discontinued the drug due to tolerability issues prior to radiologic progression. No emerging mutations or copy-number changes in PARP1 or PARP2 were observed in any of these samples.

**DISCUSSION**

In this study, we describe the clinical utility of cfDNA analyses as multipurpose biomarkers for treatment with PARP inhibition in metastatic prostate cancer. Critically, our cfDNA analyses detected all somatic HRD-associated mutations identified in tumor biopsies as well as new mutations emerging at disease progression. These new mutations likely represent tumor subclones induced by therapeutic selective pressure driving drug resistance.

The emergence of secondary mutations in BRCA1/2 germline mutation carriers has been previously described in case reports and small retrospective series of patients with breast or ovarian cancer after PARP inhibition and/or platinum chemotherapy (22–24). Here, we report the first series of patients homogeneously treated within a prospective clinical trial; mutations reverting the reading frame of mutated homologous recombination genes were detected not only in germline BRCA2 mutation carriers but also, for the first time, in tumors harboring somatic loss of BRCA2 and PALB2. These events were confirmed independently by orthogonal targeted sequencing of cfDNA and/or tumor biopsy DNA.

These multiple genomic events driving resistance emerged in parallel, indicating clonal divergence with functional convergence, restoring homologous recombination repair proficiency. A similar concept has been recently described after exposure to androgen receptor (AR)-targeting agents in prostate cancer, with emergence in parallel of multiple AR aberrations (25–30). Monitoring this subclonal equilibrium between the original clone and resistant clones merits further evaluation, as PARP inhibitor discontinuation and administration of other treatments could potentially restore the dominance of the original clone sensitive to PARP inhibitor or platinum.

Interestingly, in two cases, small tracts of DNA homology flanked all these multiple secondary mutations. These were reminiscent of similar deletions observed in preclinical models restoring the open reading frame of the gene and causing PARP inhibitor resistance, and probably arose from the use of error-prone DNA repair processes that predominate in the absence of functional BRCA2 (31–33).

In the case with the original somatic PALB2 mutation, the initial clinical response to olaparib, followed by PARPi resistance characterized by the emergence of secondary mutant PALB2 alleles with microhomology-associated intragenic deletions, is strongly suggestive of the PALB2 mutation in this patient causing a homologous recombination defect which not only drives the initial PARPi sensitivity phenotype but also the mechanism of resistance that eventually emerges. Although preclinical studies have suggested that PALB2 defects are associated with defective homologous recombination...
recombination, this is to the best of our knowledge the first evidence that the homologous recombination defect caused by a PALB2 mutation might drive not only drug sensitivity but also resistance.

These data are of major clinical relevance for subsequent treatment strategies for this subset of prostate cancers, and probably also for other cancer types. Platinum chemotherapy also has antitumor activity in BRCA2-deficient prostate cancers (34), but our data indicate likely cross-resistance at least for some patients between PARP inhibitors and platinum.

Clinical trials of PARP inhibitors and platinum-based chemotherapy in prostate cancer should therefore account for previous exposure to these drugs and the presence of these secondary reversion mutations.

Overall, our data show the potential of serial cfDNA next-generation sequencing to evaluate both temporal and spatial disease heterogeneity. In some cases, cfDNA could monitor the emergence of resistance mechanisms more comprehensively than single-site biopsies. Such analyses may allow us to truly deliver more precise patient care by integrating real-time,
noninvasive, repeated assessment of disease biology. Our study also shows that simple, inexpensive, cfDNA quantification, and tumor mutation allele frequency analyses, have clinical utility as a response biomarker to guide early treatment switch decisions in the presence of ineffective therapies. Although further validation studies are needed, these findings may be of huge importance, as earlier discontinuation of therapy for futility can spare patients the toxicity of ineffective overtrearment, allowing these men to receive alternative therapy and decreasing treatment health economic costs to fund these cfDNA studies.

A limitation of our study is that we did not collect samples between week 16 of therapy and cancer progression, which in some cases meant over a year of olaparib therapy. Therefore, we were unable to determine precisely when these restoring resistance mutations emerged; this has clinical relevance as these may appear before disease progression detection by established methods and allow earlier treatment changes. In the ongoing TOPARP-B trial, which is further evaluating responses to olaparib in patients with metastatic prostate cancer, preselected based on DNA repair aberrations, monthly samples are being collected to address this. Furthermore, our assays were limited to exon sequencing, so it remains possible that some of these tumors had alternative undetected resistance mechanisms.

In conclusion, we provide strong evidence that serial cfDNA analyses are a powerful test for guiding prostate cancer care allowing disease molecular stratification, response assessment, and the study of emerging resistant clones. Critically, we report previously undetectable divergent emerging subclones in cfDNA only at disease progression due to treatment-selective pressures, with multiple reversion genomic events restoring the DNA repair gene function, all causing acquired resistance through convergent mechanisms. Directing patient treatment through the early detection of emerging resistant tumor clones in cfDNA, either by adding other treatments or by switching therapy contingent on which clone is dominant, is envisioned. This could have a major impact on the treatment and outcome of not only prostate cancer but also other malignancies.

**METHODS**

**Study Population**

All samples were collected prospectively as part of the TOPARP-A clinical trial (CRUK/11/029, NCT01682772). Patients provided written informed consent prior to trial participation. Full details of trial design, eligibility criteria, and response to treatment have been reported previously (10). The study of cfDNA as response and resistance biomarker was included as an exploratory endpoint of the study.

**cfDNA Extraction and Quantification**

Thirty milliliters of blood was collected in CTP tubes from each patient at prespecified time points: at baseline and after 1, 4, 8, and 16 weeks of therapy, and at disease progression when possible. Circulating free DNA was extracted from 4 to 8 mL of plasma using the QIAasympometry (Qiagen) and the Circulating DNA Kit (Qiagen) and quantified by Quant-it High Sensitivity Picogreen Kit (Invitrogen).

**Targeted cfDNA Sequencing**

Targeted next-generation sequencing was performed as described previously (10). Libraries were constructed from 40 ng of cfDNA using a customized GeneRead DNAseq Mix-n-Match v2 panel (Qiagen) and sequenced on the MiSeq Sequencer (Illumina). The somatic variant calls were manually inspected in the Integrative Genomics Viewer (Broad Institute, Cambridge, MA).

**Whole-Exome cfDNA Sequencing**

WES was performed using Kapa Hyper Plus Library Prep Kits and the Agilent SureSelectXT V6 target enrichment system. Paired-end sequencing was performed using the NextSeq 500 (2 × 150 cycles; Illumina). FASTQ files were generated from the sequencer’s output using Illumina bcl2fastq2 software (v.2.17.1.14, Illumina) with the default chastity filter to select sequence reads for subsequent analysis. All sequencing reads were aligned to the human genome reference sequence (GRCh37) using the BWA (v. 0.7.12) MEM algorithm, with indels being realigned using the Stampy (v.1.0.28) package. Picard tools (v.2.1.0) were used to remove PCR duplicates and to calculate sequencing metrics for quality control check. The Genome Analysis Toolkit (GATK, v. 3.5-0) was then applied to realign local indels, recalibrate base scores, and identify point mutations and small insertions and deletions. Somatic point mutations and indels were called using MuTect2 by comparing tumor/plasma DNA to germline control, and copy-number estimation was obtained through modified ASCAT2 package.

**Statistical Analyses**

rPFS was defined as the time from trial entry to progression (RECIST 1.1, bone scan-PCWG2; refs. 35, 36) or death. OS was defined as the time from trial entry to death. Response was defined in the trial protocol as either response by RECIST 1.1, PSA decline of ≥50%, and/or conversion of CTC from baseline ≥5 cells to <5 cells/7.5 mL on treatment, requiring a confirmatory assessment at least 4 weeks later. Mann–Whitney and Fisher exact tests were used to assess the association of continuous and categorical variables with response. Kaplan–Meier curves are presented for time-to-event endpoints. The prognostic significance of changes in cfDNA at week 4 and week 8 from baseline were explored using landmark analyses. Patients experiencing rPFS and/or OS events before the landmark time were excluded. HRs were estimated utilizing Cox regression univariate and multivariable models; 95% CIs are provided. In the absence of a validated cutoff, log fold change, 30% and 50% declines in cfDNA were evaluated. The proportional hazards assumption of the Cox model was tested using Schoenfeld residuals. On the basis of the exploratory nature of these analyses, Bonferroni adjustment of P values was not pursued but a P value <0.01 was predefined as significant to account for multiple testing. Statistical analyses were conducted using STATA13 (StatCorp LP) on a data snapshot taken on May 24, 2016, when 49 of 50 patients had discontinued the trial.

**Disclosure of Potential Conflicts of Interest**

S. Sandhu has received speakers bureau honoraria from Bristol-Myers Squibb and Merck. J.S. de Bono has received speakers bureau honoraria from AstraZeneca and is a consultant/advisory board member for the same. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: J. Goodall, J. Mateo, S. Sandhu, E. Hall, S. Carreira, J.S. de Bono

Development of methodology: J. Goodall, W. Yuan, S. Miranda, S. Sandhu, S. Carreira, J.S. de Bono

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Goodall, J. Mateo, R. Perez-Lopez, D.R. Robinson, S. Sandhu, G. Fowler, B. Ebbs, P. Flohr, M. Aitkin, M. Crespo, P. Rescigno, Z. Zafeiriou, N. Tunariu, D. Bianchini, A. Gillman, E. Hall, A.M. Chinnaiyan, S. Carreira, J.S. de Bono

Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): J. Goodall, J. Mateo, W. Yuan, H. Mossop, N. Porta, D. Dolling, D.R. Robinson, S. Sandhu, G. Seed,
Goodall et al.

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