Castration Therapy Results in Decreased Ku70 Levels in Prostate Cancer

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Abstract

**Purpose:** Neoadjuvant castration improves response to radiotherapy of prostate cancer. Here, we determine whether castration therapy impairs nonhomologous end-joining (NHEJ) repair of DNA double-strand breaks (DSB) by downregulating Ku70 protein expression.

**Experimental Design:** Twenty patients with locally advanced prostate cancer were enrolled, and 6 to 12 needle core biopsy specimens were taken from the prostate of each patient before treatment. Bilateral orchidectomy was conducted in eight patients and 12 patients were treated with a GnRH agonist. After castration, two to four similar biopsies were obtained, and the levels of Ku70 and γ-H2AX were measured.

**Results:** We observed that the androgen receptor binds directly to Ku70 in prostate tissue. We also found a reduction of the Ku70 protein levels in the cell nuclei in 12 of 14 patients (P < 0.001) after castration. The reduction in Ku70 expression correlated significantly with decreased serum prostate-specific antigen (PSA) levels after castration, suggesting that androgen receptor activity regulates Ku70 protein levels in prostate cancer tissue. Furthermore, a significant correlation between the reductions of Ku70 after castration versus changes induced of castration of γ-H2AX foci could be seen implicating a functional linkage of decreased Ku70 levels and impaired DNA repair.

**Conclusions:** Castration therapy results in decreased levels of the Ku70 protein in prostate cancer cells. Because the Ku70 protein is essential for the NHEJ repair of DSBs and its downregulation impairs DNA repair, this offers a possible explanation for the increased radiosensitivity of prostate cancer cells following castration. *Clin Cancer Res;* 19(6); 1547–56. ©2013 AACR.

Introduction

Approximately one million new cases of prostate cancer are diagnosed in the world each year (1). Nonmetastatic prostate cancer is potentially curable and one treatment modality is radiotherapy, either alone or in combination with neoadjuvant castration therapy using gonadotropin-releasing hormone (GnRH) agonists for a limited period of time. Clinical studies have shown synergistic effects between castration and radiotherapy (2). A more detailed understanding of the cellular and molecular mechanisms behind this synergism would allow us to optimize treatment and may also give rise to novel therapeutic applications. We have previously shown that the hypoxia levels in prostate cancer tissue decrease following castration (3). Because hypoxia is associated with poor response to radiotherapy (4, 5), reduced hypoxia after castration may offer one plausible mechanistic explanation. The androgen receptor plays an important role in the genesis, growth, and survival of normal prostate cells. Epidemiologic studies have failed to show any relationship between high serum levels of testosterone/dihydrotestosterone or adrenal androgen and prostate cancer risk (6, 7). In a rat model, a reduction in serum dihydrotestosterone after castration resulted in a 70% loss of secretory epithelial prostate cells by means of apoptosis (8). In the cytoplasm, the androgen receptor is found in inactive complexes bound to the HSP70 and HSP90 and as a corepressor. Testosterone and dihydrotestosterone bind to the androgen receptor as ligands. Androgen receptor translocates to the nucleus and binds to androgen response elements (ARE) in the promoter regions of target genes, thus inducing cell proliferation (9).

Castration therapy by orchidectomy or pharmacologic castration adequately reduces the serum testosterone below 1.7 nmol/L. However, intraprostatic tissue testosterone levels reduce only by 75% (10) which in turn leads to reduction rather than abrogation of androgen receptor activities. Castration is not curative as a single treatment...
Translational Relevance

Clinical studies have shown improved radiotherapy response and longer overall survival for patients with prostate cancer after neoadjuvant androgen deprivation, but mechanistic insights are missing. In this study, the levels of the Ku70 protein, critical for DNA double-strand break repair, decrease after castration in parallel with corresponding reduction in serum prostate-specific antigen (PSA). This reduced level of the repair protein offers a probable explanation for the increased treatment efficiency, when radiotherapy is combined with castration therapy. Furthermore, according to our data, the castration-induced decrease in PSA may predict a successful response to radiotherapy.

modality but is the most effective palliative treatment in metastasized prostate cancer. There is no difference in long-term survival between the 2 castration methods (11, 12). Castration is achieved almost immediately after bilateral orchidectomy and approximately 4 weeks after initiating treatment with GnRH agonists (13). Randomized clinical trials have shown longer survival of patients with prostate cancer after combined castration and radiotherapy of the primary tumor than after radiotherapy alone (2, 14). This clinically observed increased radiosensitivity of prostate cancer after castration was not seen in vitro with cell lines LNCaP and PC-3 (15, 16).

The expression of prostate-specific antigen (PSA) is primarily regulated by androgen receptor via AREs (17) and is considered to be the most effective single biomarker for monitoring the metabolic activity of prostate cancer cells before, during, and after radiotherapy (18).

Nonhomologous end-joining (NHEJ) is the major DNA repair pathway involved in the repair of DNA double-strand breaks (DSB) after ionizing radiation (IR; ref. 19). Unrepaired or misrepaired DSBs lead either to cell death or chromosomal translocations and genomic instability. DNA protein kinase (DNA-PK) is a key component of NHEJ (20), which consists of the Ku70–Ku80 heterodimer and the DNA-PK catalytic subunit (DNA-PKcs) to form the DNA-PK complex. Ku proteins play a central role in NHEJ by detecting DSB ends and tethering them together (21, 22). Cells deficient in Ku70 and Ku80 proteins are extremely sensitive to IR (23, 24). Even though Ku70 is mainly located in the nucleus, a fraction of it is located in the cytoplasm. The cytoplasmic fraction of Ku70 binds the proapoptotic protein Bax, preventing its translocation to mitochondria and thereby suggesting that Ku70 suppresses mitochondrially mediated apoptosis. Depletion of the level of cytosolic Ku70 has been shown to induce apoptosis in colorectal cancer (25). Because the Ku70 protein has been shown, in prostate cell lines, to act as a coactivator of androgen receptor (26), we wanted to test whether the differential AR function after castration influences Ku70 protein levels. If so, it might offer an explanation for increased apoptosis and radiosensitivity after castration.

Using prostate needle core biopsy specimens from patients with prostate cancer, we tested whether castration downregulates the expression of Ku70 proteins in prostate cancer specimens, subsequently leading to defective DNA repair and increased cell death.

Materials and Methods

Patient material and collection

Twenty patients with newly diagnosed prostate cancer were enrolled in the study, after the approval of the regional ethics committee at Uppsala University (Uppsala, Sweden; Dnr 2007/170).

At diagnosis, the first biopsy setting, 6 to 12 prostatic needle core biopsy specimens were taken randomly from each patient. All patients were then treated with castration therapy, either with bilateral orchidectomy or with a GnRH-agonist (leuprorelin; Table 1). After castration, that is, approximately 1 month after orchidectomy and 2 months after the initiation of the GnRH agonist, the second biopsy setting, 2 to 4 prostatic needle core biopsy specimens were taken from each patient. To increase the likelihood of obtaining representative specimens, only presumed tumor areas were chosen for the second biopsy setting. Six patients were excluded from the study because biopsy specimens from their second biopsy setting did not contain representative cancer areas. Fourteen patients were included in the study and their median age was 78 years (range, 59–89 years). The median PSA level was 98 ng/mL (range, 3–1,021 ng/mL). The median serum testosterone level was 11.0 nmol/L (range, 6.6–23.0 nmol/L). The median prostate volume measured by means of transrectal ultrasound was 52 mL (range, 20–160 mL). Thirteen patients had locally advanced cT3-4 tumors and only one patient had an organ-confined cT2 tumor. Half of the patients received a GnRH agonist, whereas bilateral orchidectomy was conducted in the other half. The mean time and SD from orchidectomy to the second biopsy was 26 ± 19 days and from initiating GnRH agonist treatment to second biopsy was 54 ± 14 days. After orchidectomy, the PSA levels varied from 1 to 148 ng/mL and the serum testosterone levels from 0.3 to 0.9 nmol/L. After GnRH agonist treatment, the PSA was 0.3 to 55 ng/mL and serum testosterone 0.3 to 1.7 nmol/L.

Histological and immunofluorescence evaluation

From each patient, 2 biopsy specimens before and two after castration were chosen. The specimens were embedded in paraffin and sectioned. One section from each biopsy specimen was stained with hematoxylin and eosin (HE) and graded according to the Gleason system (27). Sections adjacent to the HE-stained sections were used for immunofluorescence studies.

Deparaffinization and rehydration of the sections from 2 series of slides were conducted before antigen retrieval in Tris/EDTA, pH 9.0, in a pressure cooker. The sections were blocked in 3% bovine serum albumin (BSA). Afterward, sections from the first set were incubated with the primary
Castration Decreases Ku70 Protein Levels in Prostate Cancer

Table 1. Patient demography and tumor characteristics of patients included in this study

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<th>Patient age</th>
<th>Prostate volume, ml</th>
<th>Clinical tumor stadium (cT)</th>
<th>Gleason score (GS)</th>
<th>PSA, ng/mL</th>
<th>Testosterone, nmol/L</th>
<th>Days between castration and biopsies</th>
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Abbreviations: cT, clinical tumor stadium; GS, Gleason score; ND, not done.

antibody KU-70 (1:500, 3C3.11 Santa Cruz) at 4°C overnight. While sections from the second set were incubated with the primary antibody γ-H2AX (1:500, 3F2, Abcam) at 4°C overnight. Extensive rinsing was conducted once the sections were incubated with the secondary antibody (donkey anti mouse IgG-Alexa 488, Molecular probe) for 1 hour at room temperature. DNA was counterstained with TO-PRO-3 iodide (Molecular probe) and slides mounted with pro long gold (Molecular probe).

An image from a tumor area with a good degree of immunofluorescence signals from each biopsy was selected. The corresponding area in the HE-stained section was identified for the histologic verification of the tumor area. In Ku70-stained slides, 3 areas containing approximately 150 to 300 cells were chosen for analysis with NIH ImageJ. Areas inside the tumor area containing approximately 150 to 300 cells were chosen for the histologic verification of the tumor area. In immunofluorescence signals from each biopsy was selected. The corresponding area in the HE-stained section was identified for the histologic verification of the tumor area. In Ku70-stained slides, 3 areas containing approximately 150 to 300 cells were chosen for analysis with NIH ImageJ. Areas inside the tumor area containing approximately 150 to 300 cells were chosen for the histologic verification of the tumor area. In

Results

Ku70 interacts with the androgen receptor in prostate cancer tissue

Radiosensitive cells have previously been isolated, and the underlying mechanism explaining the sensitivity has, in most cases, been defects in DNA repair (28). We hypothesized that castration therapy may alter the DNA repair capacity of prostate cancer cells. Androgen receptor activity is altered following castration and it has previously been reported that Ku70 interacts with the androgen receptor in the prostate cell line LNCaP (26). To investigate whether this interaction is also present in in vivo material, a co-immunoprecipitation of androgen receptor with Ku70 from

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prostate tissue extracts before and after castration indicated an interaction between androgen receptor and Ku70 both before and after castration (Fig. 1), and we also found that the castration itself did not influence the interaction between the two proteins.

Ku70 protein levels decrease following castration

Because the androgen receptor and Ku70 proteins interact, we subsequently wanted to determine whether the Ku70 protein levels are influenced by castration. We quantified the levels of Ku70 protein in cancer areas in paired slides from 14 eligible patients (Table 1) with high-grade prostate cancer before and after castration (Fig. 2). The levels of Ku70 in the nuclei were reduced by approximately half the value observed before castration ($P = 0.001$; Fig. 3A). A decrease of the same magnitude could also be seen in the Ku70 localized outside the nuclei ($P = 0.006$; Fig. 3B). However, the intensity of Ku70 was always higher in the nuclei than outside, both before and after castration (Figs. 2 and 3A and B).

We observed large individual variations in Ku70 protein levels both for the intranuclear fraction, ranging from 20 to 180, and the extranuclear fraction, ranging from 4 to 120 arbitrary intensity units (Fig. 3C and D). After castration, a decrease in the Ku70 level was seen in almost all patients in both fractions of Ku70 (Fig. 3C and D). Patients who did not respond with a Ku70 decrease after castration had very low initial Ku70 values (Fig. 3C and D). In fact, the initial Ku70 value, that is, before castration, correlated well with the decrease in Ku70 after castration in both fractions of Ku70 (intranuclear $R^2 = 0.811$, $P < 0.0001$; Fig. 3E, extranuclear $R^2 = 0.944$, $P \leq 0.0001$; Fig. 3F). Even though the level of Ku70 varied greatly between patients, the Ku70 value between the 2 fractions, intra- and extranuclear, correlated well for each patient ($R^2 = 0.817$, $P < 0.0001$; Fig. 3G).

Ku70 does not correlate with prostate size, serum PSA, serum testosterone, Gleason score, or patient age

The interindividual variations in initial Ku70 levels are large and can vary by a factor of 10 for both nuclear and cytoplasmic Ku70 (see Fig. 3C and D). We investigated whether there was any correlation between the Ku70 levels and other parameters measured in this study. There was no significant correlation to prostate size ($R^2 = 0.14$, $P = 0.97$), PSA level ($R^2 = 0.004$, $P = 0.97$), testosterone level ($R^2 = 0.0004$, $P = 0.80$), Gleason score ($R^2 = 0.09$, $P = 0.81$), or age ($R^2 = 0.007$, $P = 0.48$) versus the nucleic fraction of Ku70. This observation was also valid for the cytoplasmic fraction of Ku70 [prostate size ($R^2 = 0.20$, $P = 0.66$), PSA level ($R^2 = 0.0001$, $P = 0.94$), testosterone level ($R^2 = 0.02$, $P = 0.49$), Gleason score ($R^2 = 0.07$, $P = 0.82$), and age ($R^2 = 0.004$, $P = 0.60$)].

Ku70 reduction after castration correlates with decreased PSA, but not with decreased testosterone levels

Although there was significant decrease in Ku70 levels in prostate cancer cells after castration, the interindividual variations of the castration-induced changes in Ku70 levels were large, spanning from 2% to 126% in nuclei and 2% to 340% in cytoplasm. There was also a large variation in the testosterone and PSA reductions. The castration-induced reduction in serum PSA varied from 24% to 99% (Table 1). For patient number 2, who had a low PSA and did not respond to hormonal treatment, we noticed a 28% increase of PSA compared with the precastration value. The reduction of serum testosterone varied from 85% to 98%.

We compared pharmacologically castrated with orchiectomized patients. Patient number 8 belonged to the surgical castration group and showed atypical data in comparison with the rest of this subgroup and was, therefore, considered an outlier and was excluded from the calculations of coefficient of determination and probability values. The value of patient 8 is depicted as an unfilled circle in every figure. Between the 2 subgroups, there were no significant differences in measured parameters. Both types of castration show a significant correlation between the decrease in Ku70, both in the nuclear and the cytoplasmic fractions, and the decrease in PSA after castration (chemical nuclear $R^2 = 0.91$, $P = 0.003$, cytoplasmic $R^2 = 0.77$, $P = 0.023$ and surgical nuclear $R^2 = 0.73$, $P = 0.018$, cytoplasmic $R^2 = 0.84$, $P = 0.005$ Fig. 4A–D). No such correlation could be seen between the decrease in Ku70 and the decrease in testosterone after castration (chemical nuclear $R^2 = 0.014$, $P = 0.96$, cytoplasmic $R^2 = 0.002$, $P = 0.70$ and surgical nuclear $R^2 = 0.002$, $P = 0.96$, cytoplasmic $R^2 = 0.11$, $P = 0.62$).

Ku70 and PSA kinetics after castration are related

The kinetics of PSA and testosterone decrease depends on the castration method and this may influence the effect on Ku70 levels differently. Therefore, PSA kinetics could potentially be used to calculate the optimal time for starting radiotherapy.

To obtain an indication of the kinetics of the Ku70 decrease, the correlation between Ku70 decrease and the interval between the first and second biopsies was investigated. Patients treated with bilateral orchidectomy showed a strong significant correlation ($R^2 = 0.77$, $P = 0.02$; Fig.
5A), whereas patients treated with GnRH agonist had no significant correlation ($R^2 = 0.36, P = 0.26$; Fig. 5B). Similar results were achieved for the cytoplasmic fraction of Ku70 (Supplementary Fig. S1). This suggests that the Ku70 level in patients treated with bilateral orchidectomy is still decreasing in the time span used here, whereas the Ku70 levels in patients treated with GnRH agonist have reached a plateau.

To see whether the PSA levels had similar kinetics, the correlation between PSA decrease and the number of days after castration was analyzed. Indeed, in patients treated with bilateral orchidectomy, a significant correlation was seen between the decrease in the PSA value and the number of days after castration ($R^2 = 0.84, P = 0.019$; Fig. 5C). In contrast, patients treated with GnRH agonist did not show a significant correlation ($R^2 = 0.25, P = 0.26$; Fig. 5D).

Investigating the decrease in testosterone versus the days after castration, we found that patients treated with bilateral orchidectomy had no significant correlation ($R^2 = 0.03, P = 0.66$; Fig. 5E), which was expected because all the reduction in testosterone levels normally occurs the first few days after bilateral orchidectomy. A similar picture was seen in the group treated with a GnRH agonist ($R^2 = 0.19, P = 0.26$).
Fig. 5F). These data suggest that the kinetics of PSA decrease is the most relevant parameter to use for predicting when the minimum level of Ku70 has been reached and, thereby, the most optimal time point for radiation treatment.

Ku70 reduction after castration correlates with an increase of γ-H2AX foci

To investigate whether the initial Ku70 values or the decrease of Ku70 after castration would be reflected in the amount of unrepaired endogenous DNA damages, we stained slides from the biopsies with an antibody against γ-H2AX. Images of tumor areas were then analyzed with respect to the number of γ-H2AX foci/DNA unit with a program written in ImageJ (Fig. 6A–C). No significant difference in γ-H2AX foci could be seen in mean values of biopsies before and after castration (Fig. 6D). Neither could any correlation between the initial Ku70 values versus the amount of γ-H2AX foci be seen ($R^2 = 0.009, P = 0.3$). However, a significant correlation between the reductions of Ku70 after castration versus changes induced of castration of γ-H2AX foci could be seen (Fig. 6E, $R^2 = 0.37, P = 0.022$). These data could point to a reduced repair of DNA damage following a decrease of Ku70 levels.
Discussion

We show a reduction of the Ku70 protein in prostate cancer tissues after castration. The Ku70 protein is a major determinant of radiosensitivity. In several unbiased mutational screens in mammalian cells, the Ku70 mutant cells, defective in NHEJ (24), were isolated as the most radiosensitive (28). It is well established that the Ku70/Ku80 components of NHEJ are the most critical for effective DSB repair and they work in a DNA-PK–dependent manner after irradiation (19, 29). Hence, the severe depletion of Ku70 protein levels after castration is likely to impair NHEJ in the prostate cancer tissues and explain the increased sensitivity to radiotherapy. Several previous studies have shown that cells depleted with Ku70 are using an alternative backup pathway termed B-NHEJ in repairing irradiation-induced DSBs and PARP-1 is a major component of this alternative repair pathway (30). After castration, effective B-NHEJ mediated by PARP inhibitors would further improve radiosensitivity. However, the role of PARP-1 in prostate cancer is very complex as it has recently been shown to mediate androgen receptor function (31). Certainly, the role of PARP-1 in the repair of irradiation induced DSBs and androgen receptor transcription with and without castration warrant further studies.

To test whether the reduction of Ku70 seen after castration changed the repair kinetics of endogenous DNA lesions, we stained for the DNA DSB marker γ-H2AX. A significant positive correlation could be seen between Ku70 reductions and increase in γ-H2AX foci (Fig. 6E). However, no significant difference could be seen between biopsies before and after castration (Fig. 6D). This seemingly contradictory result may be explained by the castration effect on cell proliferation. Cells are constantly exposed to endogenous reactive oxygen species (ROS) production and replication failure leading to DNA lesions. This is particularly true for cancer cells that often have an elevated ROS level. A high ROS level leads to DNA single-strand breaks, and if not repaired, collapsed replication forks during replication (32). Collapsed replication forks are one ended DNA DSBs and will be marked with γ-H2AX. Androgen depletion leads to decreased rates of proliferation and thereby fewer collapsed replication forks. On the other hand, as shown...
Androgen deprivation leads to reduction of Ku70 levels which may slow down the repair kinetics and thereby lead to an increased amount of unrepaired DNA lesions. Thus, androgen deprivation starts processes that can lead to either increase or decrease of γ-H2AX. Indeed, as shown in Fig. 6, patients with no or minor decrease of Ku70 after androgen depletion shows a decrease of γ-H2AX, meanwhile patients with a large decrease of Ku70 shows an increase of γ-H2AX, implicating a functional linkage of decreased Ku70 levels and impaired DNA repair.

An interaction between the androgen receptor and Ku70 has been reported in the prostate cell line LNCaP (26) and we can confirm the interaction between the androgen receptor and Ku70 proteins in prostate cancer tissue. The interaction between the androgen receptor and Ku70 may unveil a mechanistic link between castration treatment and reduced Ku70 protein levels. It is indeed very difficult to test this potential mechanistic link experimentally in patients. However, the high correlation between decreased levels of PSA and decreased Ku70 levels (Fig. 4) supports the notion that the downregulation of Ku70 is related to a decrease in androgen receptor activity. We speculate that the binding between Ku70 and androgen receptor may influence the long-term stability of the Ku70 protein in prostate cancer cells. Another possibility is that androgen receptor inhibition or downregulation results in a decrease in the expression of NHEJ proteins.

In this study, none of the included patients were offered any curative therapy such as radiotherapy because they all had an advanced prostate cancer with a short life expectancy. However, all patients were treated with castration. Androgen deprivation leads to decreased rates of cell proliferation and induces apoptosis in prostate cancer cells (33). The pool of cytoplasmic Ku70 has been shown to totally disappear in cells entering senescence (34). Furthermore, the cytoplasmic fraction of Ku70 has been shown to sequester the proapoptotic protein Bax and, thereby, functions as an antiapoptotic protein (35). Because the cytoplasmic Ku70 was reduced to approximately half following castration, this may contribute to apoptosis induced by androgen depletion (36).

The optimal duration of neoadjuvant hormonal treatment is unknown. However, to achieve an optimal outcome of combined castration and radiotherapy, it might be of importance to start the radiation therapy when the level of Ku70 has reached its lowest value. Nevertheless, in most clinical studies, neoadjuvant hormonal therapy is recommended to start 2 to 6 months before the initiation of radiotherapy (37–39). To date, many studies have tried to relate the time of neoadjuvant hormonal therapy to their effect on prostate volume (40). However, other studies, have tried to find out a cutoff point for the serum PSA level (41–43).

The results of this study suggest that the PSA decrease after castration reflects the decrease in Ku70 levels in prostate cancer cells and, therefore, could be a good marker for defining the optimal time for radiotherapy.

Future studies should investigate the correlation between Ku70 and PSA decrease over time to validate the findings made in this study and, thereby, open for an improved clinical outcome of combined castration and radiotherapy. Furthermore, it would be of great interest to investigate whether the huge individual variations in Ku70 levels in cancerous prostate tissue also reflect radiation sensitivity. 

Abriratone and enzalutamide (formerly known as MDV3100) are 2 new target therapies with clinical benefits.
in treatment of castration-resistant prostate cancer (44, 45). However, any connections between these therapies and Ku70/NHEJ remain unexplored. Because the mechanism of action of these new target therapies will lead to further suppression of active androgen receptor, we presume that used neoadjuvantly they will consequently lead to further suppression of Ku70 and increase the radiosensitivity of prostate cancer cells. Obviously, this issue will be a matter of future studies.

In conclusion, we observed that the Ku70 protein level decreases in prostate cancer cells following castration and this may possibly explain the increased response to radiotherapy observed after neoadjuvant castration.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: F. Al-Ubaidi, N. Schultz, T. Granfors, T. Helleday
Development of methodology: F. Al-Ubaidi, N. Schultz, T. Helleday
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Al-Ubaidi, N. Schultz, O. Loseva, L. Egevad
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Al-Ubaidi, N. Schultz, O. Loseva, L. Egevad, T. Helleday
Writing, review, and/or revision of the manuscript: F. Al-Ubaidi, N. Schultz, O. Loseva, L. Egevad, T. Granfors, T. Helleday
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Al-Ubaidi, T. Helleday
Study supervision: N. Schultz, T. Helleday

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