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Physiologic serum 1,25 dihydroxyvitamin D is inversely associated with prostatic Ki67 staining in a diverse sample of radical prostatectomy patients

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Abstract

Purpose—To investigate the correlation between serum 25 hydroxyvitamin D, prostatic 25 hydroxyvitamin D, and serum 1,25 dihydroxyvitamin D, and their respective associations with prostatic tumor proliferation at the time of radical prostatectomy.

Methods—In this cross-sectional analysis of 119 men undergoing radical prostatectomy, serum from whole blood and expressed prostatic fluid was collected on the day of surgery. Tumor proliferation was measured in the dominant tumor on formalin-fixed prostatectomy tissues by immunohistochemical staining for Ki67 and quantified by Aperio imaging analysis.

Results—The sample included 88 African Americans (74%) and 31 (26%) European Americans. Serum and prostatic levels of 25 hydroxyvitamin D were correlated with each other (Spearman's rho (ρ) = 0.27, p = 0.004), and there was also a correlation between serum 25 hydroxyvitamin D and 1,25 dihydroxyvitamin D (ρ = 0.34, p < 0.001). Serum and prostatic 25 hydroxyvitamin D levels were not correlated with Ki67 staining in tumor cells. Serum 1,25 dihydroxyvitamin D was inversely correlated with Ki67 staining in tumor cells (ρ = -0.30, p = 0.002). On linear regression, serum 1,25 dihydroxyvitamin D was negatively associated with Ki67 staining in tumor cells (β = -0.46, 95% CI: -0.75, -0.04, p = 0.04).

Conclusion—The correlation between physiologic serum levels of 25 hydroxyvitamin D with both prostatic 25 hydroxyvitamin D and serum 1,25 dihydroxyvitamin D suggests that serum levels are reasonable biomarkers of prostatic vitamin D status. Furthermore, serum 1,25

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Conflict of interest

There are no conflicts of interest for the listed authors.

dihydroxyvitamin D has an inverse association with Ki67 staining in tumor cells at physiologic levels and may protect against tumor progression.

Keywords

Prostate cancer; Vitamin D deficiency; Proliferation; African American

Introduction

Serum 25 hydroxyvitamin D deficiency is associated with elevated risk of aggressive prostate cancer (PCa) [1–7] and is thought to contribute to disparities in PCa outcomes among African American (AA) men, a group known to have a higher prevalence of serum 25 hydroxyvitamin D deficiency than European American (EA) counterparts [8, 9]. Several preclinical experiments and some clinical trials have suggested that 25 hydroxyvitamin D may have a protective effect against PCa [10–14]. Currently, it is known that 25 hydroxyvitamin D, when converted to its active form, 1,25 dihydroxyvitamin D, exhibits important anti-proliferative effects on prostatic epithelium in vitro and has anti-proliferative effects in clinical trials [15–22].

Despite 1,25 dihydroxyvitamin D's proven effects at the cell culture level [23], serum 1,25 dihydroxyvitamin D measurements may not be associated with prostate proliferation because serum 1,25 dihydroxyvitamin D has a short half-life, is tightly regulated by parathyroid hormone and is metabolized within prostatic tissue [24, 25]. In a randomized controlled trial that included patients receiving oral 25 hydroxyvitamin D, Wagner et al. [12] have found an association between serum and prostatic vitamin D metabolites, as well as an inverse association between prostatic 1,25 dihydroxyvitamin D levels and Ki67 staining, a marker of cellular proliferation.

In the present study, we aim to validate many of these findings in a cross-sectional sample of patients not receiving exogenous 25 hydroxyvitamin D supplementation. We first investigate the correlations between physiologic serum and prostatic levels of 25 hydroxyvitamin D, as well as physiologic serum 1,25 dihydroxyvitamin D. Second, we test the associations of these metabolites with the degree of Ki67 staining, a proliferation marker in human prostatic epithelial cells, from formalin-fixed paraffin-embedded radical prostatectomy specimens. We hypothesize that serum and prostatic vitamin D metabolites are correlated and that both metabolites are inversely associated with prostatic epithelial proliferation.

Materials and methods

Study population

This is a single-center cross-sectional study of 119 men, age 40–79 years, diagnosed with clinically localized PCa who elected radical prostatectomy for initial definitive therapy. Men were continuously enrolled from 2013 to 2018 at a Chicago VA Medical Center urology clinic. Of 269 AA and European American (EA) men with PSA < 100 ng/mL who were initially approached for consent after their outpatient urology visit, we identified 219 eligible candidates. Exclusion criteria included patients opting for active surveillance or deemed to

be poor surgical candidates, history of end-stage renal or liver failure, parathyroid disorders, and treatment with androgen deprivation therapy or pelvic radiotherapy (for any pelvic malignancy). Fifty men refused enrollment and 169 men provided consent. Ki67 staining was not completed on 50 participants.

Clinical data

Clinical and demographic data were retrieved from patients' electronic health records and patient-administered questionnaires at time of enrollment or on postoperative day 1. Relevant clinical covariates obtained included age, first-degree family history of PCa, alcohol and tobacco use, marital status, and self-reported ethnicity/race, which was classified as either non-Hispanic European American, African American/ non-Hispanic Black, or Other. Body mass index was calculated using standing height (m) and weight (kg) measurements from the date of enrollment. Total vitamin D intake was obtained from a validated structured questionnaire [26, 27]. Season was modeled as a binary variable with high ultraviolet radiation exposure (May 1–October 31) and low-exposure months (November 1–April 30) [6].

Specimen handling and pathological data

Serum, whole blood and fresh prostatic secretions were collected from each patient included in the analysis. Each prostatectomy specimen was gently wiped with saline-soaked gauze. Prostatic secretions were extracted by milking both seminal vesicles and prostate from their most distal aspects to the urethral meatus. The expressed prostatic fluid was centrifuged to remove any cellular components and the supernatant was stored at -20°C . Whole blood was centrifuged for serum separation and samples were similarly stored at -20°C on the day of specimen collection. Serum and prostatic fluid were sent to Heartland Assays (Ames, Iowa) for measurement of 25 hydroxyvitamin D levels. Due to low fluid volumes, 1,25 dihydroxyvitamin D and 24,25 dihydroxyvitamin D levels in the prostate were not analyzed. Heartland assays also provided serum levels of total 1,25 dihydroxyvitamin D and total 24,25 dihydroxyvitamin D.

All prostatectomy specimens were graded and staged according to the 2014 Gleason grading system [28] and guidelines [29] by two experienced pathologists (AKB, VM). A representative block containing the largest diameter of the highest Gleason grade tumor was selected for immunohistochemistry. The corresponding tumor block was requested from the VA Pathology Department archives. The tumor blocks were then recut to produce 5- μm -thick slices.

Immunohistochemistry (IHC) and quantification of Ki67 immunostaining

To quantify proliferation, we used Ki67 labeling index, a widely established marker of tumor proliferation [30, 31] known to be associated with unfavorable PCa outcomes, including metastasis, mortality, and decreased time to progression [32–34]. Consecutive prostatic sections, each from a representative block, were stained for Ki67/CK8 + CK18. Dual Ki67/CK8 + CK18 staining was performed on Bond RX auto-stainer (Leica Biosystems) using a sequential dual-IHC protocol. In brief, sections were deparaffinized, subjected to citrate-based (Bond ER1 solution, pH 6) antigen retrieval for 40 min at 100°C and blocked

with hydrogen peroxide for 5 min. Following a 30-min incubation with Background Sniper protein block (Biocare Medical #BS966), sections were incubated with anti-Ki67 rabbit recombinant monoclonal antibody (1:100, Abcam #ab16667) for 30 min and with CK8 + CK18 rabbit monoclonal antibody cocktail (1:300, Epitomics, #AC-9002RUOC). Appropriate positive controls were used for each antibody. All cases had contralateral non-neoplastic prostatic epithelium analyzed which served as an internal negative control as appropriate. The detection was performed using Bond Polymer Refine Detection kit (Leica Biosystems, #DS9800) and Bond Polymer Refine Red Detection kit (Leica Biosystems, #DS9390). Hematoxylin was used as counterstain.

Slides were scanned using Aperio AT2 at 20 × magnification (Leica Biosystems). Regions for analysis were drawn by the research pathologist (VM) using ImageScope (Leica Biosystems) and Halo (Indica Labs), with positive regions indicating areas to be scored and negative regions indicating areas to be excluded such as folds, debris and secretions. Images were uploaded into Halo (Indica Labs, v2.1.1637.11) and the Multiplex IHC algorithm was used for analysis. The algorithm was configured to detect Hematoxylin, Ki67 and CK (blue, brown and red, respectively) and to identify the nucleus and cytoplasm of each cell. Thresholds were set for Ki67 nuclear stain and CK cytoplasmic staining to identify Ki67-positive and -negative epithelial cells. The algorithm was run on all slides and then reviewed for accuracy. Minor threshold adjustments were made if necessary to accurately identify nuclei and exclude poorly stained regions from analysis.

Statistical analysis

Spearman's bivariate correlation was used to analyze the relationship between prostatic and serum 25 hydroxyvitamin, serum 1,25 dihydroxyvitamin D, and both tumor and normal Ki67 staining percent. Kruskal–Wallis H and independent samples *t* tests were used to evaluate the relationship between quartiles of serum 1,25 dihydroxyvitamin D and tumor and normal Ki67 staining, as well as the relationship between quartiles of serum 25 hydroxyvitamin and prostatic 25 hydroxyvitamin D and serum 1,25 dihydroxyvitamin D. The percentage of cells with positive Ki67 staining was coded as a continuous measure of tumor proliferation (0–100% labeled). Linear regressions were constructed to determine the associations between tumor proliferation percentage and vitamin D status (i.e., serum 1,25 dihydroxyvitamin D, serum 25 hydroxyvitamin D and prostatic 25 hydroxyvitamin D). The linear models were adjusted for Gleason grade group [28], age, PSA, and the percent of normal epithelial cell Ki67 staining in case-matched samples. For the linear models, all three vitamin D metabolites were modeled as quartiles. The prostatic 25 hydroxyvitamin D levels for six participants were imputed. All statistical analyses were performed using the statistical package IBM SPSS version 24 (SPSS Inc., Chicago, IL, USA). The alpha = 0.05 and was two-sided for all analyses. The VA Institutional Review Board approved this protocol.

Results

The median age of our study population was 65.0 years (IQR: 59.5–69.0 years). When stratified by tertiles of serum 25 hydroxyvitamin D (0–18.0, 18.0–27.5, and 27.5 ng/ mL), we found no significant differences in median age, BMI, PSA, or prostate volume, though

intake of vitamin D was associated with higher serum 25 hydroxyvitamin D tertiles (Table 1). Median serum 1,25 dihydroxyvitamin D showed both a significant upward trend as tertiles of serum 25 hydroxyvitamin D increased ($p = 0.01$; Table 1) and a significant contrast between the first and third tertiles (39.9 vs 64.4 pg/mL, $p = 0.001$; Table 1). The median serum 1,25 dihydroxyvitamin D level was higher in AA's than in non-AA's (49.9 vs 44.5 pg/mL), though not significantly ($p = 0.88$). Prostatic 25 hydroxyvitamin D levels also showed a significant positive trend with increasing serum 25 hydroxyvitamin D tertiles ($p = 0.003$; Tables 1, 2) and a significant difference between tertiles I and III (0.31 vs. 1.82 ng/mL, $p = 0.002$; Table 1).

As expected, a significantly lower proportion of participants in the first tertile of serum 25 hydroxyvitamin D (0–18.0 ng/mL) had their blood drawn during the high-UV radiation season (May–October) than in the other two tertiles (28.9% vs 52.2% and 51.1% $p = 0.03$). The proportion of AA participants significantly decreased with increasing tertiles of serum 25 hydroxyvitamin D (87.8% vs 73.5% vs 60.4%, $p_{\text{trend}} = 0.002$, p for tertile 1 versus tertile 3 = 0.002; Table 1). Overall, AA participants had lower median serum 25 hydroxyvitamin D than non-AA (21.8 vs 27.6 ng/mL), but the difference did not reach significance ($p = 0.09$). There was no significant difference in the frequency of positive family history of PCa, lymph node invasion, or Gleason 3 + 4 between groups ($p > 0.05$).

On univariate analysis of the continuous variables in Table 2, serum and prostatic 25 hydroxyvitamin D levels were significantly correlated ($\rho = 0.27$, $p = 0.004$, Fig. 1a). Furthermore, serum 1,25 dihydroxyvitamin D was inversely correlated with both tumor ($\rho = -0.31$, $p = 0.002$, Fig. 1d) and normal ($\rho = -0.21$, $p = 0.03$, Fig. 1c) Ki67 labeling, and positively correlated with serum 25 hydroxyvitamin D quartiles ($\rho = 0.34$, $p < 0.0005$, Fig. 1b). Serum 1,25 dihydroxyvitamin D was not correlated with prostatic 25 hydroxyvitamin D ($\rho = 0.12$, $p = 0.20$).

On analysis by quartiles, we found both a significantly lower median normal Ki67 staining ($p = 0.02$, Fig. 1c) and significantly lower median tumor Ki67 staining ($p = 0.02$, Fig. 1d) in patients in the highest quartile of serum 1,25 dihydroxyvitamin D (74.8 pg/mL). There was almost no Ki67 staining in men in the highest quartile of serum 1,25 dihydroxyvitamin D. With respect to the overall trend, higher quartiles of serum 1,25 dihydroxyvitamin D were negatively associated with tumor Ki67 staining (Fig. 1d, $p = 0.03$), but only displayed a trending association with normal Ki67 staining (Fig. 1c, $p = 0.08$). The association between serum 1,25 dihydroxyvitamin D and increasing quartiles of serum 25 hydroxyvitamin D was also significant ($p = 0.01$, Fig. 1b). Finally, the association between prostatic 25 hydroxyvitamin D was not significant across quartiles of serum 25 hydroxyvitamin D ($p = 0.08$, Fig. 1a), though it was significantly lower in participants within the first quartile of serum 25 hydroxyvitamin D (15.0 ng/mL) compared to quartiles 2–4 ($p = 0.002$).

On multivariate linear regressions adjusted for age, Gleason grade group, PSA level, and normal Ki67% labeling to adjust for batch effects, we found significant associations between Ki67 labeling and serum 1,25 dihydroxyvitamin D, but not between Ki67 labeling and serum 25 hydroxyvitamin D or prostatic 25 hydroxyvitamin D (see Table 3). Serum 1,25 dihydroxyvitamin D quartiles also had the largest effect estimate ($\beta = -0.46\%$ Ki67 staining/

quartile, 95% CI: $-0.75, -0.04$, $p = 0.04$) with a 1.4% reduction in Ki67% staining from quartile 1 (median IQR) to quartile 4 (median, IQR) in tumor epithelium.

Discussion

In the largest cross-sectional epidemiologic study to date on physiologic levels of serum and prostatic vitamin D metabolites and markers of prostate tumor proliferation, we establish both an inverse correlation between serum 1,25 dihydroxyvitamin D and Ki67% labeling as well as a positive correlation between serum and prostatic 25 hydroxyvitamin D (Table 2). The significantly lower median prostatic 25 hydroxyvitamin D in participants within the first quartile of serum 25 hydroxyvitamin D (15.0 ng/mL or less), but not in the overall trend, suggests that only severe serum deficiency of 25 hydroxyvitamin D is a predictor of low-prostatic levels. The correlation between serum and prostatic levels of 25 hydroxyvitamin D has been tested and corroborated by Wagner et al. [12] in their cohort of men being treated with 25 hydroxyvitamin D. We find this same trend in men not being actively treated with 25 hydroxyvitamin D. Richards et al. [35] had a population with the majority being AA but they do not report the correlation between prostatic and serum 25 hydroxyvitamin D. However, Richards et al. do report that prostatic 25 hydroxyvitamin D is moderately correlated with serum 1,25 dihydroxyvitamin D levels (Spearman $\rho = 0.46$). Our finding that serum 1,25 dihydroxyvitamin D is negatively associated with tumor Ki67% labeling, both in unadjusted models and in an adjusted linear regression provides further evidence to support the theory that serum 1,25 dihydroxyvitamin D has a protective, anti-proliferative effect at physiologic levels (Tables 2 and 3). Our finding that the highest quartile of serum 1,25 dihydroxyvitamin had very little Ki67 labeling suggests a protective effect for men with clinically localized prostate cancer (Figure 1c, d). This also provides rationale for an intervention that would increase serum 1,25 dihydroxyvitamin D levels. This intervention could include increased 25 hydroxyvitamin D intake with or without decreased calcium intake. This strategy might mitigate the hypercalcemic side effects of 1,25 dihydroxyvitamin D and its analogs observed in other studies [36–38]. Wagner et al. [12] have shown that both serum and prostatic 1,25 dihydroxyvitamin D levels can be increased by 25 hydroxyvitamin D supplementation, and Hollis et al. [1] have shown that supplementation of 25 hydroxyvitamin D may even decrease progression of early-stage-, low-grade prostate cancers. Furthermore, Chan et al. [39] have shown that decreased calcium intake is associated with increased serum 1,25 dihydroxyvitamin D. They also show that increased calcium intake is associated with increased prostate cancer risk, a finding corroborated by a previous study in our own lab [40]. Increasing 25 hydroxyvitamin D supplementation to protect against prostate cancer is also supported by our finding in this study that serum 1,25 dihydroxyvitamin D is directly correlated with serum 25 hydroxyvitamin D. These findings are especially important for African Americans (AAs), who tend to be chronically deficient in 25 hydroxyvitamin D and have higher rates of prostate cancer relative to their EA counterparts [2], and thus may stand to benefit the most from 25 hydroxyvitamin D supplementation. Finally, while clinical trials investigating direct supplementation of 1,25 dihydroxyvitamin D or its analogs have shown conflicting results [19, 22, 36–38], direct injection of 1,25 dihydroxyvitamin D into prostatic tissue could be an effective means of delivery that merits future study.

On linear regression, we failed to find an association between prostatic levels of 25 hydroxyvitamin D and Ki67% labeling (Table 3), a result consistent with findings by Wagner et al. [12] in their predominantly EA cohort. Strengths of this study include adequate representation of clinical covariates important for PCa, such as race, age, family history, PSA, and cancer grade in our prospectively identified patient population. Pathologic results were reviewed by two pathologists with extensive experience with PCa research and diagnosis, which helped to reduce bias when determining Gleason grade and the percent of nuclei with Ki67-positive staining. Future work will include analyses stratified by race upon further recruitment.

Limitations

Limitations of this study include its relatively small sample size of non-AAs and cross-sectional design. In addition, we were only able to measure prostatic 25 hydroxyvitamin D and not 1,25 dihydroxyvitamin D or 24,25 dihydroxyvitamin D given limited prostatic fluid after robotic prostatectomy. Although our metric of proliferation, Ki67 staining, is a well-established immunohistochemistry marker [30, 32, 41], it is not as robust as a functional analysis of proliferation. 1,25 dihydroxyvitamin D impacts proliferation through various mechanisms, [21, 42, 43] though as with any observational study, it is impossible to assert that low levels of vitamin D metabolites are directly responsible for the observed differences in Ki67 staining [44]. Lastly, participants undergoing radical prostatectomy at large VA medical centers may not provide data generalizable to the rest of the population. Cohort studies are needed to longitudinally assess vitamin D metabolite status and the mechanisms through which it impacts prostatic epithelial cell proliferation.

Conclusions

Serum 25 hydroxyvitamin D is correlated with both prostatic 25 hydroxyvitamin D and serum 1,25 dihydroxyvitamin D, suggesting serum levels are a valid marker of prostatic vitamin D metabolite status. Furthermore, our current findings suggest that 1,25 dihydroxyvitamin D plays an important role in determining cancer epithelium proliferation rates at physiologic levels. Our data provides evidence that a subset of clinically localized prostate tumors has reduced prostatic proliferation at higher levels of serum 1,25 dihydroxyvitamin D and could prevent PCa progression.

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Fig. 1a–d:
Prostatic and serum vitamin D metabolites with Ki67 staining in tumor and normal epithelium. Spearman's correlation coefficient (ρ) and p values reported for continuous versions of the variables. Outliers not shown

Table 1:

Patient demographics and clinical characteristics by serum 25 hydroxyvitamin D status

	Tertile I Serum 25(OH) D < 18.0 ng/mL N = 43	Tertile II Serum 25(OH) D 18.0–27.5 ng/mL N = 40	Tertile III Serum 25(OH) D ≥ 27.5 ng/mL N = 36	P_{trend} Value^a	P_{pairwise} value
Continuous variables^a	Median [IQR]	Median [IQR]	Median [IQR]		
Age (years)	63.0 [57.0–67.5]	66.0 [59.5–69.0]	66.0 [62.5–69.0]	0.05	–
BMI (kg/m ²)	29.5 [26.0–32.4]	28.1 [24.6–31.3]	28.3 [24.4–30.9]	0.56	–
PSA (ng/mL)	8.8 [6.3–17.2]	8.4 [5.3–16.3]	7.9 [5.09–14.3]	0.52	–
Prostate volume ^d (cm ³)	31.0 [24.9–42.1]	32.9 [22.8–44.2]	31.4 [22.4–50.1]	0.98	–
Tumor Ki67 positive (%)	1.0 [0.01–3.02]	0.45 [0.01–3.11]	1.13 [0.02–3.36]	0.79	–
Normal Ki67 positive (%)	0.55 [0.01–1.02]	0.22 [0.01–1.14]	0.46 [0.01–1.23]	0.62	–
Total vitamin D intake (dietary and supplemental)	109.8 [41.4–369.4]	317.6 [48.7–535.4]	466.0 [408.0–684.3]	<0.001	<0.001 ^{I v III}
Serum 1,25 dihydroxyvitamin D (pg/mL)	39.9 [34.3–57.9]	48.6 [36.1–77.2]	64.4 [43.6–88.1]	0.01	0.007 ^{I v III}
Prostatic 25 hydroxyvitamin D (ng/mL)	0.31 [0.02–1.32]	1.20 [0.22–2.22]	1.82 [0.47–3.35]	0.003	0.04 ^{I v III}
Categorical variables^b	N (%)	N (%)	N (%)	P_{trend} value^a	P_{pairwise} value
1st degree prostate cancer family history	13 (29.5)	8 (18.2)	13 (28.9)	0.95	–
Self-reported Black race (yes)	43 (87.8)	36 (73.5)	29 (60.4)	0.002	0.002 ^{I v III}
Blood draw during high-UV period (May – October)	13 (28.9)	24 (52.2)	23 (51.1)	0.03	0.03 ^{I v III}
Lymph node invasion	3 (6.4)	1 (2.1)	2 (4.2)	0.59	–
Gleason grade > 3 + 4 ^c	17 (36.2)	22 (45.8)	21 (43.8)	0.46	–

^aKruskal–Wallis H test^bCochran–Armitage test^cHigh Gleason grade includes 3 + 4 with tertiary grade 5^dSome cases excluded pairwise.

Table 2:

Bivariate correlation matrix of vitamin D metabolites and Ki67 staining (N = 119)

	Tumor Ki67 % labeling	Normal Ki67 % labeling	Prostatic 25 (OH)D (ng/mL)	Serum 25(OH)D (ng/mL)	Serum 1,25(OH) ₂ D (pg/mL)
Tumor Ki67 % labeling	1 -	0.8 p<0.001	-0.18 p=0.08	-0.03 p=0.79	-0.3 p=0.002
Normal Ki67 % labeling	0.8 p<0.001	1 -	-0.25 p=0.01	-0.04 p=0.64	-0.21 p=0.03
Prostatic 25(OH) D (ng/mL)	-0.18 p=0.08	-0.25 p=0.01	1 -	0.27 p=0.004	0.12 p=0.20
Serum 25(OH) D (ng/mL)	-0.03 p=0.79	-0.04 p=0.64	0.27 p=0.004	1 -	0.34 p<0.001
Serum 1,25(OH)₂D (pg/mL)	-0.3 p=0.002	-0.21 p=0.03	0.12 p=0.20	0.34 p<0.001	1 -

Table 3:

Best-fit linear regressions for tumor Ki67% staining versus vitamin D status

	β estimate (95% CI)	β estimate (95% CI)	β estimate (95% CI)
Independent Variables	Prostatic 25(OH)D quartiles −0.05 (−0.54, 0.44)	Serum 25(OH)D quartiles −0.08 (−0.53, 0.36)	Serum 1,25(OH)₂D quartiles −0.46 (−0.75, −0.04)
	Gleason grade group 0.37 (−0.05, 0.78)	Gleason grade group 0.47 (0.08, 0.85)	Gleason grade group 0.35 (−0.03, 0.74)
	Age (years) 0.002 (−0.08, 0.09)	Age (years) −0.03 (−0.10, 0.05)	Age (years) −0.01 (−0.09, 0.07)
	PSA (ng/mL) 0.03 (0.00, 0.06)	PSA, ng/mL 0.02 (−0.01, 0.05)	PSA, ng/mL 0.03 (−0.001, 0.05)
	Normal Ki67% staining 1.76 (1.02, 2.50)	Normal Ki67% staining 1.85 (1.19, 2.52)	Normal Ki67% staining 1.60 (0.94, 2.25)

Bold values indicate statistical significance; Gleason grade group is modeled as a 5-level ordinal variable: 3 + 3, 3 + 4, 4 + 3, 8, 9–10; Dependent variable = % positive Ki67 staining tumor epithelial cells.