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Influence of vitamin D binding protein on the association between circulating vitamin D and risk of bladder cancer

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BACKGROUND: There is little research investigating the role of vitamin D binding protein (DBP) in the association between 25-hydroxyvitamin D (25(OH)D) and disease risk.

METHODS: Within the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study, 250 bladder cancer cases were randomly sampled and matched 1:1 to controls on age and date of blood collection. Odds ratios (OR) and 95% confidence intervals (CI) of bladder cancer were estimated by quartiles of DBP (measured by ELISA), 25(OH)D and the molar ratio of 25(OH)D:DBP, a proxy for free circulating 25(OH)D. Analyses were also conducted stratifying 25(OH)D by DBP (median split) and vice versa.

RESULTS: We found no direct association between circulating DBP levels and bladder cancer risk (*P*-trend = 0.83). The inverse association between 25(OH)D and bladder cancer risk was unchanged after adjustment for DBP (Q4 *vs* Q1 OR = 0.61, 95% CI = 0.36–1.05; *P*-trend = 0.04), and was stronger among men with lower DBP (low DBP: 25(OH)D Q4 *vs* Q1 OR = 0.47, 95% CI = 0.23–1.00; high DBP: 25(OH)D Q4 *vs* Q1 OR = 0.83, 95% CI = 0.40–1.75; *P* for interaction = 0.11).

CONCLUSION: Our findings provide additional support for an aetiologic role for vitamin D in bladder cancer and suggest that free, rather than total, circulating vitamin D may be a more relevant exposure when examining bladder and, perhaps, other cancers.

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Recent evidence suggests that vitamin D may influence the risk of urinary bladder cancer, which is an important public health concern in developed countries; for example, it is the fourth most common cancer in men and the eighth most common cause of cancer death in the United States (Jemal *et al*, 2009). One study found that vitamin D inhibits tumorigenesis and bladder cancer cell proliferation in rats (Konety *et al*, 2001), whereas another showed an increased risk of bladder cancer in persons carrying genotype variants of the vitamin D receptor *Fok1* polymorphism known to decrease the receptor's activity (Mittal *et al*, 2007). Epidemiologic studies of vitamin D intake have been mixed, with one finding no bladder cancer association (Michaud *et al*, 2000) and another finding an inverse association, but only among older individuals (Brinkman *et al*, 2010). Self-reported dietary intake of vitamin D does not capture vitamin D status as well as circulating 25-hydroxyvitamin D (25(OH)D) concentration, however, and only one study has examined serum 25(OH)D concentration in relation to risk of bladder cancer (Mondul *et al*, 2010). We conducted that analysis in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study and found that men with lower 25(OH)D levels (i.e., $<50\,\text{nmol}\,\text{l}^{-1}$) were at approximately twice the risk of bladder cancer compared with men who were replete (i.e., $\geqslant 50\,\text{nmol}\,\text{l}^{-1}$) (Mondul *et al*, 2010).

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There is a paucity of research investigating the role of vitamin D binding protein (DBP), also known as group-specific component or Gc-globulin, in the association between 25(OH)D and disease risk. Vitamin D binding protein transports both 25(OH)D and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the active hormonal form of vitamin D, in circulation (Bikle and Gee, 1989; Pike *et al*, 1997; Speeckaert *et al*, 2006), carrying 88% of 25(OH)D and 85% of $1,25(OH)_2D$, with an additional 12% of 25(OH)D and 15% of $1,25(OH)_2D$ bound to albumin (Bikle and Gee, 1989; Pike *et al*, 1997; Speeckaert *et al*, 2006). Thus, very little vitamin D circulates in a free state (Bikle and Gee, 1989; Pike *et al*, 1997; Speeckaert *et al*, 2006). Current laboratory assays of circulating vitamin D do not differentiate between the bound and free forms, and it remains unknown whether total or free vitamin D is more biologically relevant with respect to risk of bladder and other cancers. One recent study found that circulating DBP was inversely associated with the risk of pancreatic cancer, and that it modified the association between 25(OH)D and pancreatic cancer, supporting a role for unbound 25(OH)D (Weinstein *et al*, 2012). These findings suggest that DBP may be an important piece of the aetiologic puzzle when examining the association between circulating vitamin D and risk of cancer.

We conducted a nested case–control study within the ATBC Study to examine whether circulating DBP was prospectively associated with risk of bladder cancer, and whether it modified the previously reported protective association between higher circulating 25(OH)D and risk of bladder cancer (Mondul *et al*, 2010).

MATERIALS AND METHODS

Study population

The ATBC study was a randomised, double-blind, placebo-controlled, primary prevention trial designed to examine the effects of α-tocopherol and β-carotene supplementation on cancer incidence (The ATBC Cancer Prevention Study Group, 1994). From 1985 to 1988, 29 133 men were recruited from southwestern Finland. Participants were between the ages of 50 and 69 years at baseline and smoked at least five cigarettes per day as part of the enrolment criteria. Men were ineligible for the following reasons: (1) if they had previously had cancer or another serious illness at enrolment, or (2) if they reported current use of supplements containing vitamin E (>20 mg), vitamin A (>20 000 IU) or β-carotene (>6 mg). Participants were assigned to one of the four groups based on a 2 × 2 factorial design: (1) α-tocopherol (dl-α-tocopheryl acetate, 50 mg per day), (2) β-carotene (20 mg per day), (3) both supplements or (4) placebo. Men were supplemented for 5–8 years, until withdrawal from the trial, death or the end of the trial on 30 April 1993. Follow-up is ongoing through the Finnish Cancer Registry, which provides nearly 100% complete incident cancer ascertainment in Finland (Korhonen *et al*, 2002), and the Register of Causes of Death and for this analysis is complete through to 20 April 2005. This analysis was conducted in the same nested case–control sample as our previous investigation of serum 25(OH)D and risk of bladder cancer (Mondul *et al*, 2010). From the 558 total bladder cancer cases in the ATBC Study, 250 were randomly sampled by month of blood collection such that 25 cases were included from each month, with 25 cases total from June to August, because there were few clinic visits during the summer months (and none in July). We examined a subset of the cases to preserve the limited serum available for participants in this cohort; this subset did not differ from those cases not selected on a wide range of characteristics (data not shown). For cases diagnosed before May 1999, medical records were reviewed by one or two study physicians to confirm the cancer diagnosis, with subsequent cases based solely on the Finnish Cancer Registry data. To rule out any differences based on the method of case confirmation, we examined our main exposures stratified by whether the case was diagnosed before or after 1999 and found no differences (P for interaction = 0.23, 0.83 and 0.38 for 25(OH)D, DBP and 25(OH)D:DBP, respectively). Controls were sampled without replacement from ATBC Study participants who were alive and cancer free at the time the case was diagnosed and were matched 1:1 with cases on age at randomisation (±1 year) and date of blood collection (±30 days). Bladder cancer cases were not eligible to be selected as controls. The present analysis includes 245 cases and 245 controls, and excluded five pairs where the case or control (or both) had insufficient residual serum remaining after the earlier 25(OH)D assay for measurement of DBP. Written informed consent was obtained from all participants; the ATBC Study was approved by institutional review boards at both the Finnish National Public Health Institute and the US National Cancer Institute. At the time of enrolment, participants completed questionnaires providing information on general risk factors, smoking and medical history, as well as a food-frequency questionnaire. Participants were also examined by registered nurses who measured their height and weight, and collected an overnight fasting blood sample.

Laboratory measures

Fasting serum samples collected at baseline were stored at −70°C. 25-Hydroxyvitamin D was measured by Heartland Assays, LLC (Ames, IA, USA) using the DiaSorin Liaison 25(OH)D TOTAL assay (Ersfeld *et al*, 2004). Four or six blinded quality control (QC) samples were included in each batch. These QC samples came both from our study and from standard reference materials provided by the National Institute of Standards and Technology (NIST) at both level 1 (prepared from 'normal' human serum and not altered, ~60 nmol l^{-1}) and level 2 (prepared by diluting Level 1, ~35 nmol l^{-1}) ((NIST), 2009). Inter- and intrabatch CVs were as follows: 12.7% and 9.3%, respectively, for NIST level 1; 13.6% and 11.0%, respectively, for NIST level 2; 12.3% and 10.5%, respectively, for the ATBC cohort QC samples. The laboratory and QC methods are discussed in further detail elsewhere (Gallicchio *et al*, 2010).

Vitamin D binding protein was measured by the Clinical Support Laboratory, SAIC-Frederick Inc., Frederick National Laboratory for Cancer Research (Frederick, MD, USA) using the Quantikine Human Vitamin D Binding Protein Immunoassay kit (Catalog number DVDBP0, R&D Systems, Inc., Minneapolis, MN, USA). Each batch contained blinded QC samples comprising approximately 10% of the total samples. The inter- and intrabatch CVs were 10.8% and 15.2%, respectively.

Statistical analysis

Conditional logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI) of bladder cancer by quartiles of 25(OH)D and DBP, as well as the molar ratio of 25(OH)D:DBP, an estimation of free circulating 25(OH)D (Bouillon *et al*, 1981; Al-oanzi *et al*, 2006). Quartile cutpoints for DBP and the molar ratio of 25(OH)D:DBP were determined based on the distribution among controls. As 25(OH)D concentrations are known to vary by season, quartile cutpoints for 25(OH)D were based on the control distribution of each season (sunnier season = May–October, darker season = November–April); quartiles were created separately for each season and then combined into one variable. We evaluated the trend across categories by modelling the median of each category as a continuous variable and evaluating its statistical significance using the Wald test.

Based on our earlier analysis of 25(OH)D and risk of bladder cancer, we included cigarettes per day and years smoked in our multivariable models. Further, the following factors that were associated with DBP in our study were assessed as potential confounding factors: attained education, physical activity, alcohol intake and serum cholesterol. Each of the above variables was entered into the age-adjusted model to evaluate whether the point estimates for 25(OH)D categories changed by at least 10%, and none did so. Thus, our final multivariable model is conditioned on the matching factors (age and date of blood collection) and adjusted for cigarettes per day and years smoked. We also present our results mutually adjusted for 25(OH)D or DBP. Analyses were conducted stratifying DBP by 25(OH)D (season-specific quartiles 1–2 *vs* 3–4) and 25(OH)D by DBP (<median *vs* ⩾median). Stratified analyses were conducted using unconditional logistic regression adjusting for the matching factors. The main model results were unchanged when this approach was used instead of conditional logistic regression, making biased estimates unlikely. Statistical interaction was assessed using the likelihood ratio test applied to the multivariable-adjusted models.

RESULTS

Characteristics of the cases and controls have been reported previously; cases and controls were similar except for heavier smoking and lower poultry consumption among cases (Mondul *et al*, 2010). Characteristics of the control subjects by quartiles of serum DBP are shown in Table 1. Those with a higher circulating DBP concentration tended to be less educated, more physically active, consumed less dietary vitamin D, calcium and alcohol, were less likely to use supplemental vitamin D, and had slightly higher serum total cholesterol than those with lower DBP, although only the association

Table 1 Selected baseline characteristics across quartiles of vitamin D binding protein, control subjects, ATBC[a] Study

	Quartile of serum vitamin D binding protein (nmol l^{-1})				
Characteristic	**Q1 <4438**	**Q2 4438–<5606**	**Q3 5606–<7074**	**Q4 ⩾7074**	***P*-value**
Age (years)	58.5	58.0	59.0	59.0	Matched
Height (cm)	173	172	173	173	0.96
Weight (kg)	78.3	79.2	74.8	76.5	0.13
BMI (kg m^{-2})	26.7	26.5	24.8	25.7	0.09
Education (%>elementary)	19.4	21.7	16.4	16.1	0.84
Cigarettes per day	20	20	20	20	0.82
Years of smoking	39.5	37.0	39.0	38.0	0.43
Physically active during leisure time (% yes)	14.5	13.3	21.3	22.6	0.26
Dietary vitamin D (μg per day)	5.8	4.6	4.7	4.2	0.09
Dietary calcium (mg per day)	1530	1244	1231	1326	0.04
Ethanol (g per day)	12.3	10.7	7.2	8.0	0.79
Season of blood draw (% May-October)	46.8	36.7	39.3	43.6	Matched
Supplemental vitamin D use (% yes)	8.1	6.7	0	4.8	0.16
Supplemental calcium use (% yes)	9.7	10.0	3.3	9.7	0.27
Serum biomarkers					
DBP (nmol l^{-1})	3728	5037	6351	8197	NA
25(OH)D (nmol l^{-1})	30.7	41.9	35.8	32.9	0.69
25(OH)D:DBP molar ratio ($\times 10^3$)[b]	10.37	8.45	5.59	4.02	NA
Total cholesterol (mmol l^{-1})	6.0	6.1	6.2	6.3	0.36
Alpha-tocopherol (mg l^{-1})	11.3	11.8	10.9	11.5	0.22
Beta-carotene (μg l^{-1})	177	181	187	174	0.74
Retinol (μg l^{-1})	538	582	575	566	0.30

[a]Values are medians or proportions. [b]A proxy for free 25(OH)D.

Table 2 Odds ratios and 95% confidence intervals for the association between serum DBP, serum 25(OH)D and 25(OH)D:DBP molar ratio, and bladder cancer risk, ATBC Study

	Q1	**Q2**	**Q3**	**Q4**	***P*-trend**
DBP					
Range (nmol l^{-1})	<4438	4438–<5606	5606–<7074	⩾7074	
No. of cases/no. of controls	64/62	64/60	55/61	62/62	
OR[a] (95% CI)	1.0 (ref)	1.02 (0.59–1.77)	0.86 (0.50–1.50)	0.94 (0.52–1.70)	0.74
OR[b] (95% CI)	1.0 (ref)	1.05 (0.60–1.84)	0.85 (0.48–1.50)	0.98 (0.54–1.81)	0.83
OR[c] (95% CI)	1.0 (ref)	1.10 (0.62–1.95)	0.83 (0.47–1.47)	1.06 (0.57–1.98)	0.95
25(OH)D[d]					
No. of cases/no. of controls	78/62	69/63	54/63	44/57	
OR[a] (95% CI)	1.0 (ref)	0.85 (0.52–1.39)	0.66 (0.39–1.09)	0.61 (0.36–1.02)	0.02
OR[b] (95% CI)	1.0 (ref)	0.87 (0.53–1.43)	0.72 (0.42–1.21)	0.63 (0.37–1.07)	0.04
OR[c] (95% CI)	1.0 (ref)	0.87 (0.52–1.43)	0.71 (0.42–1.20)	0.61 (0.36–1.05)	0.04
25(OH)D:DBP molar ratio ($\times 10^3$)					
Range	<3.93	3.93–<6.47	6.47–<10.04	⩾10.04	
No. of cases/no. of controls	70/62	66/60	61/61	48/62	
OR[a] (95% CI)	1.0 (ref)	0.96 (0.57–1.61)	0.84 (0.51–1.38)	0.62 (0.35–1.10)	0.08
OR[b] (95% CI)	1.0 (ref)	1.02 (0.60–1.74)	0.82 (0.49–1.36)	0.67 (0.37–1.20)	0.13

[a]Conditioned on age at baseline and date of blood collection. [b]Conditioned on age and date of baseline blood draw and adjusted for number of cigarettes per day and years of smoking. [c]Conditioned on age and date of baseline blood draw and adjusted for number of cigarettes per day and years of smoking. Models are mutually adjusted for DBP and 25(OH)D. [d]Winter quartile cutpoints (in nmol l^{-1}) = Q1: <19, Q2: 19–<29, Q3: 29–<44, Q4: ⩾44; summer quartile cutpoints (in nmol l^{-1}) = Q1: <29, Q2: 29–<43, Q3: 43–<57, Q4: ⩾57; data previously published (Mondul *et al*, 2010).

with dietary calcium was statistically significant (Table 1). These associations among the controls are similar to those reported for controls in a previous study of DBP and risk of pancreatic cancer conducted in the ATBC Study (Weinstein *et al*, 2012).

We observed no direct association between serum DBP concentration and risk of bladder cancer (Table 2). Adjustment for DBP did not alter the previously reported inverse association between serum 25(OH)D and risk of bladder cancer (Table 2) (Mondul *et al*, 2010). There was an inverse association between the molar ratio of 25(OH)D:DBP, a proxy for free circulating 25(OH)D, and risk of bladder cancer that was similar in magnitude to that for total circulating 25(OH)D, although it was not statistically significant (*P*-trend = 0.08; Table 2).

We found no modification by 25(OH)D of the association between DBP and risk of bladder cancer (*P* for interaction = 0.73; Table 3). By contrast, the inverse association between 25(OH)D and bladder cancer appeared to be restricted to men with lower concentrations of DBP (e.g., for third and fourth quartiles of serum 25(OH)D compared with Q1: OR = 0.42, 95% CI = 0.21–0.84, and OR = 0.47, 95% CI = 0.23–1.00, respectively; Table 3), with little or

Epidemiology

Table 3 Odds ratios and 95% confidence intervals for the association between serum DBP and serum 25(OH)D and bladder cancer risk – stratified models, ATBC Study

	Q1	Q2	Q3	Q4	*P*-trend	*P* for interaction
DBP						
Range (nmol l^{-1})	<4438	4438–<5606	5606–<7074	⩾7074		
25(OH)D below median						
No. of cases/no. of controls	40/29	38/28	36/36	33/32		
OR[a] (95% CI)	1.0 (ref)	0.98 (0.49–1.96)	0.72 (0.37–1.41)	0.74 (0.38–1.48)	0.29	0.73
OR[b] (95% CI)	1.0 (ref)	1.02 (0.51–2.06)	0.73 (0.37–1.43)	0.78 (0.39–1.55)	0.33	
OR[c] (95% CI)	1.0 (ref)	1.01 (0.50–2.04)	0.73 (0.37–1.44)	0.77 (0.38–1.53)	0.32	
25(OH)D above median						
No. of cases/no. of controls	24/33	26/32	19/25	29/30		
OR[a] (95% CI)	1.0 (ref)	1.12 (0.54–2.35)	1.05 (0.47–2.33)	1.34 (0.64–2.78)	0.48	
OR[b] (95% CI)	1.0 (ref)	1.07 (0.51–2.26)	0.99 (0.44–2.23)	1.30 (0.62–2.72)	0.53	
OR[c] (95% CI)	1.0 (ref)	1.09 (0.52–2.31)	0.99 (0.44–2.24)	1.32 (0.63–2.78)	0.50	
25(OH)D						
Range[d]						
DBP below median						
No. of cases/no. of controls	46/25	32/32	27/38	23/27		
OR[a] (95% CI)	1.0 (ref)	0.55 (0.27–1.09)	0.39 (0.19–0.77)	0.46 (0.22–0.97)	0.05	0.11
OR[b] (95% CI)	1.0 (ref)	0.55 (0.27–1.10)	0.42 (0.21–0.85)	0.48 (0.23–1.01)	0.11	
OR[c] (95% CI)	1.0 (ref)	0.54 (0.27–1.09)	0.42 (0.21–0.84)	0.47 (0.23–1.00)	0.12	
DBP above median						
No. of cases/no. of controls	32/37	37/31	27/25	21/30		
OR[a] (95% CI)	1.0 (ref)	1.38 (0.70–2.70)	1.25 (0.61–2.57)	0.81 (0.39–1.68)	0.29	
OR[b] (95% CI)	1.0 (ref)	1.42 (0.72–2.79)	1.33 (0.64–2.75)	0.85 (0.41–1.78)	0.33	
OR[c] (95% CI)	1.0 (ref)	1.45 (0.73–2.86)	1.31 (0.63–2.71)	0.83 (0.40–1.75)	0.29	

[a]Adjusted for the matching factors: age at baseline (continuous), date of blood collection (continuous). [b]Adjusted for the matching factors: age at baseline (continuous), date of blood collection (continuous). Further adjusted for number of cigarettes per day and years of smoking. [c]Adjusted for the matching factors: age at baseline (continuous), date of blood collection (continuous). Further adjusted for number of cigarettes per day and years of smoking. Models are mutually adjusted for DBP and 25(OH)D. [d]Winter quartile cutpoints (in nmol l^{-1}) = Q1: <19, Q2: 19–<29, Q3: 29–<44, Q4: ⩾44; summer quartile cutpoints (in nmol l^{-1}) = Q1: <29, Q2: 29–<43, Q3: 43–<57, Q4: ⩾57.

no association among men with higher DBP levels. This interaction was of borderline statistical significance, however (*P* for interaction = 0.11).

DISCUSSION

We found no direct association between circulating DBP and risk of bladder cancer; however, the inverse association between total serum 25(OH)D and bladder cancer risk appeared limited to men with lower DBP levels. We further observed an inverse risk association between our estimate of free circulating 25(OH)D (i.e., the 25(OH)D:DBP molar ratio). These findings suggest that higher concentrations of free circulating 25(OH)D may be more biologically relevant to risk of bladder cancer than total 25(OH)D.

In addition to its canonical role in vitamin D transport, DBP has other important biological functions that may impact cancer risk. Vitamin D binding protein is a member of the extracellular actin scavenger system that protects the body from harmful effects resulting from release of actin into circulation following tissue injury or cell death (Pike *et al*, 1997; Speeckaert *et al*, 2006), and it is involved in chemotaxis, macrophage activation, apoptosis and angiogenesis (Pike *et al*, 1997; Speeckaert *et al*, 2006). The fact that we observed no independent association between DBP and bladder cancer risk suggests that DBP does not directly impact bladder carcinogenesis through such mechanisms. Rather, our finding that 25(OH)D was only associated with risk of bladder cancer among men with lower DBP supports the 'free hormone hypothesis', which postulates that only unbound, free hormones can have biological effects on target tissues (Pike *et al*, 1997). One possible interpretation of our results is that vitamin D may directly protect against bladder carcinogenesis, and that free vitamin D in circulation may be the more appropriate measure (and effector) of vitamin D exposure with respect to bladder cancer risk than total vitamin D (i.e., free plus bound). The 25(OH)D:DBP molar ratio, our estimate of free vitamin D, was not more strongly associated with bladder cancer than total serum 25(OH)D, as one might expect under the free hormone hypothesis. The molar ratio is, however, an imperfect estimation of free vitamin D, and it is likely subject to non-differential measurement error, which would bias our results toward the null. Thus, the true association with free vitamin D may be stronger than that we report for the 25(OH)D:DBP molar ratio. The kidney is the major site of 25(OH)D conversion to its active form, 1,25$(OH)_2$D, in humans, and the DBP-25(OH)D complex is known to be reabsorbed from the glomerular filtrate through the endocytic action of megalin, a 600-kDa protein expressed on the cell surface of absorptive epithelia including the proximal renal tubules (Willnow and Nykjaer, 2010). Mice with functional defects in the megalin gene lose an abnormal amount of vitamin D in their urine and develop vitamin D deficiency (Willnow and Nykjaer, 2010). Perhaps in individuals with lower circulating levels of DBP, less DBP-bound 25(OH)D is reabsorbed in the kidney via megalin endocytosis and more 25(OH)D is excreted in the urine, where it would be available to act on the bladder epithelial cells. Although speculative, this potential mechanism should be evaluated in future studies. Alternatively, our findings could be due to chance. Additional epidemiologic investigations are necessary to elucidate the true associations between DBP, 25(OH)D and risk of bladder cancer.

That vitamin D may protect against bladder cancer is biologically plausible: 1,25$(OH)_2$D has been shown to promote cell differentiation and decrease proliferation, invasion, angiogenesis and metastasis in a wide range of cell types and animal tumour models (Holick, 2004; Giovannucci, 2005), and one experiment found that vitamin D inhibited tumorigenesis and bladder cancer cell proliferation in rats (Konety *et al*, 2001). In addition, recent data support the idea that unbound circulating 25(OH)D may have a greater biological impact at the tissue level than total or bound

vitamin D. One study reported that calculated free 25(OH)D was more strongly correlated with bone mineral density than total 25(OH)D (Powe *et al*, 2011), while another study reported no difference in circulating total, but lower free concentrations of 25(OH)D and $1,25(OH)_2D$ in men with osteoporosis *vs* controls (Al-oanzi *et al*, 2006). The aforementioned findings regarding pancreatic cancer risk are also consistent with the free hormone hypothesis (Weinstein *et al*, 2012).

Our study has several strengths, including our prospective assessment of DBP and 25(OH)D, and our detailed information on multiple potential confounding factors. In addition, all serum samples were collected in the morning after an overnight fast, reducing the possibility of variation due to diurnal and postprandial fluctuations. One limitation of our study is that we had a relatively small sample size, because of limited biospecimen availability, which reduced our statistical power, particularly for studying the interaction between 25(OH)D and DBP. Although we did not measure serum albumin, which would have allowed us to calculate free 25(OH)D using mass action equations, only 12% of 25(OH)D is carried on albumin, compared with 88% which is carried on DBP. Thus, our analytic approach of stratifying by DBP and examining the association between the 25(OH)D:DBP molar ratio and risk of bladder cancer are meaningful, albeit imperfect, proxies for calculated or measured free circulating 25(OH)D (Al-oanzi *et al*, 2006). Another potential limitation is our measurement of 25(OH)D and DBP at only one point in time, which may not be representative of the individual's usual vitamin D status, or their status during the aetiologically relevant time period; however, studies suggest that DBP levels are stable throughout adulthood (Haddad, 1995), and that circulating vitamin D levels measured up to 14 years apart are well correlated (Hofmann *et al*, 2010). Our study population consisted of male smokers, so it remains to be determined in future studies whether our findings can be generalised to women or non-smokers. Given that smoking intensity and duration did not confound our associations, and that we observed no interaction by smoking intensity or duration, it seems unlikely that our participants' smoking status has strongly influenced our findings.

CONCLUSIONS

Our findings provide additional support for the hypothesis that vitamin D may have an aetiologic role in the prevention of bladder cancer, and suggest that free, rather than total, circulating vitamin D may be a more specific and relevant measure of vitamin D exposure when examining bladder cancer and, perhaps, other cancer outcomes. Joint examination of serum 25(OH)D and DBP concentrations in future studies, including in populations that include women and non-smokers, could shed additional light on the role of vitamin D and its pathway cofactors in the aetiology of cancer.

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