TITLE:

Vitamin D and Vitamin D Binding protein and risk of Bladder Cancer: A nested case-control study in the Norwegian Janus Serum Bank Cohort

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Conflict of interest

The authors declare no competing interests

Data availability statement

The data is available as presented in the paper. According to Norwegian legislation, our approvals to use the data for the current study do not allow us to distribute or make the data directly available to other parties.

Author contributions

HHH, TER, BKA and REG planned the study and contributed towards data analysis and drafting the paper. All authors contributed to critically revising the paper, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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List of abbreviations: 25(OH)D=25-hydroxyvitamin D, 1,25OH_2D=1-25-dihydroxyvitamin D, DBP=vitamin D binding protein, BC=urinary bladder cancer, BMI=body mass index, CRN =Cancer Registry of Norway, HR=hazard ratio, CI=confidence interval, SD=standard deviation

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ABSTRACT

Background: High circulating levels of vitamin D (25(OH)D) are suggested to reduce the risk of urinary bladder cancer (BC), but the evidence is weak, and several studies lack sufficient adjustment for potential confounders (e.g. smoking, body mass index (BMI) and physical activity). Moreover, few studies have investigated the role of vitamin D binding protein (DBP) in this context. We conducted a matched nested case-control study including 378 cases and 378 controls within the Norwegian population-based Janus cohort, using serum collected 5-41 years prior to diagnosis, to study 25(OH)D and BC risk, by taking circulating DBP into account.

Methods: Cox regression models were used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs), for 25(OH)D, DBP and the molar ratio of 25(OH)D:DBP, an estimate of unbound (free) 25(OH)D levels. We adjusted for smoking (status and pack-years), BMI, physical activity, education and (mutually) for 25(OH)D and DBP. Restricted cubic splines were employed to examine non-linear associations.

Results: High optimal levels of circulating 25(OH)D (>100 nmol/L) (HR 0.35, 95%CI 0.19-0.64) were associated with decreased BC risk, when compared with insufficient concentrations (50-75 nmol/L). This association was less pronounced for optimal levels (75-99 nmol/L) (HR=0.69, 95%CI 0.47-1.01). Moreover, estimated free 25(OH)D, was associated with decreased BC risk for molar ratio 17-21 (HR 0.66, 95% CI 0.44-0.97) and ≥ 22 (HR 0.50, 95% CI 0.29-

0.82), compared to molar ratio 11-16. The HR function for BC risk was not linear, rather reversed u-shaped, with the highest HR at 62.5 nmol/L and 13.5 molar ratio, respectively.

Conclusion: High levels of total and estimated free 25(OH)D were associated with reduced risk of BC, compared with insufficient concentrations. DBP was not associated with BC risk. We did not observe any impact of DBP or any of the studied lifestyle factors on the association between 25(OH)D and BC.

INTRODUCTION

Urinary bladder cancer (BC) is the most common genitourinary malignancy after prostate cancer worldwide [1]. The incidence rates are 3-4 times higher in men than in women and BC risk is increasing with increasing age [2, 3]. The main alterable risk factors for BC are smoking and exposure to chemicals [4-6]. Other suggested risk factors include lifestyle-related factors such as body mass index (BMI), blood pressure, physical activity, and various dietary and nutritional factors, including vitamin D [7-9].

Vitamin D is synthesized in the skin by ultraviolet radiation from the sun, or obtained from food and supplements, and must undergo activation through two steps to become the biologically active hormone; first to form 25-hydroxyvitamin D (25(OH)D) in the liver and then to form 1-25-dihydroxyvitamin D ($1,25OH_2D$) in the kidney [10]. The active hormonal form of vitamin D is vital for maintaining bone health, but does also regulate several other biological functions, including mechanisms involved in carcinogenesis, such as cell growth and differentiation [11]. Various preclinical studies have shown that $1,25(OH)_2D$ can suppress tumor progression in BC and other cancers [12]. For example, in a study performed on rats, Konety et al., found that $1,25(OH)_2D$ inhibited BC tumorigenesis and cell proliferation [13].

25(OH)D is the primary circulating form of vitamin D, and is considered the best indicator of an individual's vitamin D status [14]. The majority of circulating 25(OH)D is bound to vitamin D binding protein (DBP) (~88%) and albumin (~12%) and only a small proportion remain unbound (0.03%) [15, 16]. Most laboratory assays do not differentiate between the bound and the unbound (free) state, but a proxy of the free state can be estimated by the molar ratio of the total 25(OH)D to DBP, which is considered a reasonable measure of biologically available 25(OH)D [17]. In associations with cancer risk, it is unknown whether the total or the free state is more relevant to study.

Several observational studies report associations between circulating 25(OH)D concentrations and cancer risk at various sites, including BC [18]. The most recent meta-analysis on circulating 25(OH)D levels and BC risk, found a reduced risk of BC with higher concentrations of 25(OH)D [19, 20]. However, the individual studies did not report a clear association, and they vary according to adjustment for factors such as smoking history, BMI, and physical activity, which are related to the levels of 25(OH)D [21, 22]. Moreover, few studies have investigated the potential role of vitamin D binding protein, which is suggested to modify the association between 25(OH)D and BC risk [23].

In this study, we used stored serum from the population-based Janus Serum Bank Cohort (Janus Cohort) to examine total and free 25(OH)D as well as circulating DBP in relation to subsequent BC risk. We also examined potential interactions with smoking, BMI and physical activity.

METHODS

Study population

The Janus Serum Bank Cohort is a population-based biobank containing serum samples from 292,851 Norwegian men and women who participated in one of five large health surveys conducted between 1972 and 2004**.** Participants were aged 35-49 years at recruitment. Following this cohort by registry linkage enables, amongst others, the study of biomarkers of cancer. Detailed descriptions of the cohort and the data available have been published elsewhere [24, 25]. Our study was nested within the Janus Cohort and approvals for the study were obtained from the Janus Serum Bank Board and from the Regional Committee for Medical Research Ethics.

Identification of cancer cases and controls

The Cancer Registry of Norway (CRN) has been required by law to record cancer diagnoses since 1953, and holds complete and high quality data [26]. BC cases in the Janus Cohort were identified by linkage to the CRN and were required to be 1) histologically verified BCs of the transitional cell type (morphological codes: 8120, 8130 and 8131, according to International Classification of Disease for Oncology, 3rd revision without any previous cancer diagnosis (except basal cell carcinoma), and 3) diagnosed a minimum of 5 years after blood draw (recruitment). The selection of BC cases consisted of high-graded Ta, carcinoma in situ (Tis),

tumors invading lamina propria (T1), and tumors invading muscularis propria and further (T-stage T2-T4), thus excluding low-graded non-invasive tumors (Ta).

Follow-up began at recruitment into the Janus Cohort between 1972 and 2003 and continued until the date of BC diagnosis, emigration, death or the end of follow up at December 31st, 2016, whichever came first. During follow-up, a total of 1058 BC cases were identified (using the abovementioned case criteria). The number of included cases were limited to a random selection of 400 BC patients, based on statistical power and laboratory cost considerations [25].

Controls were required to be resident in Norway, alive and without a cancer diagnosis before index date (date of BC diagnosis of the associated case). One control was sampled at random with replacement (incidence-density sampling) and matched to each case (1:1 case-control ratio). The control was matched to each case on sex, year of birth, date of blood draw, season of blood draw within the following 3-month intervals within the same calendar year (December-February, March-May, June-August, September-November), and county of blood draw. A flow chart of the study design and exclusions is presented in Figure S1.

Vitamin D and Vitamin D binding protein

Serum was collected from non-fasting subjects, and stored at -25ºC. Serum concentrations of 25(OH)D and DBP were measured at the National Hormone Laboratory, Oslo University Hospital, participants of the vitamin D External Quality Assessment Scheme that ensures analytical reliability of 25(OH)D. Serum concentrations of 25(OH)D were measured by a liquid chromatography/tandem mass spectrometry method and DBP by a radioimmunoassay (both assays are developed at the Hormone Laboratory). The matched case-control sets were analyzed within the same batch. A blinded quality control sample was included in each of the 25 batches. The interassay coefficient of variation (CV) was 11.1% at 60.1 nmol/L for 25(OH)D, and 10.5% at 6.5µmol/L for DBP, respectively.

Since 25(OH)D concentrations are strongly affected by season, we used season-adjusted concentrations in our analysis, in addition to matching case and controls on date of blood draw [27]. We modeled the seasonal variation in our study sample by performing a least square fit of a sine function to the measured concentrations of 25(OH)D versus date of blood draw. An additional file shows this method more in detail (see supplementary including Figure S2).

The categories of 25(OH)D were defined based on previously defined clinical cut points by the endocrine society [14]; deficient <50 nmol/L, insufficient (50-75 nmol/L), optimal (75-100 nmol/L) and high optimal (>100 nmol/L), with 50-75nmol/L being the reference category reflecting the average level in the Norwegian population [28].

The molar ratio of 25(OH)D:DBP was used as an estimate for free circulating 25(OH)D, which previously has been described as a valid approximation of "free 25(OH)D"[17]. The molar ratio is a simplification of the equilibrium equation between free and bound 25(OH)D, which neglect the contribution from albumin [15, 17].

To be consistent throughout the paper, we used the clinical categories of 25(OH)D as guidance when categorizing DBP and 25(OH)D:DBP. More specifically, we applied the percentiles of the four clinically defined cut-points of 25(OH)D to the distribution of DBP and 25(OH)D:DBP leading to the following categories for DBP µmol/L (<3.9, 3.9-4.5, 4.6-5.4 and >5.4) and 25(OH)D:DBP molar ratio x 10^3(<11, 11-16, 17-21 and >22).

Covariates

All individuals in the Janus Cohort underwent health examinations and filled out health related questionnaires [25]. Information about smoking history was based on questionnaire data, and contained smoking status (never, former and current smokers), and duration and intensity of smoking. Pack-years were calculated by multiplying number of packs smoked per day with number of years smoked. We created a smoking variable consisting of five categories (never smokers, former smokers, and current smokers in 3 categories of pack-years (tertiles)). Height and weight were measured by trained health personal. BMI was calculated and categorized according to the World Health Organization's classification: underweight and normal weight (<25 kg/m^2), overweight (25–29.9 kg/m^2) and obese (≥30 kg/m^2). Information about physical activity was obtained from the questionnaires, categorized as sedentary, moderately active and active. The health examination also included measurement of blood pressure, cholesterol and triglycerides.

Information about occupation and education was obtained from Statistics Norway. Occupational working titles were categorized as high risk (yes or no) and was based on existing knowledge about chemical exposures in certain occupations that previously has shown to be related to BC risk [7, 29, 30]. More details about the categorization of high risk occupations are

published previously [7]. Education was categorized as unknown, compulsory, upper secondary, and college/university.

Statistical analysis

Descriptive statistics were used for patient characteristics. Stratified Cox regression was used to estimate hazard ratios (HRs) with 95% confidence intervals (CIs) of BC risk for four categories of 25(OH)D and DBP, and the molar ratio of 25(OH)D:DBP. Moreover, to explore the underlying shape of the effect of interest, the HR was modelled as restricted cubic splines with 4 knots dependent on 25(OH)D and 25(OH)D:DBP, using the STATA package rscgen. The knots were, following Harrell, placed at the 5^{th}, 35^{th}, 65^{th} and 95^{th} percentiles [31]. A likelihood ratio test was applied to compare the fit of the linear vs. the spline models.

In addition to be conditioned on the matching factors (age, sex and date, season and county of blood draw) in model 1, the multivariable analyses were adjusted for smoking, BMI, physical activity and education (model 2). Model 3 included in addition mutually adjustment for DBP and 25(OH)D. Occupation, blood pressure, cholesterol and triglycerides were all entered into the multivariable model to evaluate their impact on the risk estimates of DBP, 25(OH)D and 25(OH)D:DBP. However, they were not associated with BC risk (LR test, $p>0.2$) and did not change the risk estimates of interest on BC risk (HR) more than 10%, and were thus not included in the final models.

As smoking, BMI, physical activity and DBP are hypothesized to be effect modifiers of the association between 25(OH)D and BC risk, we conducted analyses stratified by these variables, and tested for interaction. Statistical interaction was evaluated using the likelihood ratio test.

To assess the influence of extreme values of 25(OH)D and DBP concentrations on the results, we performed sensitivity analyses excluding persons with values below the 2.5 percentile or above the 97.5 percentile, which did not influence the results (see supplementary Table S1 and Figure S3). All statistical analyses were performed using STATA version 15.1 (StataCorp, College Station, TX). The statistical significance level was set to 5%.

RESULTS

Distribution of population characteristics are presented for both cases and controls in Table 1 and stratified for previously defined 25(OH)D categories in Table 2. Characteristics of

cases and controls were comparable for most of the variables except for smoking with a larger proportion being current smokers among cases (59%), compared to controls (42%), in addition to higher mean of pack-years among cases. Median concentrations of 25(OH)D were slightly lower among cases 68.3 (54.8-82.9) than among controls 71.7 (53.8-88.0). Table 2 shows that individuals with 25(OH)D deficiency (<50 nmol/L) tended to be heavier smokers, have a higher BMI and were less physically active, compared with individuals with higher 25(OH)D concentrations. Median concentrations of DBP and consequently molar ratio of 25(OH)D:DBP increased with increasing 25(OH)D concentrations.

The results of the multivariable analyses investigating the association between 25(OH)D, DBP and 25(OH)D:DBP molar ratio and the risk of BC are presented in Table 3. The fully adjusted model (model 3) showed a borderline significant decreased risk of BC for optimal values of 25(OH)D (≥ 75 nmol/L) (HR 0.69, 95%CI 0.47-1.01, p=0.054), and a significant decreased risk of high optimal values of 25(OH)D (≥ 100 nmol/L) (HR 0.35, 95%CI 0.19-0.64, $p=1.0\cdot10^{-3}$), compared to the insufficient category (50-75 nmol/L). Moreover, deficient concentrations (<50 nmol/L) of 25(OH)D also showed a tendency of decreased BC risk, when compared to insufficient concentrations (HR 0.64, 95% CI 0.40-1.01, p=0.055).

No significant associations were observed between serum DBP concentrations and the risk of BC. However, an increasing 25(OH)D:DBP molar ratio, the estimate of free circulating 25(OH)D, was associated with decreased risk of BC. Compared to the molar ratio 11-16, a significantly decreased BC risk was found for molar ratio 17-21 (HR 0.66, 95% CI 0.44-0.97, p=0.036) and molar ratio ≥22 (HR 0.50, CI% 0.29-0.82, $p=9.2\cdot10^{-3}$).

We present the distribution of 25(OH)D concentrations and 25(OH)D:DBP (Figure 1A and 1B) and their impact on BC risk on a continuous scale (Figure 1C and 1D). The distribution of the 25(OH)D concentrations is ranging from 16.5-195.4 nmol with a median value of 68.6 nmol/L and the HR of its effect on BC increased from deficient concentrations to the reference concentration (the median concentration of the reference category applied above), and thereafter decreased. The distribution of 25(OH)D:DBP molar ratio is ranging from 4.0-36.5 with a median value of 15.5. The HR increased from the lowest molar ratio category (<11) to the reference level of molar ratio of 13.5 (the median level of the reference category), and decreased thereafter. The spline models showed a better fit than the linear models for 25(OH)D and for 25(OH)D:DBP (p=0.034 and p=0.065, respectively).

The results for the stratified multivariable analyses are presented in Table 4. We did not observe statistically significant interactions between 25(OH)D and any of the variables examined, including smoking status, BMI, physical activity and DBP (all P for interaction > 0.10). However, the associations between high optimal concentrations and decreased BC risk was only statistically significant among current smokers (HR 0.39, 95% CI 0.19-0.81, p= 0.014), among individuals with BMI<25kg/m^2 (HR 0.39, 95% CI 0.19-0.78, p=0.009), and individuals that are physical active (HR 0.44, 95% CI 0.25-0.80, p=0.007). In addition, the association between deficient concentrations and decreased BC risk, was only statistical significant among individuals with BMI>25kg/m^2 (HR 0.44, 95% CI 0.23-0.85, p=0.013).

DISCUSSION

In this population-based case-control study, we found that prediagnostic circulating 25(OH)D concentrations above high optimal levels (≥100 nmol/L) were associated with subsequent decreased BC risk when compared with insufficient concentrations (50-75 nmol/L). Moreover, free levels of 25(OH)D in circulation, the 25(OH)D:DBP molar ratio, was associated with decreased BC risk, when comparing high molar ratios (17-21 and ≥22) with the reference category (molar ratio 11-16). For both total and estimated free 25(OH)D, modeling the HR for the effect on BC risk by splines revealed that the effect was not linear, rather reversed u-shaped, with the highest HR at 62.5 nmol/L and 13.5 molar ratio, respectively, and with a decrease thereafter. We did not find any association between DBP and BC risk. However, the association for free circulating 25(OH)D showed slightly larger effect with BC than total 25(OH)D concentrations.

The associated decreased risk of BC found for high circulating concentrations of 25(OH)D is consistent with the most recent meta-analysis, which comprised two cohort and five case-control studies [20]. The meta-analysis showed that serum concentrations above 75 nmol/L were associated with a decreased risk of BC. Similarly, a pooling analysis of 17 cohorts recently reported that 25(OH)D concentrations above 75 nmol/L were associated with a decreased risk of colorectal cancer [32]. Circulating concentrations of 25(OH)D above 75 nmol/L have been suggested as optimal to obtain full health benefits of vitamin D. However, there is no absolute agreement in what defines optimal 25(OH)D concentrations, especially not when it comes to cancer protective concentrations [14]. In this study, concentrations above 100 nmol/L were

associated with reduced BC risk, although levels above 75 nmol/L also showed a tendency of an association.

No association between DBP and BC risk was observed, which is in agreement with other studies [23]. However, the estimate of free 25(OH)D showed a slightly stronger association with BC risk than total 25(OH)D concentrations, with decreasing risk in both categories of high molar ratios (17-21 and ≥22) compared to the reference category (molar ratio 11-16). This might indicate that the free 25(OH)D in circulation is a more relevant measure of 25(OH)D exposure with respect to BC risk than total 25(OH)D, which is supported by the free hormone hypothesis; that the biological activity is affected by the free circulating concentration[33]. In our analysis we used the 25(OH)D:DBP molar ratio as an estimation of free 25(OH)D. Errors due to an imperfect estimation would most likely not differ between case-status. Thus, the actual measured free levels of 25(OH)D could be more strongly associated with BC than we observe in our analysis.

Preclinical studies have given mechanistic evidence for a protective role of vitamin D in BC development, demonstrating that the hormone form $1,25(OH)_2D$ modulates gene transcription of antitumor genes, including genes with antiprolifereative, anti-invasive and proapoptotic properties[34]. Animal and in vitro studies have shown in various models that $1,25(OH)_2D$ suppresses BC development by reducing cell proliferation and stimulating apoptosis [13].

On the other hand, high circulating levels of 25(OH)D are suggested to reflect a healthy lifestyle, and could thereby contribute to a protective association [22]. According to our results, current smokers, those with elevated BMI and a lower physical activity levels, more frequently had low concentrations of 25(OH)D (Table 2). However, even though we incorporated solid information on various lifestyle factors into the analyses, they did not have an impact on our results. Despite of no statistical significant interactions between lifestyle factors and 25(OH)D on the risk of BC, we observed statistical significant differences in the stratified analyses on the associations between high optimal concentrations and decreased BC risk among current smokers and individuals with normal BMI and high physical activity. The reason for not finding an interaction is possibly due to limited statistical power. Even though we were not able to detect any clear interaction from any of the lifestyle variables investigated, we cannot rule out that the protective associations we observe were related to lifestyle and/or residual confounding not sufficiently captured by our variables available.

Previous studies evaluating associations between 25(OH)D concentrations and BC risk have shown a dose-dependent relationship, which might strengthen the evidence of causality (Hill's criterion) [18, 35]. We used spline functions to model the effect of vitamin D on BC risk, and found that the effect curve (HR) did not follow a linear relationship, rather a reversed u-shape with the largest HR for insufficient concentrations (50-74 nmol/L). In particular, the group with deficient concentrations was not consistent with the assumption of linearity in the risk effect across 25(OH)D concentrations. One possible explanation is that the lowest concentrations are associated with other diseases or conditions that are inversely associated with BC risk. For instance high BMI is associated with low 25(OH)D concentrations, and have in some studies showed a tendency to be inversely related to BC risk [7, 36]. In our analysis, when stratifying by BMI; a reduced BC risk for deficient 25(OH)D concentrations was only seen in the category of BMI ≥25.

A major limitation of our study is that serum samples for assessment of 25(OH)D and DBP were collected at one time point, which does not necessarily represent the individual's longitudinal vitamin D status relevant to cancer development. DBP is suggested to be relatively stable throughout adulthood [37]. However, several factors are known to affect 25(OH)D levels over time, such as changes in diet, supplement use and time spent in the sun [38]. Despite this, former studies have shown that circulating 25(OH)D measured several years apart were well correlated, although the correlation slightly declines over time [39-42]. Moreover, blood samples were collected in different periods, between 1972 and 2002, which could have affected the level and/or the quality of the samples differently. However, we accounted for the time point the blood sample was taken (including the season), by matching our cases and controls on the date and season of blood draw.

Our study has several strengths. First of all, we only sampled cases and controls without a cancer history, which together with our assessment of 25(OH)D and DBP in serum samples collected at least 5 years prior to the cancer diagnosis, reduces the risk of reverse causality. Also, we included detailed information on multiple potential confounding factors, including robust information about smoking history.

In conclusion, high serum levels of both total and estimated free 25(OH)D were associated with reduced risk of BC, when compared to insufficient levels. DBP was not

associated with BC risk. We did not observe any impact of DBP or any of the studied lifestyle factors on the association between 25(OH)D and BC.

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TABLES

Table 1 Baseline characteristics by case-control status

Characteristics	**Case**	**Control**
Male, n (%)	320 (85)	320 (85)
Female, n (%)	58 (15)	58 (15)
Year of birth, median (range)	1936 (1928-1946)	1936 (1928-1946)
Season of blood draw, n (%)		
Darker season (November-April)	179 (47)	169 (44)
Sunnier season (May-October)	199 (53)	209 (55)
Smoking status, n (%)		
Never smoker	74 (20)	100 (26)
Former smoker	80 (21)	119 (32)
Current smokers	224 (59)	159 (42)
Packyears, mean (SD)	18.0 (9.7)	13.2 (7.8)

BMI (kg/m^2), mean (SD)	24.8 (3.1)	24.9 (3.0)
BMI (kg/m^2), n(%)		
Normal (≤ 25)	208 (55)	215 (57)
Overweight (25-29)	148 (39)	139 (37)
Obese (≥ 30)	22 (6)	24 (6)
Physical activity		
Sedentary	78 (21)	65 (17)
Moderately active	217 (57)	222 (58)
Active	83 (22)	91 (24)
Hypertension		
No	213 (56)	208 (55)
Yes	165 (44)	170 (45)
High risk occupation, n (%)		
No	251(66)	269 (71)
Yes	115 (30)	100 (26)
Unknown	12 (3)	9 (2)
Education, n (%)		
Compulsory	137 (36)	144 (38)
Upper secondary	186 (49)	180 (48)
College/University	55 (15)	54 (14)
Time between blood draw and diagnosis (years), median (range)	22 (16-28)	
25-hydroxyvitamin D(nmol/L), median (range)	68.3 (54.8-82.9)	71.7 (53.8-88.0)
Vitamin D binding protein (DBP) (µmol/L), median (range)	4.5 (4.1-5.0)	4.5 (4.0-4.9)
25(OH)D:DBP molar ratio (x 10^3)	15.2 (12.0-18.4)	15.7 (12.1-19.8)
Cholesterol (mmol/L), median (range)	6.0 (5.2-6.8)	6.1 (5.3-6.9)
Triglycerides (mmol/L), median (range)	1.5 (1.2-2.3)	1.7 (1.2-2.2)

Abbreviations: 25(OH)D = 25-hydroxyvitamin D; DBP = Vitamin D binding protein; BMI = body mass index; SD = standard deviation.

Table 2 Baseline characteristics by clinical cut points of 25(OH)D

	25(OH)D (nmol/L)

Characteristics	Deficient <50	Insufficient 50-75	Optimal 75-100	High Optimal >100
Age	44 (6.2)	45 (8.3)	44 (7.2)	43 (7.5)
Sex				
Male, n(%)	107 (83)	272 (84)	185 (85)	76 (88)
Female, n(%)	22 (17)	52 (16)	32 (15)	10 (12)
Smoking status, n (%)				
Never smoker	23 (17)	84 (25)	47 (30)	22 (25)
Former smoker	32 (24)	86 (26)	67 (30)	20 (23)
Current smokers	74 (57)	157 (48)	108 (50)	44 (51)
Packyears, mean (SD)	17.2 (10.1)	16.5 (9.2)	13.8 (8.3)	15.6 (8.4)
BMI (kg/m^2), mean (SD)	25.4 (3.5)	24.9 (3.0)	24.7 (2.9)	24.3 (2.3)
BMI (kg/m^2), n(%)				
Normal (≤ 25)	63 (47)	179 (53)	135 (60)	56 (63)
Overweight (25-29)	57 (43)	133 (40)	77 (34)	29 (32)
Obese (≥ 30)	12 (9)	20 (6)	13 (5)	3 (3)
Physical activity				
Sedentary	42 (33)	58 (18)	32 (15)	11 (13)
Moderately active	67 (52)	196 (60)	129 (59)	47 (55)
Active	20 (16)	70 (22)	56 (26)	28 (33)
Hypertension				
No	67 (52)	183 (56)	119 (55)	52 (60)
Yes	62 (48)	141 (44)	98 (45)	34 (40)
High risk occupation, n (%)				
No	84 (65)	218 (67)	153 (71)	65 (76)
Yes	39 (30)	99 (31)	61 (28)	16 (19)
Unknown	6 (5)	7 (2)	3 (1)	5 (9)
Education, n (%)				
Compulsory	57 (44)	109 (34)	81 (37)	34 (40)
Upper secondary	58 (45)	170 (52)	95 (44)	43 (50)
College/University	14 (11)	45 (14)	41 (19)	9 (10)
Time between blood draw and diagnosis (years), mean (SD)	22.9 (7.8)	21.6 (8.7)	23.0 (8.3)	23.5 (8.3)

25-hydroxyvitamin D(nmol/L), median (range)	42 (17-49)	63 (50-74.9)	85 (75-99.9)	109 (100-195)
DBP (µmol/L), median (range)	4.3 (2.2-8.6)	4.4 (1.8-7.4)	4.6 (3.0-8.0)	4.7 (3.9-9.6)
25(OH)D:DBP molar ratio (x 10^3), median (range)	9.8 (4.0-17)	14 (8.0-31)	19 (11-31)	23 (13-36)
Cholesterol (mmol/L), median (range)	6.2 (1.6-11)	6.0 (3.4-9.4)	6.3 (3.4-11)	5.8 (3.9-11)
Triglycerides (mmol/L), median (range)	1.7 (0.3-10)	1.6 (0.48-7.6)	1.6 (0.34-12)	1.5 (0.39-6.1)

Abbreviations: 25(OH)D = 25-hydroxyvitamin D; DBP = Vitamin D binding protein; BMI = body mass index; SD = standard deviation.

Table 3 Hazard ratio (HR) and 95% confidence interval (CI) of bladder cancer risk by levels of 25 (OH)D, DBP and 25(OH)D:DBP molar ratio.

25OHD (nmol/L)	**<50 (Deficient)**	**50-74 (Insufficient)**	**75-99 (Optimal)**	**≥100 (High Optimal)**
Case/control	61/68	181/143	102/115	34/52
HR(95% CI)[1]	0.70 (0.46-1.07)	1 (ref)	0.70 (0.50-1.00)	0.48 (0.28-0.81)
HR(95% CI)[2]	0.62 (0.39-0.97)	1 (ref)	0.73 (0.50-1.06)	0.42 (0.24-0.74)
HR(95% CI)[3]	0.64 (0.40-1.01)	1 (ref)	0.69 (0.47-1.01)	0.35 (0.19-0.64)
DBP (umol/L)	**<3.9**	**3.9-4.5**	**4.6-5.4**	**≥ 5.4**
Case/control	48/53	182/174	100/111	48/40
HR(95% CI)[1]	0.86 (0.55-1.35)	1 (ref)	0.85 (0.57-1.26)	1.19 (0.69-2.07)
HR(95% CI)[2]	0.98(0.60-1.60)	1 (ref)	0.84 (0.55-1.27)	1.11(0.62-1.98)
HR(95% CI)[3]	0.90 (0.54-1.50)	1 (ref)	0.84 (0.56-1.28)	1.18 (0.66-2.13)
25OHD:DBP (*10^3)	**<11**	**11-16**	**17-21**	**≥22**
Case/control	63/63	193/158	84/101	38/56
HR(95% CI)[1]	0.79 (0.51-1.24)	1 (ref)	0.67 (0.46-0.96)	0.52 (0.32-0.85)
HR(95% CI)[2]	0.68 (0.42-1.10)	1 (ref)	0.66 (0.44-0.97)	0.50 (0.29-0.82)

Abbreviations: 25(OH)D = 25-hydroxyvitamin D; DBP = Vitamin D binding protein

[1] Conditioned on matching factors (age, sex, and date, season and county of blood draw)

[2] Conditioned on matching factors (age, sex, and date, season and county of blood draw) and adjusted for BMI, physical activity, smoking (status and pack-years) and education

[3] Conditioned on matching factors (age, sex, date, season and county of blood draw) and adjusted for BMI, physical activity, smoking (status and pack-years), education and DBP and 25(OH)D respectively

Table 4 Hazard ratio (HR) and 95% confidence interval (CI) of bladder cancer risk by concentrations of 25(OH)D, stratified by selected variables.

		<50 (Deficient)	**50-74 (Insufficient)**	**75-99 (Optimal)**	**≥100 (High Optimal)**	**p** -value
Smoking status						
Never smoker	Case/control	(8/15)	(38/45)	(20/26)	(8/14)	
	HR (95%CI)	0.53(0.18-1.53)	1 (ref)	0.91 (0.42-1.95)	0.61 (0.21-1.78)	
Former smoker	Case/control	(9/23)	(39/45)	(25/38)	(7/13)	
	HR (95%CI)	0.42 (0.16-1.05)	1 (ref)	0.84 (0.43-1.63)	0.56 (0.19-1.67)	
Current smoker	Case/control	(44/30)	(104/53)	(57/51)	(19/25)	
	HR (95%CI)	0.74 (0.39-1.39)	1 (ref)	0.55(0.32-0.96)	0.40 (0.19-0.83)	0.65
BMI						
< 25 kg/m^2	Case/control	(31/29)	(97/78)	(60/72)	(20/36)	
	HR (95%CI)	0.84 (0.45-1.59)	1 (ref)	0.68 (0.41-1.11)	0.40 (0.20-0.79)	
> 25 kg/m^2	Case/control	(30/39)	(84/65)	(42/43)	(14/16)	
	HR (95%CI)	0.44 (0.23-0.85)	1 (ref)	0.82 (0.46-1.47)	0.46 (0.20-1.09)	0.37
Physical activity						
Sedentary	Case/contol	(21/21)	(36/22)	(17/15)	(4/7)	
	HR (95% CI)	0.54 (0.22-1.30)	1 (ref)	0.82 (0.31-2.16)	0.24 (0.06-1.03)	
Active	Case/control	(40/47)	(145/121)	(85/100)	(30/45)	
	HR (95% CI)	0.65 (0.38-1.11)	1 (ref)	0.71 (0.47-1.05)	0.44 (0.25-0.80)	0.78
DBP						
< median	Case/control	*(38/38)*	*(84/79)*	*(40/47)*	*(10/19)*	
	HR (95%CI)	0.86 (0.47-1.56)	1 (ref)	0.81 (0.46-1.41)	0.30 (0.12-0.77)	
> median	Case/control	*(23/30)*	*(97/64)*	*(62/68)*	*(24/33)*	
	HR (95%CI)	0.40 (0.20-0.82)	1 (ref)	0.64 (0.39-1.04)	0.44 (0.22-0.87)	0.30

Abbreviations: 25(OH)D = 25-hydroxyvitamin D; DBP = Vitamin D binding protein

Conditioned on matching factors (age, sex and date, season and county of blood draw). Additionally, adjusted for BMI, physical activity, smoking (status and packyears) and education

FIGURE LEGENDS

Figure 1

Histogram distribution of A) 25(OH)D and B) 25(OH)D:DBP molar ratio (x 10^3). Restricted cubic splines displaying hazard ratios of bladder cancer risk with 95% confidence intervals according to C) 25(OH)D and D) 25(OH)D:DBP molar ratio (x 10^3) . For 25(OH)D reference was set to 62.5 nmol/L, P-value for non-linearity 0.0342. For 25(OH)D:DBP molar ratio (x10^3) reference were set to 13.5 (molar ratio x 10^3), P-value for non-linearity 0.0647. Both exposure risk curves are adjusted for matching factors (age, sex, time of blood draw) and smoking (status and pack-years) BMI, physical activity and education.

Supplementary material

Summary

1. Overview of study design (Figure S1)
2. Season adjustment (Figure S2)
3. Sensitivity analysis (Table S1, Figure S3)

1. Overview of study design

Figure S1

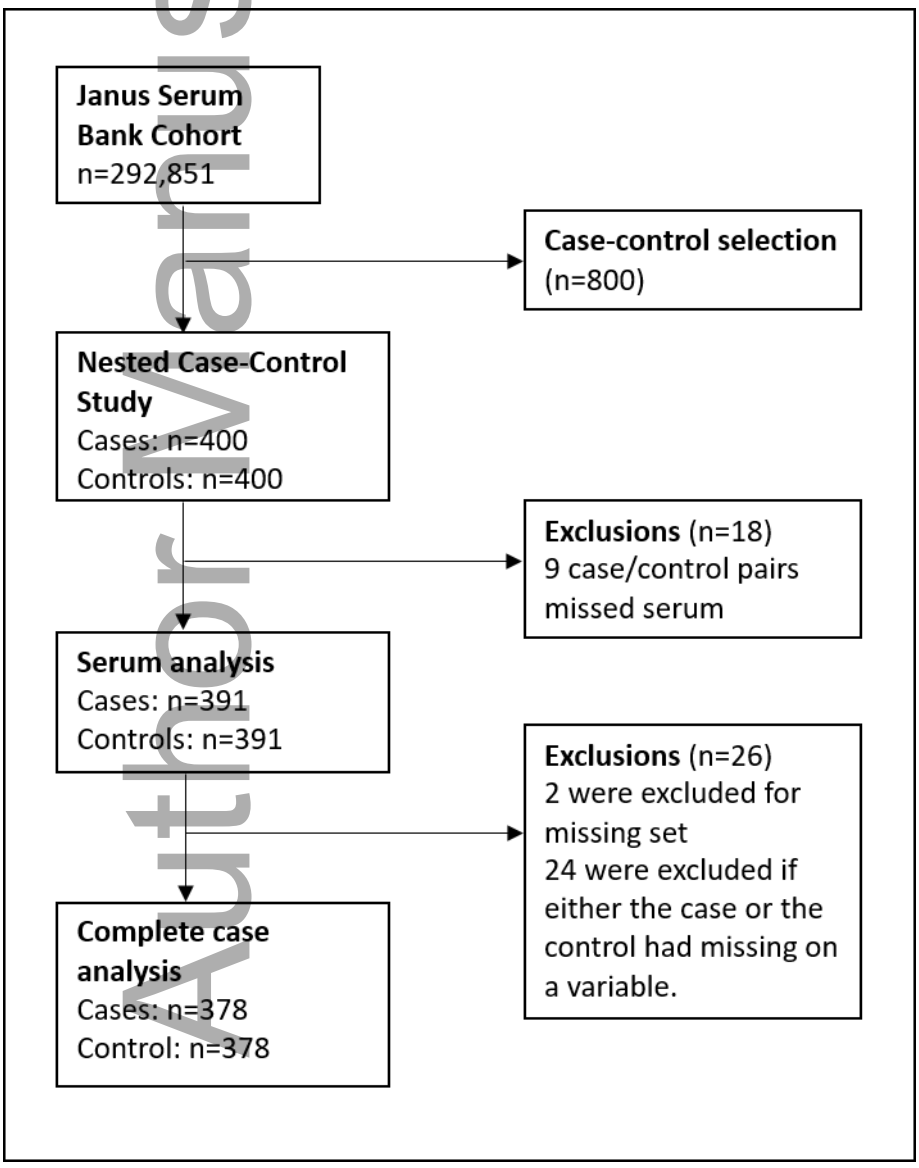

Figure S1. Overview of study design (selection of cases and controls) and exclusions.

2. Season adjustment

We modeled the seasonal variation in our study sample by performing a least square fit of a sine function to the measured concentrations of 25(OH)D versus date of blood draw.

$$y(d) = y0 + amplitude * sin\left(\frac{2\pi}{365} * d + phase\ shift\right)$$

Y(d) is the 25(OH)D concentration at d (number of days from January 1st). y0 represents the mean level of the sine curve, and thus an estimate of the annual mean of 25(OH)D of the study sample. The amplitude represents the seasonal variation and is the maximal deviation from y0. The phase shift is the translation along the x-axis. An amplitude of 14.49, y0=71.93 and phase shift=3.97 was obtained providing a maximum 25(OH)D concentration in mid august (14th) and a minimum in the end of February (29th). To adjust measured 25(OH)D values for seasonal variation, we calculated the individual deviations from the fitted sine curve to the annual mean of the study sample, thereby calculating an annual value for each participant, which mean solving the equation for y0 for each individual. The seasonal variation of the measured 25(OH)D concentrations, by month of blood draw, and the modelled sine function is shown in figure S2.

Figure S2

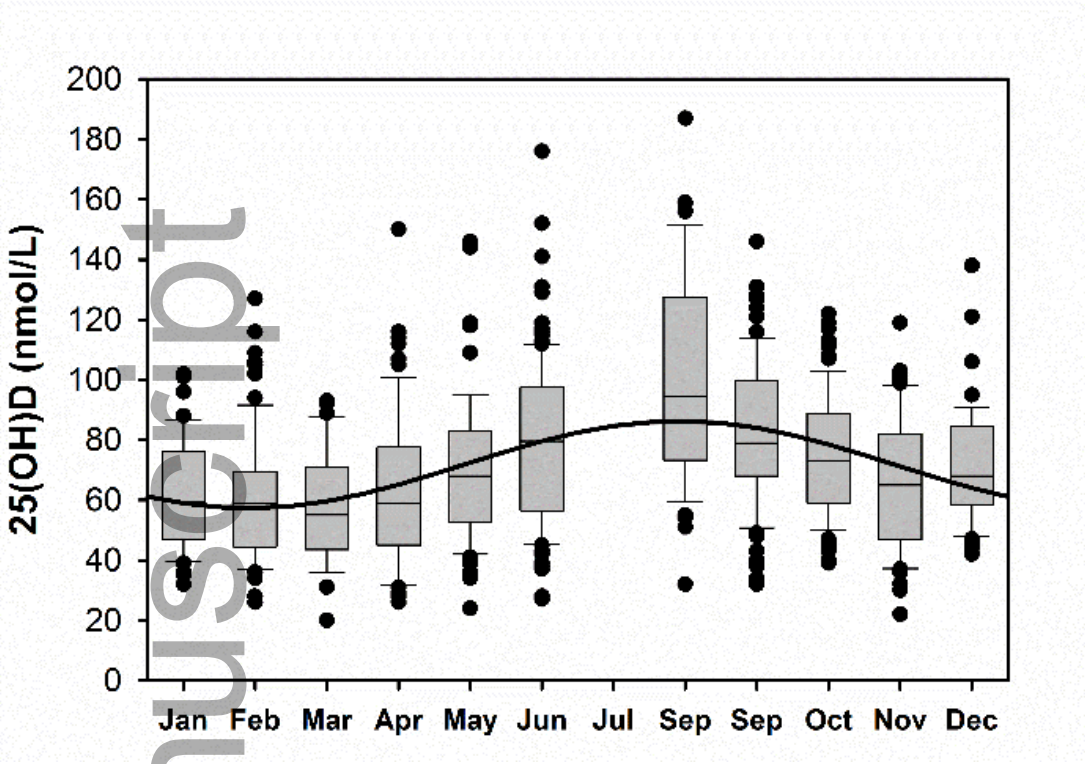

Figure S2 Seasonal variations of 25(OH)D concentrations among the whole study population, by month of blood draw. The box plot shows the median 25(OH)D concentration as a horizontal line and encompass the 25th and 75th percentiles, The solid line represents the predicted geometric mean concentrations given by date of blood draw, which was modelled as a sine function.

3. Sensitivity analysis

To assess the influence of extreme values of 25(OH)D and DBP concentrations on the results, we performed sensitivity analyses excluding values below the 2.5 percentile or above the 97.5 percentile. The results are shown in Table S1 and Figure S3.

Table S1 Hazard ratio (HR) and 95% confidence interval (CI) of bladder cancer risk by levels of 25 (OH)D, DBP and 25(OH)D:DBP molar ratio, excluding values below the 2.5 percentile or above the 97.5 percentile.

25OHD (nmol/L)	**<50 (Deficient)**	**50-74 (Insufficient)**	**75-99 (Optimal)**	**≥100 (High Optimal)**
Case/control	52/61	182/146	104/117	44/24
HR(95% CI)[1]	0.53 (0.30-0.88)	1 (ref)	0.73 (0.48-1.10)	0.29 (0.14-0.60)
DBP (umol/L)	**<3.9**	**3.9-4.5**	**4.6-5.4**	**≥ 5.4**
Case/control	54/61	167/159	94/104	45/47
HR(95% CI)[1]	0.85 (0.52-1.38)	1 (ref)	0.85 (0.55-1.32)	1.03 (0.56-1.88)
25OHD:DBP (*10^3)	**<11**	**11-16**	**17-21**	**≥22**
Case/control	50/57	184/155	81/99	28/46
HR(95% CI)[1]	0.58 (0.34-0.99)	1 (ref)	0.67 (0.44-1.03)	0.50 (0.27-0.95)

[1] Conditional on mathcing factors (age, sex, time of blood draw). Additionally, adjusted for BMI, smoking (status and packyears), education and mutually adjusted for DBP and 25OHD

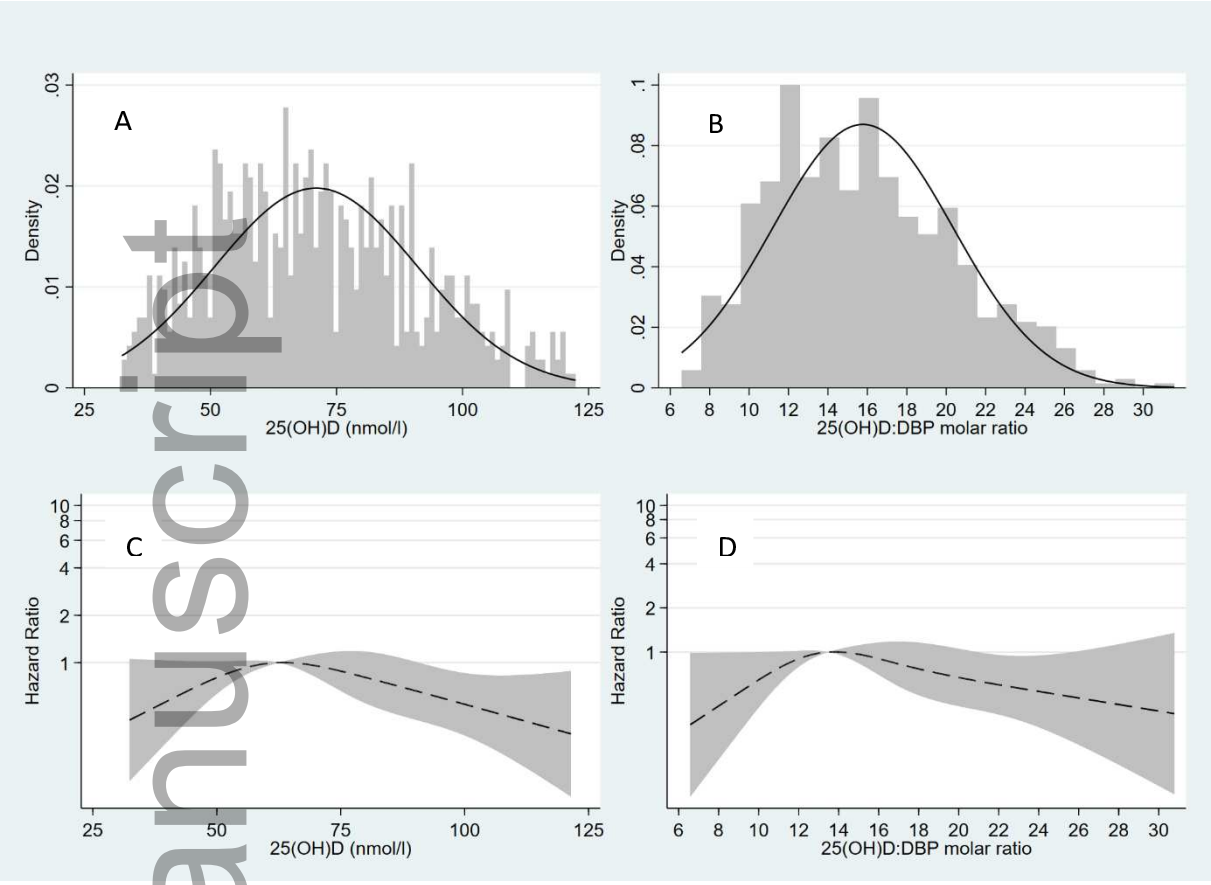

Figure S3 Histogram distribution of A) 25(OH)D and B) 25(OH)D:DBP molar ratio (x 10^3). Restricted cubic splines displaying hazard ratios of bladder cancer risk with 95% confidence intervals according to C) 25(OH)D and D) 25(OH)D:DBP molar ratio (x 10^3) . For 25(OH)D reference was set to 62.5 nmol/L, P-value for non-linearity 0.0279. For 25(OH)D:DBP molar ratio (x10^3) reference were set to 13.5 (molar ratio x 10^3), P-value for non-linearity 0.0488. Both exposure risk curves are adjusted for matching factors (age, sex, time of blood draw) and smoking (status and pack-years) BMI, physical activity and education.