

## Impact of Circulating Vitamin D Binding Protein Levels on the Association between 25-Hydroxyvitamin D and Pancreatic Cancer Risk: A Nested Case–Control Study

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### Abstract

High concentrations of circulating 25-hydroxyvitamin D [25(OH)D] have been associated with elevated pancreatic cancer risk. As this is contrary to an expected inverse association between vitamin D status and cancer, we examined whether vitamin D binding protein (DBP), the primary carrier of vitamin D compounds in circulation, plays a role in this relationship. Prediagnostic serum DBP and 25(OH)D were studied in relation to risk of pancreatic cancer in a nested case–control study of 234 cases and 234 controls in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study of Finnish men. ORs and 95% CIs were estimated using logistic regression, and statistical tests were two-sided. We found that DBP and 25(OH)D were correlated ( $r = 0.27$ ,  $P < 0.0001$ ), and DBP was inversely associated with pancreatic cancer risk (OR = 0.66, 95% CI = 0.39–1.12, for the highest vs. lowest quartile;  $P_{\text{trend}} = 0.02$ ). Importantly, this association seemed to have a threshold between quartiles 2 to 4 and quartile 1, and was primarily evident among men with concurrent high 25(OH)D concentrations (OR = 0.33, 95% CI = 0.16–0.70 for highest vs. lowest quartile;  $P_{\text{trend}} = 0.002$ ), with no association in men with lower serum 25(OH)D (OR = 1.28, 95% CI = 0.62–2.61 for highest vs. lowest quartile,  $P_{\text{trend}} 0.63$ ,  $P_{\text{interaction}} = 0.01$ ). Men with higher 25(OH)D concentrations and serum DBP below the median showed greatly elevated risk of pancreatic cancer (OR = 5.01, 95% CI 2.33–10.78, for highest vs. lowest quartile;  $P_{\text{trend}} < 0.0001$ ), while risk was weakly inversely associated with serum 25(OH)D when DBP concentrations were higher ( $P_{\text{interaction}} = 0.001$ ). Taken together, our findings indicate that higher DBP concentrations may sequester more 25(OH)D and reduce free 25(OH)D bioavailability. Simultaneous examination of DBP and 25(OH)D may be important in determining the association of vitamin D with cancer risk. *Cancer Res*; 72(5); 1190–8. ©2012 AACR.

### Introduction

Malignancies of the exocrine pancreas are the fourth most common cause of cancer death in the United States, with an estimated 44,000 new cases and 38,000 deaths in 2011 (1). The uniformly poor prognosis for pancreatic cancer further justifies efforts to identify etiologic factors, particularly those that are modifiable. For example, there is great interest in a possible role for vitamin D in several cancers, including pancreas; however, we and others have observed higher serum concentrations of 25-hydroxyvitamin D [25(OH)D], the established biomarker of vitamin D status (2), to be associated with

elevated risk of pancreatic cancer, first in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study (3), and subsequently within a large multicohort pooling project of men and women (4).

Vitamin D binding protein (DBP) is a 58 kDa polypeptide that transports vitamin D compounds in circulation (5, 6). Also known as group-specific component, or Gc-globulin, DBP binds approximately 88% of the 25(OH)D and 85% of the active hormonal form, 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D]. An additional 12% of 25(OH)D and 15% of 1,25(OH)<sub>2</sub>D are bound to albumin, leaving very little vitamin D in a free state (5–7). Current laboratory assays of vitamin D do not differentiate between free and bound vitamin D, and it is not known whether vitamin D associations with several cancers and other health outcomes are adequately captured based on total circulating 25(OH)D, the majority of which is bound to DBP, or whether the free fraction is more biologically relevant and influential in target organs.

We examined whether circulating DBP, the major vitamin D transport protein, is associated with pancreatic cancer risk, both overall and relative to 25(OH)D concentration, and whether it modifies the association between higher 25(OH)D status and elevated pancreatic cancer risk.

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## Participants and Methods

### Study population

The overall design, rationale, and objectives of the ATBC Study have been previously published (8). From 1985 to 1988, the ATBC Study recruited 29,133 Finnish male smokers between the ages of 50 and 69 years to participate in a controlled primary prevention trial. Daily supplementation with  $\alpha$ -tocopherol (50 mg dl- $\alpha$ -tocopheryl acetate/d),  $\beta$ -carotene (20 mg/d), both, or placebo continued for 5 to 8 years (median 6.1 years) until death or trial closure (April, 1993; ref. 8). Participants were followed-up passively during the postintervention period through linkage with the Finnish Cancer Registry, which provides nearly 100% case ascertainment (9). The study was approved by the Institutional Review Boards of the U.S. National Cancer Institute (NCI) and the National Public Health Institute of Finland, and written informed consent was obtained from each participant.

### Case identification and control selection

Subjects for this study were drawn from 2 existing ATBC nested case-control substudies, both of which included prior measurement of 25(OH)D. All case-control matched pairs with available residual serum were selected for DBP assay. Of the initial 200 cases and 400 controls in a nested set with serum 25(OH)D assayed in 2005 (3), 53 cases and 164 controls did not have residual serum available for the DBP assay, 24 cases and 60 controls were excluded because their matched pair case or control did not have sufficient serum, and 53 controls were randomly dropped to obtain 1:1 matching, leaving 123 matched sets. Of the initial 115 matched sets with serum 25(OH)D assayed in 2008 as part of the Vitamin D Pooling Project of Rare Cancers (4), 3 cases and 1 control did not have available residual serum, and 1 case and 3 controls were excluded because they no longer had a matched pair, leaving 111 matched sets. All cases ( $n = 234$ ) were identified through the Finnish Cancer Registry and defined as incident exocrine pancreatic tumors [International Classification of Diseases (ICD) 9, code 157, excluding islet cell carcinomas (ICD9 code 157.4)] diagnosed through April 2004. Controls were alive and cancer free at the time of case diagnosis and matched to cases (1:1) on age at randomization [ $\pm 5$  years (3) or  $\pm 1$  year (4)] and date of baseline serum collection ( $\pm 30$  days). One case and one control were missing data on 25(OH)D.

### Serum 25(OH)D and vitamin D binding protein assays

Blood samples were drawn from the antecubital vein by specially trained, registered nurses during a clinic visit prior to trial randomization, between 1985 and 1988. Serum was separated after 30 to 60 minutes by centrifuging for 10 minutes, and thereafter stored at  $-70^{\circ}\text{C}$  in 1.5-mL glass vials. Serum samples were transported from Finland to United States on dry ice in February–March 1995. All samples were aliquotted from the larger parent tubes prior to assay and were therefore thawed and refrozen at least once.

25(OH)D was previously measured in matched case-control sets using a radioimmunoassay (RIA; DiaSorin, Inc.) at Mount Sinai Hospital, Toronto, Canada (3) or a direct, competitive

chemiluminescence immunoassay (CLIA, DiaSorin Liaison 25(OH)D TOTAL assay) at Heartland Assays, Inc. (4). Matched case-control sets were assayed consecutively within the same batch along with 10% RIA- or 5% CLIA-blinded quality control samples. Samples from the same quality control pool were included in both sets of assays, with a median of 47.9 nmol/L ( $n = 30$  samples) using RIA and 53.0 nmol/L ( $n = 154$ ) using CLIA. In addition, 17 controls were included in both substudies and all but one was higher using the RIA compared with the CLIA, with a mean difference of 13.1 nmol/L between the 2 measurements. Coefficients of variation were 16.5% overall for the RIA (5.5% interbatch and 15.5% intrabatch) and 12.3% overall for the CLIA (7.1% interbatch and 10.1% intrabatch). Intraclass correlations were 86.7% and 96.0% for the RIA and CLIA, respectively.

DBP was measured in fasting baseline serum samples using the Quantikine Human Vitamin D Binding Protein Immunoassay Kit (catalog number DVDBP0; R&D Systems, Inc.) at the SAIC NCI-Frederick research facility, Frederick, MD. Each batch contained matched case-control sets and blinded quality control material comprising approximately 10% of the total samples. The overall, interbatch and intrabatch coefficients of variation, calculated using a nested components of variance analysis (10), were 18.7%, 10.8%, and 15.2%, respectively, and the intraclass correlation was 82.6%.

### Statistical analysis

Baseline descriptive data, by quartile of DBP, were calculated as medians (continuous variables) or proportions (categorical variables) among controls. ORs and 95% CIs were determined using conditional logistic regression models. Because the unconditional logistic regression results were similar, this approach was used for stratified modeling to retain subjects who were not in the same stratum as their matched case or control. Both 25(OH)D and DBP were categorized as quartiles on the basis of distribution among the controls and entered into the models as indicator variables. Quartiles rather than quintiles were used for greater stability in estimation of the ORs, especially for stratified models, but findings for quintiles were similar. Because of the slight differences in the 25(OH)D data from the 2 laboratories, and because 25(OH)D levels are known to fluctuate throughout the year, the 25(OH)D quartiles are calculated separately for each laboratory by sunnier and less sunny season categories (May–October and November–April) and then merged into one quartile variable. Results were essentially unchanged when 25(OH)D was instead categorized using clinically defined cut points or adjusted for season using the residual method (11). Exclusion of participants reporting a history of diabetes did not materially change the risk estimates, therefore, these subjects were retained.

A molar ratio of 25(OH)D:DBP was created as a proxy for free circulating 25(OH)D (12, 13). In addition, we estimated "free 25(OH)D" on the basis of total 25(OH)D and DBP using the affinity constants of albumin and DBP (12, 14), both by ignoring albumin (based on its relatively smaller vitamin D transport function) and by assigning a fixed albumin value to every subject (because of limited biospecimen availability, we did

not measure albumin). As described previously (12), estimating "free 25(OH)D" without albumin provides a valid approximation that is comparable with the 25(OH)D:DBP molar ratio. We also adjusted 25(OH)D for DBP through the residual method used to adjust for season (11). Tests for linear trend were obtained by assigning to each category an ordinal value (1–4) and treating this parameter as a continuous variable. DBP was also modeled as a continuous variable of 1,000 nmol/L increments.

Variables assessed for confounding included those identified in previous publications (3, 4) and those associated with DBP as identified in Table 1. These included body mass index (BMI), number of cigarettes smoked per day, years of smoking, smoking cessation during the trial period [reporting to have quit smoking >3 consecutive visits (i.e., >1 year)], history of diabetes, occupational physical activity, education; serum  $\alpha$ -tocopherol, retinol, and cholesterol; and intake of fat, vitamin D, calcium, and ethanol. Both forward and backward stepwise regression procedures were used to determine whether the addition or removal of a potential confounder produced more than 10% change in the 25(OH)D or DBP coefficients. As none of the identified factors met

the definition of a confounder, we present a crude model (Model 1) conditioned on the matching factors, and a multivariate-adjusted model (Model 2), which includes known pancreatic cancer risk factors and factors strongly associated with DBP in this analysis (i.e., BMI, number of cigarettes smoked per day, number of years of smoking, history of diabetes, occupational physical activity, education, ethanol intake, and serum retinol, cholesterol,  $\beta$ -carotene, and  $\alpha$ -tocopherol). A third model (Model 3) builds on Model 2 and includes mutual adjustment for 25(OH)D or DBP. Additional testing of several medical history variables (history of renal failure, pancreatitis, liver cirrhosis, lung emphysema, chronic bronchitis, stroke, cerebral hemorrhage, cerebral infarction, valvular heart disease, myocardial infarction, coronary heart disease, angina pectoris, or heart failure) did not alter the DBP results.

To evaluate effect modification, we stratified DBP by 25(OH)D, and 25(OH)D and the ratio of 25(OH)D:DBP by DBP. We also examined DBP stratified by age, BMI, number of cigarettes per day, occupational physical activity, season of blood collection, intakes of fat (total, monounsaturated, saturated, and trans fat), folate, vitamin D, calcium, and ethanol, and follow-up

**Table 1.** Selected baseline characteristics across DBP quartiles in controls, ATBC Study, 1985–2004<sup>a</sup>

Characteristic	Quartile of serum DBP <sup>b</sup>			
	Q1	Q2	Q3	Q4
Age, y	58	58	58	59
Height, cm	175	172	172	173
Weight, kg	81.8	76.8	77.7	77.7
BMI, kg/m <sup>2</sup>	26.4	26.3	26.0	25.6
Education, % >elementary	28.8	22.4	25.4	6.9
Cigarettes/day	20	20	20	20
History of diabetes, %	11.9	1.7	5.1	5.2
Occupational physical activity, % heavy	6.8	5.2	13.6	8.6
Dietary vitamin D, $\mu$ g/day	4.7	4.1	5.0	4.6
Vitamin D supplement use, %	10.2	6.9	5.1	1.7
Total vitamin D intake (diet and supplements), $\mu$ g/day	6.0	4.1	5.4	4.7
Calcium intake, mg/day	1,411	1,311	1,396	1,343
Total fat intake, g/day	118	112	115	113
Ethanol intake, g/day	11.4	13.3	6.1	6.1
Season of blood draw, % May–October	20.3	27.6	37.3	36.2
Serum biomarkers				
DBP, nmol/L	3,163	4,745	5,851	8,243
25(OH)D, nmol/L	31.5	39.2	45.1	49.9
25(OH)D:DBP molar ratio ( $\times 10^3$ ) <sup>c</sup>	10.45	8.29	7.27	6.16
$\alpha$ -tocopherol, mg/L	10.9	11.2	10.8	11.7
$\beta$ -carotene, $\mu$ g/L	164	159	159	181
Total cholesterol, mmol/L	6.1	6.2	5.9	6.4
HDL cholesterol, mmol/L	1.08	1.09	1.13	1.09
Retinol, $\mu$ g/L	514	548	613	568

<sup>a</sup>Values are medians or proportions.

<sup>b</sup>Quartile cuts for DBP were Q1:  $\leq 4,026$ , Q2:  $>4,026$ , and  $\leq 5,329$ , Q3:  $>5,329$  and  $\leq 6,721$ , Q4:  $>6,721$  nmol/L.

<sup>c</sup>A proxy for free 25(OH)D.

time. Effect modification was tested by including the cross-product term of the biomarker quartiles and the effect modifier (split at the median or yes/no), and by stratified analyses conducted using unconditional logistic regression, adjusted for the matching factors only. Statistical analyses were done using SAS software version 9.1.3 (SAS Institute, Inc.) and all *P* values were 2-sided.

**Results**

Serum DBP concentrations in controls ranged from 2,973 to 8,544 nmol/L (for 10th–90th percentiles) and did not vary by season of blood draw. Baseline characteristics of controls by quartile of DBP are presented in Table 1. Median DBP values for quartiles 2, 3, and 4 were 50%, 85%, and 161% higher, respectively, compared with those in the first quartile. Men with higher DBP had higher circulating 25(OH)D,  $\alpha$ -tocopherol, cholesterol, retinol, and  $\beta$ -carotene, and lower 25(OH)D:DBP molar ratio and ethanol intake. In addition,

men with higher DBP had lower levels of education, were less likely to have a history of diabetes or used vitamin D supplements, and were more likely to have had their blood sampled in the sunnier months. The prevalence of diabetes and vitamin D supplement use in this population was low; therefore, the patterns observed are based on small numbers of subjects and may or may not indicate important biologic relationships.

Spearman correlation coefficients (and related *P* values) for DBP and selected factors were 0.08 (0.22) for age,  $-0.08$  (0.20) for BMI,  $-0.08$  (0.22) for cigarettes per day, 0.10 (0.13) for years of smoking, 0.14 (0.03) for serum  $\alpha$ -tocopherol, 0.11 (0.09) for serum cholesterol, 0.18 (0.005) for serum retinol, 0.06 (0.37) for serum  $\beta$ -carotene,  $-0.20$  (0.003) for alcohol intake, 0.27 ( $< 0.0001$ ) for total 25(OH)D, and  $-0.42$  ( $< 0.0001$ ) for the 25(OH)D:DBP molar ratio. The correlations between total 25(OH)D and both the 25(OH)D:DBP molar ratio and the calculated "free 25(OH)D" were 0.72 ( $P < 0.0001$ ).

**Table 2.** ORs and 95% CIs for the association between DBP, 25(OH)D, 25(OH)D:DBP molar ratio, and risk of pancreatic cancer, ATBC Study, 1985–2004<sup>a</sup>

	Quartile 1 OR (95% CI)	Quartile 2 OR (95% CI)	Quartile 3 OR (95% CI)	Quartile 4 OR (95% CI)	<i>P</i> <sub>trend</sub>
<b>DBP</b>					
Range, nmol/L	≤4,026	>4,026 & ≤5,329	> 5,329 & ≤6,721	>6,721	
<i>N</i> <sup>b</sup>	87/59	58/58	31/59	58/58	
Model 1 <sup>c</sup>	1.00 (reference)	0.69 (0.42–1.12)	0.36 (0.21–0.63)	0.66 (0.39–1.12)	0.02
Model 2 <sup>d</sup>	1.00 (reference)	0.74 (0.44–1.24)	0.35 (0.19–0.62)	0.70 (0.40–1.22)	0.03
Model 3 <sup>e</sup>	1.00 (reference)	0.70 (0.42–1.19)	0.35 (0.20–0.64)	0.67 (0.37–1.19)	0.02
<b>25(OH)D</b>					
Range <sup>f</sup>					
<i>N</i> <sup>b</sup>	48/60	61/58	50/59	74/56	
Model 1 <sup>c</sup>	1.00 (reference)	1.33 (0.79–2.24)	1.14 (0.65–1.98)	1.78 (1.02–3.11)	0.08
Model 2 <sup>d</sup>	1.00 (reference)	1.51 (0.87–2.62)	1.21 (0.68–2.17)	1.91 (1.05–3.46)	0.07
Model 3 <sup>e</sup>	1.00 (reference)	1.42 (0.80–2.49)	1.28 (0.71–2.33)	1.81 (0.97–3.37)	0.10
<b>25(OH)D:DBP molar ratio (x10<sup>3</sup>)<sup>g</sup></b>					
Range	≤4.91	>4.91 & ≤8.07	>8.07 & ≤11.03	>11.03	
<i>N</i> <sup>b</sup>	55/59	52/58	38/58	88/58	
Model 1 <sup>c</sup>	1.00 (reference)	1.09 (0.59–2.03)	0.81 (0.43–1.53)	1.89 (1.01–3.54)	0.04
Model 2 <sup>d</sup>	1.00 (reference)	1.00 (0.52–1.92)	0.77 (0.39–1.50)	1.86 (0.97–3.56)	0.04

<sup>a</sup>All models used conditional logistic regression.

<sup>b</sup>*N* for cases/controls.

<sup>c</sup>Model 1 is conditioned on the matching factors of age and date of blood collection.

<sup>d</sup>Model 2 is conditioned on the matching factors of age and date of blood collection, and adjusted for BMI (continuous), number of cigarettes smoked/d (continuous), years of smoking (continuous), history of diabetes (yes/no), occupational physical activity (heavy vs. no heavy activity), education ( $>$  elementary vs. elementary), ethanol intake (quartiles, with separate categories for nondrinkers and missing), and serum retinol (continuous), cholesterol (continuous),  $\beta$ -carotene (continuous) and  $\alpha$ -tocopherol (continuous).

<sup>e</sup>Model 3 is the same as Model 2, with additional adjustment for either 25(OH)D or DBP, respectively (quartiles).

<sup>f</sup>Season-specific/laboratory-specific quartiles for the 25(OH)D RIA data were as follows: Q1:  $\leq 31.5$ , Q2:  $>31.5$ , and  $\leq 45.1$ , Q3:  $>45.1$  and  $\leq 56.7$ , Q4:  $>56.7$  nmol/L for the darker months; Q1:  $\leq 40.9$ , Q2:  $>40.9$  and  $\leq 54.7$ , Q3:  $>54.7$  and  $\leq 73.4$ , Q4:  $>73.4$  nmol/L for sunnier months. Season-specific/laboratory-specific quartiles for the 25(OH)D CLIA data were as follows: Q1:  $\leq 18.4$ , Q2:  $>18.4$  and  $\leq 26.8$ , Q3:  $>26.8$  and  $\leq 40.3$ , Q4:  $>40.3$  nmol/L for the darker months; Q1:  $\leq 29.0$ , Q2:  $>29.0$  and  $\leq 41.5$ , Q3:  $>41.5$  and  $\leq 61.8$ , Q4:  $>61.8$  nmol/L for sunnier months.

<sup>g</sup>A proxy for free 25(OH)D.

Serum DBP concentration was inversely associated with risk of pancreatic cancer (Table 2, Model 1,  $P_{\text{trend}} = 0.02$ ), with an apparent threshold effect such that risk in quartiles 2 to 4 were lower than for quartile 1 (OR = 0.57, 95% CI = 0.38–0.85 for quartiles 2–4 combined vs. quartile 1). Multivariate-adjustment for pancreatic cancer risk factors and factors associated with DBP (Model 2), or further adjustment for 25(OH)D (Model 3) did not alter the risk estimates as compared with the base model, indicating little confounding by these factors. The OR for a 1,000 nmol/L increment in DBP was 0.92 (95% CI = 0.84–1.00,  $P = 0.04$ ).

Serum 25(OH)D was positively associated with risk of pancreatic cancer (Table 2), although none of the patterns seem monotonic, and statistical significance was achieved for the

highest quartile only. When the data were examined as quintiles, as was done in the earlier study of vitamin D (3), the OR for the highest versus lowest quintile was 2.01 (95% CI = 1.08–3.72,  $P_{\text{trend}} = 0.03$ ). Risk estimates were slightly stronger with multivariate adjustment or further adjustment for circulating DBP. The association between 25(OH)D and pancreatic cancer was attenuated when 25(OH)D was residually adjusted for DBP (OR = 1.51, 95% CI = 0.82–2.78 for highest vs. lowest quartile).

The risk of pancreatic cancer was significantly elevated in the highest quartile of the 25(OH)D:DBP molar ratio [a proxy for free 25(OH)D], with risks similar to those for 25(OH)D alone (Table 2, Model 1), although again, the patterns did not seem monotonic. Multivariate adjustment did not alter the risks. Using the estimation of "free 25(OH)D" without albumin

**Table 3.** ORs and 95% CIs for the association between serum DBP and 25(OH)D and risk of pancreatic cancer—stratified models, ATBC Study, 1985–2004<sup>a</sup>

	Quartile 1 OR (95% CI)	Quartile 2 OR (95% CI)	Quartile 3 OR (95% CI)	Quartile 4 OR (95% CI)	$P_{\text{trend}}$	$P_{\text{interaction}}$
DBP, nmol/L	≤4,026	>4,026 & ≤5,329	>5,329 & ≤6,721	>6,721		
25(OH)D below median						
$N^b$	44/41	21/33	13/23	28/20		
Model 1 <sup>c</sup>	1.00 (reference)	0.59 (0.30–1.19)	0.51 (0.23–1.14)	1.28 (0.62–2.61)	0.63	0.01
Model 2 <sup>d</sup>	1.00 (reference)	0.61 (0.29–1.26)	0.56 (0.23–1.34)	1.42 (0.67–3.05)	0.59	
Model 3 <sup>e</sup>	1.00 (reference)	0.63 (0.30–1.33)	0.60 (0.25–1.45)	1.41 (0.65–3.05)	0.59	
25(OH)D above median						
$N^b$	43/18	37/25	17/36	30/37		
Model 1 <sup>c</sup>	1.00 (reference)	0.62 (0.29–1.31)	0.20 (0.09–0.44)	0.33 (0.16–0.70)	0.002	
Model 2 <sup>d</sup>	1.00 (reference)	0.59 (0.26–1.33)	0.17 (0.07–0.41)	0.32 (0.14–0.71)	0.001	
Model 3 <sup>e</sup>	1.00 (reference)	0.64 (0.28–1.44)	0.18 (0.08–0.43)	0.30 (0.13–0.68)	0.001	
25(OH)D, nmol/L <sup>f</sup>						
DBP below median						
$N^b$	28/40	40/37	28/26	49/14		
Model 1 <sup>c</sup>	1.00 (reference)	1.55 (0.81–3.00)	1.53 (0.74–3.15)	5.01 (2.33–10.78)	<0.0001	0.001
Model 2 <sup>d</sup>	1.00 (reference)	1.62 (0.79–3.34)	1.54 (0.70–3.41)	5.72 (2.46–13.30)	0.0001	
Model 3 <sup>e</sup>	1.00 (reference)	1.57 (0.77–3.25)	1.62 (0.73–3.60)	5.76 (2.46–13.48)	0.0001	
DBP above median						
$N^b$	20/20	21/21	22/33	25/42		
Model 1 <sup>c</sup>	1.00 (reference)	0.96 (0.40–2.31)	0.65 (0.29–1.49)	0.59 (0.27–1.31)	0.12	
Model 2 <sup>d</sup>	1.00 (reference)	1.01 (0.39–2.57)	0.71 (0.29–1.74)	0.50 (0.21–1.21)	0.08	
Model 3 <sup>e</sup>	1.00 (reference)	0.90 (0.34–2.36)	0.79 (0.31–1.99)	0.42 (0.17–1.04)	0.05	

<sup>a</sup>All models used unconditional logistic regression.

<sup>b</sup> $N$  for cases/controls.

<sup>c</sup>Model 1 is adjusted for the matching factors of age (continuous) and date of blood collection (continuous).

<sup>d</sup>Model 2 is adjusted for the matching factors of age (continuous) and date of blood collection (continuous), as well as BMI (continuous), number of cigarettes smoked/d (continuous), years of smoking (continuous), history of diabetes (yes/no), occupational physical activity (heavy vs. no heavy activity), education (> elementary vs. elementary), ethanol intake (quartiles, with separate categories for nondrinkers and missing), and serum retinol (continuous), cholesterol (continuous),  $\beta$ -carotene (continuous) and  $\alpha$ -tocopherol (continuous).

<sup>e</sup>Model 3 is the same as Model 2, with additional adjustment for either 25(OH)D or DBP, respectively (quartiles).

<sup>f</sup>Season-specific/laboratory-specific quartiles for the 25(OH)D RIA data were as follows Q1: ≤31.5, Q2: >31.5 and ≤45.1, Q3: >45.1 and ≤56.7, Q4: >56.7 nmol/L for the darker months; Q1: ≤40.9, Q2: >40.9 and ≤54.7, Q3: >54.7 and ≤73.4, Q4: >73.4 nmol/L for sunnier months. Season-specific/laboratory-specific quartiles for the 25(OH)D CLIA data were as follows Q1: ≤18.4, Q2: >18.4 and ≤26.8, Q3: >26.8 and ≤40.3, Q4: >40.3 nmol/L for the darker months; Q1: ≤29.0, Q2: >29.0 and ≤41.5, Q3: >41.5 and ≤61.8, Q4: >61.8 nmol/L for sunnier months.

resulted in the exact same risk estimates as those for the 25(OH)D:DBP molar ratio. Including a constant to represent albumin in the estimation of "free 25(OH)D" yielded similar patterns.

We found strong and statistically significant interactions between circulating DBP and 25(OH)D in relation to pancreatic cancer risk (Table 3). Higher DBP was associated with a significantly reduced risk of pancreatic cancer only among men with 25(OH)D concentrations above the median (OR = 0.33, 95% CI = 0.16–0.70, for highest vs. lowest quartile;  $P_{\text{trend}} = 0.002$ ), with no association in men with lower serum 25(OH)D ( $P_{\text{interaction}} = 0.01$ ). Similarly, men in the highest quartile of 25(OH)D concentrations who also had DBP levels below the median were at substantially elevated pancreatic cancer risk (OR = 5.01, 95% CI = 2.33–10.78, for highest vs. lowest quartile;  $P_{\text{trend}} < 0.0001$ ), and risk was inversely associated with serum 25(OH)D when DBP concentrations were above the median ( $P_{\text{interaction}} = 0.001$ ). These patterns persisted when laboratory-specific 25(OH)D data were examined separately. Finally, the pattern of interaction for the 25(OH)D:DBP molar ratio mirrored that of 25(OH)D, with elevated risks when DBP was low (OR = 3.40, 95% CI = 1.53–7.54 for the highest vs. lowest quartile) and reduced risks when DBP was high (OR = 0.31, 95% CI = 0.11–0.86 for the highest vs. lowest quartile;  $P_{\text{interaction}} = 0.002$ ).

The inverse association between DBP and pancreatic cancer was stronger among older men, those with lower intakes of fat (all types examined), vitamin D, calcium, and ethanol, those who smoked less, and for cases with at least 10 years between blood collection at study entry and diagnosis (data not shown). However, none of the interaction tests for these factors were significant.

## Discussion

To our knowledge, this is the first investigation of the role of circulating DBP and risk of pancreatic cancer. We found that serum DBP was inversely associated with pancreatic cancer risk, particularly among men with higher serum 25(OH)D concentrations. Serum 25(OH)D concentration was positively associated with risk overall, as previously reported (3), but we observed greatly elevated risk (i.e., OR > 5) for men in the highest 25(OH)D quartile whose DBP levels were below the median, possibly representing a stronger impact of higher vitamin D status relative to the plasma binding capacity of DBP. Among men with higher DBP concentrations, 25(OH)D seemed inversely associated with pancreatic cancer risk, although this was not statistically significant. The 25(OH)D:DBP molar ratio, which is a proxy for free 25(OH)D (12, 13), displayed risk patterns similar to those of 25(OH)D. There was no evidence of confounding by any of the factors we examined, and inclusion of both serum DBP and 25(OH)D in the regression models did not attenuate the risk associations for either factor.

Two previous studies of prostate cancer risk reported no association with serum DBP concentrations (15) or the 25(OH)D:DBP molar ratio (what the authors referred to as the "free 25-D index"; ref. 16), although an interaction between the

vitamin D receptor polymorphism *BsmI* and the free 25-D index was observed in the latter study (17).

Previously within the ATBC Study cohort, we observed a positive association between serum 25(OH)D and pancreatic cancer risk (OR = 2.92, 95% CI = 1.56–5.48, for the highest vs. lowest quintile;  $P_{\text{trend}} = 0.0001$ ; ref. 3). In the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO), circulating 25(OH)D was not associated with pancreatic cancer risk overall, but elevated risks for higher serum levels were noted among those whose blood was collected in the fall or winter (OR = 3.91, 95% CI = 1.19–12.85, for highest vs. lowest quintile;  $P_{\text{trend}} = 0.10$ ), and those residing in areas with low residential sun exposure (OR = 4.03, 95% CI = 1.38–11.79;  $P_{\text{trend}} = 0.02$ ; ref. 18). Within the Vitamin D Pooling Project of Rare Cancers, which included 8 cohorts of men and women and 952 pancreatic cancer cases, higher concentrations of circulating 25(OH)D were associated with elevated risk (OR = 2.12, 95% CI = 1.23–3.64 for  $\geq 100$  nmol/L vs. 50–<75 nmol/L), and elevated risk persisted when the previously reported data from ATBC and PLCO were excluded (4).

DBP binds and transports vitamin D compounds in circulation, including, importantly, 25(OH)D and 1,25(OH)<sub>2</sub>D, as well as other molecules (2, 6, 19). The plasma concentration of DBP is orders of magnitude greater than that of 25(OH)D, its half-life is approximately 2.5 to 3 days, and its affinity for 25(OH)D is substantially greater than that for 1,25(OH)<sub>2</sub>D (6, 19–21). As a consequence, only 0.4% of 1,25(OH)<sub>2</sub>D and 0.04% of 25(OH)D is in a free state (6, 21), and less than 5% of circulating DBP is associated with vitamin D compounds (19, 20). The large excess of DBP compared with vitamin D provides a reservoir for 25(OH)D, prolongs its half-life, and protects against short-term vitamin D deficiency; it may also relate to other functions of DBP that are described below (5, 20). Circulating DBP concentrations show some diurnal variation with a morning nadir and rapid daytime increase in levels, but in contrast to vitamin D levels, they do not vary by season (13, 22). Fatty acids also bind to DBP, thereby reducing its capacity for vitamin D compounds (5, 6); of note in this regard, the inverse pancreatic cancer association for DBP was marginally stronger among those with lower fat intake in our study.

In addition to its role in vitamin D transport, DBP functions as an actin scavenger, as a precursor to a macrophage activating factor and as a chemotactic factor (5, 6, 23, 24). Actin is released into circulation following cellular injury or death and can result in platelet aggregation, vascular obstruction, and organ dysfunction. By binding actin, DBP attenuates these effects (5, 6), which may be one reason for very high DBP concentrations compared with vitamin D (5). The DBP–macrophage activating factor, a selectively deglycosylated form of DBP, has antiangiogenic, antitumorigenic, immunomodulatory, and proapoptotic properties (5, 25, 26) and has been shown to inhibit pancreatic (25) and prostate tumor growth (26). DBP also plays a role in inflammation, acting as a chemotactic factor by attracting neutrophils to sites of inflammation through its influence on complement-derived peptides (C5a; refs. 5, 6, 24). Although these other properties of DBP may impact cancer, the strong interaction with 25(OH)D

concentrations supports the notion that its role in binding 25(OH)D is important for pancreatic cancer risk.

Although direct measurement of unbound 25(OH)D in circulation is currently limited to small laboratory investigations and has not been undertaken in larger scale epidemiologic studies, our finding of a much stronger vitamin D–pancreatic cancer risk association among men with lower DBP levels, as well as strengthening of the inverse association for DBP in the setting of higher 25(OH)D concentrations, may be indicative of higher concentrations of free 25(OH)D available to cells to initiate a range of transcriptional events through conversion to 1,25(OH)<sub>2</sub>D and binding the nuclear vitamin D receptor in target organs. This would be consistent with the "free hormone theory" that only unbound hormones are able to enter target cells and influence transcriptional and downstream biologic events (5, 6). Two recent studies support this concept with respect to vitamin D, with one showing calculated "free 25(OH)D" to be more highly correlated with bone mineral density ( $r = 0.41$ ) than was total 25(OH)D alone ( $r = 0.17$ ; ref. 27), and the other finding no difference in plasma total 25(OH)D or 1,25(OH)<sub>2</sub>D but significantly lower "free" concentrations of these metabolites in men with osteoporosis, compared with controls (12). Although we did not measure serum albumin and therefore could not calculate the concentration of "free 25(OH)D" in the same manner, because approximately 88% of 25(OH)D is carried on DBP and only approximately 12% on albumin, our stratification for DBP levels and examination of the 25(OH)D:DBP molar ratio represent reasonable proxies for free versus bound vitamin D in circulation (12).

Cellular uptake of the DBP-bound 25(OH)D is also mediated by the plasma membrane receptor megalin, known also as low-density lipoprotein receptor protein 2, or LRP2 (28). This is particularly important in the renal proximal tubules for clearance of DBP–25(OH)D from the glomerular filtrate and conversion of 25(OH)D to 1,25(OH)<sub>2</sub>D; for example, megalin knockout mice are unable to resorb the 25(OH)D–DBP complex from urine, resulting in excretion of vitamin D, drastically reduced circulating 25(OH)D and 1,25(OH)<sub>2</sub>D levels, and rachitic bone disease (23, 28). Megalin-mediated, endocytic internalization of vitamin D has also been shown in other epithelial tissues, including the small intestine, breast, uterus, epididymis, and brain (23, 24, 28), but as yet not in the pancreas. It is therefore possible that higher circulating DBP levels promote greater renal tubule DBP–25(OH)D resorption and higher vitamin D status, which, if vitamin D were inversely associated with pancreatic cancer, as experimental data support, could explain both the inverse DBP–pancreatic cancer association and the greatly elevated risk for high 25(OH)D in the low DBP stratum we observed. However, the fact that mutually adjusting for 25(OH)D and DBP does not alter the associations argues against this. Establishing whether megalin is expressed in pancreatic tissue may help elucidate any role cellular absorption of DBP-bound 25(OH)D might play in the interrelations among DBP, vitamin D status, and pancreatic cancer risk.

The present finding of an inverse association between 25(OH)D and pancreatic cancer, which was evident only when DBP concentrations were high, is not easily explained in light of consistent findings of elevated risk for 25(OH)D in several studies. Because 25(OH)D is preferentially bound to DBP (14), in a setting of high DBP, the high 25(OH)D could potentially displace 1,25(OH)<sub>2</sub>D, which has been shown experimentally to have several antitumorigenic properties (29, 30).

Our study has several strengths and limitations. The prospective evaluation of 25(OH)D and DBP and the long follow-up greatly reduce any effect of pancreatic cancer on these biochemical measures and the possibility of reverse causality. Although the ATBC Study participants were male smokers, our pancreatic cancer–25(OH)D findings have been replicated in other populations that included women and were predominantly nonsmokers (4). It is unclear whether the present findings for DBP are generalizable to women or to nonsmoking populations. DBP concentrations were not correlated with our smoking measures, and smoking did not confound any of our observed associations, suggesting that residual confounding from smoking is unlikely to explain our findings. In addition, limited data on participants who reported cessation of smoking during follow-up does not indicate that this behavior change influenced the findings. We found no evidence of confounding by any of the factors examined, including those associated with DBP concentrations; however, we cannot completely rule out residual confounding by smoking, smoking behavior change, or factors associated with circulating DBP. Circulating 25(OH)D is considered the accepted biomarker of vitamin D status that integrates exposure from diet, supplements, and sunlight (31). The vitamin D status within the ATBC Study population was relatively low as a result of the high latitude of Finland, low use of vitamin D supplements in the study population, and few blood collections during the summer months when vitamin D levels tend to peak. Within the Vitamin D Pooling Project of Rare Cancers, however, risk of pancreatic cancer was elevated at 25(OH)D concentrations greater than those in ATBC ( $\geq 100$  nmol/L; ref. 4). The DBP concentrations we measured were within the range of those reported by others (5, 6, 19, 23). We measured 25(OH)D and DBP at only one point in time, but DBP concentrations seem to be stable during the lifetime of an individual (32), and evidence indicates that 25(OH)D in samples collected up to 14 years apart are well correlated (33–35). The relatively high coefficients of variation for the 25(OH)D and DBP assays indicate variation in the laboratory assays and may have reduced our ability to observe stronger associations. All serum samples were collected in the morning after an overnight fast, thereby reducing the potential impact of diurnal and postprandial variation.

In conclusion, the simultaneous examination of the vitamin D carrier protein, DBP, and 25(OH)D supports previous findings of an adverse relationship between vitamin D and pancreatic cancer. Specifically, circulating levels of the DBP were inversely associated with pancreatic cancer risk, particularly when the concentration of 25(OH)D—which itself was directly associated with risk—was high. This may indicate that higher

levels of DBP lead to more bound 25(OH)D and less 25(OH)D freely available to impact target tissues. Studies of vitamin D-related genes, including *GC*, the gene encoding DBP, and risk of pancreatic and other cancers could provide further evidence to test this hypothesis, and DBP should be examined in other investigations of vitamin D, including, particularly, women and nonsmokers. Simultaneous examination of DBP and 25(OH)D seems warranted in future risk association studies of vitamin D.

#### Disclosure of Potential Conflicts of Interest

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of

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