Serum Concentrations of Emerging Vitamin D Biomarkers and Detection of Prevalent High-Risk HPV Infection in Mid-adult Women

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ABSTRACT

Background: Vitamin D has potential immunomodulating benefits in infection. One prior population-based cross-sectional study showed a protective association between serum concentrations of 25(OH)D and high-risk human papillomavirus (hrHPV) detection. Additional biomarkers present at different stages along the vitamin D metabolic pathway may more completely characterize vitamin D status but have not yet been evaluated in relation to hrHPV infection.

Methods: Stored sera from women aged 30–50 years ($N = 404$) enrolled in an HPV natural history study from 2011–2012 were tested for 25(OH)D and 4 novel vitamin D biomarkers: $1,25(OH)_2D$, $24,24(OH)_2D_3$, free vitamin D, and vitamin D–binding protein. Cross-sectional associations between vitamin D serum concentrations and cervicovaginal hrHPV detection were estimated using logistic regression.

Results: 25(OH)D serum concentrations were not associated with hrHPV. After adjusting for age, race, season, education, oral contraceptive use, smoking status, body mass index, and serum concentrations of calcium and phosphate, each 1 ng/mL increase in $24,25(OH)_2D_3$ was nearly statistically significantly associated with higher likelihood of hrHPV detection [aOR = 1.22; 95% confidence interval (CI), 0.97–1.52]. No significant associations were observed for other biomarkers.

Conclusions: 25(OH)D serum concentrations were unassociated with prevalent hrHPV. Higher levels of one novel biomarker, $24,25(OH)_2D_3$, were positively associated with hrHPV, an unexpected finding.

Impact: Inconsistent with previous findings of a protective association between 25(OH)D and prevalent hrHPV infection, these results suggest serum concentrations of 4 vitamin D biomarkers are unassociated with detection of hrHPV in mid-adult women.

Introduction

High-risk human papillomavirus (hrHPV) infections are causally linked to anogenital and oropharyngeal cancers (1). While approximately 90% of hrHPV infections become undetectable within 1–2 years, the minority that persist are associated with increased risk for cervical cancer (2). Understanding factors that may contribute to acquisition and persistence of infection (i.e., vitamin D deficiency) are critical to efforts to prevent HPV infections and HPV-related carcinogenesis.

Vitamin D sufficiency is associated with improved bone and intestinal health, reduced risk and severity of respiratory and influenza-like infections (3), and lower incidence of certain cancers (4). However, data collected by the population-based National Health and Nutrition Examination Survey (NHANES) in 2005–2006 showed 42% of adults in the United States are vitamin D deficient or insufficient with 25(OH)D ≤20 ng/mL (5). Previous studies have investigated the potential biological pathways by which vitamin D acts as an immune modulator in the body (6, 7). Lower concentrations of serum 25(OH)D may lead to diminished innate immune function and increased susceptibility to and duration of infection. Specifically, insufficient and deficient levels of vitamin D may limit the body's ability to produce and regulate the expression of antimicrobial peptides (AMP; ref. 8), which inactivate pathogens and boost the innate immune response by attracting phagocytes to the site of infection (9). AMPs are produced in endothelial cells in the vagina and the cervix, where they can specifically defend against sexually transmitted infections (10, 11). In response to HPV, certain AMPs can eliminate the virus before it establishes infection in the host (12). Optimal AMP production and activity (associated with sufficient serum concentrations of 25(OH)D; ref. 13) may be critical in bolstering the innate immune response to HPV and, thus, defend against infection with a virus capable of evading adaptive immune response (14).

Metabolism of vitamin D occurs in the bloodstream, kidney, and liver; this process generates several biomarkers indicative of an individual's vitamin D status (15, 16). Most epidemiologic studies have estimated vitamin D status by measuring serum concentrations of total 25-hydroxyvitamin D, or 25(OH)D, but this biomarker is only one means (albeit a stable one; ref. 17) of characterizing vitamin D status (18). Additional biomarkers, including (i) calcitriol [the hormonally active metabolite of D_3 otherwise known as $1,25(OH)_2D$], (ii) $24,25(OH)_2D_3$ (the most concentrated product of $25(OH)D_3$ catabolism), (iii) vitamin D–binding protein (DBP), and (iv) free vitamin D (circulating unbound to DBP or albumin; refs. 17, 18), are present at different stages along the vitamin D metabolic pathway, and have potential to characterize vitamin D status more fully than with 25(OH)D alone (18). However, the absence of established cut-points

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of sufficiency for these additional biomarkers makes comparisons across biomarkers difficult. Additional vitamin D biomarkers have historically not been factored into recommendations (19).

To our knowledge, only one study has evaluated the association between serum 25(OH)D and HPV. Using cross-sectional data from NHANES, investigators reported that concentrations of serum 25(OH)D were inversely associated with HPV infection (20). To expand on these findings, we evaluated the cross-sectional associations between five different vitamin D biomarkers and hrHPV infection among mid-adult women in Washington State (21). Given previous literature suggesting differences in vitamin D metabolism and bioavailability of the micronutrient among racial groups (22, 23), we also evaluated whether this association varied by race.

Materials and Methods

Study population

We used baseline data and stored specimens from a 6-month longitudinal cohort study of HPV infections in healthy mid-adult women in Seattle, WA. Details of the parent study design were described previously (21). Briefly, women aged 30–50 years who were affiliated with the University of Washington (Seattle, WA; faculty, staff, or student) were enrolled from March 2011–January 2012. Women who were pregnant, had a hysterectomy, or had a current medical condition that prohibited participation were excluded. At enrollment, participants self-collected vaginal swabs for HPV DNA testing and provided venous blood specimens; residual serum specimens were stored at −80°C. Demographic (age, race/ethnicity, education level, marital status), health [body mass index (BMI), alcohol intake, smoking status, oral contraceptive use, HPV vaccination history], and sexual behavior characteristics (history of genital warts and/or other sexually transmitted infections, history of pregnancy, and number and recency of male sex partners) were collected through a combination of an interview with a study coordinator and a self-administered online questionnaire. Information on vitamin D supplementation was not collected. The protocol was reviewed and approved by the University of Washington Institutional Review Board.

Laboratory methods

HPV testing

As part of the parent study, self-collected vaginal swabs were HPV genotyped using the Roche Linear Assay (Roche Diagnostics; ref. 21). Our outcome of interest was detection of one or more of 19 hrHPV strains: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82, and IS39 (24, 25).

Vitamin D biomarker testing

Vitamin D biomarker testing was conducted on stored sera at the University of Washington's Nutrition and Obesity Research Center Analytic Core. Sera specimens were thawed and 750 μL aliquoted and transported on dry ice. Serum concentrations of albumin, calcium, and phosphate were determined using an automated clinical chemistry analyzer (Beckman AU5812, Beckman Coulter). Concentrations of $1,25(OH)_2D_3$, $1,25(OH)_2D_2$, $24,25(OH)_2D_3$, $25(OH)D_3$, and $25(OH)D_2$ were determined using immunoaffinity enrichment LC/MS-MS, as described previously (26, 27). Concentrations of vitamin D binding globulin and the haplotypes present in each sample were determined using trypsin digestion–LC/MS-MS, as previously described (28, 29).

Exposure definitions

Specifically, we measured as exposures serum concentrations of (i) 25(OH)D, (ii) $1,25(OH)_2D$, (iii) $24,25(OH)_2D_3$, (iv) free/unbound vitamin D, and (v) 25(OH)D adjusted for DBP and stratified by DBP haplotype. 25(OH)D and $1,25(OH)_2D$ are composite measures and are calculated as the sums of $25(OH)D_2$ and $25(OH)D_3$, and $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$, respectively. We calculated free vitamin D using an established equation that factors in affinity constants for albumin and DBP (30). Free vitamin D was measured in 1,000 nmol/L given its presence in low concentrations.

Our primary exposure was 25(OH)D, analyzed as a continuous increase of 10 ng/mL of serum 25(OH)D. As a secondary analysis of 25(OH)D, we generated two binary exposure variables using clinically meaningful cut-points of sufficiency: ≥20 ng/mL and ≥30 ng/mL (31, 32). These cut-points were based on recommendations from the Institute of Medicine (IOM; ref. 31), the Endocrine Society (32), and previous literature (20), although clinical cut-points for sufficiency remain contested (33). The other four biomarkers were secondary exposures and were each analyzed as a continuous variable.

Post hoc 25(OH)D analyses: exposure definitions

To explore the potential implications of modeling 25(OH)D as a linear versus categorical measure, we conducted a *post hoc* analysis using additional cut-points. First, we evaluated hrHPV prevalence among five subgroups of women categorized by 25(OH)D serum concentration: <12 ng/mL (severe deficiency, per IOM), 12–19 ng/mL (deficiency, per IOM), 20–29 ng/mL (sufficiency, per IOM), 30–49 ng/mL (sufficiency, per Endocrine Society), and ≥50 ng/mL. Distinguishing concentrations around the 50 ng/mL cut-point was also of interest because serum concentrations at or above this level in Seattle, WA typically indicate vitamin D supplementation (34). Second, we generated a binary exposure variable (≥50 ng/mL, <50 ng/mL).

Statistical analysis

Using logistic regression with robust SEs (to account for potential model misspecification), we estimated ORs with 95% confidence intervals (CI) for associations between serum vitamin D and hrHPV detection. Because serum concentration of vitamin D fluctuates during the year, we adjusted for season of enrollment using a B-spline with five degrees of freedom (to account for 10 months of enrollment). We considered additional variables *a priori* for inclusion. In an effort to present a reliable, precise estimate of an unconfounded relationship, we performed our modeling in a phased approach. For descriptive purposes, we produced a minimally adjusted model adjusting only for age (continuous), self-reported race [White, African American, Asian, Other (American Indian or Alaska Native, Native Hawaiian or Other Pacific Islander, other, or multiple races)], and season. Our moderately adjusted model is our primary model. This model incorporated additional biological and behavioral risk factors: oral contraceptive use (never, former, current), highest level of education (less than college, bachelor's degree, masters/doctoral degree), smoking status (never, former, current smoker), BMI (<18.5, 18.5 to <25, 25 to <30, or ≥30), and continuous serum concentrations of calcium (ng/mL) and phosphate (mg/dL). Finally, we ran a fully adjusted model that also included sexual behavior risk factors evaluated as precision variables: lifetime number of male sex partners (quintiles) and number of male sex partners in the 6 months prior to enrollment (none, non-new only, one new, ≥2 new).

We evaluated potential effect modification by race/ethnicity. Comparisons were made between White and non-White women due to small sample sizes of non-White race subgroups.

Post hoc 25(OH)D analysis: statistical analysis

We ran generalized additive models for each exposure to assess evidence of non-linearity in the relationship between hrHPV and our predictors.

Using logistic regression with robust SEs to estimate ORs and 95% CIs, we measured the association between serum 25(OH)D ≥50 ng/mL and hrHPV. We conducted this analysis using the three stages of modeling outlined above.

All analyses were conducted in R version 3.4.3 (35).

Results

Characteristics of the study population

One woman was excluded from analyses due to a specimen that was insufficient for HPV testing. After completing laboratory results quality assurance, four additional women were excluded due to uncertainty in patient-to-specimen matching attributable to specimen labeling errors. The mean age of the remaining 404 participants was 38.3 years (SD = 6.1 years; **Table 1**). The study population was predominantly White, with a mean BMI of 25.9 (SD 5.8). One third of participants reported current hormonal contraceptive use, and 26% were current or former smokers. The median lifetime number of male partners was seven (IQR 3–15). hrHPV prevalence was 22%.

Table 1. Characteristics of study population of mid-adult women in Seattle, WA, 2011–2012 ($N = 404$).

Demographic, health, and behavioral characteristics	
Age, mean (SD)	38.3 (6.1)
Lifetime number of male sex partners, median (interquartile range)[a]	7 (3–15)
Race, *n* (%)	
White	320 (79.2)
African American	11 (2.7)
Asian	45 (11.1)
Other or multiple races[b]	28 (6.9)
Highest level of education, *n* (%)	
Less than college	67 (16.6)
Bachelor's degree	151 (37.4)
Master's or doctoral degree	186 (46.0)
Smoking status, *n* (%)	
Never smoked	297 (73.7)
Former smoker	88 (21.8)
Current smoker	18 (4.5)
Missing	1
Hormonal contraceptive use, *n* (%)	
Current use	139 (34.4)
Former use	225 (55.7)
Never	40 (9.9)
BMI, *n* (%)	
Underweight (<18.5)	6 (1.5)
Normal (18.5–24.9)	216 (53.6)
Overweight (25–29.9)	113 (28.0)
Obese (30+)	68 (16.9)
Missing	1
Male sex partner(s) within 6 months prior to enrollment, *n* (%)	
None	86 (21.7)
Non-new partners only	217 (54.7)
1 new partner	77 (19.4)
2+ new partners	17 (4.3)
Missing	7
High-risk HPV positive, *n* (%)	89 (22.0)
>1 type detected	37 (41.6)[c]

[a]Four women excluded due to missing sexual history data ($n = 400$).
[b]Includes women who self-reported as American Indian or Alaska Native, Native Hawaiian or Other Pacific Islander, other races, or multiple races.
[c]Denominator is number of women with any high-risk HPV detected.

Biomarker summary statistics

Median and interquartile ranges for all vitamin D biomarkers evaluated were within normal ranges (ref. 36; **Table 2**). Mean serum concentrations of calcium (37) and phosphate (38) were normal, at 9.5 ng/mL (SD 0.4 ng/mL) and 3.5 mg/dL (SD 0.5 mg/dL), respectively.

Associations between serum concentrations of vitamin D and hrHPV infection

Associations between serum concentrations of 25(OH)D and hrHPV detection were inconsistent and not statistically significant (**Table 3**). Controlling for season of enrollment, age, race, BMI, smoking status, education level, and oral contraceptive use, each 10 ng/mL increase in 25(OH)D was not associated with significantly increased odds of hrHPV infection (aOR 1.08; 95% CI, 0.83–1.41). Estimates produced using minimally and fully adjusted models were also not significantly different from 1.0.

Secondary analyses of categorical exposure variables designed to classify 25(OH)D as sufficient or deficient also yielded non-significant results. After adjustment under our primary model, compared with women with deficient serum 25(OH)D concentrations below 20 ng/mL, those with serum concentrations ≥20 ng/mL (indicating sufficiency) had estimated odds of hrHPV infection of 0.85 (95% CI, 0.38–1.89). Under the same model, the estimated odds of infection for a higher 25(OH)D cut-point of 30 ng/mL was also 0.85 (95% CI, 0.50–1.43).

Upon examination of $1,25(OH)_2D$, free vitamin D, and 25(OH)D adjusted for DBP and DBP haplotype, we found no significant associations between serum concentrations of vitamin D biomarkers and odds of hrHPV infection. However, in a minimally adjusted model, serum $24,25(OH)_2D_3$ was significantly associated with hrHPV infection. Each 1 ng/mL increase in $24,25(OH)_2D_3$ was associated with higher odds of infection (aOR 1.28; 95% CI, 1.03–1.58), although the significance of this association waned upon further adjustment (under primary model, aOR 1.22; 95% CI, 0.97–1.52).

Evaluation of effect modification by race

Analyses examining potential interaction between 25(OH)D levels and race did not reveal significant differences in odds of hrHPV infection between White and non-White women. After adjustment with our primary model, each 10 ng/mL increase in 25(OH)D suggested higher odds of infection among both White (aOR 1.05; 95% CI, 0.77–1.41) and non-White women (aOR 1.23; 95% CI, 0.66–2.28); however, these results were not statistically significant.

Post hoc 25(OH)D analysis

Although results of a generalized additive model did not provide strong evidence of a nonlinear effect of continuous 25(OH)D (Supplementary Fig. S1), our *post hoc* analysis indicated that hrHPV prevalence varied by serum concentrations of 25(OH)D. There were no hrHPV-positive women in our sample with 25(OH)D <12 ng/mL. hrHPV prevalence was nearly identical among women with serum

Table 2. Vitamin D biomarkers: descriptive statistics among the study population ($N = 404$) and summary of biologic mechanisms.

Vitamin D measure	Units	Mean	Standard deviation	Median	Inter-quartile range	Categorical definitions of sufficiency, *n (%)*	Summary of biomarker mechanism in the metabolic pathway of overall vitamin D synthesis
25(OH)D	ng/mL	31.54	10.69	30.32	24.37–37.84	Institute of Medicine (30) <12 ng/mL: 9 (2.2) ≥12 ng/mL and <20 ng/mL: 45 (11.1) ≥20 ng/mL: 350 (86.6) Endocrine Society (31) <12 ng/mL: 9 (2.2) ≥12 ng/mL and <30 ng/mL: 186 (46.0) ≥30 ng/mL: 209 (51.7)	• Primary circulating form of vitamin D, the sum of D_2 and D_3 generated in the liver through metabolism (14) • Most commonly measured to assess deficiency (17) • Inversely associated with parathyroid hormone (PTH) levels (9) • Cut-points to establish sufficiency remain contested (32)
$1,25(OH)_2D$	pg/mL	53.07	18.31	51.45	39.35–64.76	N/A	• Active form of vitamin D after metabolism of 25(OH)D in the kidney (a process regulated tightly by PTH, calcium, and phosphorous levels) • Higher levels improve efficiency of absorption of calcium and phosphorous • Induces expression of enzyme CYP24A1 (15) • Charged with keeping cellular proliferation and differentiation in check (39) • Concentration varies by race/ethnicity (15)
$24,25(OH)_2D_3$	ng/mL	2.14	1.17	1.95	1.34–2.75	N/A	• Biomarker of 25(OH)D and 1,25(OH)D catabolism • Levels may indicate local 1,25(OH)D activity (15)
Free vitamin D	nmol/L x (1000)	19.20	6.28	18.88	14.98–22.39	N/A	• Circulating vitamin D unbound to DBP or albumin • Calculated using equations that incorporate 25(OH)D, DBP, albumin, and genotypic differences in DBP (though the validity of these equations has been questioned; ref. 18) • May be most relevant in populations showing variation in DBP levels (i.e., women using oral contraceptives; ref. 23) • Accurate measurement difficult due to low concentration (<1%) of circulating vitamin D (29)
Vitamin D Binding Protein (DBP)	μg/mL	261.49	49.31	250.85	229.20–279.55	N/A	• Binding protein that transports most (85%–90%) circulating vitamin D in blood (29) • Higher levels observed in pregnant women or women taking oral contraceptives (15)

concentrations 12–19 ng/mL (24.4%; 95% CI, 12.9–39.5) and 20–29 ng/mL (24.1%; 95% CI, 17.3–32.0). Prevalence was lower among women with 25(OH)D concentrations 30–49 ng/mL (18.3%; 95% CI, 13.0–24.6). However, hrHPV prevalence was highest in women with the highest serum 25(OH)D concentrations (≥50 ng/mL; 43.5%; 95% CI, 23.2–65.5). Finally, we evaluated the odds of prevalent hrHPV among women with 25(OH)D ≥50 ng/mL (**Table 3**). Under our primary model, the odds of hrHPV infection among women who likely supplement [25(OH)D ≥50 ng/mL] were significantly higher than the odds among women in whom supplementation is unlikely [25(OH)D <50 ng/mL; aOR 2.74; 95% CI, 1.14–6.59].

Discussion

To our knowledge, this is the first study to analyze an association between serum vitamin D status and hrHPV prevalence using five different biomarkers of vitamin D status, four of which are novel in this context. We aimed to understand the relationship between

Table 3. Associations between increasing levels of serum vitamin D biomarkers and high-risk HPV positivity at study enrollment among mid-adult women ($N = 404$).

	Minimally adjusted[a]			Moderately adjusted[b]			Fully adjusted[c]		
Vitamin D measure	***n***	**OR (95% CI)**	***P***	***n***	**OR (95% CI)**	***P***	***n***	**OR (95% CI)**	***P***
25(OH)D, continuous serum level									
Per 10 ng/mL increase	404	1.12 (0.87–1.45)	0.37	402	1.08 (0.83–1.41)	0.55	393	1.11 (0.85–1.45)	0.45
25(OH)D, categorical (IOM) (30)									
<20 ng/mL	54	1.00 (Ref)	NA	53	1.00 (Ref)	NA	52	1.00 (Ref)	NA
≥20 ng/mL	350	0.94 (0.43–2.04)	0.87	349	0.85 (0.38–1.89)	0.69	341	0.92 (0.41–2.07)	0.85
25(OH)D, categorical (ES) (31)									
<30 ng/mL	195	1.00 (Ref)	NA	194	1.00 (Ref)	NA	191	1.00 (Ref)	NA
≥30 ng/mL	209	0.86 (0.53–1.42)	0.57	208	0.85 (0.50–1.43)	0.54	202	0.80 (0.45–1.42)	0.45
25(OH)D, categorical (*post hoc* analysis)									
<50 ng/mL	381	1.00 (Ref)	NA	379	1.00 (Ref)	NA	370	1.00 (Ref)	NA
≥50 ng/mL	23	2.88 (1.18–7.02)	0.02	23	2.74 (1.14–6.59)	0.02	23	2.60 (1.07–6.33)	0.04
$1,25(OH)_2D$	404	1.01 (0.99–1.02)	0.49	402	1.01 (0.99–1.02)	0.45	393	1.00 (0.99–1.02)	0.59
$24,25(OH)_2D_3$	404	1.28 (1.03–1.58)	0.03	402	1.22 (0.97–1.52)	0.09	393	1.19 (0.94–1.51)	0.14
Free vitamin D	404	1.01 (0.97–1.06)	0.56	402	1.02 (0.97–1.07)	0.47	393	1.02 (0.97–1.07)	0.39
25(OH)D adjusted for Vitamin D Binding Protein (DBP), stratified by DBP haplotype[d]	404	1.11 (0.84–1.48)	0.45	402	1.12 (0.84–1.50)	0.43	393	1.15 (0.85–1.55)	0.36

[a]Adjusted for race, age, and season of enrollment.
[b]Adjusted for race, age, season of enrollment, BMI, highest education level, oral contraceptive use, smoking status, and calcium (ng/mL) and phosphate (mg/dL) levels.
[c]Adjusted for race, age, season of enrollment, BMI, highest education level, oral contraceptive use, smoking status, calcium (ng/mL) and phosphate (mg/dL) levels, number of lifetime sexual partners, and number of new sexual partners in last 6 months.
[d]Primary predictor was 25(OH)D, with additional adjustment for DBP levels and stratification by DBP haplotype.

serum concentrations of vitamin D and detection of hrHPV through evaluation of not only 25(OH)D, but other forms of the micronutrient along the metabolic pathway as well. These biomarkers included byproducts of 25(OH)D metabolism tasked with keeping cellular proliferation and differentiation in check [$1,25(OH)_2D$ and $24,25(OH)_2D_3$; refs. 8, 17] and a measure of circulating vitamin D unbound to binding proteins and other nutrients (free vitamin D). We did not observe significant associations between 25(OH)D and prevalent hrHPV in our analyses of 25(OH)D as both a continuous and categorical variable, as defined *a priori*. *Post hoc* analysis of 25(OH)D revealed women who likely supplement [25(OH)D ≥50 ng/mL] had significantly higher odds of prevalent hrHPV compared with other women. Apart from our findings on $24,25(OH)_2D_3$, we did not detect significant relationships between additional biomarkers and prevalence of hrHPV. Similarly, our analysis of race did not yield evidence of effect modification.

Higher serum concentrations of $24,25(OH)_2D_3$ were significantly associated with higher odds of hrHPV infection. Although statistical significance waned in the moderately and fully adjusted models (which may be related, in part, to including a large number of covariates in models with relatively few outcomes), this hint of an association warrants closer examination. Low concentrations of $24,25(OH)_2D_3$, the most plentiful byproduct of 25(OH)D metabolism by the enzyme CYP24A1 (found in most tissues throughout the body), have previously been associated with chronic kidney disease (39). Unsurprisingly, serum concentrations of $24,25(OH)_2D_3$ are positively correlated with concentrations of 25(OH)D (40). Thus, the direction of the association we observed between increasing serum concentrations of $24,25(OH)_2D_3$ and detection of hrHPV is in the opposite direction than we would expect.

Our hypothesis of an inverse association between serum concentrations of 25(OH)D and detection of hrHPV was motivated by prior research of the relationship (20) and suggestions of vitamin D's immunomodulating properties in the context of chronic (4) and acute disease (3). However, our selection of cut-points for our categorical analyses, though clinically motivated, perhaps oversimplified the association and resulted in attenuated estimates. By grouping very deficient and deficient women together [all with 25(OH)D <20 ng/mL or <30 ng/mL, depending on the analysis], our original *a priori* categorical analyses generated comparison groups of women who looked very similar with regards to hrHPV prevalence. Among women whose 25(OH)D concentrations were ≥30 ng/mL, two distinct subgroups exist; those whose serum concentrations suggest supplementation (≥50 ng/mL) had the highest odds of prevalent hrHPV in the study population, and this association was statistically significant. It is possible women who (likely) supplement were previously diagnosed as deficient, and an association between hrHPV status and 25(OH)D level *prior* to supplementation might better reflect a relationship between vitamin D status and detection of hrHPV.

In addition to the difference in the distribution of subjects' 25(OH)D serum concentrations, differences in age distribution may also explain the difference in our primary findings compared with the population-based NHANES sample. While the NHANES study (20) included women aged 20–59, we restricted our analyses to a narrower age range of mid-adult women aged 30–50 years. In the NHANES study, younger women were the most likely, relative to all other age groups, to be severely 25(OH)D deficient and to have prevalent hrHPV. It is possible these younger women with low mean serum concentrations of 25(OH)D and relatively high prevalence of hrHPV drove the observed association between 25(OH)D deficiency and detection of hrHPV. However, because results of the NHANES study are not stratified by age, it is difficult to discern whether the association between vitamin D status and detection of hrHPV varies between age groups.

Our study had several strengths, including secondary analyses with four novel vitamin D biomarkers. Further research into these biomarkers, particularly in longitudinal analyses, would be useful in better describing potential associations in less healthy populations.

In addition, our use of multiple stages of regression models enabled comparison across studies and allowed for transparent inclusion of confounders and precision variables, the latter of which can increase the precision of effect estimates and lead to tighter confidence intervals.

Our study is not without limitations. Previous literature (41) suggests low serum concentrations of 25(OH)D may be a consequence of chronic infection (for example, with hrHPV), rather than a contributing factor in the development or persistence of disease. Our cross-sectional study design precludes causal inference and prevents understanding of whether serum 25(OH)D influences hrHPV acquisition or if concentrations of the micronutrient are a marker of inability to clear an existing infection. In future analyses, we will use longitudinal data from the parent cohort of mid-adult women to evaluate associations between vitamin D status and persistent detection of hrHPV in monthly samples. In addition, information on vitamin D supplementation was not collected as part of the parent study, the focus of which was unrelated to vitamin D. Future longitudinal studies would benefit from capturing information on supplementation at baseline and could evaluate whether supplementation affects acquisition or clearance of hrHPV. Finally, while NHANES data from 2005–2006 estimated that 42% of US women were deficient at <20 ng/mL of serum 25(OH)D, our data revealed a healthy cohort in which only 13% of women had serum concentrations <20 ng/mL. Over half of our study population was 25(OH)D sufficient at ≥30 ng/mL and another third were sufficient at the cut-point of ≥20 ng/mL, reducing power to detect associations between 25(OH)D insufficiency and hrHPV. Future studies would benefit from larger sample sizes in which detection of an association would be possible at smaller effect sizes.

In conclusion, evaluation of the association between vitamin D status, as measured by five biomarkers measured along the micronutrient's metabolic pathway, and detection of prevalent hrHPV yielded mixed results in a population of healthy mid-adult women in Seattle, WA. Findings from longitudinal studies in populations with both more variability in vitamin D status and data on supplementation may be useful in motivating clinical recommendations for subpopulations of women.

Disclosure of Potential Conflicts of Interest

A.N. Hoofnagle reports receiving a commercial research grant from Waters. No potential conflicts of interest were disclosed by the other authors.

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