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14. ABSTRACT African American (AA) men are disproportionally affected by prostate cancer (PCa). AA men are not only at increased risk of PCa compared to American men of European descent (EA), but also are at the highest risk of aggressive PCa and death from PCa. Vitamin D3 deficiency increases PCa mortality, highlighting the importance of maintaining adequate vitamin D3 status for prostate health. Vitamin D3 is acquired in the diet or via UVB/sunlight-initiated synthesis in the skin. Cutaneous melanin absorbs UVB radiation, which leads to reduced vitamin D3 synthesis in darker pigmented skin. Consequently, ~65% of AA men are vitamin D3 deficient compared to ~20% of EA men. The level of skin pigmentation is correlated with the extent of African ancestry and serum vitamin D3 status. Besides vitamin D3 status, the activity of vitamin D3 is mediated by the vitamin D receptor (VDR) and determined by several cytochrome P450 metabolism enzymes that bioactivate/inactivate the active form of the hormone, 1,25-dihydroxyvitamin D3 (1,25D).					
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1. Introduction

African American (AA) men are disproportionately affected by prostate cancer (PCa). AA men are not only at increased risk of PCa compared to American men of European descent (EA), but also are at the highest risk of aggressive PCa and death from PCa. Vitamin D3 deficiency increases PCa mortality, highlighting the importance of maintaining adequate vitamin D3 status for prostate health. Vitamin D3 is acquired in the diet or via UVB/sunlight-initiated synthesis in the skin. Cutaneous melanin absorbs UVB radiation, which leads to reduced vitamin D3 synthesis in darker pigmented skin. Consequently, ~65% of AA men are vitamin D3 deficient compared to ~20% of EA men. The level of skin pigmentation is correlated with the extent of African ancestry and serum vitamin D3 status. Besides vitamin D3 status, the activity of vitamin D3 is mediated by the vitamin D receptor (VDR) and determined by several cytochrome P450 metabolism enzymes that bioactivate/inactivate the active form of the hormone, 1,25-dihydroxyvitamin D3 (1,25D).

We hypothesized that the high prevalence of vitamin D3 deficiency in AA men is associated with reduced prostatic concentrations of vitamin D3, which leads to lower expression of vitamin D pathway genes and suppress pro-differentiating actions of vitamin D3 in the prostate; this ultimately abrogates the chemoprotective effects of this natural hormone and raises the susceptibility of AA men to aggressive PCa.

2. Keywords

Vitamin D, prostate cancer, African-American

3. Accomplishments

TASK 1: Using fresh frozen prostate tissue to determine the relationship between prostatic vitamin D₃ status and the tissue expression of vitamin D₃ pathway genes in AA and EA men.

Experiment 1.1: Identify patients from cohort for the study and collect all samples for analysis (months 1-6)

Experiment 1.2: Analysis of vitamin D₃ metabolites (25D and 1,25D) in patient specimens (months 1-12)

Experiment 1.3: Measure the expression of vitamin D₃ pathway genes and African ancestry SNPs in patient specimens (months 1-12)

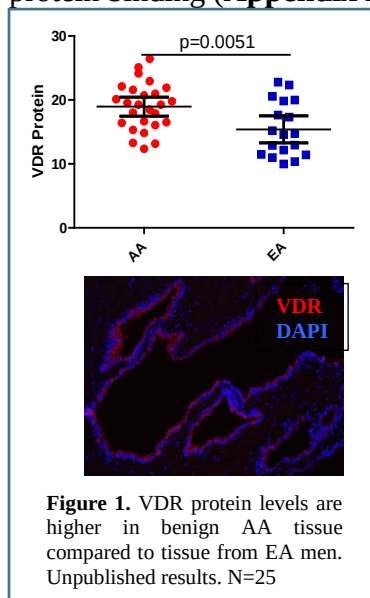
Analysis: Perform statistical analysis (months 12-24)

Within Task 1 Experiment 1.1 was completed in Y1 and Experiments 1.2 and 1.3 in Y2-3. The results of Task 1 were recently published in the Journal of Clinical Investigations Insight (**Appendix A**).

We ended up with a group of 60 PCa patients (AA n=31, EA n=29) who underwent radical prostatectomy (**Appendix A, Figure 1A-B, Table 1**). DNA isolated from whole blood was genotyped for West African ancestry markers and vitamin D-related SNPs. In our cohort, the percentage of African Ancestry in men ranged from 2-95% (**Appendix A, Figure 1C**), which demonstrates the diverse ethnic background of self-declared black men and underscores the value of this additional analysis in interpreting our final data sets.

The vitamin D measurement was done in collaboration with Heartland Assays, a lab that only measures vitamin D metabolites and was started by the world renowned vitamin D expert Bruce Hollis. Heartland Labs measured all of our serum samples and in the frozen human prostate tissues. Vitamin D metabolites 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were measured in the serum and tissue by uHPLC-MS-MS. Serum measurement of 25-hydroxyvitamin D (25D) is used to determine vitamin D status and the AA patients had significantly lower 25D (**Appendix A, Figure 2A**). The levels of 1,25-dihydroxyvitamin D (1,25D), the active hormone, were also measured and not significantly different (**Appendix A, Figure 2A**). The 25D and 1,25D only correlated in the EA men, not the AA men (**Appendix A, Figure 2B**).

The tissue findings were quite surprising and show that the AA prostate has *higher* 1,25D compared to the EA, despite having lower 25D (**Appendix A, Figure 2C**). The 25D and 1,25D levels in the prostate correlated in all men (**Appendix A, Figure 2D**). Serum levels of the vitamin D binding protein (DBP) were measured by ELISA and did not differ between groups (**Appendix A, Figure 2E**). Interestingly, DBP levels correlated with vitamin D metabolite levels only in EA group, demonstrating race-specific differences in the protein binding (**Appendix A, Figure 2F-G**).



We completed the laser-capture microdissection (LCM) on the 60 frozen prostate tissues and determined there was sufficient RNA from all patients to complete gene expression analysis by whole transcriptome amplification followed by PCR. The expression of key genes in the vitamin D axis was examined; VDR, CYP2R1, CYP27A1, CYP27B1, CYP24A1, DHCR7. VDR mRNA levels were higher in the AA group (**Appendix A, Figure 3A**). We further examined VDR protein expression in a validation set of prostate tissues from AA and EA men. These unpublished results confirm higher expression of VDR protein in the AA men (**Figure 1**). *This finding is the basis for the pending R01 proposal.* None of the other genes showed a difference between AA and EA, but CYP27B1 was positively correlated with % WAA (**Appendix A, Figure 3B-F**). Expression of the extracellular receptor of vitamin D binding protein, megalin (gene name *LRP2*), was positively associated with West African ancestry and inversely associated with tissue 25-hydroxyvitamin D concentrations in AAs (**Appendix A, Figure 4A**). *LRP2* levels correlated with 25D only in AAs and serum and tissue levels of 1,25D did not correlate (**Appendix A, Figure 4B-C**). Since the data suggested presence of an active megalin transport system we examined megalin protein expression in prostate tissue and observed membrane staining in both benign and PCa epithelium (**Appendix A, Figure 4D**).

The relationships between vitamin D binding protein, *LRP2*, and vitamin D metabolites suggest that the prohormone is actively transported into the prostate followed by intra-prostatic conversion to the active hormone, rather than passive diffusion. These findings support the presence of a compensatory response in prostate tissue to vitamin D deficiency in AAs and reveal a previously unknown complexity involving tissue distribution of vitamin D metabolites.

DNA isolated from whole blood was also genotyped for vitamin D-related SNPs. This was not an original aim, but we had the material. Although our N was too small for significance, several intriguing race-specific relationships between gene expression and SNPs can be seen for DBP (GC), CYP27B1, DHCR7, VDR and CYP24A1 ((**Appendix A, Table 2**).

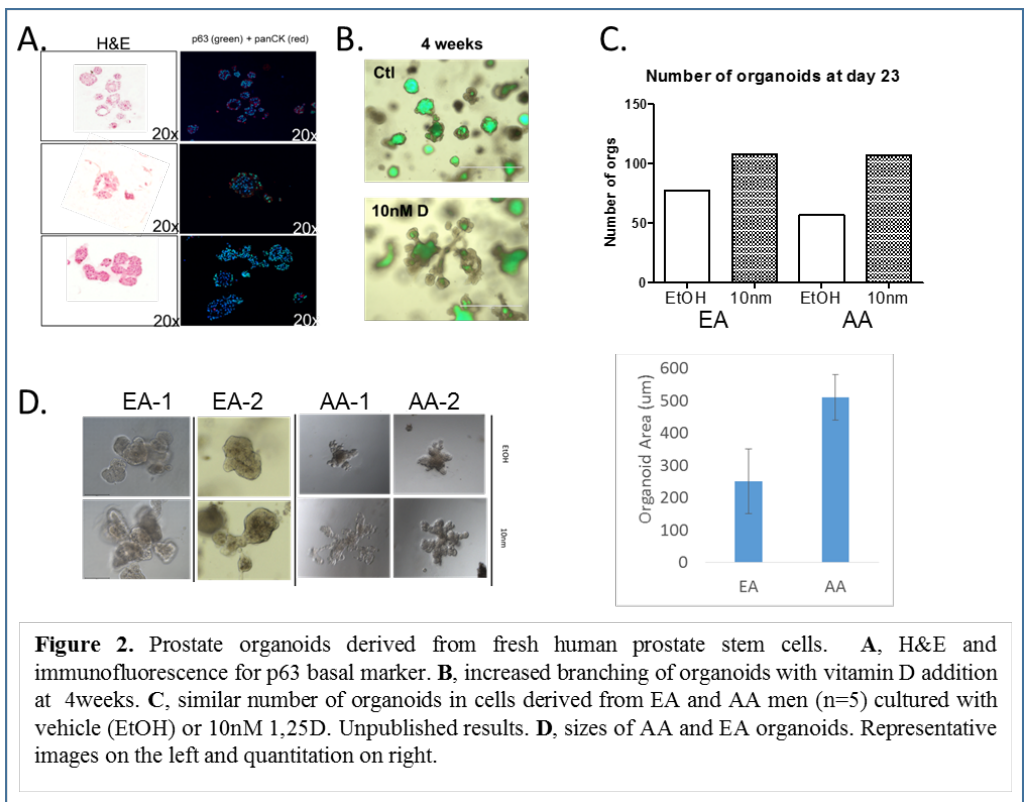
TASK 2: To examine and compare the activity of vitamin D3 in prostatic stem-progenitor cells derived from AA and EA patients.

Experiment 2.1: Characterize prostatic stem-progenitor cells derived from AA and EA men (months 12-24)

Experiment 2.2: Compare the response of the prostatic stem-progenitor cells to vitamin D3 (months 18-36)

Significant progress has been made on Task 2 in all years of the grant. However, the methodology for growth of prostatic stem-progenitor cells has rapidly advanced since conception of the grant and our methods have evolved to stay current. We are now growing from single stem cells in 3D. These organoids can be grown up to 4 weeks and show epithelial differentiation (**Figure 2A**). Upon addition of vitamin D the organoids

undergo extensive branching (**Figure 2B**). Analysis of organoid phenotype in AA and EA organoids showed that AA organoids are larger than EA under normal conditions, but there was no difference in response to 1,25D (**Figure 2C-D**). However, given that 25D is the circulating form of D in our bodies, and our recent findings with the differential regulation of megalin (to import 25D) and CYP27B1 (to bioactivate 25D), the more biologically relevant question is how do they respond to 25D. These experiments have been completed and we are in the process of analysis.

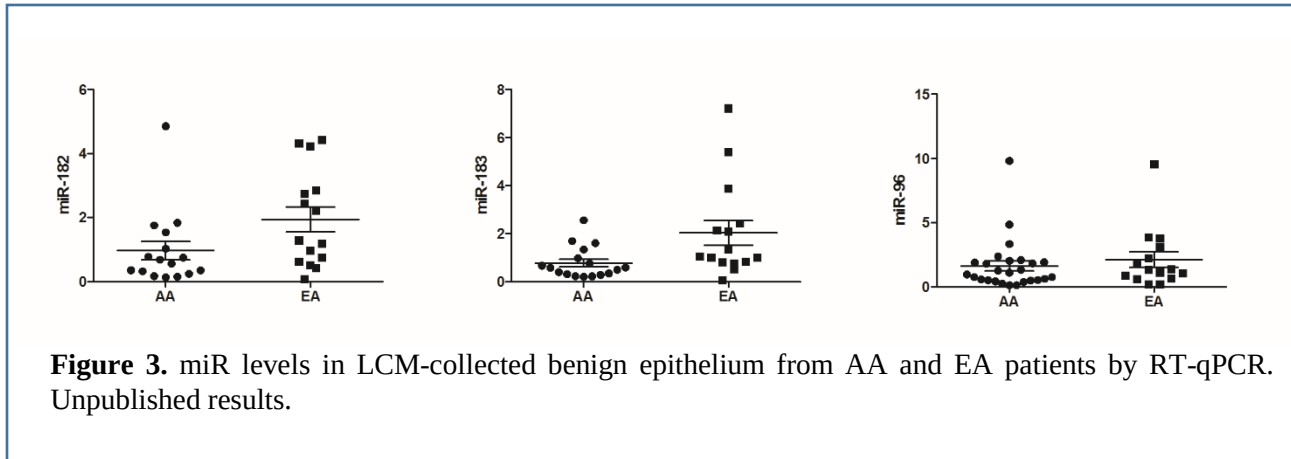


TASK 3: To identify ethnicity-specific differences between vitamin D3 status and the expression of vitamin D-regulated microRNAs in normal and PCa LCM-collected epithelium from AA and EA patients.

Experiment 3.1: Measure the expression of vitamin D3-regulated microRNAs in patient specimens from Task 1 (months 24-36)

Experiment 3.2: Measure the expression of vitamin D3-regulated microRNAs in prostatic stem-progenitor cells from Task 2 (months 24-36)

Experiment 3.1 was partially completed in Y3 for the microRNAs miR-183, 96 and 182. MiRs-182 and 183 were lower in AA, and were not regulated by vitamin D (**Figure 3**). No other miRs in our panel showed differential expression in the patient samples (data not shown). The results shown in Figure 3 were unexpected and as these miRs are considered oncogenic. This finding is the foundation for a new project by a pathology resident in the lab, Dr. Andy Acosta, to determine if this may be a subtype of cancer that correlates with prognosis.



4. IMPACT

This study has provided novel insight into the distribution of vitamin D in the prostate and the results challenge our previous assumptions, in particular The Free Hormone Hypothesis. The Free Hormone Hypothesis is that only “free” hormones unbound to serum globulins are available to tissues via diffusion. Our data contradict that and show that the prostate can maintain levels of active vitamin D in the setting of serum deficiency suggesting a mechanism of active transport. We observed membrane expression of the endocytic receptor megalin (gene LRP2) and hypothesize that it is active in the prostate. *These findings are foundation for a recently funded F31 and a pending DOD grant.*

In addition, the findings support a compensatory response occurring in AA men with deficiency, underscoring the essential role for this hormone in the prostate and perhaps other tissues. The relationships between vitamin D binding protein, LRP2, and vitamin D metabolites suggest that the prohormone is actively transported into the prostate followed by intra-prostatic conversion to the active hormone, rather than passive diffusion. This reveals the paucity of knowledge about how vitamin D deficiency and/or ancestry alters the mechanisms and regulation of tissue levels of vitamin D. It is premature at this point to speculate on the clinical implications of these findings in regards to vitamin D deficiency and prostate health.

Not be overlooked, this project has enabled the training of 2 graduate students and one resident in the area of prostate cancer health disparities. It is also the foundation for two funded grants and three pending projects. Additionally, it led to a fruitful collaboration with Dr. Sarki Abdulkadir at Northwestern and we have already published on this project (see below).

5. CHANGES/PROBLEMS

Aim 1 was completed almost exactly as planned with the following changes which were fully detailed in prior Progress Reports. The tissue and serum source change due to Dr. Freeman suffering freezer meltdown. The specimens were acquired from Dr. Gann and the Cooperative Human Tissue Network. We also chose to use Heartland Assays to optimize and complete the measurement of the vitamin D metabolites in the specimens. This change was initiated in Y2 when the planned lab had not made progress on the optimization. Heartland Assays was very active in the project and the lead scientist, Drew Makowski, is a co-author on the published

manuscript. We also added measurement of vitamin D Binding protein (by Heartland) and vitamin D-related SNPS (bu Kittles) to the patient analysis as we had sufficient samples and funds.

Aim 2 (Tasks 2 and part of Task 3), which is *in vitro*, was slightly behind schedule and we have completed experiments during this year of no cost extension.

6. PRODUCTS

Publication: Richards Z, Batai K, Farhat R, Makowski A, Gann PH, Kittles R, **Nonn L**. Prostatic Compensation of the Vitamin D Axis in African American men. *JCI Insight*, 2(2):e91054, 2017. PMID: 28138564

Publication: Unno K, Roh M, Yoo Y, Al-Shraideh Y, Wang L, **Nonn L**, and Abdulkadir SA. Modeling African American prostate adenocarcinoma by inducing defined genetic alterations in organoids. *Oncotarget*, 8(31):51264-51276, 2017. PMID: 28472770

Thesis: Farhat, Rachael S. Title: Examination of Vitamin D Disparities in African American and Caucasian Prostate Cancer Patients and Cells. URL <http://hdl.handle.net/10027/19512>
Publication Date 2015

Poster: “Addition of Stroma Enhances Branching Morphogenesis and AMACR Expression in a 3D Organoid Co-Culture Model of Prostate Cancer.” *SBUR Fall Symposium*, Nov. 9-12, 2017. Tampa, FL

Invited Oral Presentation: “Challenging the free hormone hypothesis for vitamin D has implications for the prostate cancer disparity in African American men.” *2nd Annual GEMS Research Symposium*, Sep. 29, 2017. Chicago, IL

Poster, Honorable Mention Award Winner: “Analyses of the prostate vitamin D axis in African American and Caucasian men reveals that ancestry associates with differences in local regulation of the active hormone in the prostate,” *UIC College of Medicine Research Forum*, Nov. 18, 2016. Chicago, IL

Poster: “Analyses of the prostate vitamin D axis in African American and Caucasian men reveals that ancestry associates with differences in local regulation of the active hormone in the prostate,” *SBUR Fall Symposium*, Nov. 10-13, 2016. Scottsdale, AZ

Poster: “Serum and Prostatic Levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D in a Cohort of African American and European-American Prostate Cancer Patients,” *The 19th Workshop on Vitamin D*, Mar. 29-13, 2016. Boston, MA

Abstract and Invited Oral Presentation: “Vitamin D and prostate Cancer in African-American Men,” *American Association for Cancer Research (AACR)- The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved*, Dec 2013, Atlanta, GA.

Poster: “Prostatic Concentrations of Vitamin D Differ Between African-American and European-American Prostate Cancer Patients,” *3rd International Congress on Steroid Research*, Nov. 15-18, 2015. Chicago, IL

Inventions, Patents and Licenses

none

Grant applications

- 1) FUNDED- F31 “Vitamin D and Prostate Cancer Disparities in African American Men” with Mr. Richards (grad student) as the PI. Percentile 1 , Impact Score 10

- 2) FUNDED- Chicago Cancer Health Equity Collaborative-Pilot Projects Catalyst Grant with Dr. Nonn as PI. (Four PI grant with NEIU, NU and Project Brotherhood) \$240,000 “Prostate Cancer Disparity in Chicago-Area African American Men: Patient-derived models and biomarkers of cancer risk”
- 3) R01CA220278 resubmitted June 2017-“The Mechanisms of African Ancestry-Associated Differences in Prostatic Vitamin D” with Dr. Nonn as PI and Dr. Kittles as co-I.
- 4) DOD PCRP HDRA submitted August 2017 “Vitamin D deficiency leads to increased intra-prostatic hormones in African American men” PC170484 Dr. Nonn as PI
- 5) R21CA231610 “Vitamin D Deficiency Leads to Increased Intra-Prostatic Androgens in African American Men” with Dr. Nonn as PI.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

UIC Pathology: Zachary Richards
 Rachael Farhat
 Peter H. Gann

University of AZ: Ken Batai
 Ebony Shah
 Rick Kittles (Co-I)

Heartland Assays: Andrew Makowski

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

JCI Insight publication

Prostatic compensation of the vitamin D axis in African American men

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BACKGROUND. African American (AA) men are disproportionately affected by both prostate cancer (PCa) and vitamin D deficiency compared with European American (EA) men. Vitamin D deficiency is linked to increased PCa aggressiveness and mortality. Therefore, it has been hypothesized that vitamin D deficiency may contribute to the PCa disparity between AA and EA men.

METHODS. We studied a cross sectional group of 60 PCa patients (AA, $n = 31$; EA, $n = 29$) who underwent radical prostatectomy. Vitamin D metabolites 25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)₂D) were measured in the serum and tissue by uHPLC-MS-MS. Tissue was laser capture microdissected, and gene expression was quantified by microarray. DNA isolated from whole blood was genotyped for West African ancestry markers and vitamin D-related SNPs.

RESULTS. Serum concentrations of 25(OH)D were lower in AAs, but concentrations of 1,25(OH)₂D in the prostate tissue were higher compared with EAs. Expression of the vitamin D receptor was higher in prostate tissue from AAs. Expression of the extracellular receptor of vitamin D binding protein, *LRP2*, was positively associated with West African ancestry and inversely associated with tissue 25(OH)D concentrations in AAs.

CONCLUSIONS. The relationships between vitamin D binding protein *LRP2* and vitamin D metabolites suggest that the prohormone is actively transported into the prostate, followed by intraprostatic conversion to the active hormone, rather than passive diffusion. These findings support the presence of a compensatory response in prostate tissue to vitamin D deficiency in AAs and reveal a previously unknown complexity involving tissue distribution of vitamin D metabolites.

FUNDING. Department of Defense Prostate Cancer Research Program Idea Award for Disparities Research PC121923 (LN and RK) and the NIH 1R01MD007105 (RK).

Introduction

Vitamin D is an essential regulatory hormone for normal human physiology (1). The canonical role for vitamin D is calcium homeostasis; however, vitamin D deficiency has been associated with both calcium-related conditions, including rickets and osteoporosis (2), as well as non-calcemic-related diseases such as diabetes (3), cardiovascular disease (4), autoimmune disease, and cancers of the colon, breast, and prostate (5). Prostate cancer (PCa) is of particular interest, since African American (AA) men are disproportionately affected by both PCa and risk of vitamin D deficiency (6–8). AAs not only present with PCa at a younger age, but they also have 50% higher incidence and twice the mortality compared with European American (EA) men (9). Skin pigmentation is the largest predictor of vitamin D deficiency in the USA, as UV-induced cutaneous synthesis of vitamin D is the primary source of the vitamin D prohormone and is inhibited by melanin (10). Consequently, >90% of AA men are vitamin D insufficient by current standards (11), and 65% are deficient with serum levels below 20 ng/ml (12).

There is experimental, epidemiological, and clinical trial evidence to support chemopreventive and sometimes even anticancer activity of vitamin D. In vitro and in vivo studies of the active hormone 1,25-dihydroxyvitamin D (1,25(OH)₂D), as well as analogs, consistently demonstrate inhibition

Conflict of interest: The authors have declared that no conflict of interest exists.

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of cancer-related phenotypes (13–15). In genetic mouse models of PCa, such as the NKX3.1 model, vitamin D can delay the formation of preneoplastic lesions (16). Although epidemiologic studies on the relationship between PCa incidence and vitamin D serum concentration have produced mixed results, analyses restricted to aggressive or lethal cases have more consistently shown inverse associations (17–19). Interventional studies with vitamin D supplements have reported lower prostate-specific antigen levels (20), reduced number of positive biopsies (21), and decreased prostate proliferation markers (22). These studies are limited in their applicability to the racial disparity in PCa, since none included a diverse population of AA men. Also, it is not clear whether the positive results of these trials in PCa patients are mediated through systemic effects, such as immune regulation (23), or local effects of vitamin D within the tissues.

Serum concentration of the prohormone 25-hydroxyvitamin D (25(OH)D) is the clinical indication of vitamin D status, and the Endocrine Society defines vitamin D deficiency as serum levels below 20 ng/ml, insufficiency between 21–29 ng/ml, and replete status above 30 ng/ml (24). However, these cut points were formed solely in the context of bone health, and applicability to overall health and cancer prevention remain unclear. Additionally, whether serum and tissue levels of vitamin D correlate remains unclear, and there is only one comparative report on serum and intraprostatic levels of vitamin D (22). This pivotal study showed that oral supplementation with vitamin D can alter levels in the prostate tissue, but it also lacked diversity since all patients were Canadian men of European descent.

The effects of vitamin D are mediated by 1,25(OH)₂D, which is both the active hormone and high-affinity ligand for the vitamin D receptor (VDR), a classical nuclear hormone receptor transcription factor that regulates transcription via vitamin D response elements located throughout the genome (25–27). A direct readout of VDR activation is challenging to assess in patients and is dependent not only on ligand, but also on recruitment of coactivators and removal of corepressor binding (28). The circulating level of 1,25(OH)₂D is under tight regulatory control by calcium and parathyroid hormone (29). 1 α -hydroxylation of prohormone 25(OH)D to active hormone 1,25(OH)₂D takes place in the kidney by the enzyme CYP27B1. There is also substantial evidence of extrarenal 1 α -hydroxylase activity in prostate cells (30–32). Additionally, 1 α -hydroxylase activity has been reported to be reduced in primary cells derived from PCa tissue (33), suggesting a decrease in local levels of 1,25(OH)₂D in tumors. Yet, *in vivo* evidence of prostatic CYP27B1 activity has not been demonstrated, and there remains only one report of 1,25(OH)₂D in human prostate tissue (22).

Currently, multiple knowledge gaps exist regarding (i) the relationship between intraprostatic and serum vitamin D metabolites, (ii) the regulation of prostatic vitamin D metabolites, and (iii) intraprostatic levels of vitamin D metabolites in AA men. This knowledge is essential to understanding the proposed chemopreventive role of vitamin D in PCa and how it may contribute to the increased risk of aggressive PCa in AA men. In the present study, we describe the first evaluation of the serum and prostatic vitamin D axis in samples from a diverse cross sectional population of radical prostatectomy (RP) patients. Vitamin D metabolites were quantified in both the serum and prostate tissue. Expression of genes involved in vitamin D metabolism, transport, and VDR were measured in the prostatic epithelial tissues.

Results

AA PCa patients were vitamin D deficient in the serum and had higher levels of the active hormone in their prostate tissues. A diverse group of RP patients (*n* = 60) was assembled retrospectively to examine the serum-prostatic vitamin D axis (Figure 1A) by African ancestry (Table 1 and Figure 1B). Fresh frozen RP tissue, serum, and whole blood were used for analysis (Figure 1B). Of the patients, 32 self-identified as AA and 28 as EA. West African ancestry was determined by analysis of 105 ancestry informative markers (AIMs) (34) in DNA isolated from the whole blood and showed a heterogeneous population of EAs and a range of 53.0%–95.5% in the AA group (Figure 1C). One patient who self-identified as AA was reclassified as EA (2.2% West African, 95.5% European ancestry).

Vitamin D metabolites 25(OH)D and 1,25(OH)₂D were measured in serum and frozen prostate tissue by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS-MS). The mean serum 25(OH)D concentration was lower in the AA group compared with the EA group (AA: 20 ng/ml, 95% CI, 16–23 ng/ml; EA: 33 ng/ml, 95% CI, 28–37 ng/ml; *P* < 0.0001) (Figure 2A). Serum levels of the active hormone 1,25(OH)₂D did not differ between the 2 groups but did correlate with serum concentration of 25(OH)D in the EA group only (Spearman *r* = 0.46; *P* = 0.02) (Figure 2, A and B).

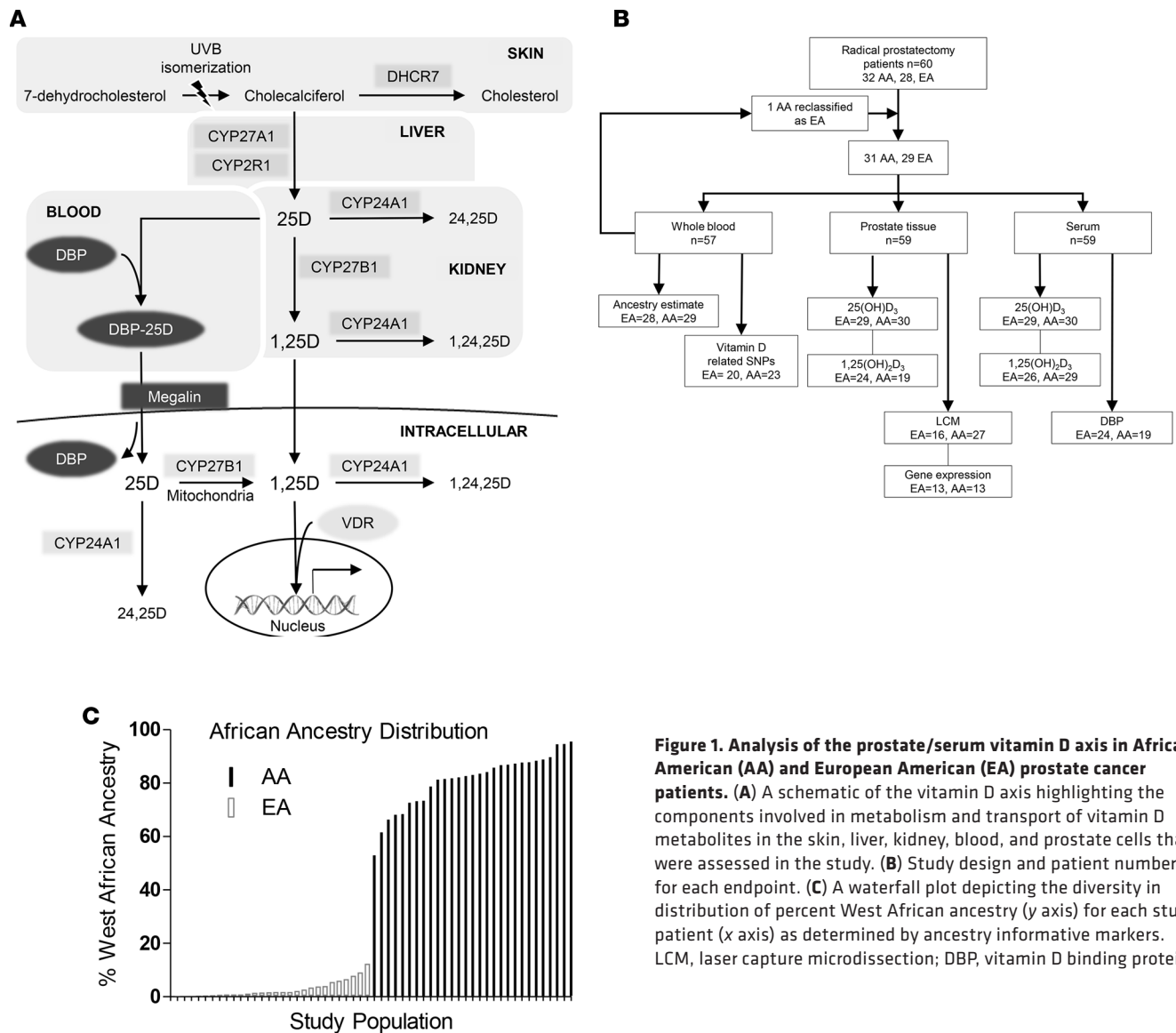


Figure 1. Analysis of the prostate/serum vitamin D axis in African American (AA) and European American (EA) prostate cancer patients. (A) A schematic of the vitamin D axis highlighting the components involved in metabolism and transport of vitamin D metabolites in the skin, liver, kidney, blood, and prostate cells that were assessed in the study. (B) Study design and patient numbers for each endpoint. (C) A waterfall plot depicting the diversity in distribution of percent West African ancestry (y axis) for each study patient (x axis) as determined by ancestry informative markers. LCM, laser capture microdissection; DBP, vitamin D binding protein.

Extraction and measurement of 25(OH) $_2$ D and 1,25(OH) $_2$ D by uHPLC-MS-MS in prostate tissue has not been previously reported and was thoroughly optimized for input and quality controls. Benign fresh frozen RP tissue was used for all patients. Vitamin D metabolite levels in the benign RP tissue did not differ from transurethral resection of the prostate samples that were used to optimize the assay (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/jci.insight.91054DS1). Similar to serum levels, prostatic 25(OH) $_2$ D was lower in the AA group compared with EA group (AA: 11 ng/g, 95% CI, 8.4–15 ng/g; EA: 15 ng/g, 95% CI, 12–18 ng/g; $P = 0.04$) (Figure 2C). In contrast, the active hormone 1,25(OH) $_2$ D was significantly higher in the AA group's prostate tissue compared with the EA group's prostate tissue (AA: 34 pg/g, 95% CI, 27–40 pg/g; EA: 23 pg/g, 95% CI, 18–28 pg/g; $P = 0.009$) (Figure 2C). In all patients, 25(OH) $_2$ D and 1,25(OH) $_2$ D were positively correlated in the prostate tissue (AA: $r = 0.49$, $P = 0.08$; EA: $r = 0.50$, $P = 0.01$) (Figure 2D). Serum and tissue levels of vitamin D metabolites did not correlate with West African ancestry (data not shown). Vitamin D $_2$ metabolites were also measured but were below the limit of detection in 88% of patients, and the contribution of D $_2$ to total vitamin D was negligible in the 7 patients who had detectable levels (data not shown).

Most 25(OH) $_2$ D in circulation (~90%) is bound to the vitamin D binding protein (DBP) and is thought to affect the fraction available for conversion to 1,25(OH) $_2$ D (35). DBP was measured in serum using a polyclonal antibody ELISA, which has been demonstrated to mitigate bias caused by the presence of functional SNPs in DBP in AAs compared with the monoclonal assay (36, 37). Serum levels of DBP did

Table 1. Population Characteristics

	European American		African American		P value ^c
	n ^a	Mean ± SD or [range] ^b	n ^a	Mean ± SD or [range] ^b	
Age	29	63.6 ± 5.6	31	61.5 ± 7.2	0.22
%WA ancestry	28	2.9 [0.2–12.4]	29	81.0 [53.0–95.5]	< 0.001
%Euro ancestry	28	93.1 [79.1 to 99.2]	29	14.3 [1.6–45.1]	< 0.001
%NatAmer ancestry	28	4.0 [0.5–14.9]	29	4.8 [0.8–11.3]	0.45
PSA (ng/ml)	25	6.8 ± 3.1	31	8.1 ± 4.5	0.22
Season ^d of blood draw					
High UVR	17		13		
Low UVR	12		18		0.30
Season ^d of surgery					
High UVR	17		17		
Low UVR	12		14		0.80
Radical prostatectomy tissue					
Gleason					
≤ 6	7		11		
7	14		15		
8	0		2		0.47
Stage ^e					
I	1		1		
IIA	2		2		
IIB	14		16		
III	3		8		0.72
TURP tissue					
BPH	8		3		0.10
Biorepository					
UIC	9		21		
CHTN	20		10		0.009

AA, African American; EA, European American; UIC, University of Illinois at Chicago; CHTN, Cooperative Human Tissue Network; %WA, percent West African; %Euro, percent European ancestry; %NatAmer, percent Native American ancestry; PSA, prostate specific antigen; TURP, transurethral resection of the prostate; BPH, benign prostatic hyperplasia; UVR, UV radiation. ^aGenotype data, Gleason score, or stage not available or not applicable to all individuals.

^bMean and ±SD reported for age and PSA; range reported for ancestries. ^c $P < 0.1$, 2 sided student's t test for age, ancestry, and PSA; 2-tailed Fisher's exact test for season, tumor, and site comparisons. ^dSeasons categorized into high UVR (June to November) and low UVR (December to May). ^eTumor staging not available for 2 patients (1 AA, 1 EA).

not differ between AAs and EAs (Figure 2E). Correlations of DBP with serum and prostate 25(OH)D levels were significantly positive for EAs, and they trended negative for AAs but did not reach significance (Figure 2, F and G).

Gene expression of VDR in benign prostate epithelium was higher in AAs compared with EAs. A homogenous population of benign prostate epithelium was collected from the frozen tissue by laser capture microdissection (LCM) for RNA isolation and gene expression analysis. Conditions for optimal RNA stability and recovery during LCM were previously described by our group (38, 39). Gene expression was quantified by whole transcriptome amplification followed by Affymetrix Human Gene 1.0 ST array as described by others for prostate tissues (40, 41). Thirteen patients from each group were selected for the gene expression analysis based on sufficient RNA input. Gene expression of *VDR*, the vitamin D metabolism genes — *CYP27A1*, *CYP2R1*, *CYP27B1*, *CYP24A1*, and *DHCR7* — was determined (Figure 3, A–F). There was significantly higher expression of *VDR* in the AA population (Figure 3A). Of the vitamin D metabolism enzymes, only *CYP2R1* differed by ancestry group, with AAs having significantly lower expression (Figure 3B). *CYP2R1* and *CYP27B1* both correlated with percent West African ancestry in AAs, but in the negative and positive direction, respectively (Figure 3, B and C).

Megalin protein is present in prostate epithelium, and gene expression of Megalin (LRP2) correlates with ancestry and prostatic vitamin D. Megalin, encoded by the gene *LRP2*, is an abundantly present cell surface protein in the kidney that binds DBP to mediate internalization of 25(OH)D into the cytosol (42).

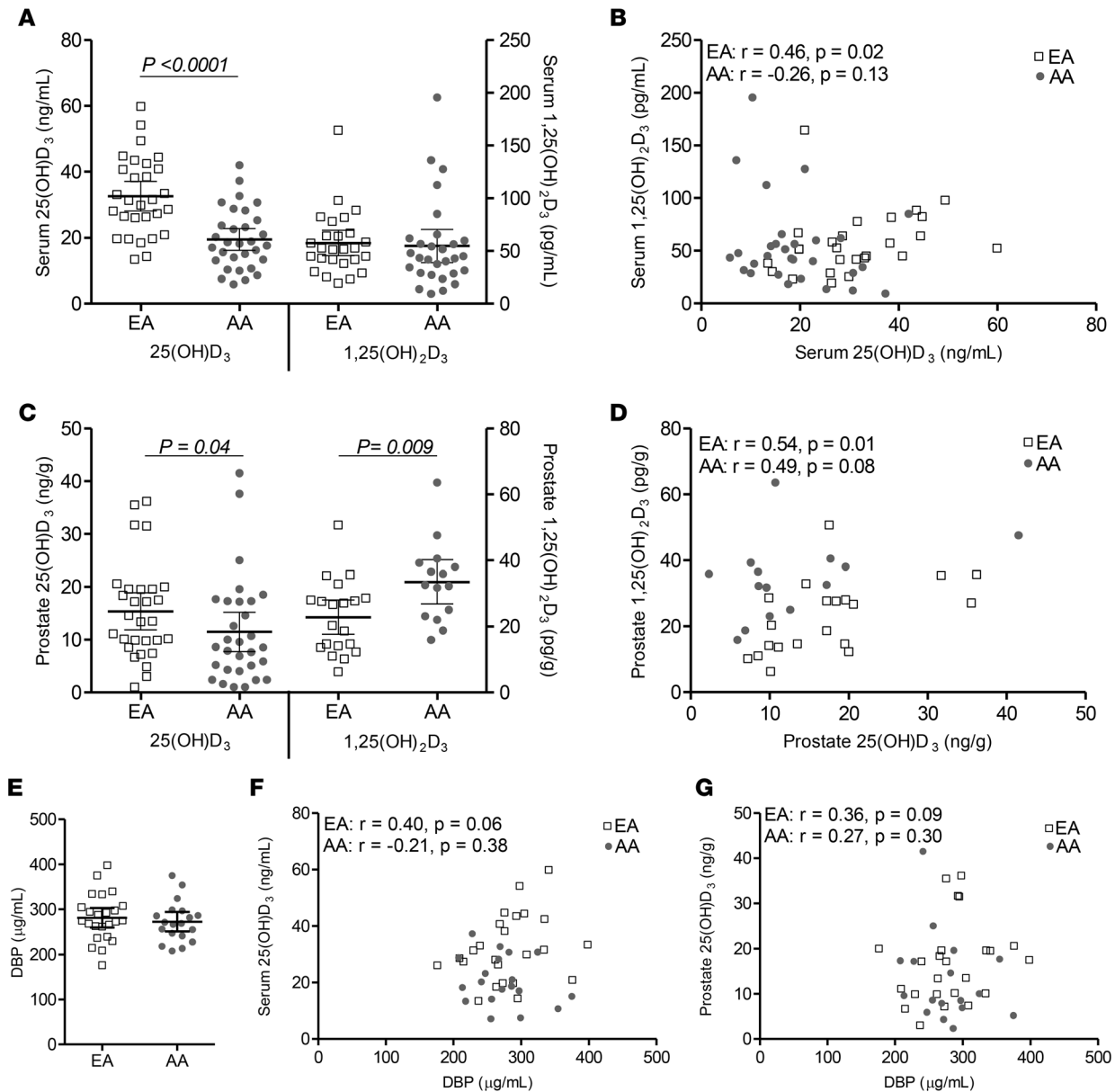


Figure 2. Radical prostatectomy patients of African ancestry had lower 25-hydroxyvitamin D (25(OH)D) in the serum but higher 1,25-dihydroxyvitamin D (1,25(OH)₂D) in the prostate. (A) comparison of serum levels of 25(OH)D (European American [EA], $n = 29$; African American [AA], $n = 30$) and 1,25(OH)₂D (EA, $n = 26$; AA, $n = 29$) measured by uHPLC-MS-MS between radical prostatectomy patients of AA and EA ancestry. (B) Correlation between vitamin D² metabolites 25(OH)D and 1,25(OH)₂D in the serum by ancestry (EA, $n = 26$; AA, $n = 29$). (C) Comparison of prostate tissue (benign area) levels of 25(OH)D (EA, $n = 29$; AA, $n = 30$) and 1,25(OH)₂D (EA, $n = 24$; AA, $n = 19$) measured by uHPLC-MS-MS. (D) Correlation between prostatic levels of 25(OH)D and 1,25(OH)₂D (EA, $n = 24$; AA, $n = 19$). (E) Serum levels of vitamin D binding protein (DBP) quantified by polyclonal antibody ELISA and correlation with 25(OH)D levels in the (F) serum and (G) prostate tissue (EA, $n = 24$; AA, $n = 19$). $P < 0.1$; differences by ancestry were determined by 2-sided Wilcoxon signed-rank test and correlations determined by Spearman's rank coefficient.

The role of extrarenal Megalin and effect on tissue levels of 25(OH)D has not been reported. Given the essential function of Megalin in retaining 25(OH)D-DBP, *LRP2* gene expression was examined in the prostate tissues. In our study, expression of *LRP2* was significantly positively correlated with West African ancestry; however, expression overall was not statistically different between the two populations (Figure 4A). In AAs only, *LRP2* expression was significantly negatively correlated with tissue 25(OH)D, and there was a similar negative trend with serum 25(OH)D (Figure 4B). Comparison of vitamin D metabolites between serum and prostate showed that 1,25(OH)₂D did not correlate, suggesting active transport and local 1 α -hydroxylase activity by CYP27B1 rather than passive diffusion of 1,25(OH)₂D

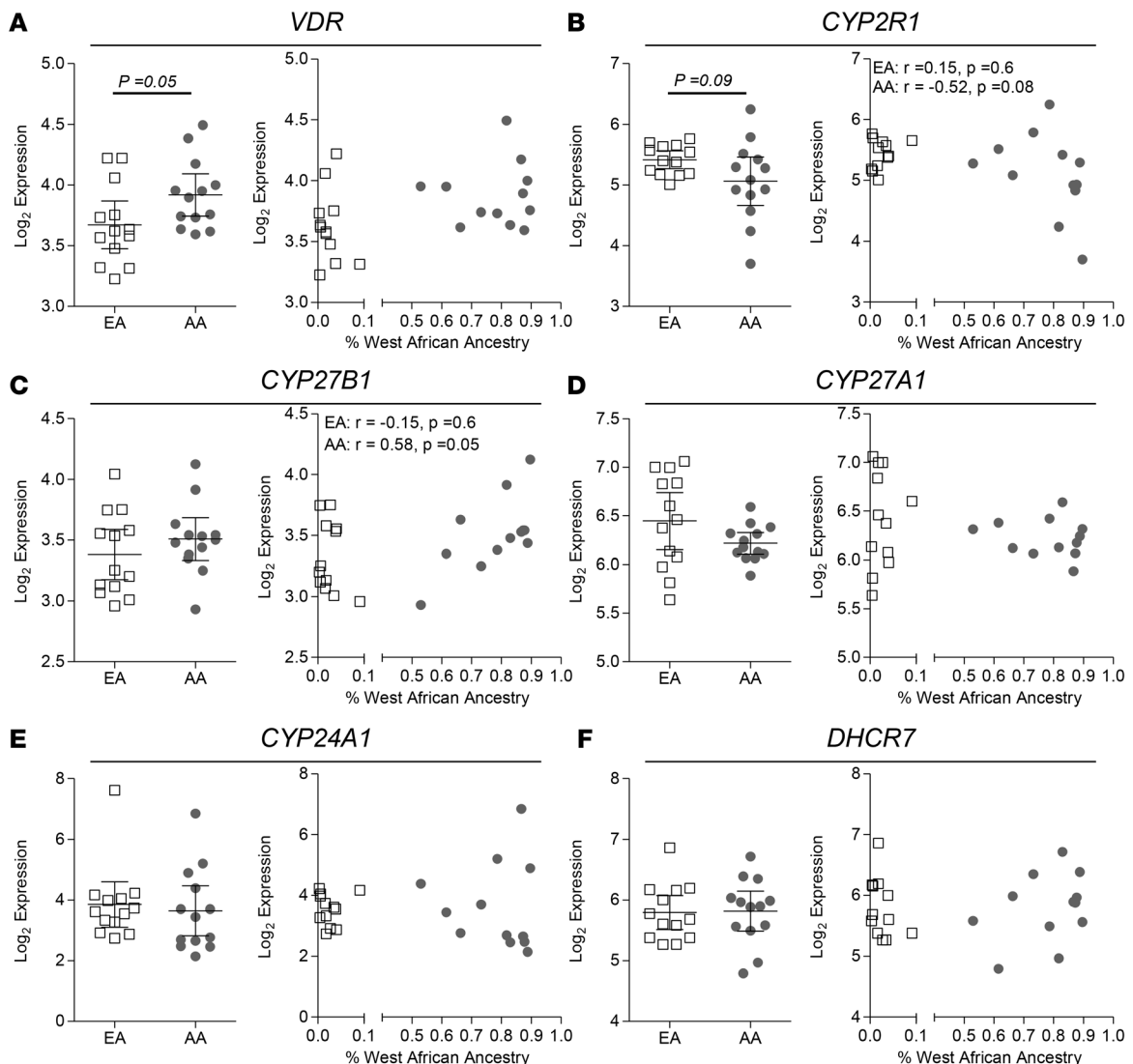


Figure 3. Expression of several vitamin D-related genes differ by ancestry and correlate to West African ancestry percentage. Affymetrix array expression (reported as log₂) of selected vitamin D-related genes collected by laser capture microdissection from the benign epithelium of fresh frozen radical prostatectomy specimens. Gene expression by ancestry group and correlation to the percent West African ancestry for (A) VDR, (B) CYP2R1, (C) CYP27B1, (D) CYP27A1, (E) CYP24A1, (F) and DHCR7 (European American [EA], $n = 13$; African American [AA], $n = 13$). $P < 0.1$; differences between ancestry determined by 2-sided Wilcoxon signed-rank test, and correlations between gene expression and percent ancestry determined by Spearman's rank coefficient.

into the prostate (Figure 4C). Megalin protein was abundant in the prostate epithelium by immunofluorescent staining in both benign and PCa regions, and it was premembranous in some glands and cytosolic in others (Figure 4D).

SNPs in vitamin D-related genes associate with phenotypes. Patients were genotyped for 38 SNPs in 8 vitamin D metabolic and signaling pathway genes that were previously explored for associations with serum 25(OH)D levels in AA and EA men (43, 44). Several significant associations between SNPs and phenotypes were identified. SNP relationships to gene expression were analyzed for both self-regulation (*cis*-expression quantitative trait loci [*cis*-eQTL]) and regulation of other genes (*trans*-eQTL) (Table 2 and Supplemental Table 1). A missense variant in *GC* (the coding gene for DBP), rs7041, was significantly associated with DBP levels in AAs ($P = 0.006$), and the minor allele G reduced DBP levels. SNPs in the *DHCR7*/*NADSYN1* region were associated with several phenotypes, including prostatic 25(OH)D levels, and the minor allele A variant of rs382951 significantly reduced *DHCR7* expression levels ($\beta = -1.68$, $P = 0.002$).

Although the remaining associations were not statistically significant after correcting for multiple testing, we identified several interesting SNPs that may influence vitamin D metabolite levels and/or gene

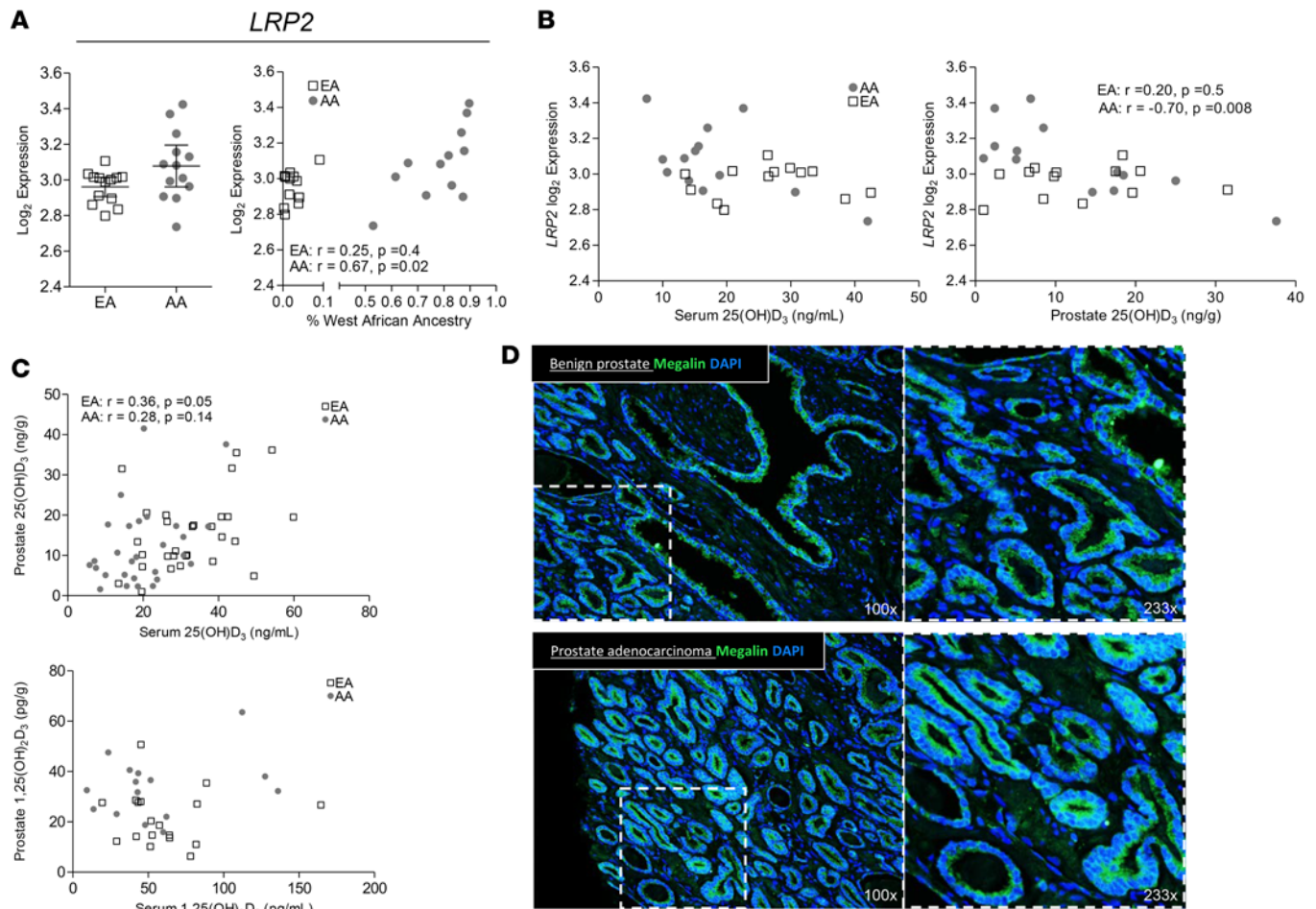


Figure 4. Megalin is present in prostate epithelium, and gene expression of Megalin (*LRP2*) correlates with ancestry and prostatic 25-hydroxyvitamin D. (A) Affymetrix array expression (reported as log₂) of Megalin (*LRP2*) in benign epithelium collected from fresh frozen radical prostatectomy specimens by laser capture microdissection (European American [EA], $n = 13$; African American [AA], $n = 13$). Comparison of gene expression by ancestry group and correlation to percent West African ancestry for *LRP2*. $P < 0.1$; differences between ancestry determined by 2-sided Wilcoxon signed-rank test, and correlations determined by Spearman's rank. (B) Comparison by ancestry group of correlation between *LRP2* and 25(OH)D in the serum and prostate tissue. (C) Comparison by ancestry group of correlation between the vitamin D metabolites 25(OH)D (EA, $n = 28$; AA, $n = 28$) and 1,25(OH)₂D (EA, $n = 18$; AA, $n = 15$) between the serum and prostate tissue. (D) Benign and cancer areas of prostate tissue from a radical prostatectomy tissue specimen immunofluorescently stained for Megalin (green) and DAPI (blue) nuclear counterstain.

expression of vitamin D–related genes, given a larger sample size. Notably, among EAs only, 1 SNP in the *CYP27B1* gene was associated with serum 1,25(OH)₂D levels ($P = 0.03$) and another SNP in *CYP27B1* was associated with *CYP27B1* gene expression in AAs only ($P = 0.03$). Variants in the *VDR* associated with expression of *CYP2R1* in EAs ($P = 0.03$) and *CYP27A1* in AAs ($P = 0.011$). The results of all tests with $P < 0.05$ are shown in Supplemental Table 1.

Discussion

Our study presents the first report of vitamin D metabolite measurement in the prostate tissue from a diverse population of AA and EA men. Analysis of the entire prostatic vitamin D axis points to several potential compensatory mechanisms unique to the AA population.

Comparison of serum and prostate tissue vitamin D levels in AAs and EAs revealed both familiar and unexpected findings. AAs had significantly lower serum 25(OH)D than EAs, which corroborates epidemiologic reports in larger patient populations (44–46). Additionally, serum levels of 1,25(OH)₂D did not differ by race, which was expected, given the tight regulatory control of the parathyroid gland on 1,25(OH)₂D levels in circulation. Unexpectedly, AAs had higher levels of the active hormone 1,25(OH)₂D in the prostate tissue, despite having lower levels of the circulating metabolite 25(OH)D in the serum. AAs also had

Table 2. SNP associations between vitamin D binding protein and vitamin D metabolites levels or vitamin D pathway gene expression

	SNP	Chromosome	Position ^A	Gene	MA ^B	MAF ^C	n	β	P
Vitamin D Binding Protein									
<i>African Americans</i>									
DBP	rs7041	4	72618334	GC	G	0.3	15	-0.068	0.006 ^D
Vitamin D Metabolites									
<i>European Americans</i>									
Serum 1,25(OH) ₂ D	rs10877012	12	58162085	CYP27B1	T	0.34	19	0.142	0.03
Prostatic 25(OH)D	rs3794060	11	71187679	DHCR7/NADSYN1	C	0.47	19	0.238	0.02
Prostatic 1,25(OH) ₂ D	rs11234027	11	71234107	DHCR7/NADSYN1	A	0.12	13	0.241	0.009
<i>African Americans</i>									
Prostatic 25(OH)D	rs12800438	11	71171003	DHCR7/NADSYN1	A	0.48	21	-0.291	0.007
trans-eQTL									
<i>European Americans</i>									
CYP2R1	rs731236 (Taql)	12	48238757	VDR	C	0.31	8	0.236	0.03
CYP27A1	rs2282679	4	72608383	GC	C	0.31	8	-1.096	0.003
<i>African Americans</i>									
CYP27A1	rs731236 (Taql)	12	48238757	VDR	C	0.4	10	0.221	0.011
VDR	rs6022990	20	52775532	CYP24A1	G	0.1	10	0.587	0.005
cis-eQTL									
<i>African Americans</i>									
CYP27B1	rs4646537	12	58157281	CYP27B1	C	0.2	10	0.359	0.027
DHCR7	rs3829251	11	71194559	DHCR7/NADSYN1	A	0.3	10	-1.678	0.002 ^D

^AGRCh37, base-pair position. ^BMA, minor allele. ^CMAF, minor allele frequency. ^DStatistically significant based on *P* value cutoff for testing association by linear regression Spearman correlation coefficient.

lower 25(OH)D in the prostate compared with EAs. Importantly, prostatic 25(OH)D and 1,25(OH)₂D positivity correlated in both AAs and EAs, providing confidence in the uHPLC-MS-MS data and showing that intraprostatic 1,25(OH)₂D levels are likely dependent on tissue 25(OH)D and not passive diffusion from the circulation, as previously thought (47). Despite this positive correlation, tissue metabolite concentrations diverged in AAs — lower 25(OH)D but higher 1,25(OH)₂D — which was not observed in EAs and suggests a race-specific perturbation of vitamin D equilibrium in the prostate.

The intraprostatic metabolite data support the presence of 1α-hydroxylase activity in the prostate. Circulating 1,25(OH)₂D likely does not dictate tissue levels via passive diffusion, since serum 1,25(OH)₂D levels did not correlate with prostatic 1,25(OH)₂D. Although local production of 1,25(OH)₂D has been demonstrated in prostate cells in vitro, our data provides the first evidence in human tissue. Additionally, this revelation provides evidence that active hormone levels in the tissues do not mirror those in the circulation and suggests that “vitamin D status” is more complex than previously thought.

The lack of correlation between serum and tissue vitamin D levels also exposes a gap in the current understanding of vitamin D metabolite regulation in the tissues. Higher intraprostatic levels of the active hormone in AAs suggest compensatory differences in vitamin D delivery and metabolism that may be race specific. Nearly all 25(OH)D in circulation is bound to DBP and sequestered in the serum, thus preventing passive diffusion into the tissue. Racial differences in DBP levels have been postulated to alter the concentration of bioavailable vitamin D and, therefore, alter availability to the tissues (37). However, serum DBP was not different in our cross sectional group, which corroborates other recent studies (48) and associations between variants in *GC* and DBP serum levels in our study emulate those reported by others (37, 49). This, in combination with our metabolite data, suggests serum DBP levels do not regulate tissue concentrations of vitamin D.

Circulating 25(OH)D bound to DBP can enter the cell via Megalin-mediated endocytosis, a process that is well understood in the kidney and functions to resorb 25(OH)D from the glomerular filtrate (50, 51). Expression of *LRP2* has been reported in the thyroid, kidney, brain, lung, breast, and adipose tissue. Here, we show the first report of Megalin protein expression in human prostate tissue. Previous reports of extrarenal DBP-Megalin-mediated uptake in the prostate are limited; just one in vitro study has reported

Megalin protein in the immortalized and transformed LNCaP and PC-3 cell lines (52). We observed prominent membrane expression of Megalin protein in the prostate epithelium.

Not only was Megalin protein and RNA (*LRP2*) present in the prostate, but *LRP2* expression had a strong positive correlation with West African ancestry in the AA patients. Skin pigmentation increases with West African ancestry and vitamin D synthesis depends on UV penetration of the skin; therefore, vitamin D status negatively correlates with West African ancestry (53). Megalin expression was also significantly correlated with prostate concentrations of 25(OH)D and trended negative with serum 25(OH)D in AAs only. Taken together, our data challenge the dogma of passive diffusion of bioavailable vitamin D and suggest that tissue levels of the hormone are not dependent on the unbound fraction of 25(OH)D in the serum, but instead point to a role for Megalin-mediated endocytosis of 25(OH)D-DBP in the prostate. Additionally, the absence of racial differences in serum DBP levels, the presence of Megalin in prostate epithelium, and the correlation of Megalin expression with tissue vitamin D metabolites in AAs point to a compensatory mechanism conserved by evolution to ensure adequate vitamin D in the prostate tissue. The free hormone hypothesis posits that DBP-bound 25(OH)D is not bioavailable (54), but our results suggest that Megalin expression in the prostate may be modulated to capture DBP-25(OH)D and challenges the assumption of vitamin D bioavailability.

Analysis of intraprostatic gene expression of *VDR* and vitamin D metabolism enzymes also points to tissue compensation with evolutionary undertones. *VDR* expression was significantly higher in AAs but may not equate to higher *VDR* activity for several reasons. First, there are known SNPs in *VDR* that may affect activity and, second, analysis of coregulatory proteins that interact with *VDR* on the DNA were not included in our study. Expression of *CYP27B1* did not differ between AAs and EAs, but was positively correlated with West African ancestry and may lead to increased 1 α -hydroxylation as West African ancestry increases. The expression of *CYP2R1*, the enzyme that generates 25(OH)D from pre-vitamin D, was present at significantly higher levels in EAs and also negatively correlated with West African ancestry. However, the biological significance of this observation remains unclear, as extrahepatic 25(OH)D synthesis has not been previously reported. Similarly, we observed an association between an SNP in *DHCR7* that associated with gene expression of *DHCR7*, suggesting *cis*-regulation via a functional SNP; however, extracutaneous roles for *DHCR7* have not been previously described.

Measurement of vitamin D metabolites in the prostate tissue presents a new paradigm in vitamin D and PCa disparities research. Only one prior study has measured vitamin D metabolites in prostate tissue (22) in a population composed almost entirely of European ancestry and predominantly replete vitamin D status. In that study, 1,25(OH)₂D was measured in both serum and tissue by enzyme immunoassay, whereas we utilized uHPLC-MS-MS to measure both 25(OH)D and 1,25(OH)₂D. Compared with standard HPLC-MS and radioimmunoassay, uHPLC-MS-MS has superior sensitivity and specificity, and it also enables differentiation between D₂ and D₃ isoforms.

The sum of our findings support the presence of a compensatory mechanism in AAs that ensures prostate levels of 1,25(OH)₂D remain adequate, perhaps even higher, in the setting of low serum 25(OH)D. The discrepancy in serum 25(OH)D levels between AAs and EAs — with AAs being deficient — is central to the hypothesis that vitamin D is a chemopreventive agent and a biological contributor to the racial disparities in PCa. However, higher active hormone in the prostate tissue of AAs, association of *CYP27B1* and *LRP2* expression with West African ancestry, association of *LRP2* expression with prostatic 25(OH)D in AAs, and increased *VDR* expression in AAs challenge this notion and are highly suggestive of an evolutionary process of compensation.

Despite these findings, one must exercise caution before concluding that no vitamin D disparity exists. Our study had some limitations and leaves several potential key areas to be explored. For one, our study had an underrepresentation of vitamin D-deficient EAs, as well as replete AAs, which is needed to separate ancestry from deficiency. Second, our study does not rule out regulation of innate and adaptive immunity by vitamin D, which has been demonstrated to significantly influence inflammation-related pathways (31, 55) in AA men (56). We examined gene expression solely in prostatic epithelium, which does not include immune cells or prostate stroma, both essential modulators of inflammatory gene signaling (31). The activity of *VDR* was not assessed by the endpoints of our study, and it is not only regulated by ligand but also transcriptional coregulators that were not included here (28).

In conclusion, this study provides insight into the distribution of vitamin D in the prostate, and the results challenge our previous assumptions. Our data show that the prostate can maintain levels of

active vitamin D in the setting of serum deficiency, underscoring the essential role for this hormone in the prostate and perhaps other tissues. This reveals the paucity of knowledge about how vitamin D deficiency and/or ancestry alters the mechanisms and regulation of tissue levels of vitamin D. It is premature at this point to speculate on the clinical implications of these findings in regards to vitamin D deficiency and prostate health.

Methods

Patient biospecimens

Fresh frozen prostate, whole blood, and serum were retrospectively collected from RP patients. Specimens from 60 patients were included for analysis: 30 from the UIC Hospital (Chicago, Illinois, USA) and 30 from the Cooperative Human Tissue Network Western Division at Vanderbilt University (Nashville, Tennessee, USA). Criteria for inclusion were self-declared race data, >500 mg of benign frozen prostatectomy specimen, serum, and whole blood. All patients had localized cancer without prior chemotherapy or hormonal therapy.

Ancestry estimate

DNA was isolated from 1 ml of whole blood. One hundred and 5 AIMs were genotyped to assess ancestral admixture proportion (34). Agena Bioscience MassARRAY was used for genotyping. Ancestral admixture proportion, West African ancestry, European ancestry, and Native American ancestry were estimated using STRUCTURE 2.3 (57).

LCM collection

Prostate specimens were removed from liquid nitrogen storage and thawed to -20°C in a cryostat prior to sectioning. RNase free conditions for LCM was carried out as previously described by our group (38). Briefly, 3 or more 10 micron cryosections per specimen were placed onto PEN membrane $4.0\text{ }\mu\text{m}$ LCM slides from Leica Biosystems and stained with Toluidine blue in RNA and RNase free solutions for visualization under the microscope. An adjacent section was H&E stained for markup by a board-certified pathologist. Only benign epithelium $>1\text{ cm}$ from cancer was used for this study. LCM was done for 1 hour only to prevent RNA degradation and resulted in 100–200 acini per specimen. Tissue was stored in lysis buffer (RNAqueous-Micro kit, Ambion) at -80°C until ready for RNA isolation.

RNA extraction and amplification

Extraction and isolation of RNA from the LCM samples was completed using the RNAqueous-Micro kit, and the RNAqueous-Micro procedure for LCM protocol was followed with DNase treatment. Quantity and quality of RNA was assessed via NanoDrop ND-1000 (Thermo Scientific), and yields ranged from $99.6\text{ ng}/\mu\text{l}$ to $602.8\text{ ng}/\mu\text{l}$. For each sample, 10 ng of RNA was used for whole transcriptome amplification using the Ovation PicoSL WTA System V2 (NuGEN) and purified with the DNA Clean & Concentrator-25 kit (Zymo Research). Quantity and purity of cDNA was also assessed via NanoDrop.

Gene expression analysis

Amplified cDNA from 26 LCM patient samples (13 EA, 13 AA) was biotin labeled using the Encore Biotin Module (NuGEN) and hybridized to GeneChip Human Gene 1.0 ST Arrays (Affymetrix). Use of microarray enabled optimal and accurate quantification of gene expression, given the limited nature of the LCM samples. Hybridization and chip scanning were completed by The Core Genomics Facility at the UIC using standard Affymetrix protocols. The samples were divided into two batches for labeling, hybridization, and scanning, and samples were evenly distributed across batches by race and collection site to mitigate any potential bias. Principal component analysis confirmed no batch effect was present. Microarray data from CEL files were read, background corrected, and normalized. Robust multi-array averages were generated using the Affy (58) and Oligo (59) packages from Bioconductor (<https://www.bioconductor.org/>) in R (<https://cran.r-project.org/>). The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (60) and are accessible through GEO Series accession number GSE91037.

Histology

A formalin-fixed and paraffin-embedded human tissue microarray containing 3 benign and 3 PCa cores was used to stain for Megalin protein. Sections of 5 microns were incubated with rabbit polyclonal anti-Lrp2/Megalin antibody (ab76969) diluted 1:100 (Abcam) and then incubated with secondary antibody AlexaFluor-488 goat anti-rabbit diluted 1:200 (Invitrogen), counterstained with DAPI, and imaged on the PerkinElmer Vectra 3.

Vitamin D Metabolite Measurement

Extraction. Samples were weighed and spiked with the internal standards d_6 -1,25(OH) $_2$ D $_3$, d_3 -25(OH)D $_2$, and d_3 -25(OH)D $_3$, then saponified with methanolic potassium hydroxide. Both deuterated and nondeuterated neat reagents were purchased from Sigma-Aldrich. After saponification for 2 hours, the samples were vortexed and extracted with methylene chloride. The organic layer was dried and reconstituted with hexane/methylene chloride, and both 25(OH)D and 1,25(OH) $_2$ D were isolated on a 0.5 g silica SPE column. The metabolites 1,25(OH) $_2$ D and 25(OH)D were eluted and then reconstituted into vitamin D-stripped serum (DiaSorin).

25(OH)D. From this point, the 25(OH)D was extracted via liquid-liquid extraction using hexanes alongside a serum-based standard curve with 972a serum (National Institute of Standards and Technology) serum 25(OH)D controls. The samples were reconstituted into LCMS-grade methanol and LCMS-high purity water both with 0.1% formic acid, and then injected onto an Agilent 1290 HPLC with an Agilent C18 Poroshell Column coupled to an Agilent 6460 Triple-quad mass spec with electrospray ionization source (ESI) in positive mode and analyzed using Masshunter software. The 25(OH)D measurements had a percent coefficient of variation of 8.6% for tissue and 6% for serum.

1,25(OH) $_2$ D. The samples, standard curve, and 1,25(OH) $_2$ D QC calibrator (DiaSorin) were then extracted from the serum and serum reconstitution by acetonitrile protein precipitation, followed by SPE isolation using C-18OH DiaSorin columns. Samples were eluted and dried in a savant dryer. The 1,25(OH) $_2$ D fraction from the samples/standard curve/QC were derivatized using 100 μ l of 0.75 mg/ml PTAD in acetonitrile for 2 hours at room temperature. The samples were quenched with 50 μ l LCMS-grade water, loaded into LCMS vials, and injected onto an Agilent 1290 HPLC with an Agilent ZORBAX C18 Eclipse plus RRHD column coupled to an Agilent 6460 Triple-quad mass spec with ESI in positive mode and analyzed using Masshunter software. The 1,25(OH) $_2$ D assay had a percent coefficient of variation of 13.0% for tissue and 10.8% for serum.

DBP measurement

Serum levels of DBP were measured by the total Human Vitamin D Binding Protein polyclonal ELISA test kit (Genway Biotech Inc.) using 50 μ l of input, per manufacturer's instructions. This ELISA is a highly sensitive 2-site enzyme linked immunoassay (ELISA) for measuring DBP in human biological samples.

Vitamin D-related SNPs

SNPs, 38 in total, in 8 vitamin D metabolic and signaling pathway genes were genotyped as previously described (43). Agena Bioscience MassARRAY was used for genotyping. Four specific hypotheses were tested: (i) associations between GC (DBP alias) SNPs and DBP protein levels, (ii) associations between CYP27B1 SNPs and 25(OH)D and 1,25(OH) $_2$ D levels, (iii) associations between VDR SNPs and prostatic gene expression of *CYP2R1*, *CYP24A1*, *CYP27A1*, and *CYP27B1* (*trans*-eQTL of VDR targets), and (iv) associations between SNPs and expression of the gene in which SNPs are located (*cis*-eQTL), in addition to associations of all the genotyped SNPs with DBP and vitamin D metabolites level and gene expression.

Statistics

The month of sample collection was recorded and plotted against each of the serum and tissue vitamin D metabolites (Supplemental Figure 2) and did not show a consistent difference in the UV-high months. Therefore, vitamin D metabolite levels were not adjusted for seasonality in the group comparisons. For comparison of metabolite levels between the AA and EA groups in both the serum and prostate tissue, a 2-sided Wilcoxon rank-sum test was used, as data did not follow a normal distribution. Spearman's rank coefficient, which compensates for nonparametric data and allows a nonlinear relationship, was calculated

to quantify correlations between serum and prostate metabolites. Analysis of gene expression data also used a 2-sided Wilcoxon rank-sum test for comparisons between groups and the Spearman rank coefficient for correlations between gene expression and percent West African ancestry estimate. The mean and 95% CI is reported for all data sets. $P < 0.1$ was considered significant for all analyses. Linear regression was used to test the association between SNPs and DBP, as well as serum and prostatic vitamin D metabolite levels adjusted for age in EAs and age and West African ancestry for AAs. DBP and vitamin D metabolite levels were log-transformed to normalize the data. To test if genotyped SNPs are eQTL with vitamin D metabolic genes, we used log₂-normalized gene expression levels as dependent variables in a linear regression model. The Spearman correlation coefficient is reported for all SNP statistical analysis and was performed using PLINK (61) and SPSS statistical software version 24.0 (IBM Corp.).

Study Approval

The samples were obtained from 2 deidentified biorepositories; Cooperative Human Tissue Network (CHTN) and UIC. The research was determined to not fit the definition of human subjects research by the UIC IRB (2013-0341).

Author Contributions

ZR contributed to LCM gene expression, statistical analyses, immunofluorescence, and preparation of the manuscript and figures. KB contributed to SNP analyses and interpretation. ES contributed to ancestry and SNP genotyping and analyses. RF contributed to patient sample preparation. AM performed all metabolite measurements. PHG provided the UIC patient specimens and statistical guidance in data analyses. RK contributed to genetic ancestry and SNP analyses and interpretation. LN provided study conception, organization among collaborators, funding, data analysis and interpretation, and preparation of the manuscript.

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