The Free Hormone Hypothesis and Ancestry-related Differences in Prostate Vitamin D Uptake and Metabolism

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THESIS

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DEDICATION

This work is dedicated to cancer patients who have donated their tissue to research.

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CONTRIBUTION OF AUTHORS

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Chapter III utilized the microarray gene expression data set generated from the published work in **Chapter II.** The TMA histology mark-up in Figure 10A was completed by Dr. Andre Acosta, and the slide scanning and data organization in Figure 10B-E was completed by Ryan Deaton. The data in Figure 11C was generated by Jason Garcia. Androgen metabolites in Figure 13 were measured by Yue-Ting Wang in the UIC Mass Spectrometry, Metabolomics & Proteomics Facility.

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ABBREVIATIONS AND NOMENCLATURE

1,25D	1,25-dihydroxyvitamin D
25D	25-hydroxyvitamin D
1,24,25D	1,24,25-dihydroxycholecalciferol
24,25D	24,25-dihydroxycholecalciferol
3D	Three-dimensional
AA	African American
AR	Androgen Receptor
BPH	Bengin Prostatic Hyperplasia
CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1
CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1
CYP27A1	Cytochrome P450 Family 27 Subfamily A Member 1
CYP27B1	Cytochrome P450 Family 27 Subfamily B Member 1
CYP2R1	Cytochrome P450 Family 2 Subfamily R Member 1
CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4
DBP	Vitamin D Binding Protein
DHCR7	7-Dehydrocholesterol Reductase
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
EA	European American
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GC	Group-Specific Component (Vitamin D Binding Protein)
LCM	Laser-capture Microdissection
LRP2	DL Receptor Related Protein 2
NADSYN1	NAD Synthetase 1
PCa	Prostate Cancer
PrE	Primary Epithelial Cells
PrS	Primary Stromal Cells
PSA	Prostate Specific Antigen
RNA	Ribonucleic Acid
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RXR	Retinoid X Receptor
SHBG	Sex-hormone Binding Globulin
SNP	Single Nucleotide Polymorphism
SRD5A2	Steroid 5 Alpha-Reductase 2
T	Testosterone
TMA	Tissue Microarray
TURP	Transurethral Resection of the Prostate
uHPLC-MS/MS	Ultra-high Performance Liquid Chromatography tandem Mass Spectrometry
UIC	University of Illinois at Chicago
UVB	Ultraviolet Radiation-B
UVR	Ultraviolet Radiation
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element

SUMMARY

Prostate cancer is the leading cause of cancer-related deaths in men and 1 in 7 men will be diagnosed in their lifetime. African American men are disproportionately at risk for prostate cancer and have twice the mortality compared to Caucasian men. African American men are also at increased risk of vitamin D deficiency as higher levels of skin melanin inhibit cutaneous UV penetration required for vitamin D synthesis. Vitamin D is a chemoprotective hormone that has anti-cancer actions in the prostate and deficiency has been hypothesized to contribute to the prostate cancer disparity in African American men, however, associations between vitamin D status and cancer outcomes are limited to serum assessment of vitamin D status. This thesis challenges the concept of vitamin D status by exploring intra-prostatic vitamin D metabolites and presents an ancestry-specific role for megalin-mediated endocytosis of both vitamin D and androgens in the prostate.

CHAPTER I: INTRODUCTION

A. Ancestry-related Disparities in Prostate Cancer

Prostate cancer is the most common cancer and second leading cause of cancer-related deaths among men in the United States (Siegel et al., 2018). Prostate cancer disproportionately affects African American men with 60% higher incidence and 2.1 times the rate of mortality compared to American men of European ancestry (SEER*StatDatabase). This disparity is present throughout the entirety of the disease; African American men are diagnosed at an earlier age (Cotter et al., 2002), present with more advanced stage (Hoffman et al., 2001), have larger tumor volume (Sanchez-Ortiz et al., 2006), higher PSA (Moul et al., 1995), are quicker to progress to advanced disease (Powell et al., 2010), and have lower 5-year survival (SEER*StatDatabase).

The inconsistency in prostate cancer incidence and mortality between men of African and European ancestry can be attributed to both biological and non-biological factors. Several non-biological factors such as socio-economic and socio-cultural differences have been identified to partly contribute to the disparity; African Americans are less likely to receive radical prostatectomy or treatment with hormone deprivation therapies (Imperato et al., 1996; Moses et al., 2010). Controlling for non-biological factors including life-style, access to healthcare, education, and insurance coverage greatly reduces the magnitude of the race-stage odds ratio disparity in incidence (Jones et al., 2008). However, adjusting for similar factors accounted for only 25% of the racial-gap in mortality from data analyzed in the Surveillance, Epidemiology, and End Results-Medicare database (Taksler et al., 2012).

Although non-biological factors are partially responsible, the disparity in ancestry-related prostate cancer outcomes persists even when confounders are taken into account. Ancestry-specific genetic and molecular differences in prostate biology have been identified and may explain the remaining disparity-gap. Age and family history are the strongest risk factors for developing prostate cancer in all men, however, a prostate cancer susceptibility locus at 8q24 has been identified and admixture mapping

showed increased risk for prostate cancer in African Americans with African ancestry versus European ancestry at this locus (Freedman et al., 2006; Yeager et al., 2009).

Numerous genomic alterations comprise the prostate cancer genetic landscape making the disease genetically heterogeneous (Barbieri et al., 2013). Recent studies have demonstrated that African Americans are more likely to fall into genomic subclasses prognostic of more aggressive disease (Khani et al., 2014). Early prostate cancer is driven by androgens and androgen signaling. Mutations in *SRD5α2,* the gene encoding for the enzyme 5α-reductase that metabolizes testosterone to the more potent AR ligand dihydrotestorone, and polymorphisms in the androgen metabolism enzymes *CYP17A1* and *CYP3A4* associate with prostate cancer in African American men (Karakas et al., 2017). Reports of total serum testosterone levels between African Americans and European Americans are mixed, (Richard et al., 2014; Ross et al., 1986) however, as are reports of associations with prostate cancer outcomes (Eaton et al., 1999; Gann et al., 1996a; Hsing and Comstock, 1993). Additionally, African Americans have fewer CAG repeats in the AR compared to European men, which has been attributed to increased susceptibility to prostate cancer (Bennett et al., 2002).

B. Vitamin D, Prostate Cancer, and African Ancestry

The link between vitamin D and cancer was first proposed by Cedric and Frank Garland who reported an inverse correlation between mean daily solar radiation and colon cancer mortality in major metropolitan and non-metropolitan cities across the United States (Garland et al., 1985). This correlation has been coined “The Sunshine Hypothesis” and posits that increased vitamin D production due to UVB exposure provides chemo-protective effects. Indeed, prospective ecological and observational studies have demonstrated this correlation for cancers of the colon, breast, kidney, lung, prostate, bladder, thyroid, and pancreas (Grant and Garland; Lin et al., 2012; Moukayed and Grant, 2017). Overall, the association between higher vitamin D status and reduced cancer risk remains strongest for colorectal cancer and is mixed for others including prostate (Gandini et al., 2011).

Vitamin D is an essential hormone and numerous health organizations have provided guidelines for recommended dosing as well as cut-offs to define deficiency. The recommended daily dose set by the Institute of Medicine is 600 to 800 IUs and deficiency defined as serum 25D <20ng/mL (Ross et al., 2011). These guidelines and cut-offs, however, are based solely on studies of Caucasian and European populations in the context of bone health outcomes and relevance to cancer chemoprevention in diverse populations remains unclear.

Epidemiological evidence of a beneficial role for vitamin D in reducing prostate cancer risk is largely mixed. Prospective and case-control studies have reported negative (Ahonen et al., 2000; Corder et al.; Li et al., 2007), null (Braun et al., 1995; Gann et al., 1996b; Travis et al., 2009), and positive associations between serum vitamin D status and prostate cancer risk (Ahn et al., 2008; Xu et al., 2014). Additionally, a U-shaped curve where both high and low levels of vitamin D confer increased risk has also been reported (Tuohimaa et al., 2004). Analyses restricted to disease mortality and aggressiveness, however, do indicate a protective effect as men with higher vitamin D status have less aggressive tumors and longer survival (Brändstedt et al., 2016; Mondul et al., 2016; Schenk et al., 2014a).

Observational studies of UVB exposure and prospective studies of vitamin D status and prostate cancer outcomes are predominantly confined to populations of white Caucasian men. Whether the conclusions regarding vitamin D and prostate cancer outcomes reported in the epidemiologic literature also pertain to ancestrally diverse populations is limited to relatively few studies. Increased skin melanin correlates with African ancestry and inhibits vitamin D synthesis; therefore, African Americans have an increased risk of vitamin D deficiency (Forrest and Stuhldreher, 2011; Signorello et al., 2010). The few studies that included African American populations reported that decreased serum vitamin D status associated with disease aggressiveness (Steck et al., 2015), higher Gleason grade and tumor stage on biopsy (Murphy et al., 2014), and adverse pathology upon radical prostatectomy (Nyame et al., 2016). Additionally, several variants unique to African Americans in vitamin D-related genes including *GC, CYP27B1, CYP2R1,* and *DHCR7/NADSYN1* are reported to account for a portion of the variance in serum vitamin D status and highlight ancestry-related differences in the vitamin D pathway (Batai et al., 2014;

Signorello et al., 2011). Overall, vitamin D deficiency in African American men has been hypothesized as a biological contributor to the disparity in prostate cancer outcomes.

C. Vitamin D Metabolism and Chemoprotective Effects

Vitamin D is an essential hormone for normal human physiology (Lips, 2006). Vitamin D exists in two biologically relevant isoforms: D_3 (cholecalciferol) – obtained primarily through cutaneous exposure to UVB radiation and animal dietary sources; and D_2 (ergocalciferol) – obtained from dietary plant sources. Although both isoforms are biologically relevant, the D_3 isoform constitutes the overwhelming majority (>90%) of total vitamin D in most populations, has a longer half-life, and is more effective at raising and maintaining serum 25D levels (Heaney et al., 2011; Lehmann et al., 2013).

First discovered as the curative agent for rickets, the canonical role of vitamin D is in calcium homeostasis for which the mechanism has been well described (DeLuca, 1988; Hume et al., 1927). Vitamin D is a pleiotropic hormone that targets nearly every tissue in the body and its role in phosphate homeostasis, cellular metabolism, innate and adaptive immunity, and chemoprotective effects have been reviewed extensively (Christakos et al., 2016; Feldman et al., 2014; Hewison et al., 2012). *In vitro* studies using prostate cancer cells have shown that treatment with calcitriol (1,25D) or analogs decrease cellular proliferation (Barreto et al., 2000), induce apoptosis (Simboli-Campbell et al., 1997), promote differentiation (Pendás-Franco et al., 2007), decrease inflammation (Nonn et al., 2006), inhibit angiogenesis (Bao et al., 2006), and reduce invasion and metastasis (Sung and Feldman, 2000). Additionally, *in vivo* studies have reported decreased tumor volume after vitamin D_3 dietary supplement using mouse xenograft models of tumors derived from hormone insensitive human prostate cancer PC-3 (Swami et al., 2012) and DU-145 cells (Ray et al., 2012).

Synthesis of vitamin D begins in the skin where UVB exposure isomerizes 7-dehydrocholesterol to cholecalciferol. Cholecalciferol is then sequentially metabolized by members of the cytochrome P450 family through a series of hydroxylation reactions forming the active hormone and inactivated metabolites (**Figure 1**). The first hydroxylation reaction occurs in the liver where cholecalciferol is hydroxylated by CYP2R1, or alternatively CYP27A1, forming 25-hydroxyvitamin D (25D).

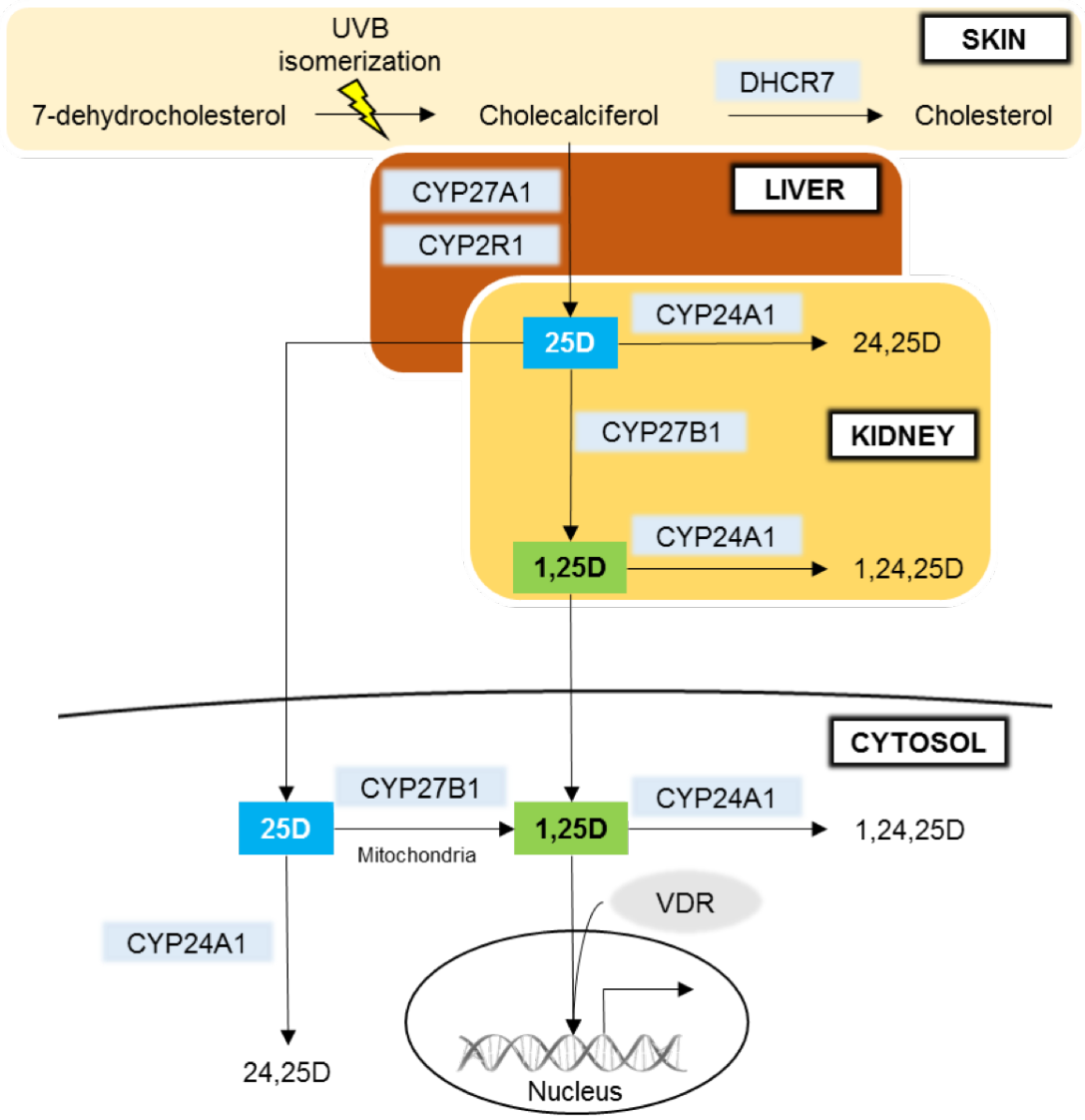

Figure 1. The vitamin D axis.
The vitamin D axis consists of the kidney and liver cytochrome P450 monooxygenases, vitamin D metabolites, extra-renal enzymes, and the vitamin D receptor (VDR).

25D is the major circulating form of vitamin D and the clinical measure of vitamin D status. The active hormone, 1,25-dihydroxyvitamin D (1,25D), is generated through the addition of a hydroxyl group on carbon 1 of the 25D metabolite and is catalyzed by the mitochondrial enzyme CYP27B1 in the proximal tubule of the kidney. Both 25D and 1,25D are inactivated by hydroxylation at carbon 24 by CYP24A1 in the mitochondria of the proximal tubule forming 24,25D and 1,24,25D, respectively (Bikle, 2014). In addition to liver and renal metabolism, numerous tissues, including the prostate, harbor the vitamin D hydroxylating enzymes and can locally control metabolite levels (Schwartz et al., 1998).

Activity of vitamin D is mediated through the vitamin D receptor (VDR); a member of the steroid receptor family. The VDR contains a zinc-finger DNA binding domain, hinge region, and ligand binding domain. Upon binding 1,25D, the VDR forms an obligate heterodimer with the retinoid X receptor (RXR) and targets vitamin D response elements (VDREs) located throughout the genome (Pike and Meyer, 2010). ChIP-seq and *in silico* analyses have elucidated thousands of VDREs comprising the VDR cistrome, many of which have been validated *in vitro* and *in vivo* (Haussler et al., 2011; Pike et al., 2016). The VDR regulates transcription by recruiting coregulatory machinery such as chromatin remodeling and histone acetylation complexes and ultimately RNA Polymerase II. Recruitment of corepressors and transcriptional inhibition has also been reported, however, the specific mechanism of gene suppression remains poorly understood (Demay et al., 1992).

D. The Free Hormone Hypothesis, Vitamin D Binding Protein, and Megalin-mediated Endocytosis

i. The Free Hormone Hypothesis

Originally developed to describe the effects of testosterone actions in the prostate, the free hormone hypothesis states that the activity of a hormone is due to its bioavailable fraction rather than its bound fraction (Mendel, 1989). Because vitamin D circulates bound to its carrier protein, the vitamin D binding protein (detailed below), the free hormone hypothesis is thought to apply to VDR activation by vitamin D binding.

ii. Vitamin D Binding Protein

The primary transporter of 25D in the serum is the vitamin D binding protein (DBP), also known as group-specific component (GC) globulin. DBP can bind both 25D and 1,25D, but is known as the carrier protein for 25D as it has ~20-fold higher affinity for 25D compared to 1,25D and concentrations of 25D are three orders of magnitude higher than 1,25D in the serum (Chun et al., 2012). DBP is solely responsible for maintaining the 14-day half-life of vitamin D in circulation. In a *dbp* knockout mouse model, homozygous knockout mice are phenotypically identical to the wild-type when fed a vitamin D replete diet but produce symptoms of bone disease after 4 to 6 weeks when switched to a vitamin D deficient diet. Similarly, total 25D levels are dependent on the number of functional *dbp* alleles, where $dbp^{+/+}$ mice have higher 25D than heterozygous $dbp^{+/-}$, and both have higher 25D than the homozygous $dbp^{-/-}$ knockout (Safadi et al., 1999).

Mathematical models from *in vitro* studies have calculated that ~90% of all 25D in the serum circulates bound to DBP with the remaining bound to serum albumin; <1% is predicted to exist in a "free" or unbound state (Bikle et al., 1986). Unbound 25D, and the fraction bound to albumin, is termed "bioavailable" and thought to determine vitamin D activity per the free hormone hypothesis (**Figure 2**). Therefore, vitamin D status may be confounded by DBP concentration. The relevance of bioavailable versus total vitamin D in determining vitamin D status, and the implications for health outcomes and deficiency cut-offs remain a topic of much debate (Chun, 2012; Chun et al., 2014; Tsuprykov et al., 2017; Yousefzadeh et al., 2014).

Adding to the complexity, there are three major genetic variants of DBP in humans – GC1F, GC1S, and GC2 – and they differ in both ancestral distribution and 25D affinity. The GC1F variant is most abundant in individuals with African ancestry and has the highest affinity for 25D, but is exceedingly rare in populations of European ancestry where the GC1S variant predominates and has the lowest affinity for 25D (Arnaud and Constans, 1993; Braithwaite et al., 2015). One study reported individuals homozygous for the GC1F variant had a reduced risk of cancer (Jorde et al., 2015), and another found that higher circulating DBP associated with decreased prostate cancer risk independent of

25D status in African American men (Layne et al., 2017). Differences in DBP concentration have been hypothesized to alter the amount of bioavailable vitamin D and one study reported that although African American men had lower serum concentration of DBP compared to European Americans, the bioavailable fraction of vitamin D was not different (Powe et al., 2013). A follow-up study, however, found the assay used a monoclonal antibody that failed to detect the DBP isoform most common to African ancestry, and reported no difference in DBP levels between African Americans and European Americans when a polyclonal assay was performed (Nielson et al., 2016a).

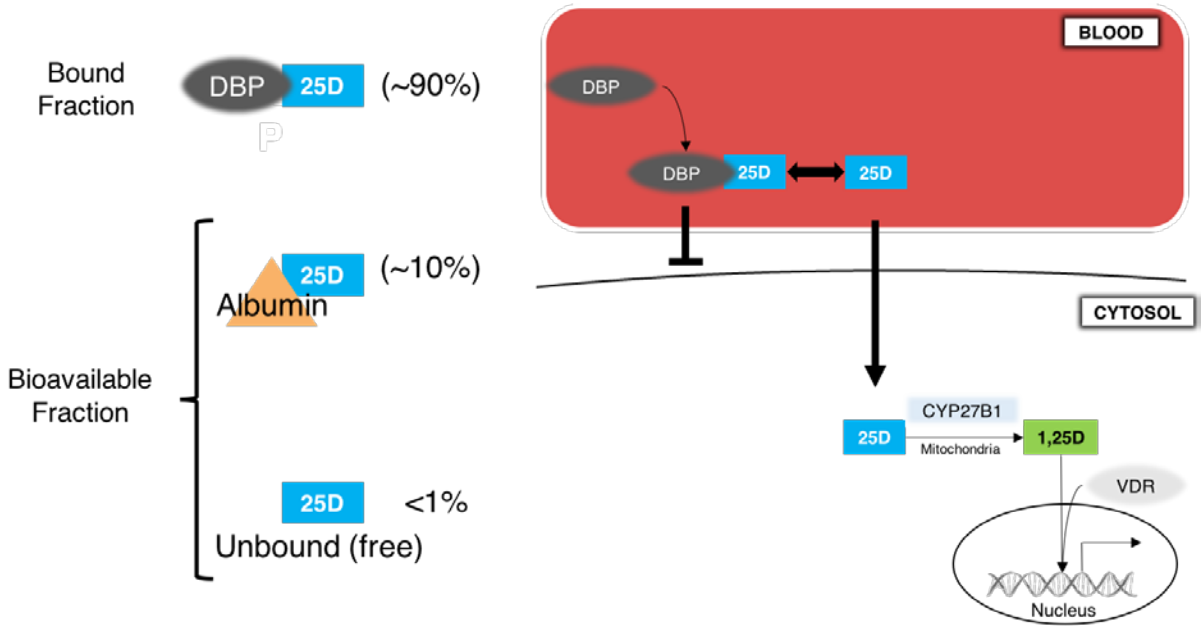

Figure 2. DBP is the carrier protein for 25D in the blood
25D is the high affinity ligand for DBP and ~90% of 25D circulates with the carrier protein, known as the "bound" fraction. The remaining ~10% is termed "bioavailable" and can passively diffuse across the plasma membrane to be converted to 1,25D and activate the vitamin D receptor (VDR).

iii. Megalin-mediated Endocytosis

DBP is the primary ligand for the transmembrane receptor megalin. Megalin is multi-liganded, 4600 amino acid, single-spanning transmembrane glycoprotein of the low-density lipoprotein receptor-related protein (LRP) family. The extracellular region of megalin consists of four cysteine rich ligand binding domains separated by 17 EGF-like repeat domains and eight pH sensitive spacer regions (Willnow et al., 1999). The short cytoplasmic tail of megalin is distinct from other LRP family members, except for three conserved NPXY-motifs responsible for plasma membrane sorting to clathrin-coated pits and mediation of endocytosis (Christensen and Birn, 2002). Megalin is found primarily on the apical surface of absorptive epithelia of several tissues including the kidney, ileum, thyroid, and epididymis. Dozens of ligands for megalin have been identified and consist of vitamin-binding proteins, carrier proteins, lipoproteins, hormones, toxins, and enzymes and enzyme inhibitors (Christensen and Birn, 2002; Eshbach and Weisz, 2017; Moestrup and Verroust, 2001; Nielsen et al., 2016) (**Table 1**).

Megalin has been described as a "scavenger receptor" as it binds dozens of ligands and its function has been well characterized in the proximal tubule of the kidney where it is responsible for re-uptake of low-molecular-weight proteins from the glomerular filtrate (Kerjaschki et al., 1984). In megalin deficient mice the primary phenotype is lethality due to improper forebrain development; however, 1 in 50 mice that do survive exhibit massive proteinuria for which the primary species is vitamin D binding protein (DBP) (Leheste et al., 1999). Indeed, DBP is a high affinity ligand for megalin and, in addition to reduced serum 25D levels, megalin deficient mice also display severe bone defects and a rachitic phenotype (Nykjaer et al., 1999). Therefore, the primary function for megalin is thought to be prevention of vitamin D loss to the urine.

Cubilin is another large glycoprotein receptor that shares DBP as a ligand with megalin (**Table 1**). Cubilin is also located on the extracellular side of the plasma membrane through a GPI-anchored complex, but lacks a cytoplasmic tail and, therefore, cannot mediate endocytosis (Christensen and Birn, 2002). Instead, cubilin has been demonstrated to co-localize with megalin and facilitate the sequestration

of 25D at the membrane surface before megalin-mediated endocytosis, and cubilin dysfunction results in abnormal metabolism of 25D (Nykjaer et al., 2001).

Expression of megalin in human prostate tissue has not been previously reported. Megalin and the clathrin-associated adaptor disabled-2 (Dab2) expression has been reported in LNCaP and PC-3 cells *in vitro* and modulated uptake of DBP after low-dose retinoic acid (RA) treatment (Ternes and Rowling, 2013). Vitamin D has been reported to stimulate megalin gene expression in rat kidney proximal tubule cells, human placental cells (JEG-3), and a mouse embryonic carcinoma cell line (Liu et al., 1998), however, regulation of megalin and cubilin gene expression is still not well understood.

Megalin	Cubilin
Vitamin carrier proteins	
Transcobalamin–vitamin B12	Intrinsic factor vitamin B12
Vitamin D–binding protein	***Vitamin D–binding protein***
Retinol-binding protein	
Folate-binding protein	
Other carrier proteins	
Albumin	Albumin
Myoglobin	Myoglobin
Hemoglobin	Hemoglobin
Lactoferrin	Transferrin
Selenoprotein P	
Metallothionein	
Neutrophil gelatinase-associated lipocalin	
Odorant-binding protein	
Transthyretin	
Liver-type fatty acid-binding protein	
Sex hormone-binding globulin	
Lipoproteins	
Apolipoprotein B	Apolipoprotein A-I
Apolipoprotein E	High-density lipoprotein
Apolipoprotein J/clusterin	
Apolipoprotein H	
Apolipoprotein M	
Hormones and signaling proteins	
Parathyroid hormone	Fibroblast growth factor
Insulin	
Epidermal growth factor	
Prolactin	
Thyroglobulin	
Sonic hedgehog protein	
Angiotensin II	
Leptin	
Bone morphogenic protein 4	
Connective tissue growth factor	
Insulin-like growth factor	
Survivin	
Enzymes and inhibitors	
Plasminogen activator inhibitor type I	
Plasminogen activator inhibitor type I–urokinase	
Plasminogen activator inhibitor type I–tissue plasminogen activator	
Pro-urokinase	
Lipoprotein lipase	
Plasminogen	
α-Amylase	
Lysozyme	
Cathepsin B	
α-Galactosidase A	
Cystatin C	
Recombinant activated factor VIIa	Recombinant activated factor VIIa
Immune and stress-related proteins	
Ig light chains	Ig light chains
Pancreatitis-associated protein 1	Clara cell secretory protein
α1-Microglobulin	a1-Microglobulin
β2-Microglobulin	
Drugs and toxins	
Aminoglycosides	Aminoglycosides
Polymyxin B	
Aprotinin	
Trichosanthin	
Colistin	
Others	
Receptor-associated protein	Receptor-associated protein
Ca2+	
Cytochrome c	
Seminal vesicle secretory protein II	
Coagulation favor VII	Coagulation favor VII
Coagulation favor VIII	

Table adapted from Christensen and Birn, 2002; Eshbach and Weisz, 2017; and Nielson et al., 2016

Table 1. Megalin and cubilin ligands

E. Hypothesis and Goals of the Thesis

African American men are at an increased risk for both prostate cancer and vitamin D deficiency. Vitamin D has well documented anti-cancer actions *in vitro* and *in vivo*, however, the epidemiologic evidence consists of conflicting reports between vitamin D status and prostate cancer outcomes. To date, epidemiologic studies investigating vitamin D status and prostate cancer have largely been confined to European and Caucasian populations and relied solely on serum measurement of 25D to assess vitamin D status. The relevance of intra-prostatic vitamin D metabolites and vitamin D-related gene expression in the prostate to ancestry-related disparities in prostate cancer is unknown. Therefore, we hypothesized that vitamin D deficiency in African American men results in alterations to the entire prostate vitamin D-axis.

The thesis aims to reveal how intra-prostatic vitamin D metabolite levels and vitamin D-related gene expression fit into the current understanding of serum deficiency and ancestry-related disparities in prostate cancer outcomes. **Chapter II** presents the first analysis of intra-prostatic vitamin D metabolite levels in an ancestrally diverse group of prostate cancer patients. The results both question how vitamin D status is defined and introduce a paradox regarding the hypothetical anti-cancer action of vitamin D for prostate cancer. **Chapter III** begins to characterize the role of megalin and DBP-25D sequestration in the prostate and reports associations between intra-prostatic androgen and vitamin D metabolites.

CHPATER II: PROSTATIC COMPENSATION OF THE VITAMIN D AXIS IN AFRICAN AMERICAN MEN

A. Introduction

This work was previously published as an article in *JCI Insight*. Richards, Z., Batai, K., Farhat, R., Shah, E., Makowski, A., Gann, P., Kittles, R., Nonn, L. Prostatic Compensation of the Vitamin D Axis in African-American Men. *JCI Insight.* (2017) 26;2(2).

As outlined in **Chapter I**, African American men are disproportionately affected by prostate cancer and vitamin D deficiency; therefore, vitamin D deficiency has been hypothesized to contribute to ancestry-related disparities in prostate cancer outcomes. Although laboratory studies have provided strong evidence for the chemo-preventive activity of vitamin D, epidemiologic evidence is mixed and the association between vitamin D status and prostate cancer remains unclear. Most epidemiologic studies are limited in their applicability to the racial disparity in prostate cancer as they lacked inclusion of a diverse population of African American men. As well, it is not clear whether the studies that reported positive results that suggest a chemoprotective role for vitamin D are mediated through systemic effects, such as immune regulation, or local effects of vitamin D within the tissues.

Serum concentration of the prohormone, 25D, is the clinical indication of vitamin D status and the Endocrine Society defines vitamin D deficiency as serum levels below 20ng/mL, insufficiency between 21-29ng/mL, and replete status above 30ng/mL (Holick et al., 2011). 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 is unknown and there is only one comparative report on serum and intra-prostatic levels of vitamin D (Wagner et al., 2013). This pivotal study showed that high-dose oral supplementation with vitamin D can raise levels in the prostate tissue, but also lacked diversity since all patients were Canadian Caucasian men.

Currently, multiple knowledge gaps exist regarding 1) the relationship between intra-prostatic and serum vitamin D metabolites, 2) the regulation of prostatic vitamin D metabolites, and 3) intra-prostatic levels of vitamin D metabolites in African American men. This knowledge is essential to understand 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 chapter, we describe the first evaluation of the serum and prostatic vitamin D axis in samples from a diverse cross-sectional group of radical prostatectomy patients. Vitamin D metabolites were quantified in both the serum and prostate tissue, and expression of genes involved in vitamin D metabolism, transport, and VDR were measured in the prostatic epithelial tissues.

B. Materials and Methods

i. Patient biospecimens

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

ii. Ancestry Estimate

DNA was isolated from 1 mL of whole blood. One hundred and five ancestry informative markers (AIMs) were genotyped to assess ancestral admixture proportion (Giri et al., 2009). 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 (Falush et al., 2003).

iii. LCM collection

Prostate specimens were removed from liquid nitrogen storage and thawed to -20C in a cryostat prior to sectioning. RNase free conditions for LCM was carried out as previously described by our group(Lugli et al., 2015). Briefly, three or more 10 micron cryosections per specimen were placed onto PEN membrane 4.0μm LCM slides from Leica Biosystems (Wetzlar, Germany) and stained with Toluidine blue in RNA and RNase free solutions for visualization under the microscope. An adjacent

section was hematoxylin and eosin stained for markup by a board-certified pathologist. Only benign epithelium >1cm from cancer was used for this study. LCM was done for one hour only to prevent RNA degradation and resulted in 100-200 acini per specimen. Tissue was stored in lysis buffer (RNAqueous®-Micro kit, Ambion, USA) at -80C until ready for RNA isolation.

iv. RNA extraction and amplification

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

v. Gene Expression Analysis

Amplified cDNA from 26 LCM patient samples (13 EA,13 AA) was biotin labeled using the Encore® Biotin Module (NuGEN, USA) and hybridized to GeneChip® Human Gene 1.0 ST Arrays (Affymetrix, USA). Use of microarray enabled optimal and accurate quantification of gene expression given the limited and precious nature of the LCM samples. Hybridization and chip scanning were completed by The Core Genomics Facility at the University of Illinois at Chicago (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(Gautier et al., 2004) and Oligo(Carvalho and Irizarry, 2010) 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 (Barrett et al., 2013) and are accessible through GEO Series accession number GSE91037.

vi. Histology

A formalin fixed and paraffin embedded human tissue microarray containing three benign and three PCa cores per patient 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, Cambridge, UK), then incubated with secondary antibody AlexaFluor-488 goat anti-rabbit diluted 1:200 (Life Technologies, Carlsbad, CA, USA), counterstained with DAPI and imaged on the PerkinElmer Vectra3 in the Research Histology and Tissue Imaging Core facility at UIC.

vii. Vitamin D Metabolite Measurement

Extraction: Samples were weighed and spiked with the internal standards d6-1,25$(OH)_2D_3$, d3-25$(OH)D_2$ and d3-25$(OH)D_3$), then saponified with methonalic potassium hydroxide. Both deuterated and non-deuterated neat reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). After saponification for two 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 then reconstituted into Vitamin D stripped serum (DiaSorin, Stillwater, MN, USA).

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 NIST 972a serum 25(OH)D controls. The samples were re-constituted 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, Stillwater, MN, USA) 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

100µl of 0.75mg/ml PTAD in acetonitrile for 2 hours at room temp. The samples were quenched with 50µl LCMS grade water and loaded into LCMS vials and injected onto a 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 $1,25(OH)_2D$ assay had a percent coefficient of variation of 13.0% for tissue and 10.8% for serum.

viii. DBP measurement

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

ix. Vitamin D-related SNPs

Single nucleotide polymorphisms (SNPs), 38 in total, in eight vitamin D metabolic and signaling pathway genes were genotyped as previously described(Batai et al., 2014). Agena Bioscience MassARRAY was used for genotyping. Four specific hypotheses were tested: 1) associations between GC (DBP alias) SNPs and DBP protein levels, 2) associations between CYP27B1 SNPs and 25(OH)D and $1,25(OH)_2D$ levels, 3) associations between VDR SNPs and prostatic gene expression of CYP2R1, CYP24A1, CYP27A1, and CYP27B1 (trans-eQTL of VDR targets), and 4) 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.

x. Statistical analyses

The month of sample collection was recorded and plotted against each of the serum and tissue vitamin D metabolites (Figure 8) 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 two-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 two-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% confidence interval is reported for all data sets. A p-value <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 $\log_2$-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(Purcell et al., 2007) and SPSS statistical software version 24.0 (IBM Corp., Armonk, NY).

C. Results

i. 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 radical prostatectomy (RP) patients (n= 60) was assembled retrospectively to examine the serum-prostatic vitamin D axis (**Figure 3A**) by African ancestry (**Table 2, Figure 3B**). Fresh frozen RP tissue, serum, and whole blood were used for analysis (**Figure 3B)**. Of the patients, 32 self-identified as AA, 28 as EA. West African ancestry was determined by analysis of 105 ancestry informative markers (Giri et al., 2009) in DNA isolated from the whole blood and showed a heterogeneous population of EAs and a range of 53.0 to 95.5% in the AA group (**Figure 3C**). One patient that 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 uHPLC-MS-MS. The mean serum 25(OH)D concentration was lower in the AA group compared to the EA group (AA: 20ng/mL, 95%CI [16 - 23ng/mL]; EA: 33ng/mL, 95%CI [28 - 37ng/mL]; $p < 0.0001$)(**Figure 4A**). Serum levels of the active hormone, $1,25(OH)_2D$, did not differ between the two groups, but did correlate with serum concentration of 25(OH)D in the EA group only (Spearman $r = 0.46$; $p = 0.02$) (**Figure 4A-B**).

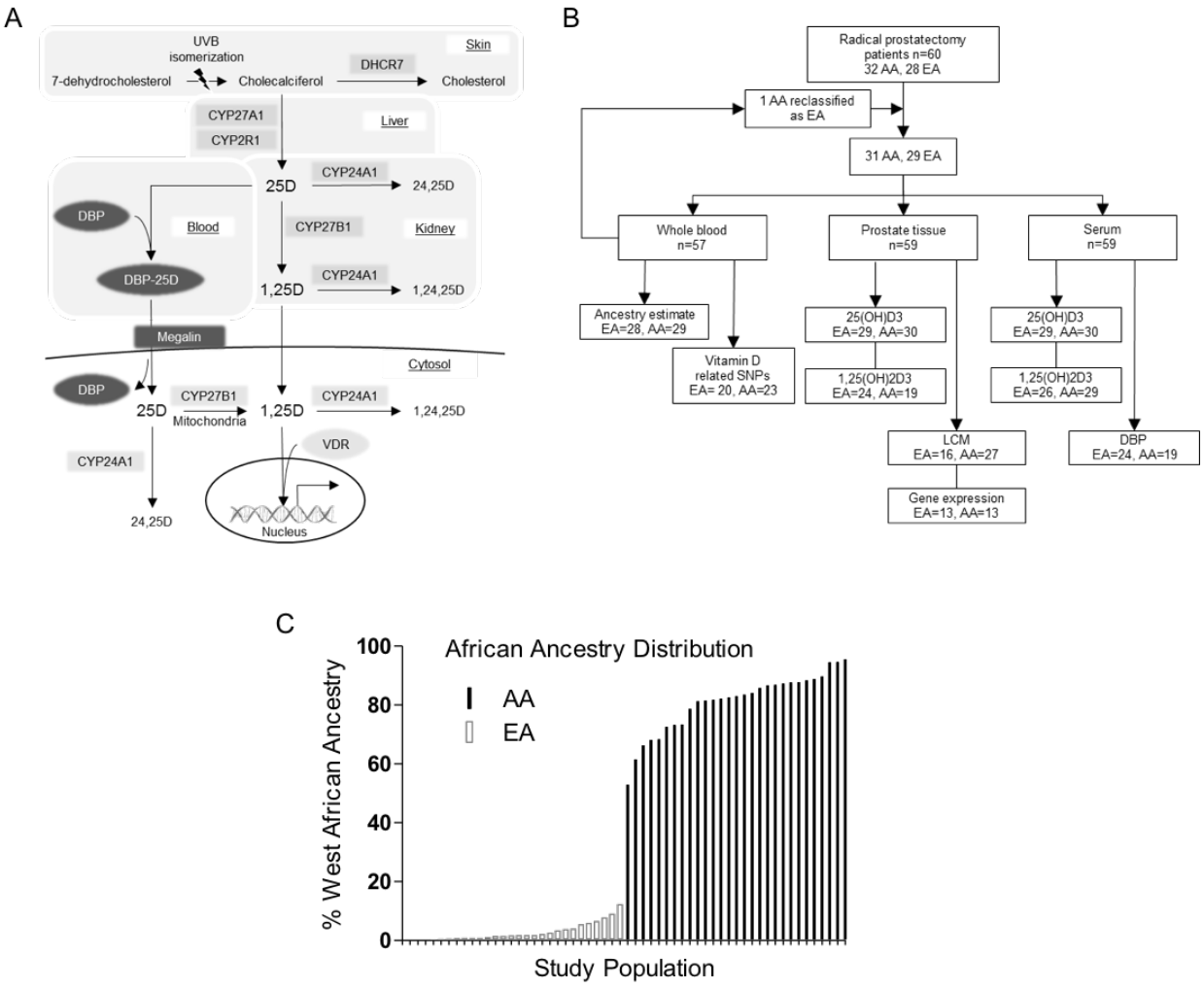

Figure 3. Analysis of the prostate-serum vitamin D axis in African-American and European-American prostate cancer patients.

A, A schematic of the vitamin D axis components that were assessed in the patients. **B,** Study design and patient numbers for each endpoint. **C,** A waterfall plot of West African ancestry percentage (Y-axis) for the patients (x-axis) as determined by ancestry informative markers.

	European American		African American		
	n[A]	Mean ± SD or [range][B]	n[A]	Mean ± SD or [range][B]	*P* value[C]
Age	29	63.6 ± 5.6	31	61.5 ± 7.2	0.22
%WA ancestry	28	2.9 [0.2 to 12.4]	29	81.0 [53.0 to 95.5]	<0.001
%Euro ancestry	28	93.1 [79.1 to 99.2]	29	14.3 [1.6 to 45.1]	<0.001
%NatAmer ancestry	28	4.0 [0.5 to 14.9]	29	4.8 [0.8 to11.3]	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

Abbreviations: 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

[A]Genotype data, Gleason score, or stage not available or not applicable to all individuals

[B]Mean and standard deviation reported for age and PSA, range reported for ancestries

[C]P values <0.1 considered significant, two sided student's t test for age, ancestry, and PSA; two tailed Fisher's exact test for season, tumor, and site comparisons.

[D]seasons categorized into high UVR: June to November, and low UVR: December to May

[E]tumor staging not available for 2 patients (1 AA, 1 EA)

Table 2. Population Characteristics

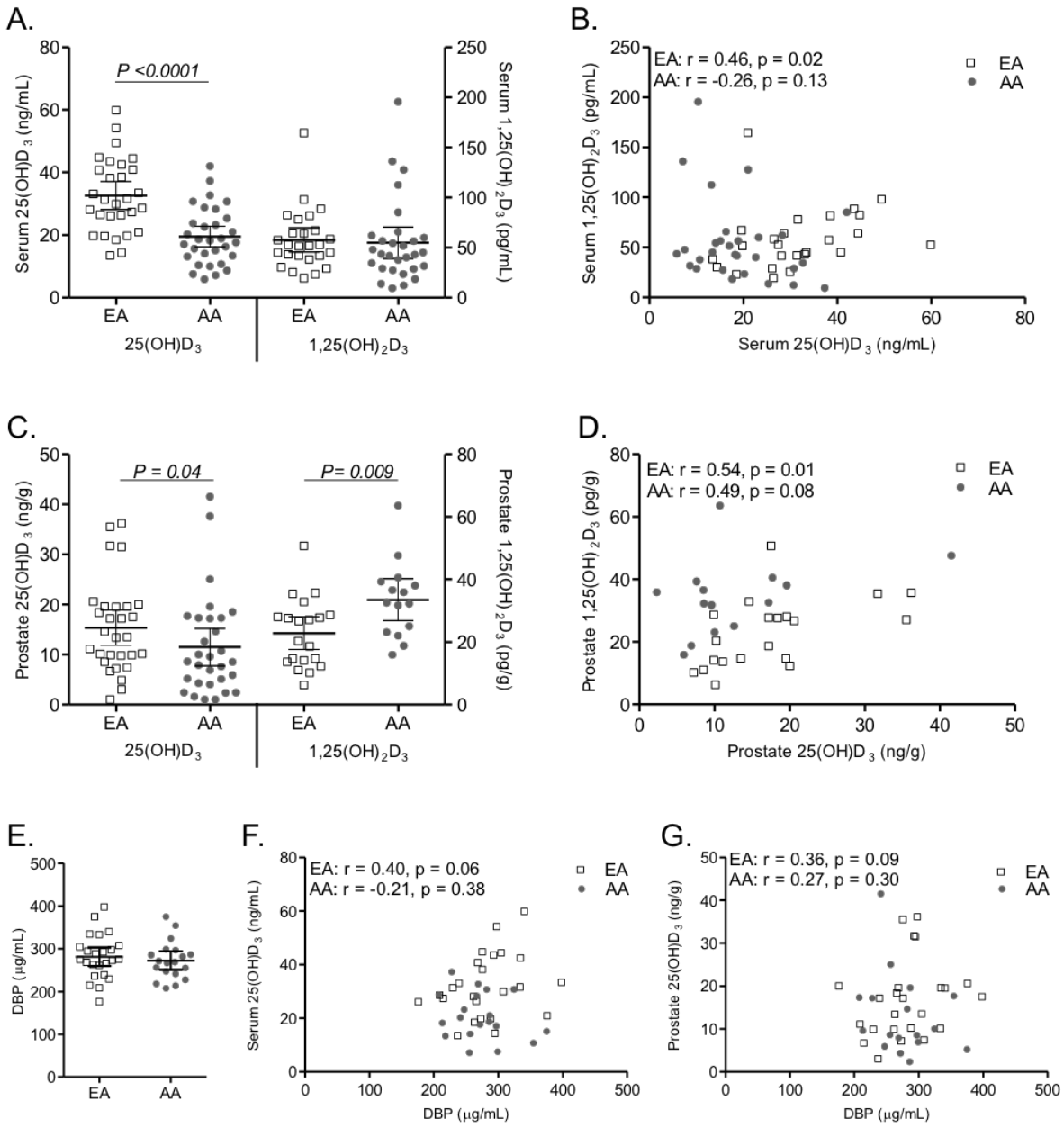

Figure 4. Radical prostatectomy patients of African ancestry had lower 25-hydroxyvitamin D in the serum, but higher 1,25-dihydroxyvitamin D in the prostate.

A, comparison of serum levels of 25(OH)D (European American (EA) n=29, African American (AA) n=30) and 1,25$(OH)_2$D (EA n=26, AA n=29) measured by uHPLC-MS-MS between radical prostatectomy patients of African (AA) and European (EA) ancestry. **B**, correlation between vitamin D metabolites 25(OH)D and 1,25$(OH)_2$D 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$(OH)_2$D (EA n=24, AA n=19) measured by uHPLC-MS-MS**. D,** correlation between prostatic levels of 25(OH)D and 1,25$(OH)_2$D (EA n=24, AA n=19). **E,** serum levels of 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) . A p-value <0.1 was considered significant and differences by ancestry were determined by two-sided Wilcoxon signed-rank test and correlations determined by Spearman's rank.

Extraction and measurement of 25(OH)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 (**Figure 5**). Like serum levels, prostatic 25(OH)D was lower in the AA group compared to EA group (AA: 11ng/g, 95%CI [8.4 - 15ng/g]; EA: 15ng/g, 95%CI [12 -18ng/g]; p =0.04) (**Figure 4C**). In contrast, the active hormone 1,25$(OH)_2$D was significantly higher in the AA group's prostate tissue compared to EA group's prostate tissue (AA: 34pg/g, 95%CI [27 - 40pg/g]; EA: 23pg/g 95%CI [18 - 28pg/g]; p =0.009) (**Figure 4C**). In all patients 25(OH)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 4D**). 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 that had detectable levels (data not shown).

Most 25(OH)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 (Bikle et al., 2009). 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 to the monoclonal assay (Nielson et al., 2016a; Powe et al., 2013). Serum levels of DBP did not differ between AAs and EAs (**Figure 4E)**. Correlations of DBP with serum and prostate 25D levels were significantly positive for EAs and trended negative for AAs, but did not reach significance (**Figure 4F-G**).

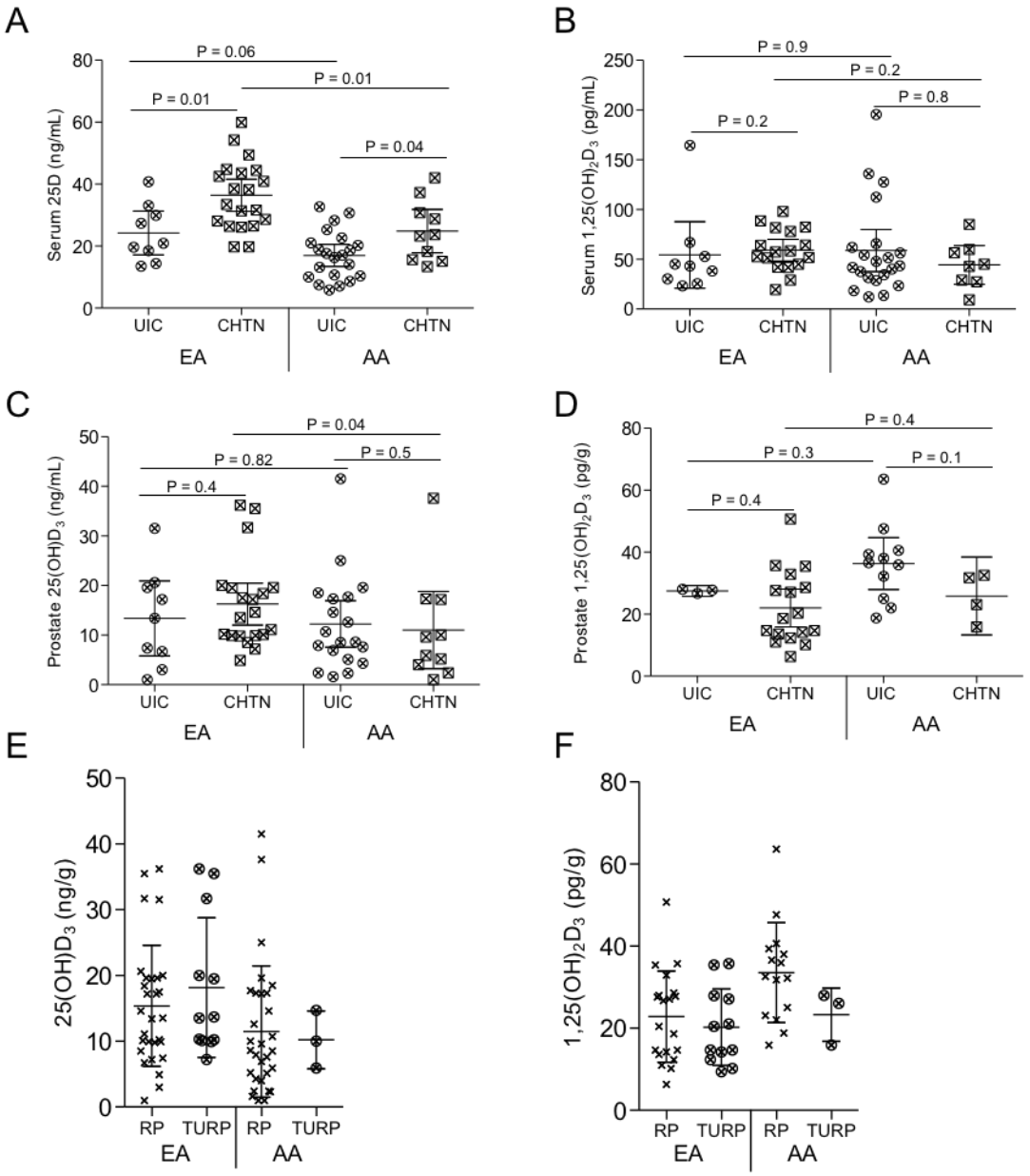

Figure 5. Quality control analyses for the vitamin D metabolite measurement.
Comparison of vitamin D metabolite levels in serum and prostate by collection site **A-D.** Serum and prostate levels of 25(OH)D and $1,25(OH)_2D$ were measured by uHPLC-MS-MS in serum and prostate tissue specimens collected at the University of Illinois at Chicago (UIC) or the Cooperative Human Tissue Network (CHTN) Western Division in Nashville, TN. **A,** Serum 25(OH)D by collection site in African Americans (AA) and European Americans (EA\). **B,** Serum $1,25(OH)_2D$, **C,** prostate tissue 25(OH)D and **D,** $1,25(OH)_2D$ did not differ by site of collection. **E-F**, comparison between radical prostatectomy (RP) and transurethral resection of the prostate (TURP) specimens. Comparison of RP and TURP 25(OH)D, **E**, and $1,25(OH)_2D$, **F**, in EAs and AAs. All comparisons made by two sided Wilcoxon signed-rank test.

ii. 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 (Lugli et al., 2015; Nonn et al., 2010). Gene expression was quantified by whole transcriptome amplification followed by Affymetrix Human Gene 1.0 ST array as described by others for prostate tissues(Erho et al., 2013; Karnes et al., 2013). 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, DHCR7* — was determined (**Figure 6A-F**). There was significantly higher expression of *VDR* in the AA population (Figure 6A). Of the vitamin D metabolism enzymes, only *CYP2R1* differed by ancestry group with AAs having significantly lower expression (**Figure 6B**). *CYP2R1* and *CYP27B1* both correlated with %West African ancestry in AAs, however, in the negative and positive direction, respectively (**Figure 6B-C**).

iii. 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 kidney that binds DBP to mediate internalization of 25(OH)D into the cytosol(Christensen and Birn, 2002). The role of extra-renal 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 7A**). 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 7B**). Comparison of vitamin D metabolites between serum and prostate showed that $1,25(OH)_2D$ did not correlate, suggesting active

transport and local 1α-hydroxylase activity by CYP27B1 rather than passive diffusion of $1,25(OH)_2D$ into the prostate (**Figure 7C**). Megalin protein was abundant in the prostate epithelium by immunofluorescent staining in both benign and PCa regions and was pre-membranous in some glands and cytosolic in others (**Figure 7D**).

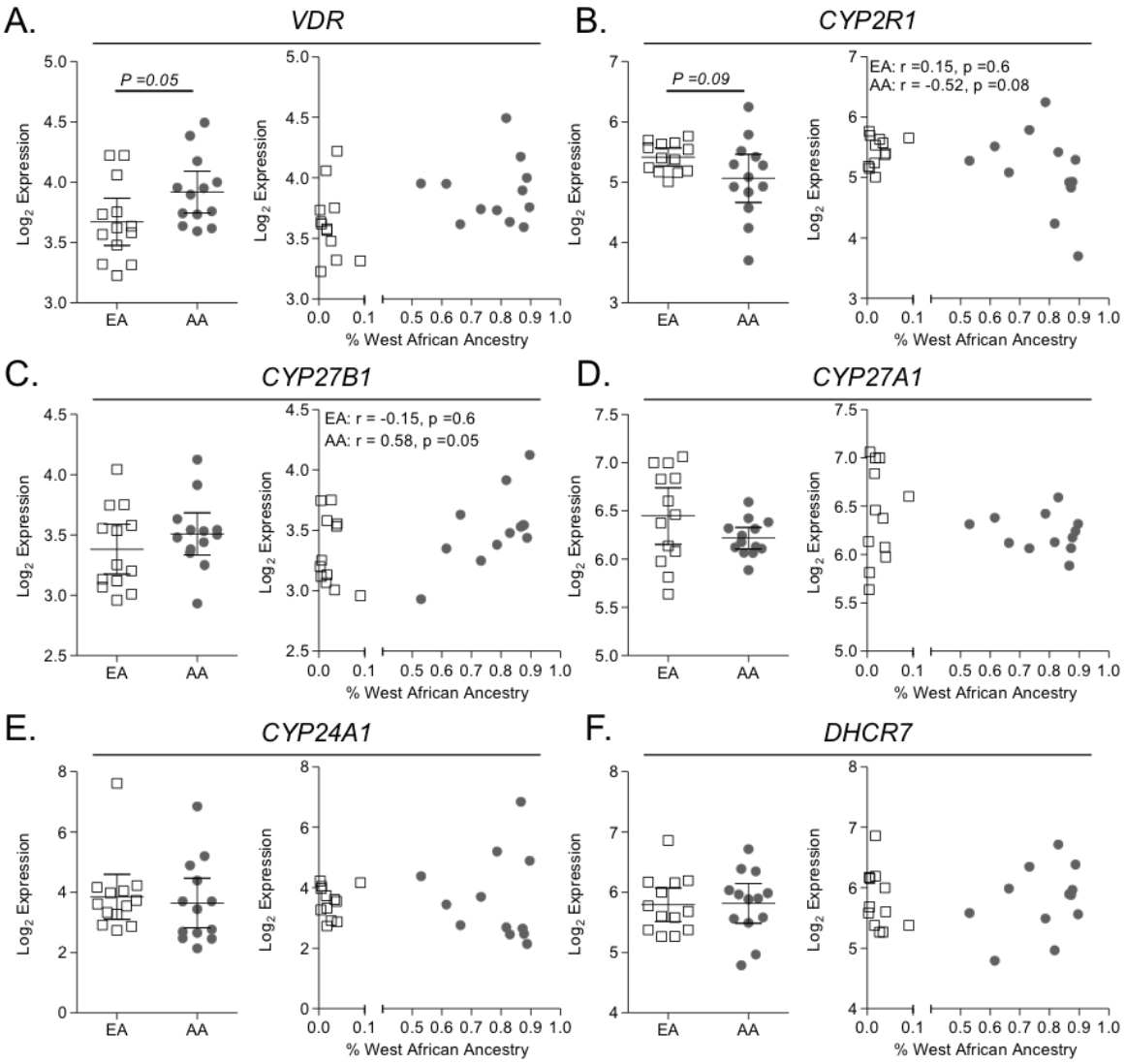

Figure 6. Expression of several vitamin D-related genes differ by ancestry and correlate to West African ancestry percentage.

Affymetrix array expression (reported as $\log_2$) 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). A p-value <0.1 was considered significant and differences between ancestry determined by two-sided Wilcoxon signed-rank test and correlations between gene expression and percent ancestry determined by Spearman's rank

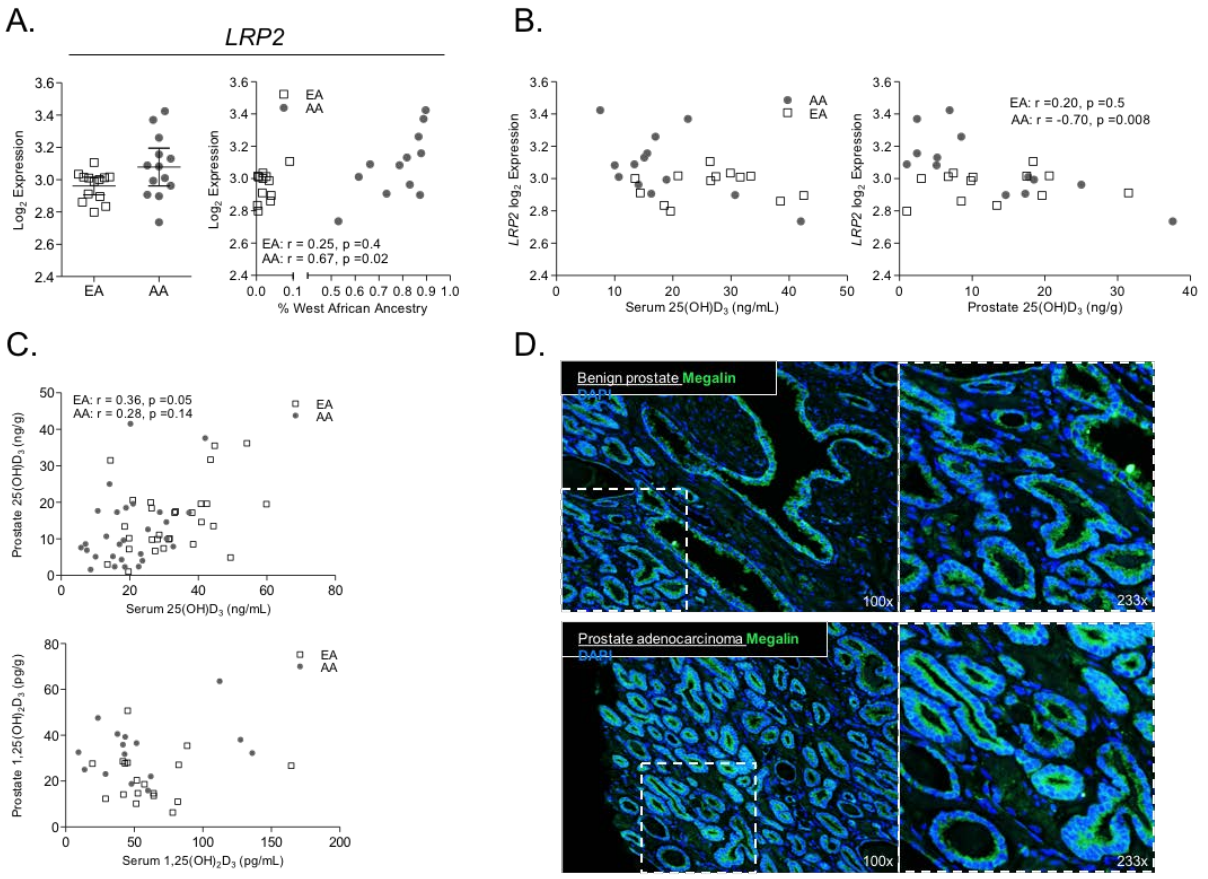

Figure 7. Megalin is present in prostate epithelium and gene expression of megalin (LRP2) correlates with ancestry and prostatic 25-hydroxyvitamin D.
A, Affymetrix array expression (reported as $\log_2$) of Megalin *(LRP2)* in benign epithelium collected from fresh frozen radical prostatectomy specimens 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 African ancestry for *LRP2*. A p-value <0.1 was considered significant and differences between ancestry determined by two-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.

iv. SNPs in vitamin D–related genes associate with phenotypes

Patients were genotyped for 38 SNPs in eight vitamin D metabolic and signaling pathway genes that were previously explored for associations with serum 25(OH)D levels in AA and EA men (Ahn et al., 2009; Batai et al., 2014). Several significant associations between SNPs and phenotypes were identified. SNP relationships to gene expression were analyzed for both self-regulation (cis-eQTL) and regulation of other genes (trans-eQTL) (**Table 3**). 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 *DHCR7*/*NADSYN1* were associated with several phenotypes including prostatic 25(OH)D levels and the minor allele A variant of rs382951 significantly reduced *DHCR7* expression levels (β =-1.68, p =0.002).

	SNP	Chromosome	Position[A]	Gene	MA[B]	MAF[C]	N	*β*	*P*
Vitamin D Binding Protein									
African Americans									
DBP	rs7041	4	72618334	*GC*	G	0.3	15	-0.068	**0.006**[D]
Vitamin D Metabolites									
European Americans									
Serum 1,25D	rs10877012	12	58162085	*CYP27B1*	T	0.34	19	0.142	0.03
Prostatic 25D	rs3794060	11	71187679	*DHCR7/NADSYN1*	C	0.47	19	0.238	0.02
Prostatic 1,25D	rs11234027	11	71234107	*DHCR7/NADSYN1*	A	0.12	13	0.241	0.009
African Americans									
Prostatic 25D	rs12800438	11	71171003	*DHCR7/NADSYN1*	A	0.48	21	-0.291	0.007
***trans*-eQTL**									
European Americans									
CYP2R1	rs731236 (*Taq*I)	12	48238757	*VDR*	C	0.31	8	0.236	0.03
CYP27A1	rs2282679	4	72608383	*GC*	C	0.31	8	-1.096	0.003
African Americans									
CYP27A1	rs731236 (*Taq*I)	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**									
African Americans									
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]

[A] Base-pair position (GRCh37), [B] Minor Allele (MA), [C] Minor Allele Frequency (MAF), [D]Statistically significant based on *P*-value cutoff for testing association.

Table 3. SNP associations with vitamin D binding protein and vitamin D metabolites levels and vitamin D pathway gene expression

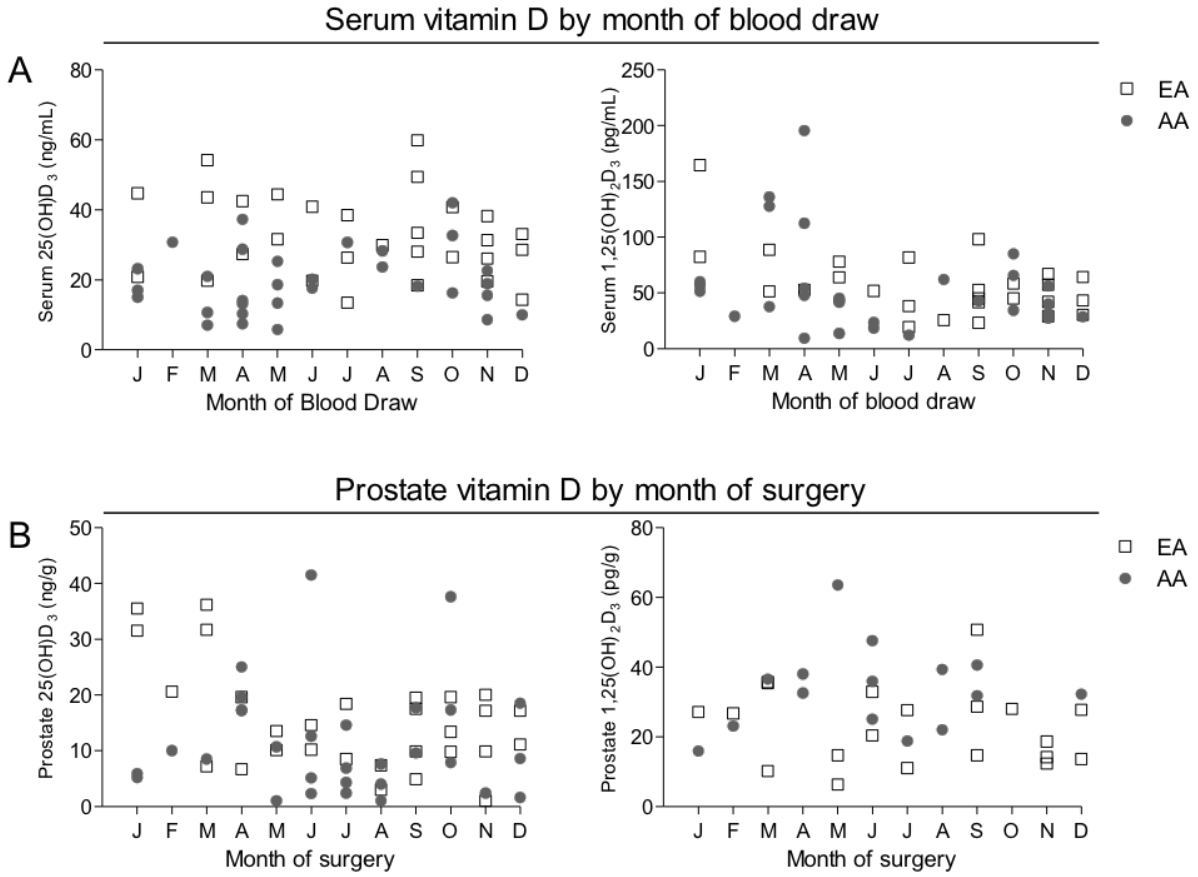

Figure 8. Serum and prostate vitamin D status stratified per month of blood draw and surgery. Comparison of vitamin D metabolite levels between European American (EA) and African American (AA) populations in serum and prostate by collection month**. A,** Serum 25(OH)D and 1,25$(OH)_2$D, by collection month in AAs and EAs. **B,** prostate tissue 25(OH)D and 1,25$(OH)_2$D by collection month.

D. 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 25D than EAs, which corroborates epidemiologic reports in larger patient populations (Ahn et al., 2009; Murphy et al., 2012; Schenk et al., 2014b). 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 lower 25(OH)D in the prostate compared to 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 intra-prostatic $1,25(OH)_2D$ levels are likely dependent on tissue 25(OH)D and not passive diffusion from the circulation as previously thought (Chun et al., 2014). 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 intra-prostatic 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 suggest 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 intra-prostatic 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 (Powe et al., 2013). However, serum DBP was not different in our cross-sectional group, which corroborates other recent studies (Nielson et al., 2016b) and associations between variants in *GC* and DBP serum levels in our study emulate those reported by others (Moy et al., 2014; Powe et al., 2013). 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 (Hilpert et al., 2002; Perez Bay et al., 2016). 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 extra-renal 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 (Ternes and Rowling, 2013). 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 WA ancestry in the AA patients. Skin pigmentation increases with West African ancestry and vitamin D synthesis depends on UVR penetration of the skin, therefore, vitamin D status negatively correlates with West African ancestry (Signorello et al., 2010). 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 suggests 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, presence of megalin in prostate epithelium, and correlation of megalin expression with tissue vitamin D metabolites in AAs points 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, 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 intra-prostatic gene expression of *VDR* and vitamin D metabolism enzymes also point 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 co-regulatory 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 1α-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 negatively correlated with West African ancestry. However, the biological significance of this observation remains unclear as extra-hepatic 25(OH)D synthesis has not been previously reported. Similarly, we observed an association between a SNP in DHCR7 that associated with gene expression of *DHCR7* suggesting cis-regulation via a functional SNP, however, extra-cutaneous 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 (Wagner et al., 2013) 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 to standard HPLC-MS and radioimmunoassay, uHPLC-MS-MS has superior sensitivity and specificity and can better differentiate between D_2 and D_3 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 under-representation 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 (Batai et al., 2016; Giangreco et al., 2015) in AA men (Hardiman et al., 2016). We examined gene expression solely in prostatic epithelium, which does not include immune cells or prostate stroma, which are essential modulators of inflammatory gene signaling (Giangreco et al., 2015). The activity of VDR was not assessed by the endpoints of our study, which is not only regulated by ligand but also transcriptional co-regulators that were not included here (Haussler et al., 1998).

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.

CHAPTER III: THE FREE HORMONE HYPOTHESIS, MEGALIN, AND ANDROGENS IN THE PROSTATE

A. Introduction

The previous chapter revealed novel findings regarding the associations between serum and tissue vitamin D metabolites between African American (AA) and European American (EA) prostate cancer patients. The unexpected finding that AAs had higher intra-prostatic 1,25D despite having lower serum 25D compared to EAs cannot be explained by passive diffusion alone and infers a mechanism of active transport of vitamin D into the prostate.

As detailed in **Chapter I,** megalin has been well characterized to carry out vitamin D uptake in the kidney and **Chapter II** is the first report of its presence in prostate tissue at both the RNA and protein levels. In addition to DBP, megalin can also bind and endocytose sex hormone binding globulin (SHBG), the carrier protein for testosterone (T) and other sex hormones in the blood. The finding that *LRP2* expression – the gene that encodes megalin – was inversely correlated with intra-prostatic 25D levels in AAs suggests megalin is upregulated in the setting of vitamin D deficiency in an ancestry-specific manner. This chapter will explore the new hypothesis that vitamin D deficiency in AAs increases intra-prostatic androgens via megalin-mediated endocytosis of T-SHBG (**Figure 9**).

The inter-play between the hormone receptors VDR and AR is currently unknown. Prostate tumors are initially dependent on androgens prior to progressing to castration resistance in advanced and metastatic disease (Feldman and Feldman, 2001). Because AR activity is dependent on DHT binding in early disease, intra-prostatic androgen levels have been studied as a possible correlate with prostate cancer outcomes. High intra-prostatic testosterone levels in needle biopsy cores have been reported to associate with higher Gleason score (Miyoshi et al., 2014). However, low serum testosterone has been attributed to promote high grade prostate cancer (Schatzl et al., 2001). Overall, there is a poor correlation between serum and prostate androgen levels (Cook et al., 2017) and tissue distribution and uptake of androgens is not well understood. Adding to the complexity, associations between serum androgens and prostate cancer risk are modified when levels of SHBG are adjusted for (Gann et al., 1996a). In the

context of ancestry-related disparities in prostate cancer, only two studies have directly compared intra-prostatic androgen levels between African American and Caucasian men and neither reported a difference in T or DHT concentration by ancestry, however, SHBG was higher in African Americans (Marks et al., 2006; Mohler et al., 2004).

The interaction between vitamin D and androgens in the prostate is limited to just a single study that reported DHT is required for 1,25D-mediated growth inhibition of LNCaP cells *in vitro* (Murthy et al., 2005). Since serum vitamin D and androgens do not correlate with intra-prostatic levels, and share megalin as a receptor for active uptake, the inter-play between the two hormones warrants study and may have implications for both normal prostate physiology and disease. The studies presented in this chapter propose a function for megalin in the prostate, provide a basis for DBP sequestration of 25D, quantify DHT in the prostate tissue of an ancestrally diverse group of prostate cancer patients, and explore intra-prostatic DHT-vitamin D metabolite and gene interactions.

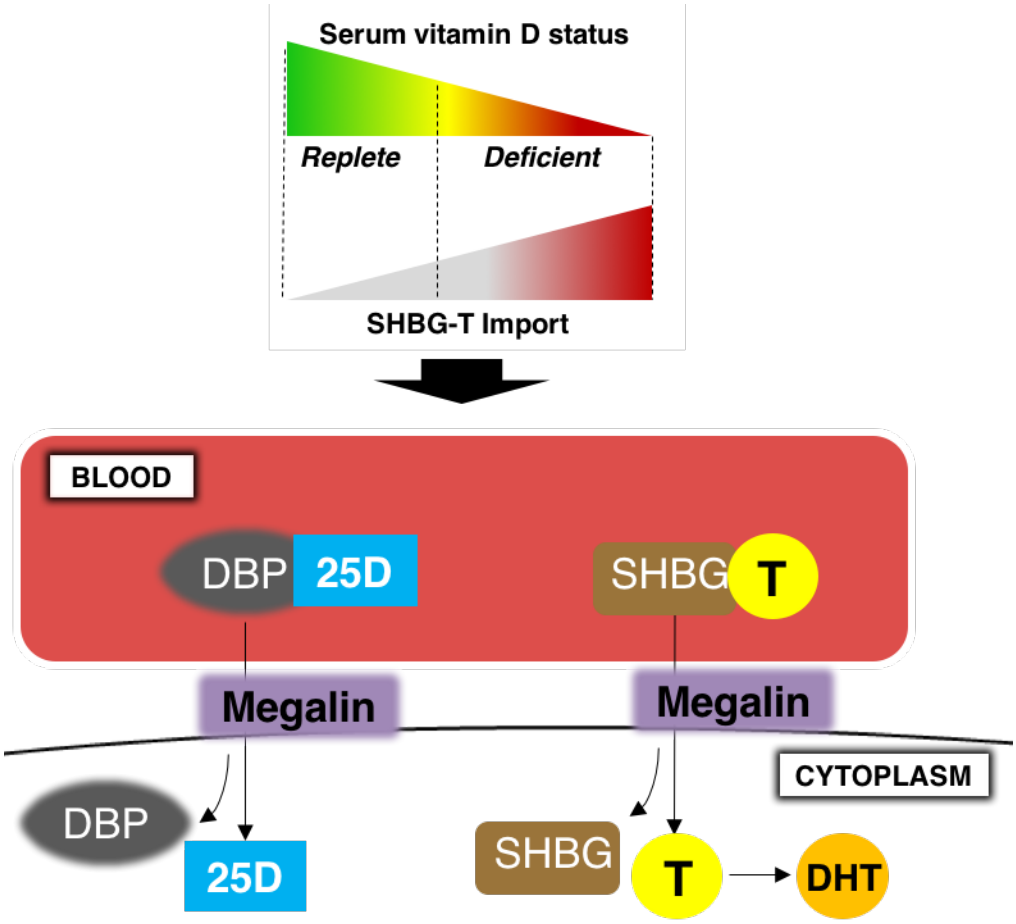

Figure 9. Vitamin D deficiency may increase testosterone import via megalin upregulation in African American men

B. Materials and Methods

i. Tissue microarray immunostaining and analysis

A formalin fixed and paraffin embedded human tissue microarray containing 118 prostate biopsy cores from 29 patients (20 AA, 9 EA) and consisted of at least 2 benign and cancer cores from each patient. Pathology of and mark-up of each core was completed by a board-certified pathologist. Sections of 5 microns were incubated with rabbit polyclonal anti-Lrp2/megalin antibody (ab76969) diluted 1:100 or rabbit polyclonal anti-VDR antibody (C-20) diluted 1:100 (abcam, Cambridge, UK), and mouse monoclonal anti-PanCK (AE1/AE3) diluted 1:2000 then incubated with secondary antibody AlexaFluor-488 goat anti-rabbit diluted 1:200 and AlexaFluor-555 goat anti-mouse diluted 1:200 (Life Technologies, Carlsbad, CA, USA), counterstained with DAPI and imaged on the PerkinElmer Vectra3. Epithelial areas were identified and segmented by PanCK marker automatically using PerkinElmer Inform software (version 2.3) and adjusted manually to ensure accuracy. Megalin and VDR fluorescence intensity were quantified and reported as average intensity per pixel of the segmented area of each core using Inform software.

ii. Cell culture

Primary prostate epithelial cells used for immunostaining and organoid culture were isolated from radical prostatectomy tissue as previously described (Giangreco et al., 2013; Nonn et al., 2006). Briefly, tissue from benign regions of the peripheral zone was collected according to an Internal Review Board-approved protocol. Tissue histology was confirmed by a board-certified pathologist. Tissue was digested in collagenase and cultured in either Prostate Cell Growth Media (Lonza) or MCDB-105 media (Sigma-Aldrich) to select for epithelial or stromal cell populations. Epithelial population purity was authenticated by expression of the epithelial specific markers *CK5, CK8, CK18,* and *p63* by RT-qPCR. Stromal cell purity was authenticated by lack of epithelial marker expression and expression of the stroma specific marker *TIMP3*.

For 3D organoid cultures, primary prostate epithelial cells were seeded in 33% Matrigel™ in Keratinocyte Serum Free Media (KSFM) supplemented with 5% Charcoal stripped FBS and 10nM DHT. Media was replaced every 3 days. All cells cultured in humidified conditions at 37°C and 5% CO_2.

iii. *in vitro* immunostaining and imaging

Cells derived from the benign area of an AA patient were grown on a chamber slide, fixed in 4% paraformaldehyde, and stained with guinea pig polyclonal anti-cytokeratins/keratins 8/18 antibody diluted 1:200 (American Research Products, MA, USA), rabbit polyclonal anti-Lrp2/megalin antibody (ab76969) diluted 1:100 then incubated with secondary antibody AlexaFluor-488 goat anti-rabbit diluted 1:200 and AlexaFluor-555 goat anti-mouse diluted 1:200 (Life Technologies, Carlsbad, CA, USA), counterstained with DAPI and imaged on the PerkinElmer Vectra3.

Organoids grown in 3D culture derived from the same patient were fixed in 4% paraformaldehyde and suspended in Histogel™ for paraffin embedding. Sections of 5 microns were stained with rabbit polyclonal anti-Lrp2/megalin antibody (ab76969) diluted 1:100 then incubated with secondary antibody AlexaFluor-488 goat anti-rabbit diluted 1:200 and counterstained with DAPI and imaged on the PerkinElmer Vectra3.

iv. 2D vitamin D treatment

Primary prostate stromal cells (referred to as megalin deficient cells) were cultured in MCDB-105 media containing 10%FBS and supplemented with 1nM DHT, 5µg/mL Insulin, and 1nM FGFb. Cells were grown to ~70% confluent and treated with ethanol, 1,25D (1, 10, or 50nM), or 25D (10, 50, or 100nM) for 16 hours and harvested into Trizol for RNA extraction. In FBS titrating conditions, primary prostate stromal cells were cultured in serum-free media, 0.1, 1, 5, or 10% FBS and treated with 10nM 25D. Cells were harvested into Trizol for RNA extraction after 16 hours of treatment and stored at -80°C. Activation of VDR was determined by *CYP24A1* induction quantified by RT-qPCR.

v. 3D organoid vitamin D treatment time course

Primary prostate epithelial cells were grown in 3D culture seeded at a density of $5x10^3$ cells/well in a 96-well ultra-low attachment microplate to establish organoids. After 7 days, media was replaced

with either serum-free media or media containing 5% charcoal stripped FBS and treated with 10nM 25D or ethanol. Cells were harvested after 0.5, 1, 4, or 16 hours of treatment by addition of Dispase (1U/mL) to dissociate Matrigel and then collected into Trizol and stored at -80°C until RNA extraction. Activation of VDR was determined by *CYP24A1* induction quantified by RT-qPCR.

vi. RNA isolation and RT-qPCR

RNA collected from experiments detailed above was extracted using the Trizol chloroform method according to the manufacture's specifications (Invitrogen). RNA quantity and quality were determined by the NanoDrop Spectrophotometer (Thermo Fisher Scientific). 500ng of RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacture's instructions and qPCR performed on the QuantStudio6 machine. *CYP24A1* expression was quantified using the forward primer 5'-GGCAACAGTTCTGGGTGAAT-3' and reverse primer 5'-ATTTGAGGACAATCCAACA-3', and normalized to the reference gene *HPRT* using forward primer 5'-TGCTGACCTGCTGGATTACA-3' and reverse primer 5'-CTGCATTGTTTTGCCAGTGT-3'. Fold-change was calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

vii. Intra-prostatic androgen quantification

Calibration curve: Standard compounds T and DHT (1 mg/ml in MeOH), internal standard (IS) d3T (0.1 mg/ml in ACN) were purchased from Cerilliant (Round Rock, TX, USA). Spiking solutions of T and DHT used to prepare calibrators were diluted with MeOH to 10 µg/ml, 5 µg/ml, 1 µg/ml, 500 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, and 6.25 ng/ml. The working solution of IS was prepared by diluting the original stock to a final concentration of 500 ng/ml with ACN. Nine calibrators: 0.0625, 0.125, 0.25, 0.5, 1, 5, 10, 50 and 100 ng/ml in MeOH were used to establish calibration curves. The final concentration of IS was 50 ng/ml. All solutions were stored at -40°C in amber vials. Curves were fitted by linear regression with a weighting factor of 1/x.

Tissue homogenization and extraction: Approximately 20 to 200mg of tissue from each patient was bead homogenized using the Mikro-Dismembrator II (Handelskontor Freitag, Germany), collected

into pre-weighed tubes chilled on dry ice, and resuspended in 5 volumes of 10% MeOH (m : v) immediately prior to extraction. Sample homogenate (500 µl) was transferred into glass tube, 10 µl of IS working solution was immediately spiked in. After mixing each sample was extracted 3 times, each time using 3 volumes of hexane : ethyl acetate (60 : 40, v:v) followed by vigorous vortexing (10 sec x 3). After vortexing samples were centrifuged at 2000 pm for 5 min, the organic layer from each extraction was collected, combined and brought to dryness under high purity nitrogen flow. The residue was reconstituted into 1 ml of MeOH : H2O (20 : 80, v:v) and subjected to solid phase extraction (SPE) using ISOLUTE C18 SPE cartridge (100mg, 1 ml) following vendor's protocol. Briefly, prior to sample loading, cartridges were activated with 3 ml of MeOH, and equilibrated with 3 ml of H_2O. Once samples were loaded onto the cartridges 2 ml of H_2O was used to wash. The target analytes were then eluted into 2 ml of MeOH. This elute was dried under nitrogen then reconstituted into 100 µl of MeOH for LC/MS analysis.

LC-MS/MS analysis: Quantification of T and DHT was achieved using SCIEX Qtrap 6500 spectrometer coupled with Agilent 1290 UPLC system. Five µl of sample was injected and eluted by Waters ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 x 100 mm) maintained at 45 degrees, at a flow rate of 450 µl/min. Elution started with 60% of mobile phase A (5% MeOH in H_2O, 0.1% FA), followed by a linear gradient increase of mobile phase B (ACN with 0.1%FA) from 40 to 80%. MS data were acquired by MRM scan at positive mode. The ESI spray voltage and source temperature was set at 5.0kV and 450 degrees. T, DHT and D3T were detected by monitoring their transitions to signature product ions 289>97 (T), 291>255 (DHT), and 292>97 (D3T) respectively. Data were analyzed using Analyst software.

viii. Statistical analyses

Comparison of megalin and VDR protein immunofluorescence by race and pathology in the TMA was by two-sided unpaired t-test. Spearman's rank coefficient is reported for correlations between protein immunofluorescence intensities. For comparison of androgen metabolite levels between the AA and EA groups in prostate tissue a two-sided Wilcoxon rank-sum test was used. Analysis of gene

expression data also used a two-sided Wilcoxon rank-sum test for comparisons between groups and the Spearman rank coefficient for correlations between gene expression and percent West African ancestry, European ancestry, age, and PSA. The mean and 95% confidence interval is reported for all data sets. A p-value <0.1 was considered statistically significant. Statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software).

C. Results

i. Megalin protein correlates with VDR and is down in cancer

In **Chapter II**, *LRP2* expression was quantified at the RNA level in benign tissue and positively correlated with percent West African ancestry. To validate this finding at the protein level and determine if megalin is altered in cancer a tissue microarray composed of 118 prostate biopsy cores from 29 patients (20 AA, 9 EA), with 2 cores from benign and cancer regions for each patient, was immunostained for megalin. Protein levels were quantified by fluorescence intensity in the epithelial regions and normalized to the epithelial marker Pan-CK (**Figure 10A**). The pathology and tissue mark-up of each epithelial region was determined by a board-certified pathologist. Megalin protein levels were significantly lower in the cancer tissue compared to benign in AA patients ($p = 0.006$) and did not differ in EA patients (**Figure 10B**). Megalin was also significantly lower in cancer compared to benign tissue when AA and EA patients were analyzed together ($p = 0.02$) and the effect was driven by the AA group as the TMA was unevenly weighted and contained more AA patients. Overall, megalin protein levels were lower in prostate cancer.

Next, protein levels of VDR were quantified in the TMA described above to validate the finding that expression of *VDR* was significantly higher in the benign epithelium of the AA group at the RNA level reported in **Chapter II**. Quantification of VDR by immunofluorescent staining revealed that levels were significantly higher in the AA group ($p = 0.08$ for all AAs, $p = 0.005$ when restricted to AAs with >80% West African ancestry) and validated the aforementioned gene expression data (**Figure 10C**). VDR did not differ between benign and cancer cores in either group, however, levels were highly variable in the cancer cores in both the AA and EA groups and indicative of possible dysregulation (**Figure 10C**). Additionally, VDR and megalin levels were significantly positively correlated in benign tissue in the AA group and the correlation was not present in cancer tissue or EA patients (**Figure 10D**). Taken together, although VDR is significantly higher in the benign epithelium of AAs, levels appear to be dysregulated in cancer and the correlation between VDR and megalin in AAs is suggestive of an ancestry-specific regulatory mechanism that is dysregulated in cancer.

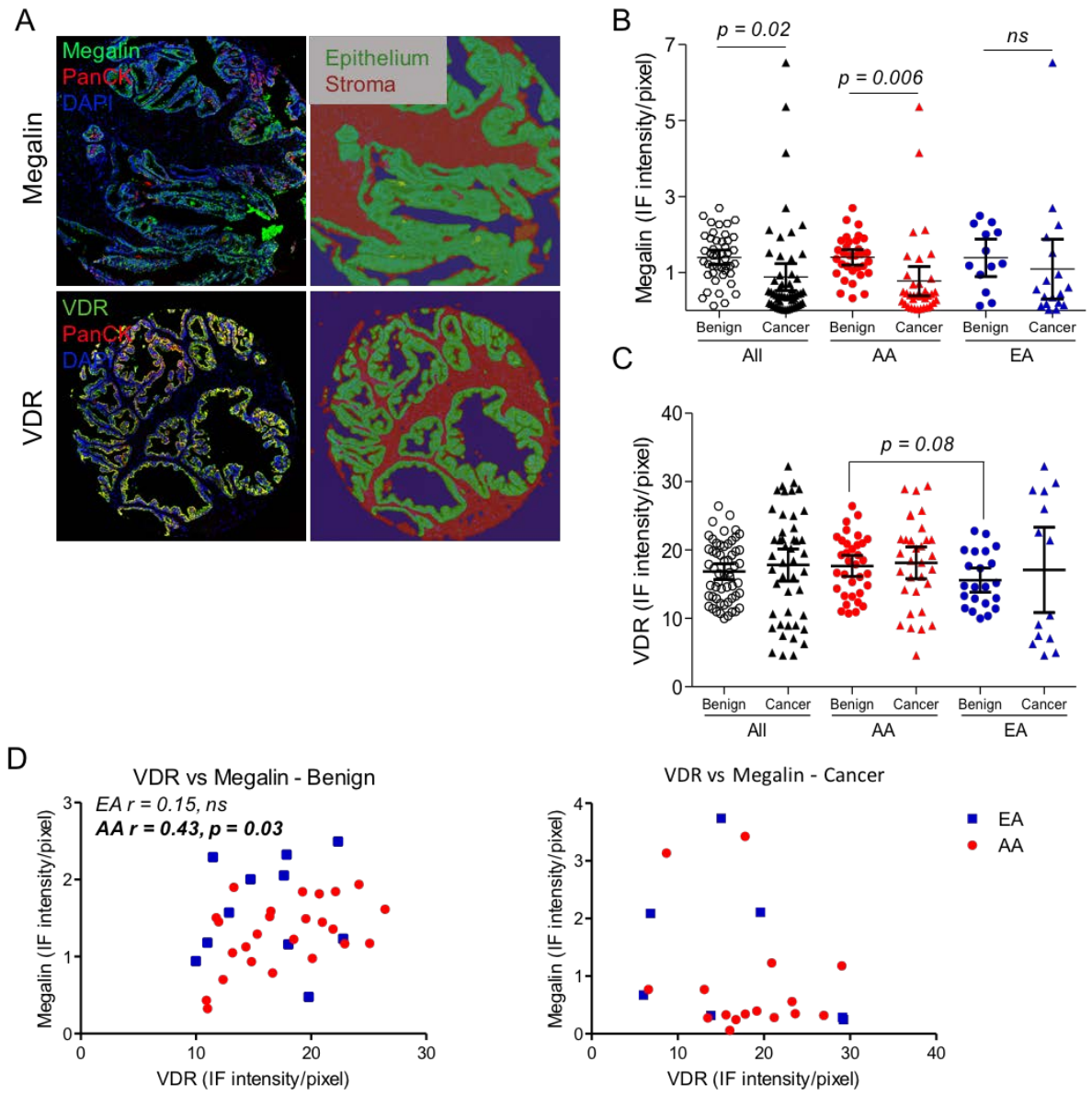

Figure 10. Megalin is down in prostate cancer.
A tissue microarray of 118 prostate biopsy cores from 29 patients (20 AA, 9 EA) was stained for megalin and VDR. **A**, Epithelial regions were identified by PanCK staining and benign and cancer regions determined by a board-certified pathologist. Immunofluorescence intensity per pixel was quantified for **B**, megalin and **C**, VDR. **D**, Correlation of megalin and VDR protein in benign (left) and cancer (right) regions.

ii. Extracellular sequestration of 25D by DBP

To determine whether DBP can sequester 25D outside of the cell, megalin deficient primary prostate stromal cells (PrS) were treated with 25D in media containing 10% fetal bovine serum (FBS); FBS is a rich source of DBP. The effect of DBP sequestration of 25D was first tested in megalin deficient cells to determine whether diffusion or other import mechanisms occur without megalin. Absence of megalin in PrS cells was confirmed by immunostaining (data not shown). Expression of *CYP24A1* was used to quantify VDR activation, which is highly induced upon 1,25D binding to VDR and, therefore, *CYP24A1* induction would imply successful cellular entry and conversion of 25D to 1,25D. In +FBS (DBP rich) conditions, VDR activation was not observed after treatment with 10nM, 50nM, or 100nM 25D for 16 hours. However, VDR was activated in a dose-dependent manner after treatment with 1,25D indicating a functional VDR and DBP specificity for 25D (**Figure 11A**). Treatment of megalin deficient cells with 50nM 25D in FBS titrating conditions restored VDR activation in media containing ≤1% FBS (**Figure 11B**). To verify DBP was specifically responsible for the observed results, experiments were repeated using 10nM 25D in serum free media containing 0.02g/L DBP and showed similar results (**Figure 11C**).

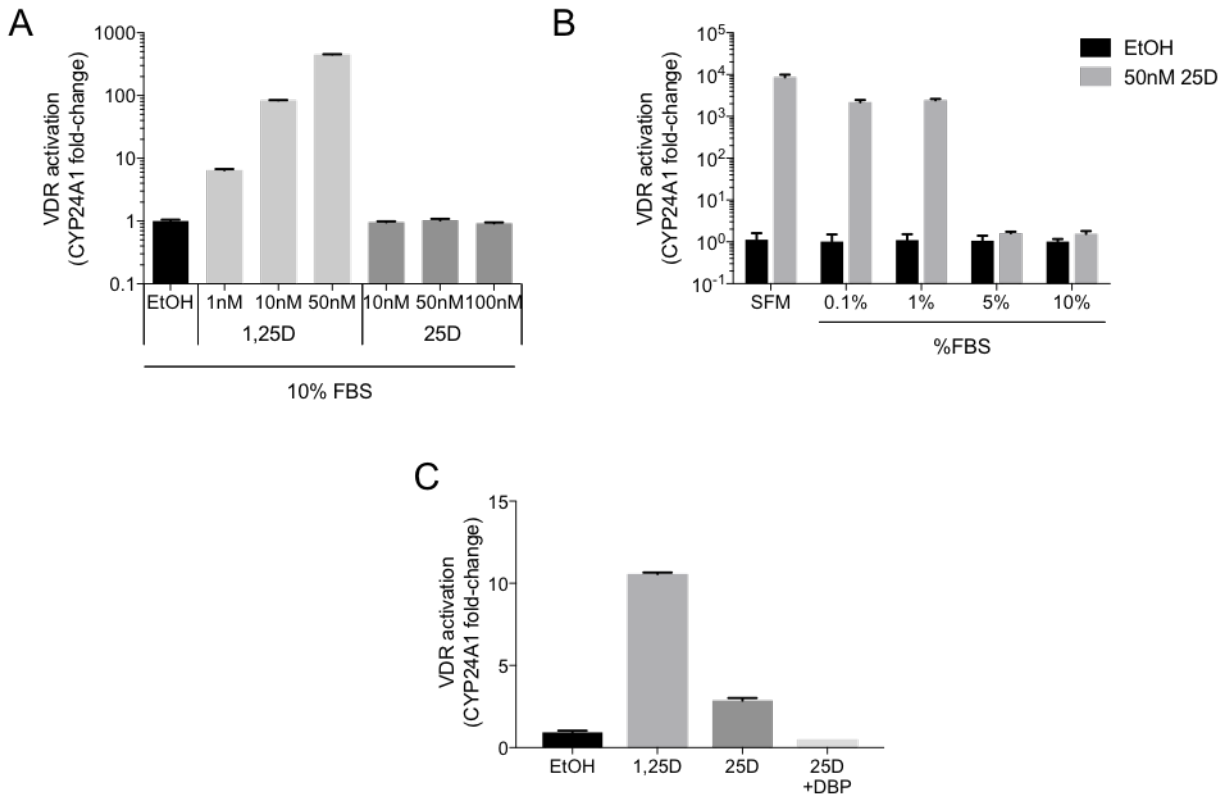

Figure 11. Extracellular sequestration of 25D by DBP in megalin deficient cells.
A, VDR activation after treatment with 1,25D or 25D in megalin deficient PrS cells in DBP rich conditions. **B**, VDR activation in megalin deficient PrS cells in DBP titrating conditions. **C**, VDR activation by 10nM 1,25D or 50nM 25D in serum free media +/-DBP.

iii. 25D-DBP uptake in primary prostate organoids

Next, the ability of megalin expressing primary prostate epithelial cells (PrE) to uptake 25D in the presence of DBP was tested. Although immunostaining of prostate tissue showed megalin protein is abundant in the epithelium, expression in 2D culture of primary prostate epithelial cells *in vitro* exhibited a different staining pattern (**Figure 12A**, top panel). Organoids derived from benign regions of radical prostatectomy tissue cultured in 3D conditions, however, exhibited a staining pattern that more closely resembled that seen in the tissue and were used for the following experiments (**Figure 12A**, bottom panel). To assess DBP sequestration of 25D in megalin expressing cells, PrE cells were cultured in 3D conditions for 7 days to form organoids and treated with 25D in +/-FBS conditions. VDR activation was assessed by quantification of *CYP24A1* induction at 0.5, 1, 4, and 16 hours after treatment (**Figure 12B**). Activation of VDR was more robust and occurred faster in +FBS conditions, thus providing evidence of cellular entry and metabolism of 25D in the presence of DBP (**Figure 12C**). Overall, megalin expressing organoids have faster activation of VDR when treated with 25D in DBP rich conditions, indicating a role for megalin in 25D uptake and metabolism in the prostate epithelium.

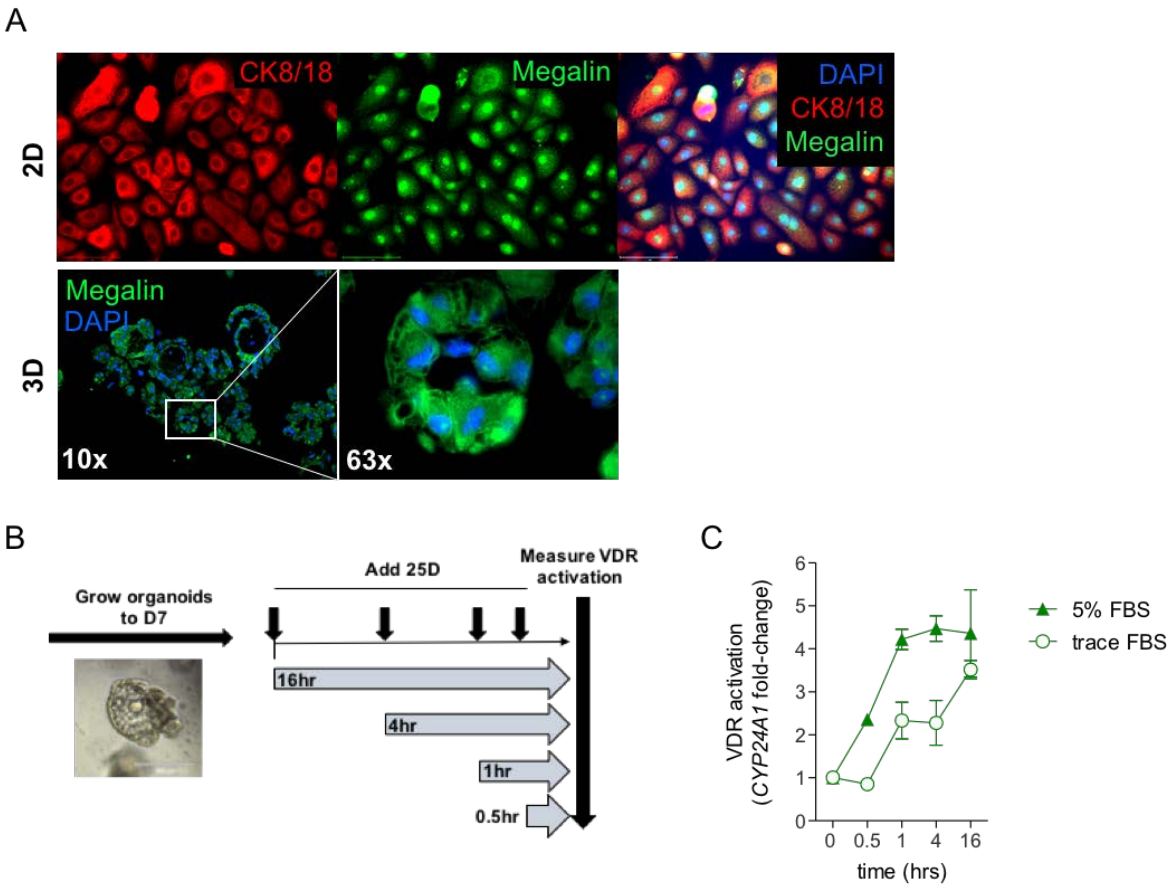

Figure 12. VDR activation occurs faster in prostate organoids expressing megalin. Immunofluorescent staining for megalin in primary prostate epithelial cells grown in **A,** 2D (top panel) and 3D (bottom panel) culture conditions. **B,** Experimental diagram of 25D treatment time course to asses VDR activation in megalin expressing organoids in the presence or absence of DBP. **C**, VDR activation in megalin expressing organoids in the DBP rich (+FBS) and DBP depleted conditions (serum-free).

iv. Intra-prostatic androgens in African American and European American prostate cancer patients.

To further test the hypothesis that vitamin D deficiency in AAs results in increased androgen import into the prostate, intra-prostatic androgen levels were quantified in the RP tissue used in **Chapter II.** The RP tissue was homogenized and testosterone (T) and dihydrotestosterone (DHT) were quantified by uHPLC-MS/MS (see methods). DHT was the more abundant metabolite in the prostate tissues as it made up the greater majority of intra-prostatic androgen in 24/29 EA men and 26/28 AA men (**Figure 13A**, top panel). The intra-prostatic ratio of T:DHT had no effect on total concentration of intra-prostatic androgen (**Figure 13A**, bottom panel). Comparison of T and DHT between the two groups revealed that DHT was significantly higher in AAs compared to EAs and levels of T did not differ (**Figure 13B**). To determine if the difference in DHT levels was due to differences in abundance of 5α-reductase, the enzyme that metabolizes T to DHT, expression of *SRD5A2* – the gene encoding for 5α-reductase – was compared between the groups from laser-capture microdissected gene expression data reported in **Chapter III**. Expression of *SRD5A2* did not differ by ancestry (data not shown), however, intra-prostatic T was inversely associated with *SRD5A2* expression in AAs while DHT did not associate with either group (**Figure 13C**). Overall, DHT is the more abundant androgen in the prostate and significantly higher in AAs compared to EAs. Although expression of 5α-reductase did not differ by ancestry, lower expression in the AA group associated with a higher T:DHT ratio, and suggests T is rapidly converted to DHT when the enzyme is more highly expressed.

Serum androgen levels have been shown to decrease with age even as PSA rises during prostate cancer progression (Zakaria et al., 2018). In our study population, intra-prostatic DHT did not correlate with age, PSA, percent West African ancestry, or percent European Ancestry (**Figure 14A**) and highlights the dissociation of serum clinical measures of T and PSA with tissue androgens. Additionally, levels of DHT did not differ when stratified by Gleason grade, however, DHT was significantly higher in RP

tissues compared to benign TURP specimens from patients with benign prostatic hyperplasia (**Figure 14B**).

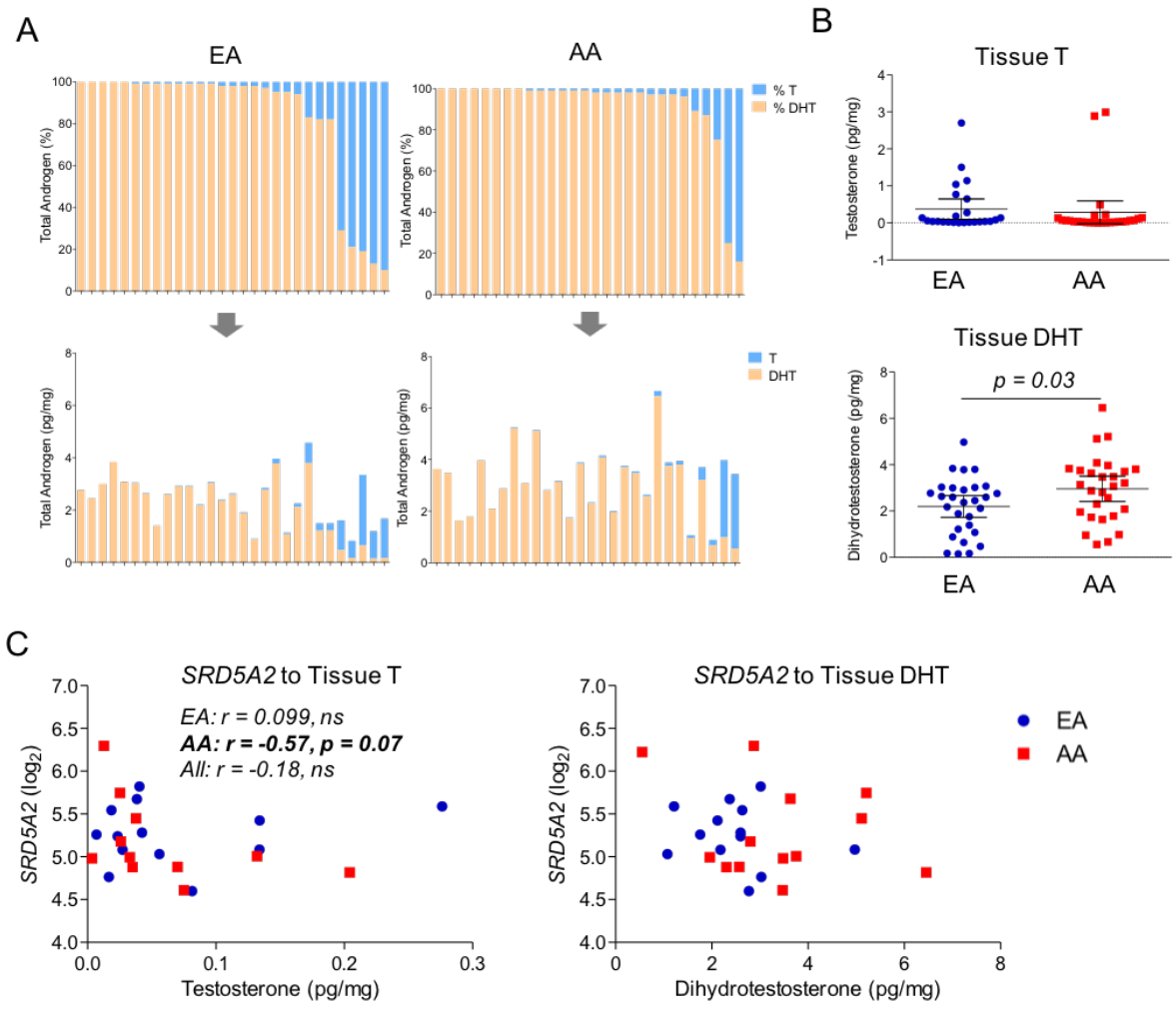

Figure 13. Intra-prostatic androgens levels in an ancestrally diverse group of prostate cancer patients.
Radical prostatectomy tissue from an ancestrally diverse group of prostate cancer patients was homogenized and testosterone (T) and dihydrotestosterone (DHT) were quantified by uHPLC-MS/MS. **A,** Total androgen distribution of T:DHT in each patient. **B,** Intra-prostatic T (top) and DHT (bottom) in EAs and AAs. **C**, Correlation of *SRD5A2* with intra-prostatic T (left) and DHT (right).

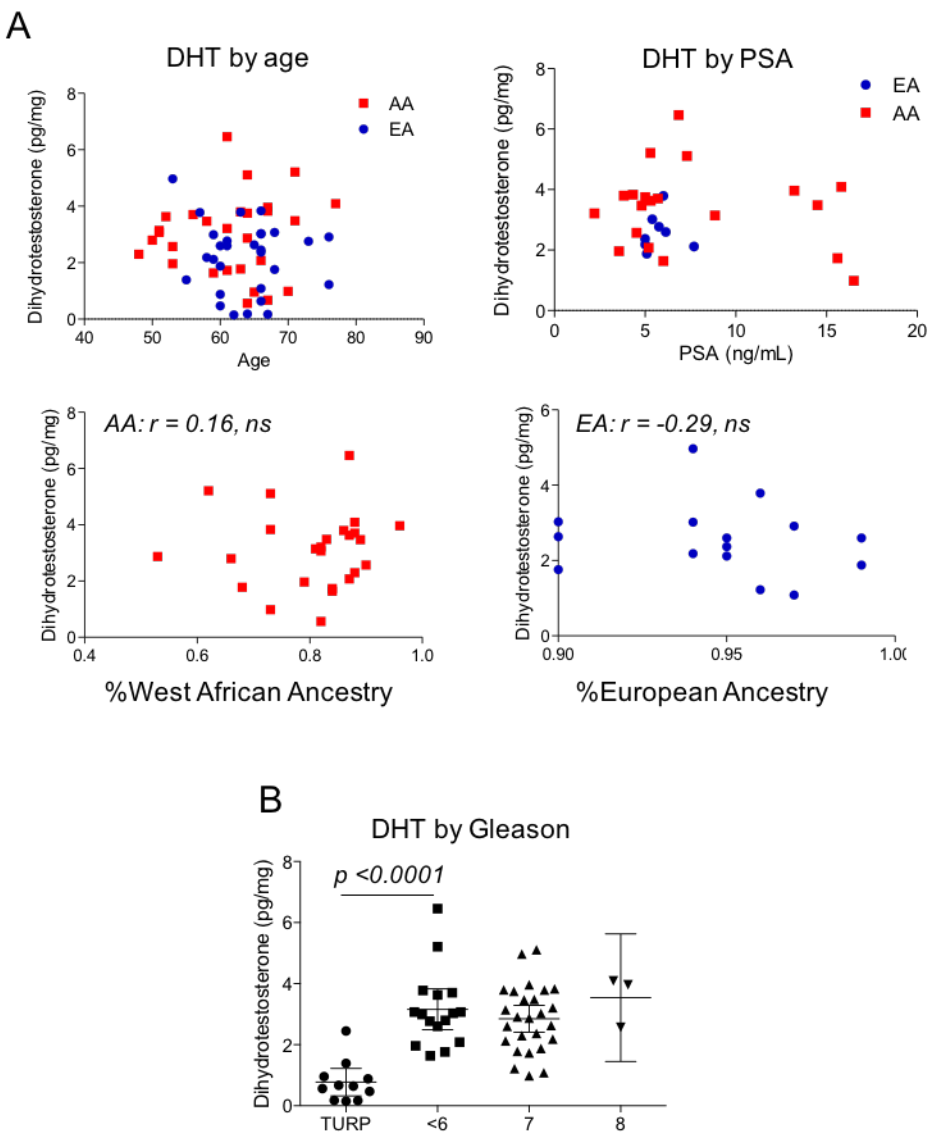

Figure 14. Intra-prostatic DHT does not correlate with ancestry.
A, Levels of DHT by age, PSA, percent West African ancestry, and percent European ancestry. **B,** DHT stratified by Gleason grade and compared to TURP specimens.

v. Intra-prostatic DHT and 1,25D positively correlate in African American men

As both DBP and SHBG share megalin as a ligand, import of vitamin D and androgens may be affected by one another. Comparison of intra-prostatic DHT to intra-prostatic vitamin D metabolites revealed DHT was significantly positively correlated with 25D and 1,25D in AAs (**Figure 15A,B**). This correlation was not present in the EA group. Additionally, analysis of serum 25D to intra-prostatic DHT revealed a significant inverse correlation (**Figure 15C**). However, this correlation was not observed when the population was analyzed by ancestry and may reflect the lack of vitamin D deficient EAs and replete AAs in the study population. Serum 1,25D did not correlate with intra-prostatic DHT (data not shown). **Chapter III** revealed that *LRP2* was inversely correlated with intra-prostatic 25D in AAs, but no association of *LRP2* with intra-prostatic DHT was observed here (**Figure 15D**). Interestingly, intra-prostatic DHT positively correlated with *VDR* expression EAs and the population as whole (**Figure 15E**). Overall, the inverse correlation of serum 25D with intra-prostatic DHT and positive correlation of intra-prostatic 25D with DHT suggests serum vitamin D deficiency could be a driver of higher prostate DHT levels. Additionally, these correlations underscore ancestry-specific differences in intra-prostatic vitamin D and androgens and allude to possible inter-play between the two metabolites.

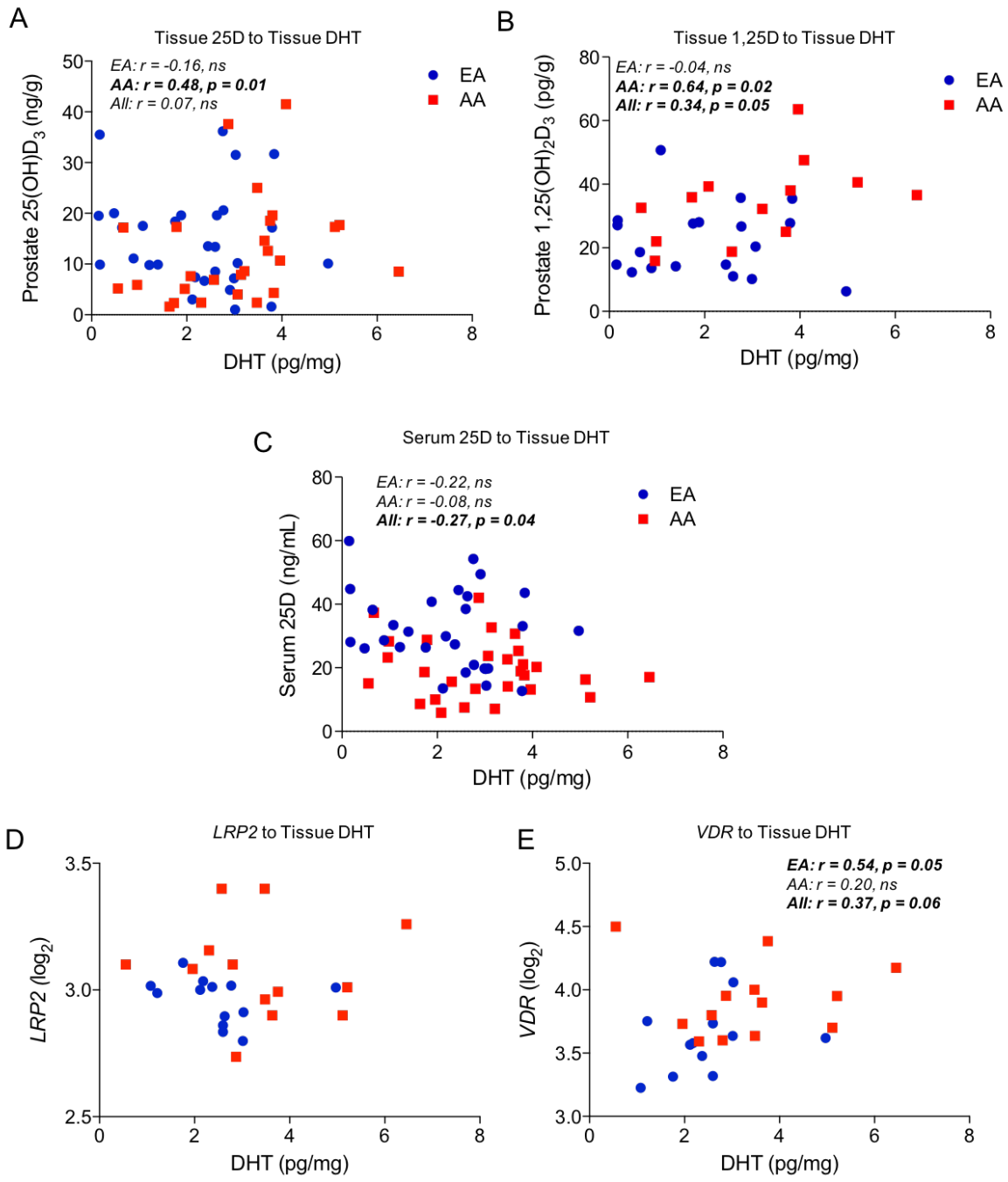

Figure 15. Serum and tissue vitamin D metabolite associations with intra-prostatic DHT. Correlation of intra-prostatic DHT with **A,** tissue 25D; **B,** tissue 1,25D; **C,** serum 25D. Correlation of laser-capture microdissected gene expression in the benign epithelium with intra-prostatic DHT of **D,** *LRP2* and **E**, *VDR*.

D. Discussion

This chapter provides evidence of DBP sequestration of 25D, megalin-mediated endocytosis, and increased intra-prostatic DHT in vitamin D deficient AAs. The presence and function of megalin has not been previously investigated in the prostate, and the studies within this chapter challenge the free hormone hypothesis and suggest an interplay between vitamin D and androgens with important implications for prostate cancer and vitamin D disparities.

The presence of megalin in prostate tissue was first reported by our lab (Richards et al., 2017), but its expression in cancer and relevance to prostatic uptake of DBP-25D has not been previously assessed. Megalin protein levels were significantly lower in prostate cancer tissue, a finding not previously reported. The temporality of megalin down regulation cannot be determined from our study but could be of importance regarding progression of castration resistant disease. While androgens are necessary to drive early prostate cancer, lower serum testosterone in late-stage disease has been associated with high grade prostate cancer (Schatzl et al., 2001). Loss of megalin in early disease may eliminate a mechanism of androgen import and further exacerbate decreased androgen availability by the prostate, thus providing a selection pressure towards androgen independence.

The mechanism of androgen and vitamin D import into the prostate has not been explored and the free hormone hypothesis is thought to determine hormone bioavailability and consequent downstream activity. In the present chapter, we validated 25D sequestration by DBP, but challenge the notion that it is not bioavailable by demonstrating uptake in prostate organoids. Inhibition of VDR activation in megalin deficient cells after treatment with 25D in the presence of DBP demonstrates extracellular sequestration of vitamin D by DBP and suggests active transport is required for tissue uptake of vitamin D. In PrE-derived organoids expressing megalin, VDR activation was more robust and occurred faster in the presence of DBP and hints at a potential mechanism of endosomal trafficking of DBP-25D that streamlines into vitamin D metabolism and/or VDR ligand binding. Megalin is rapidly recycled from endosomes at the plasma membrane and may facilitate cytosolic accumulation of its ligands (Perez Bay et al., 2016). Whether megalin-mediated endocytic vesicles are trafficked to the mitochondria, which harbor

enzymes to activate 25D to 1,25D is currently unknown and warrants further study. Whether megalin is necessary for DBP-25D uptake needs to be confirmed in using a knockdown model. Additionally, primary cells are heterogeneous in nature, and while the effect was observed in cells derived from one patient validation in other patients is necessary.

We successfully showed 25D-DBP activation of VDR in megalin expressing organoids, but whether the same holds true for testosterone and AR activity remains to elucidated. The interplay between androgens and vitamin D has not been studied other than one observation of decreased 1,25D growth inhibition in the absence of DHT in LNCaP cells (Murthy et al., 2005). Here, we present the first study to quantify both vitamin D and androgen metabolites in the prostate tissue. In our study population, AAs had higher intra-prostatic DHT, which positively correlated with tissue 25D and 1,25D levels. The association was ancestry-specific as it was not observed in the EA group, however, AAs in our study population were almost entirely vitamin D deficient and measurements should be repeated in a vitamin D replete population to discern between ancestry and deficiency. Similarly, serum 25D inversely associated with intra-prostatic DHT in the population as a whole, but the association was lost when analyzed by ancestry. This could be due again, in part, to the incomplete distribution of vitamin D status within our population.

The association between serum 25D and tissue DHT is intriguing, but the mechanism of interaction, if any, is currently speculative and warrants further study. Testosterone is rapidly converted to DHT in the prostate, as evidenced by the inverse correlation between intra-prostatic testosterone and *SRD5A2* expression, and DHT levels were ~5 to 20 fold higher than intra-prostatic testosterone in our patient tissues. DBP and SHBG are reported to have similar binding affinity for megalin (Hammes et al., 2005). Therefore, if megalin is the de facto regulator of both vitamin D and androgen import into the prostate then the possibility of competition for binding and uptake exists. If one assumes no difference in binding affinity for megalin between DBP and SHBG, then high serum 25D conditions would result in lower SHBG-T uptake and therefore lower intra-prostatic DHT. Conversely, serum vitamin D deficiency would result in increased testosterone uptake and higher intra-prostatic DHT. If megalin is indeed

upregulated in conditions of serum deficiency, as reported in **Chapter II**, the effect would enhance the aforementioned uptake of testosterone into the prostate.

Overall, **Chapter III** provides evidence of megalin function in the prostate and shows megalin expression is required for VDR activation when 25D is sequestered by DBP. Additionally, we report that DHT is significantly higher in the prostate tissue of AA men and levels correlate with tissue vitamin D metabolites. Taken together, megalin may be a central player in the inter-play between serum and tissue androgen and vitamin D actions in the prostate, and its function presents an attractive avenue for future studies in prostate cancer disparities due to its potential ancestry-specific regulation by vitamin D status.

CHAPTER IV: CONCLUSIONS

Presently, vitamin D status is assessed through serum measurement of 25D and acquired during routine blood draw. The studies presented here are the first report of vitamin D metabolite distribution in the prostate tissues of an ancestrally diverse group of prostate cancer patients. The finding that African American men have higher intra-prostatic active hormone 1,25D, despite being vitamin D deficient in the serum, is not only surprising but presents a paradox for cancer disparities that challenges our current understanding of vitamin D status.

Differences in intra-prostatic vitamin D levels are particularly relevant in the context of ancestry-related disparities in prostate cancer outcomes and vitamin D deficiency. There is strong evidence that vitamin D has anti-cancer actions *in vitro* and *in vivo*, and deficiency in African Americans has been hypothesized as a biological contributor to increased prostate cancer risk and more aggressive disease. However, there is much controversy and confusion in the epidemiologic data as evidence of negative, null, and even positive associations between high versus low quantiles of vitamin D status and increased prostate cancer risk have been reported. The finding that AAs had higher intra-prostatic 1,25D and higher VDR compared to EAs presents a paradox. Anti-cancer actions of vitamin D are mediated through 1,25D activation of VDR, therefore, one would expect higher levels of both 1,25 and VDR in the prostate to confer adequate, if not increased, chemoprotective benefit; yet AAs still have an increased risk of prostate cancer and mortality.

The combination of higher intra-prostatic 1,25D and VDR in AAs also demonstrates a clear ancestry-specific difference in the prostate vitamin D-axis that is further bolstered by correlations of vitamin D-related gene expression – *CYP27B1* and *LRP2* – with percent West African ancestry. CYP27B1 is the 1α-hydroxylase that metabolizes 25D to 1,25D and higher expression may increase conversion of 25D to 1,25D and contribute, in part, to the higher 1,25D in AA tissue. Gene expression in the benign epithelium was quantified in the present study, but translational regulation and enzyme activity of CYP27B1 likely affect 1,25D production as well and should be assessed in the future. *LRP2* encodes for the transmembrane receptor megalin for which the carrier protein of 25D, DBP, is a ligand. Presence

of megalin in the prostate tissue is reported here by RNA expression of *LRP2* in the epithelium and protein at the plasma membrane confirmed by immunostaining of prostate tissue. Expression of *LRP2* was not only positively correlated with West African ancestry, but also inversely correlated with intra-prostatic 25D. This correlation was unique to the AA group and suggests an ancestry-specific mechanism of megalin upregulation when intra-prostatic 25D is low; perhaps to ensure adequate levels of vitamin D in the prostate. Regulation of megalin is not well understood in general, and the findings presented here provide rationale for future exploration of megalin function in the prostate.

The presence of megalin in the prostate suggests uptake of 25D in the prostate is not limited to passive diffusion and more nuanced and regulated than previously thought. This is further supported by the lack of association between serum and tissue 1,25D metabolite levels as one would expect levels to correlate if 1,25D was passively diffused from serum to tissue. Intra-prostatic 25D and 1,25D levels significantly correlated and support a mechanism of local metabolism of 25D to 1,25D as the source of intra-prostatic 1,25D. However, 25D must be imported prior to conversion to 1,25D and nearly all 25D is bound to DBP the serum, therefore, this finding directly challenges the free hormone hypothesis, which states that the activity of a hormone is due to its free, rather than bound fraction. The importance of free, or "bioavailable", vitamin D is currently a topic of debate; however, the evidence reported here suggests a method of active transport must occur to enable uptake of vitamin D into the prostate even when serum levels are low.

Indeed, a mechanism of active transport was observed in organoids derived from primary prostate epithelial cells and supports megalin activity. Organoids expressing megalin treated with 25D in the presence of DBP had faster activation of VDR than those treated in the absence of DBP; megalin deficient cells had no VDR activation. This supports a process of active 25D uptake in the prostate that may be megalin-mediated, however, inhibition or knockdown experiments should be completed to confirm megalin necessity. Additionally, cubilin shares several ligands with megalin and cubilin dysfunction has been demonstrated to increase 25D loss to the urine in humans (Nykjaer et al., 2001); therefore, necessity and sufficiency of cubilin in the uptake of 25D-DBP should also be assessed.

Megalin-mediated endocytosis has been demonstrated to traffick through early endosomes for apical recycling or directed to the lysosomal pathway (Perez Bay et al., 2016). Since VDR activation occurred faster in organoids when 25D was bound to DBP, 25D-DBP may traffick directly to the mitochondria where CYP27B1 resides and facilitate a streamlined process for 25D metabolism and subsequent 1,25D activation of VDR. However, this remains to be determined as 25D-DBP intracellular trafficking in the prostate remains unknown.

The rationale for studying the role of megalin for vitamin D transport in the prostate was based on its well-established role in kidney reuptake of vitamin D from the glomerular filtrate. However, in addition to DBP, megalin has numerous ligands including the androgen carrier protein SHBG, which is particularly relevant to the prostate as androgens are required for normal glandular function and drive early prostate cancer. The ancestry-specific upregulation of megalin in the setting of vitamin D deficiency may not only increase 25D-DBP endocytosis but also T-SHBG uptake into the prostate and, therefore, may solve the paradox that AAs have higher 1,25D and VDR in the prostate tissue yet have higher incidence and mortality of prostate cancer. In the present study, intra-prostatic T and DHT were quantified by uHPLC tandem MS-MS and revealed that AAs do indeed have significantly higher levels of DHT in the prostate tissue compared to EAs. Additionally, intra-prostatic DHT and 25D were positively correlated in the AA group and suggest DBP and SHBG import is linked, perhaps through megalin. Interestingly, expression of *VDR* significantly positively correlated with intra-prostatic DHT levels. It is tempting to speculate of an inter-play or cross-regulation between AR and VDR, perhaps involving megalin, but the direction of regulation cannot be inferred from the data presented here.

Overall, the studies presented here are steeped in the context of ancestry and provide a novel view into the prostate vitamin D-axis. Indeed, numerous ancestry-specific differences and correlations were observed, which highlights the importance of studying cellular function and disease in the context of ancestry and evolution. These ancestry-specific correlations are likely a direct consequence of evolutionary selective pressures and the balance between vitamin D production and UV radiation exposure are theorized to be the primary drivers of the distribution of skin pigmentation in humans

(Jablonski and Chaplin, 2010). Humans evolved from equatorial Africa where sunlight exposure is most abundant and dark skin pigmentation is theorized to have evolved as protection from UV-radiation alongside the gradual loss of protective fur. As humans migrated out of sun-rich Africa to northern latitudes with less sun exposure, vitamin D function in immunity and reproductive success selected for lighter skin pigmentation to increase vitamin D synthesis (Mead, 2008).

Given the pleiotropic effects of vitamin D in humans, it would be naïve to assume skin pigmentation as the only physiological adaptation to migratory chanages in UV exposure over evolutionary time. The carrier protein for 25D, DBP, exists in three major isoforms and is unevenly distributed by ancestry. Curiously, the isoform with the highest affinity for 25D is most abundant in AAs, while the isoform most abundant in EAs has the lowest affinity (Arnaud and Constans, 1993). DBP is necessary to prevent 25D loss in the urine and the abundance of a higher affinity variant in sun-soaked, vitamin D replete Africans seems contrary. Perhaps this high affinity variant functions to prevent overexposure of vitamin D to the tissues, and megalin is the regulatory gate-keeper.

Hormone carrier proteins not only prevent loss of hormone to the urine, but sequester hormones in the circulation to prevent tissue overexposure. In this respect, the free hormone hypothesis is backwards and should be modified to state that the activity of a hormone is dependent on the highly-regulated uptake of the bound fraction of a hormone, rather than passive diffusion of the free or unbound fraction. The free hormone hypothesis was created from observations that SHBG limited tissue uptake of T (Mendel, 1989). However, AR activity was recently reported to be induced 3-fold higher when T was bound to SHBG compared to T alone (Li et al., 2016). The similarities between T versus T-SHBG activation of AR in this study, and 25D versus 25D-DBP activation of VDR reported here are especially intriguing for the relationship between vitamin D deficiency and increased prostate cancer risk in AAs given that SHBG and DBP share megalin as a ligand and megalin expression is correlated with West African ancestry.

Overall, the studies presented here demonstrate that the relationship between serum and tissue hormones are more complex than previously thought and introduce a role for megalin-mediated

endocytosis of vitamin D and androgens in the prostate. The differences in intra-prostatic 1,25D and DHT between AAs and EAs challenge the relevance of serum assessment of hormones, suggest vitamin D deficiency is related to increased androgens in the prostate, and highlight ancestry-specific differences in the prostate vitamin D-axis. The implications of the research are aimed at the understudied African American minority group and are of immediate relevance to public health regarding vitamin D supplementation. The thesis presents the new hypothesis that vitamin D deficiency in AAs results in increased intra-prostatic androgens as a biological explanation for the vitamin D and prostate cancer disparity and provides a starting point for further exploration.

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APPENDIX

Copyright Clearance Center

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Order Date: 03/27/2018

Customer Information

Customer: Zachary Richards
Account Number: 3001266296
Organization: Zachary Richards
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Phone: +1 (312) 996-7441
Payment Method: Invoice

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APPENDIX (continued)

Determination Notice - Revised
Research Activity Does Not Involve "Human Subjects"

May 3, 2013 **(Revised November 15, 2013)**

Larisa Nonn, PhD
Pathology
840 S. Wood Street, Rm. 130 CSN
M/C 847
Chicago, IL 60612
Phone: (312) 355-3726 / Fax: (312) 996-4812

RE: Research Protocol # 2013-0341
"Disparate Vitamin D Activity in the Prostate of Men with African Ancestry"

Sponsor:	Department of Defense
PAF#:	2012-00487
Grant/Contract No:	PC121923
Grant/Contract Title:	Disparate Vitamin D Activity in the Prostate of Men with African Ancestry

Dear Dr. Nonn:

The UIC Office for the Protection of Research Subjects received your "Determination of Whether an Activity Represents Human Subjects Research" application, and has determined that this activity **DOES NOT meet the definition of human subject research** as defined by 45 CFR 46.102(f)/ 21 CFR 50.3(g) and 21 CFR 56.102(e)/ 38 CFR 16.102(f).

DOD Human Research Protection Officer (HRPO) concurrence: November 6, 2013

You may conduct your activity without further submission to the IRB.

If this activity is used in conjunction with any other research involving human subjects or if it is modified in any way, it must be re-reviewed by OPRS staff.

APPENDIX (continued)

Determination Notice
Research Activity Does Not Involve "Human Subjects"

February 27, 2018

Zachary Richards
Pathology
840 S. Wood St.
CSN 130, M/C 847
Chicago, IL 60612
Phone: (312) 996-7441

RE: Research Protocol # 2018-0086
"Vitamin D and Prostate Cancer Disparities in African American Men"

Sponsor:	NIH
PAF#:	00374242
Grant/Contract No:	1F31CA221073-01A1
Grant/Contract Title:	Vitamin D and Prostate Cancer Disparities in African American Men (PI of Grant: Zachary Richards)

Dear Zachary Richards:

The UIC Office for the Protection of Research Subjects received your "Determination of Whether an Activity Represents Human Subjects Research" application, and has determined that this activity **DOES NOT meet the definition of human subject research** as defined by 45 CFR 46.102(f)/ 21 CFR 50.3(g) and 21 CFR 56.102(e)/ 38 CFR 16.102(f).

Specifically, this project utilizes existing frozen prostate tissues and cell lines currently in the lab. The tissue is left over from a previous project. The tissue was originally acquired from a biorepository and deemed "not human subjects research" per determination #2013-0341. The tissue contain no patient identifiers and numbered from biorepository. The cells are from #2011-1138 "The University of Illinois Hospital and Health Sciences Systems (UI Health System) Biorepository: Accepting Samples from IRB Approved Research Studies." The cells contain no patient identifiers and numbered from biorepository.

You may conduct your activity without further submission to the IRB.

If this activity is used in conjunction with any other research involving human subjects or if it is modified in any way, it must be re-reviewed by OPRS staff.

All the documents associated with this proposal will be kept on file in the OPRS and a copy of this letter is being provided to your Department Head for the department's research files.

If you have any questions or need further help, please contact the OPRS office at (312) 996-1711 or me at (312) 355-2908.

VITA

Zachary A. Richards

EDUCATION

University of Illinois at Chicago, *Chicago, IL*
Ph.D., Pathology, *2018*

Northwestern University, *Chicago, IL*
Post-Bacc., Biological Sciences, *2013*

University of Wisconsin-Madison, *Madison, WI*
B.S., Biochemistry, *2011*

PUBLICATIONS

Richards Z, Marsili J, McCray T, Manlucu J, Zenner M, Garcia J, Voisine C, Murphy A, Abdulkadir S, Prins G, Nonn L. Prostate stroma supports branching of human prostate organoids and maintains the tissue phenotype of tumoroids. (*In Review*).

Dambal S, Baumann B, McCray T, Williams L, **Richards Z**, Prins G, Nonn L. The miR-183 family cluster contains a secondary transcription site and induces oncomiR-like changes in zinc and prostate growth. *Sci. Rep.* (2017) 9;7(1):7704. PMID: 28794468

Dambal S, Giangreco AA, Acosta AM, Fairchild A, **Richards Z**, Deaton R, Wagner D, Vieth R, Gann PH, Kajdacsy-Balla A, Van der Kwast T, Nonn L. (2017) microRNAs and DICER1 are regulated by 1,25-dihydroxyvitamin D in prostate stroma. *J Steroid Biochem Mol Biol.* (2017) 167:192-202. PMID: 28089917

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Lugli G, Kataria Y, **Richards Z,** Gann P, Zhou X, Nonn L. Laser-capture Microdissection of Human Prostatic Epithelium for RNA Analysis. *J Vis Exp*. (2015) 105. PMID: 26651078

PROFESSIONAL RESEARCH EXPERIENCE

WIL Research Laboratories, LLC, *Skokie, IL (acquired by Charles River Laboratories Apr. 2016)*
Scientist II – Jun. 2013 to Aug. 2014
Scientist I – Aug. 2011 to Jun. 2013

FELLOWSHIPS & AWARDS

NRSA (F31) Individual Predoctoral Fellowship (2018-2020)
CCTS Pre-doctoral Education for Clinical and Translation Scientists Fellowship (2017-2019)
Honorable Mention Award Winner, UIC College of Medicine Research Forum (2011)

LEADERSHIP, CERTIFICATIONS, AND PROFESSIONAL MEMBERSHIPS

Graduate Student Council – Department of Pathology Representative (2016-2017)
Workshop, NIH BD2K Initiative, Purdue (2016)
Member – SBUR (2015-2018)
Lean Six Sigma Green Belt – Asher Associates (2013)

Presentation, "Building a better prostate in a dish: Prostate stroma alters branching of human prostate organoids and maintains transcriptional phenotype of tumoroids." *PCF Young Investigator Tumorigenesis Working Group*, Feb. 14, 2018. Webinar.

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

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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

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