Examination of Vitamin D Disparities in African American and Caucasian Prostate Cancer Patients and Cells

BY

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B.S. Iowa State University, 2012

THESIS

Submitted as partial fulfillment of the requirements
for the Masters of Science in Pathology
in the Graduate College of the
University of Illinois at Chicago, 2015

Chicago, Illinois

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ACKNOWLEDGEMENTS

First and foremost I have to thank Dr. Larisa Nonn. Thank her for her guidance during my time in the lab, her advising with all my projects and this thesis, and for creating an environment where I felt secure and free to explore. I would also like to give a tremendous thanks to the other members of my committee, Dr. Maarten Bosland and Dr. Alan Diamond, for their help navigating my graduate degree and for being outstanding Directors of Graduate Study, who I always knew had my best interest in mind.

Additionally, I of course have to thank all the members of the Nonn lab who helped teach me, give me feedback and guidance, and who made a terrific work environment. Thank you to Dr. Giovanni Lugli, who patiently and joyfully advised me with all my work. Thank you to Shea Dambal, who remained calm and helped me manage when things went awry, and who provided laughs and lightheartedness. Thank you to Dr. Brittney Mihelich, Shaozia Lin, Shuangping Zhao, and LaTanya Williams, for contributing to a warm and enjoyable work environment, and for your help.

I must also extend that thanks beyond the Nonn lab to the entire Department of Pathology. I feel very fortunate to have been a part of a department that fostered collaboration and comradery amongst all the labs and members, and I am thankful for the friendships that developed.

Finally, I have to thank my family and friends. My dear loved ones who support and encourage me through all my life's adventures. You know who you are, and I would never have accomplished this (or anything) without you. I especially have to thank Thomas, for his patience, support, and help with my graduate work and this thesis, and for encouraging me to always follow my heart.

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LIST OF ABBREVIATIONS

1,25(OH)D	Calcitriol, Active Vitamin D
25(OH)D	Circulating Vitamin D
3D	Three Dimensional
AA	African American
AR	Androgen Receptor
CO_2	Carbon Dioxide
CRPC	Castration Resistant Prostate Cancer
CT	Cholera Toxin
EA	European American/Caucasian
EGF	Epidermal Growth Factor
EtOH	Ethanol
FBS	Fetal Bovine Serum
H&E	Hematoxylin and Eosin
HBS	Hepes Buffered Saline
LCM	Laser Capture Microdissection
MCDB	Spz cell culture media
PCR	Polymerase Chain Reaction
PIN	Prostatic Intraepithelial Neoplasia
PrE	Primary Prostate Epithelial Cells
PrEBM	PrE cell culture media
R1881	Androgen
RA	Retinoic Acid
ROS	Reactive Oxygen Species
RT-qPCR	Real Time Qualitative PCR

LIST OF ABBREVIATIONS (continued)

RXR	Retinoid X Receptor
SNP	Single Nucleotide Polymorphism
Spz	Primary Prostate Stromal Cells
UIC	University of Illinois at Chicago
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element

SUMMARY

African American and African-Caribbean men have the highest rates and poorest outcomes of prostate cancer of any population worldwide. African American men are 60% more likely to develop prostate cancer than American men of European decent, and 240% more likely to die from the disease. Additionally, African American men have significantly lower levels of vitamin D, in large part due to decreased endogenous production of active vitamin D during exposure to UVB.

Vitamin D has been extensively studied and shown to have anti-inflammatory and chemopreventative effects in tissues through the body, and in the prostate specifically. Here we looked at the disparities of vitamin D status between African American and European American men in clinical samples. We also used cell culture to further examine whether there are prostate-specific disparities in the effect of vitamin D between African American and European American men. What we found is a decrease in response to active vitamin D in stromal prostate cells derived from African American men compared to cells derived from European American men.

Beyond disparities, we also examined the effects of cross-talk between the different cell types of the prostate. The two major cells types that make up the prostate gland are stromal and epithelial cells, which signal to one another to influence development, maintain the microenvironment of the prostate, and potentially influence cancer development. Although prostate cancer arises from the epithelial cells, there is substantial evidence that the micro-environment of the prostate and reciprocal communication between the cell types is important for prostate development, and development of prostate cancer. To study the effects of communication between the cell types we used a co-culture of stromal and epithelial cells that resulted in luminal differentiation of basal epithelial progenitor cells. Though this differentiation effect

was expected, we saw no significant difference in differentiation when these cultures were treated with vitamin D, which was contrary to our hypothesis and requires more observations to understand this lack of effect.

CHAPTER 1:

Background and Introduction

Prostate

Prostate Histology

The prostate is a glandular, encapsulated exocrine organ that sits at the base of the bladder and wraps around the male urethra[1–3]. Ducts in the prostate gland are formed by three types of epithelial cells: luminal cells form the interior of the duct and secrete prostatic fluid, basal cells rest between the luminal cells and basement membrane, and rare neuroendocrine cells[4]. Outside of the ducts, the musculature of the gland is formed by stromal cells[5]. The prostate consists of three zones: the central, peripheral and transitional zones[1]. Prostate cancer (PCa) generally arises from the epithelial cells of the peripheral zone[6], though it is not currently known which specific type of epithelial cell PCa originates from. In contrast, benign prostatic hyperplasia, a common but non-malignant condition often arises from the transition zone[7].

Cross-talk between the stromal and epithelial cells of the prostate is a current topic of much interest and exploration. Unique stem cells for the basal and luminal epithelia of the prostate may exist to self-maintain each distinct population, but each type of stem cell may have the capacity of producing all prostate cell types[8]. Recent research in the mouse prostate indicates a symbiotic relationship between the stroma and epithelia of the prostate, with important signaling from the epithelial cells to the stroma, and vice versa[8]. For example, stromal factors such as Wnt and TGF-β signal to epithelial cells to maintain homeostasis in a healthy prostate, or conversely initiate tumorigenic transformation when inappropriately upregulated[9,10]. Additionally, stromal growth factors have been shown to play a role in maturation and AR expression of the epithelial cells[11]. In the reverse direction, mouse prostate epithelial cells have been shown to produce sonic hedgehog (SHH), which impacts the stromal cells of the prostate and is

implicated in prostate development and androgen production. SHH production in the prostate is unique to epithelial cells, whereas the gene activated downstream of SHH in a paracrine fashion, *Gli1,* is expressed solely in the prostate stroma[4]. This indicates that reciprocal communication between both populations of cells are crucial for the development and maintenance of the prostate.

Prostate Cancer

PCa is the second most common cancer in men in the United States[12] . The American Cancer Society estimates that in 2015 there will be 220,800 new cases, which accounts for 26% of new cancer cases in men this year[12]. They also estimate 27,540 deaths due to PCa this year, which accounts for 9% of all cancer deaths in men and makes PCa the second leading cause of male cancer death[12]. Two of the biggest risk factors for PCa development are age and Sub-Saharan African ancestry[13]. Currently, one in seven men will develop PCa during their lifetime, and 97% of these cases will be in men over the age of 50[12].

Due to recent advances in early detection and screening of PCa that allows identification of the disease when it is still localized, the 5 year survival rate is over 99%. However, this survival rate drops down to 28% once the tumor has metastasized[12]. In the early stages of the disease, PCa has few symptoms and therefore frequent screening is important for prevention of advanced metastatic disease[14]. Because this disease is so prevalent and has such a stark contrast in survival rates based on the stage of the disease, screening and prevention are highly desirable approaches for management of the disease.

Cellular and molecular changes during prostate carcinogenesis often mimic those in development, therefore understanding the embryonic development of prostate aids in understanding PCa development and will be briefly covered here. Development of the human prostate begins during late embyogenesis[7]. The gland forms from ductal budding from the urogenital sinus epithelium and is stimulated by the presence of androgen in utero[3,7]. However, androgen is not the only hormone or chemical mediator of development. Both estrogen and retinoic acid (RA) have been shown to play crucial roles in prostate development as well[3]. Additionally, signaling pathways such as Wnt, SHH, and fibroblast growth factor

(FGF) are active in prostate development[3,7]. SHH signaling operates in a negative-feedback loop with FGF[3], whereas Wnt operates through β-catenin. The loss of any of these signaling pathways leads to defective development of the prostate[7].

Initially in the development of PCa, as in prostate development, tumors are highly dependent on androgen signaling, which drives the growth and survival of the tumor[3]. At this stage, androgen deprivation therapy can be useful for treatment of the disease. However, over time many PCa tumors become independent of the presence of androgen, which is known as castration-resistant prostate cancer (CRPC). There seems to be several causes of castration resistance in these cells resulting in various forms of constitutive androgen signaling, including androgen receptor (AR) gene mutations, amplification of AR, or alterations in the co-repressors or co-activators of AR[3].

PCa frequently progresses slowly from premalignant prostatic intraepithelial neoplasia (PIN), to high-grade PIN, to adenocarcinoma[7]. PIN is characterized by hyperplasia of the luminal epithelia, reduction in basal epithelial cells, atypical enlargement of the nuclei and increased proliferation[7]. In PIN lesions, many PCa-associated genetic changes are already present including chromosomal rearrangement that places the ERG oncogene under control of an androgen-regulated gene TMPRSS2[3,7]. This TMPRSS2-ERG fusion occurs in about 50% of human PCa[3]. However this is not the only rearrangement associated with PIN and PCa. Multiple chromosomal rearrangements and copy number variations have been identified. Some of these variations are in regions for genes such as NKX3.1, PTEN, and Myc[7]. This finding corresponds with common signs of disease progression including NKX3.1 down-regulation, Myc overexpression, PTEN inactivation or loss, ERK/MAPK activation, and Ezh2 methyltransferase overexpression[7].

In addition to these genetic alterations, many epigenetic variations can be seen in PCa as well. Abnormal DNA methylation, chromatin modifications, and microRNA variations all are present in PCa. A histone modification that is particularly important in PCa is the trimethylation of H3K27 (lysine residue 27 on histone 3) mediated by the Ezh2 histone methyltransferase, which causes repression of the tumor suppressor gene DAB2IP[7].

Vitamin D

Sources

Despite its deceiving name, vitamin D is actually a steroid hormone rather than a vitamin[15]. Vitamins by classical definition are organic substances that can be acquired in the diet and are necessary in very small amounts for normal cell and body function[16,17]. Vitamin D can be acquired in the diet[18], however it is also endogenously produced in the melanocytes of the body when they are exposed to UVB radiation[19]. Therefore, vitamin D status is determined by both diet (including supplementation) and exposure to sunlight[18].

The current RDA for vitamin D is 600-4,000 IU (15-100ug) for both men and women greater than 9 years old[18]. This recommendation is controversial however, and broadband variation of sunlight exposure and skin color should be considered for individual supplementation recommendations. Few foods are naturally good sources of vitamin D. Vitamin D is obtained in the diet primarily from fortified dairy products, fatty fish, beef liver, sardines, egg yolks, and some several types of mushrooms[18]. For many people, however, the diet alone may not be sufficient and supplementation is recommended.

Because skin exposure to UV radiation is necessary to produce vitamin D in the body, population trends in vitamin D status follow longitude, with deficiency being more common the farther a population is from the equator. This is especially true in cold winter months when very little skin is exposed to the sunlight and sun light is less intenset[19]. Additionally, risk of deficiency is greater in cultures where attire that covers a large portion of the body is traditional, regardless of whether sunlight is plentiful[19]. Unfortunately, an additional factor that decreases endogenous vitamin D production is daily use of sunscreen that protects against skin cancer. As sunscreen has become more common, circulating vitamin D levels have decreased[20]. Finally, having darker complexion that makes a person less sensitive to the UV rays of the sun can lead to lower levels of circulating vitamin D[19].

Metabolism

Vitamin D exists in several forms in the body. Cholecalciferol, the precursor to the active hormone vitamin D, is the form of vitamin D that is ingested from animal dietary sources and generated in the melanocytes of the body[19]. Plants provide a different form of vitamin D, vitamin D_2 (ergosterol), which is less active but similar in function to vitamin D_3.

The production of vitamin D in the body starts when the skin is exposed to UV radiation[19]. The energy from the UVB radiation is utilized to convert the substrate 7-dehydrocholesterol to vitamin D_3. Regardless of the source, vitamin D then travels to the liver where it is hydroxylated on C25 by CYP2R1 to form 25-hydroxvitamin D (25(OH)D). $25(OH)D_2$ or D_3 is the circulating form of vitamin D in the body and is the form that is commonly measured to monitor a person's vitamin D status[19]. A person who has a circulating vitamin D level less than 20 ng/mL is said to have a deficiency by the Institute of Medicine[19] and the recommended circulating vitamin D level to receive benefit is between 40-70ng/mL[19]. Vitamin D deficiency is linked to increased incidence and poorer outcomes of cardiovascular disease, diabetes, and various cancers, including prostate, breast, colon and others[21,22].

Circulating $25(OH)D_3$ is hydroxylated a second time on C1 by the enzyme CYP27B1 in the kidneys or various other tissues to generate $1,25(OH)_2D_3$, the active form of vitamin D known as calcitriol[19]. Importantly, the prostate is one of the sites in the body aside from the kidneys where CYP27B1 is found. Therefore, the prostate itself is capable of vitamin D metabolism and is not necessarily dependent on levels of $1,25(OH)_2D_3$ synthesized by the kidneys, which could have very organ specific implications[19]. Calcitriol and $25(OH)D_3$ are both inactivated in the cell by the enzyme CYP24A1 that hydroxylates vitamin D at C24. CYP24A1 is transcriptionally induced by vitamin D itself, and therefore this hormone physiologically acts as its own negative regulator within the cell[19]. The CYP27B1 enzyme is very tightly regulated, and along with CYP24A1, is responsible for maintaining steady intracellular levels of vitamin D[19]. In cancer, CYP24A1 may be upregulated, and CYP27B1 down-regulated, leading to decreased effects of vitamin D in cancer cells[19].

Functions

Classically, vitamin D is known for its role in calcium and phosphate homeostasis and bone mineralization[19]. In the kidneys, three calcitropic hormones control 25(OH)D and 1,25(OH)D metabolism by regulating CYP27B1 and CYP24A1. These hormones are parathyroid hormone (PTH), calcitriol, and fibroblast growth factor 23 (FGF23). However, calcitriol has numerous functions in various tissues of the body that go far beyond bone mineralization. Vitamin D receptor (VDR) is present in almost all cells of the body, indicating that vitamin D has widespread general effects, in addition to possible tissue-specific effects[23]. In recent decades, both epidemiological studies and controlled scientific experiments have illuminated the role of vitamin D as a potent anti-cancer agent, and linked vitamin D deficiency to increased risk of cancer development, as well as other diseases[19]. In extrarenal sites where CYP27B1 is present (such as the prostate), the calcitropic hormones do not play a role in regulating the enzyme, and rather the levels of 25(OH)D itself seem to be the most important in regulating CYP27B1[19].

The canonical function of vitamin D is carried out by calcitriol entering the cell, binding to the VDR, and activating transcription of vitamin D regulated genes. Both 25(OH)D and calcitriol are lipophilic molecules that readily cross the cell membrane[23]. Once inside the cell, calcitriol binds to the nuclear VDR[23] and forms an obligate heterodimer with the retinoid X receptor (RXR) liganded by 9-*cis*-rtinoic acid, the complex then binds to vitamin D response elements (VDREs) on the genome[19]. These VDREs lie in the regulatory regions of a variety of vitamin D regulated genes.

A prominent mechanism of calcitriol related to cancer prevention is reducing inflammation. Inflammation is widely accepted as a risk factor for cancer, and inflammatory cells and mediators are commonly found at tumor sites[23]. Some of these inflammatory mediators include reactive nitrogen species and reactive oxygen species (ROS), inflammatory cytokines, chemokines, and prostaglandins[23]. In the human prostate, calcitriol has been shown to reduce inflammation by inhibiting the synthesis of pro-inflammatory prostaglandins[23]. Calcitriol decreases levels of cyclooxygenase 2 (COX-2), the enzyme that converts arachidonic acid to inflammatory prostaglandins. By reducing COX-2 expression, calcitriol thus reduces prostaglandin formation and inflammatory signaling[23]. Additionally, calcitriol decreases pro-

inflammatory cytokines by increasing the expression of the inflammatory cytokine inhibitor, mitogen-activated protein kinase phosphatase 5 (MAPK5)[23]. Other possible anti-inflammatory mechanisms of calcitriol include inhibition of nuclear factor kappa B (NFκB), IL-6 and TNFα[23].

Not only is calcitriol anti-inflammatory, but it has been shown to have a host of other chemopreventative properties. 1,25(OH)D has been shown to prevent cell proliferation, cause cell cycle arrest, stimulate differentiation, induce apoptosis, and inhibit metastasis and angiogenesis in cell culture and animal tumor models[23]. It induces cell cycle arrest and thus reduces proliferation through increased expression of cyclin dependent kinases (CDK) inhibitors. Additionally, it reduces the effect of mitogenic signals, and increases the expression of growth inhibiting proteins, such as TGFβ[19]. The pro-differentiation effects of calcitriol seem to be dependent on the specific cell type, but include pathways such as PI3K, NFκB and β-catenin. 1,25(OH)D induces apoptosis both by suppressing anti-apoptotic proteins, and by increasing pro-apoptotic proteins. Metastasis is reduced by calcitriol via increased expression of E-cadherin, and through suppression of several other proteins. Lastly, calcitriol suppresses angiogenesis through the NFκB and COX-2 interactions mentioned previously[19].

In addition to the potential anticancer therapeutic and chemopreventive effects listed above, in the prostate specifically, calcitriol has been shown to inhibit PCa both through AR regulation and independently of AR. In various PCa cell lines, high levels of androgen have been linked to reduced cell proliferation and used as a marker of differentiation. $1,25(OH)_2D_3$ has been shown to increase AR expression in these cells thereby acting through AR to prevent growth and proliferation[11]. Calcitriol has also been shown to co-regulate androgen responsive genes, increase androgen-inducible growth inhibitor AS3 levels, and cause differentiation of PCa cells[19]. A person's 25(OH)D status mediates the level of these benefits they see, with lower levels of 25(OH)D associated with increased risk of PCa. Compared with other racial/ethnic groups in the United States, AA men and women have the lowest levels of circulating 25(OH)D.

Disparities

Vitamin D Status

Skin pigmentation is a major factor to vitamin D production in the body, and therefore can play a large role in determining vitamin D status of an individual. The National Health and Nutrition Examination Surveys (NHANES) from 2001-2004 revealed that 97% of non-Hispanic Black Americans have vitamin D insufficiency (<20ng/uL)[22]. This could be related to factors such as dietary intake of foods that contain vitamin D, and reduced rate of supplementation, in addition to darker skin pigmentation, but regardless of the cause correlates with poor prognosis[21]. Though the innate ability of the body to produce vitamin D varies by race, racial disparities in circulating vitamin D status can be overcome by supplementation[24]. When men were given a vitamin D supplement of 4000 IU/d for two months, differences in circulating vitamin D between African Americans (AA) and European Americans (EA) were eliminated[24].

Prostate Cancer

A very strong disparity exists in PCa incidence and mortality between EA and AA men. Between 2007 and 2011 the PCa incidence for Non-Hispanic white men in America was 133.2 per 100,000 versus 219.8 per 100,000 in Non-Hispanic black men[12]. This equals an incidence nearly 60% higher for AA men than their EA counterparts[12]. Not only are AA men at higher risk of developing PCa, but those that do are more likely to die from the disease. The age-adjusted death rate per 100,000 for EA men is 20.7 versus 49.8 for AA men[12], which translates to an increased death rate of 240% for AAs. Though incidence and death rates have decreased dramatically in all races since the discovery of PSA, this disparity in rates based on race still exist (ACS, 2015, **Figure 1**).

AA and African Caribbean men have the highest incidence of PCa in the world. Because American men of African descent share genetic similarities to African men living in Africa and share an environment with EA men, but have higher incidence of PCa than either of these groups, it seems that an

interplay of the environment and genetics are contributing to this phenomena. A study at Wayne State University that examined high grade PIN in AA and EA men found no significant differences in zonal distribution, number of cancer foci or Gleason score between the two races[13]. However, they found that AA men develop high-grade PIN at younger ages compared to their EA counterparts. Additionally, upon initial diagnosis, AA men have higher levels of PSA and more aggressive tumors[13].

Though environmental factors, such smoking, and a diet high in processed meat have been shown to increase PCa incidence or mortality[12], these environmental and socio-economic factors alone do not account for the stark racial disparities seen in the United States. According to a study published in the American Journal of Epidemiology in 2000, when adjusted for age, stage, education, and income, the death ratio of AA men was still 1.41 times greater than that of EA men when the age at diagnosis less than 65 years, and 1.20 times greater when the men were older than 65 years[25]. All these data suggest that racial disparities in both PCa incidence and mortality has a genetic link that puts men with Sub-Saharan African ancestry at higher risk.

Hypothesis

AA men have reduced levels of and response to vitamin D in the prostate, which leads to a reduction in the chemopreventative effects of vitamin D and an increase in PCa risk and severity compared to EA men.

Age-Adjusted SEER Incidence Rates
By Race/Ethnicity
Prostate, All Ages, Male
2000-2011 (SEER 18)
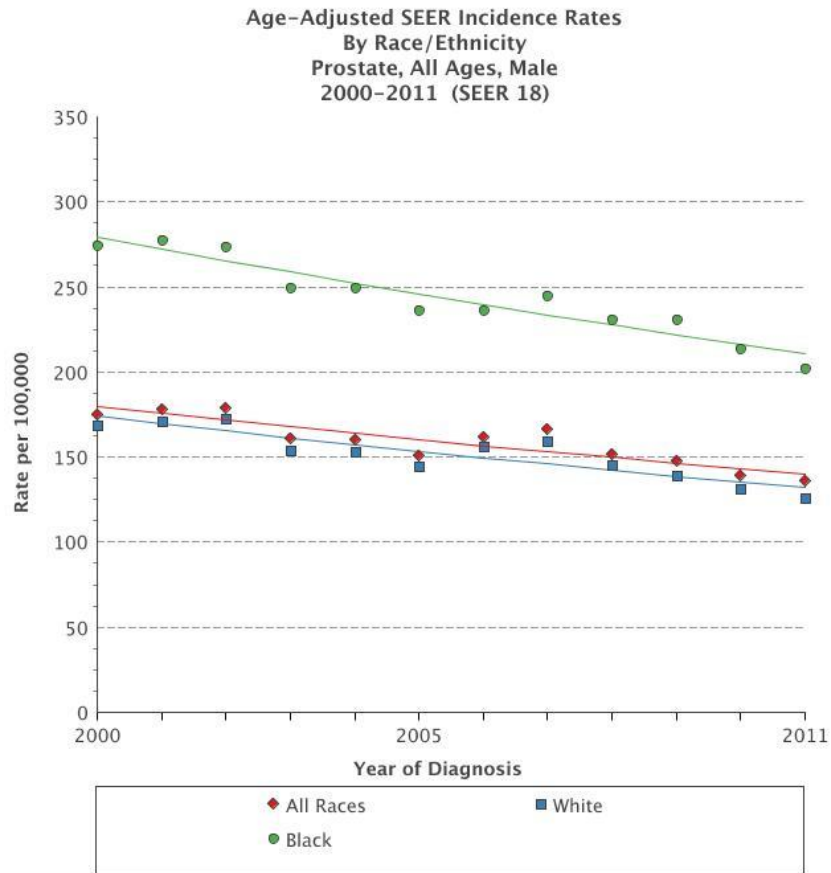
Cancer sites include invasive cases only unless otherwise noted.
Rates are per 100,000 and are age-adjusted to the 2000 US Std Population (19 age groups – Census P25-1130). Regression lines are calculated using the Joinpoint Regression Program Version 4.1.0, April 2014, National Cancer Institute.
Incidence source: SEER 18 areas (San Francisco, Connecticut, Detroit, Hawaii, Iowa, New Mexico, Seattle, Utah, Atlanta, San Jose-Monterey, Los Angeles, Alaska Native Registry, Rural Georgia, California excluding SF/SJM/LA, Kentucky, Louisiana, New Jersey and Georgia excluding ATL/RG).

Figure 1: Trends in prostate cancer incidence by race from 2000-2011, adjusted for age. Graph generated by *faststats* at cancer.gov.

CHAPTER 2:

Materials and Methods

Cell Culture

Primary prostate epithelial (PrE) and primary prostate stromal (Spz) cells were cultured from tissue samples acquired from the University of Illinois at Chicago Hospital and Health Center. PrE cells were grown in PrEBM (Lonza) supplemented with 50uL CT and 50uL EGF. Spz cells were grown in MCDB supplemented with 10% FBS. Cell cultures were maintained in an incubator with 5.0% CO2 at 37 °C and grown until they reached 70% confluence. When not being used, cells were stored in liquid nitrogen suspended in 70% FBS/10% DMSO/20% media.

Primary Cell Culture

All experiments were done with primary prostate cells obtained from radical prostatectomy tissue provided by the University of Illinois at Chicago Hospital and Health Center. Immediately after the operation, four small pieces of prostate tissue were removed using a metal punch (Fischer Scientific) and were placed in four separate 15mL tubes filled with PrEBM. A small slice of each tissue section received was stored in formalin overnight, then moved to 70% EtOH the following morning and given to the UIC Research Resource Histology Core where it was H&E stained to determine whether the tissue section was cancerous or benign. The remaining tissue was processed according to the Nonn lab "Prostate Tissue Processing" protocol. Tissue samples were washed several times with HBS with antibiotic, then mechanically cut into small pieces and washed in HBS again. Finally, tissue samples were treated with collagenase and put on a rotator in the incubator overnight.

The next morning, tissue was once again washed with HBS, then resuspended in 10mL of either PrEBM or MCDB/10% FBS with antibiotic and plated. The cell type, epithelial versus stromal, was determined by the media used to grow the cells. PrEBM has the growth factors necessary to sustain

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epithelial prostate cells, whereas MCDB/10% FBS is suitable for stromal cell growth. Therefore, though the tissue samples we received were not solely stromal or epithelial, we could select for which cells would grow from each tissue sample and thus culture two different types of cells from the same prostate[26]. Following processing, tissue samples were left completely undisturbed for one week to allow cells to dissociate and attach to the plate. At one week the media was changed and cells were either cultured for experimental use or stored in liquid nitrogen until they were needed.

Prostasphere Co-culture

Co-cultures of Spz and Pre cells were started by seeding 18,000 PrE cells in 100uL PrEBM combined with 100uL BD matrigel (Fischer Scientific) along the outside edge of a 12-well dish. Once seeded, the matrigel was allowed to sit for at least one hour in the 37 °C incubator. The day prior to seeding PrEs in matrigel, 10,000 Spz cells were seeded in hanging inserts (Millipore). Once the matrigel and PrEs had set, the hanging inserts were placed into the wells, along with 2mL of PrEBM.

The media in the co-cultures was changed every two days. After four or twenty-one days of co-culture, PrE cells were collected in Trizol and stored at -80C. Spz cells were collected about every 7-10 days, as needed to prevent the inserts from becoming 100% confluent. Spzs were also collected in Trizol and stored at -80 °C. RNA was purified from all samples using chloroform and RNA concentration was determined by Nanodrop optical density at 260 nanometer wavelength. RNA was stored at -80C.

Vitamin D Treatment

To analyze the effects of vitamin D on differentiation, growth, and gene expression of the cells, 1,25(OH)D3 was added directly to the media of the cells. 1,25(OH)D3 was purchased from Enzo Life Sciences in the form of a powder, and was then dissolved and diluted in 100% molecular grade EtOH to 500nM stocks which were stored, protected from light, at -80 °C. This stock of 1,25(OH)D3 was then diluted in the media each time media was changed, to either 10nM or 50nM concentrations, and incubated

with the cells. Controls were given an equal volume of EtOH that was used to dilute the highest concentration (50nM) of vitamin D.

PCR

Gene expression was analyzed by quantitative real-time qPCR (qPCR). RNA that had been extracted and purified from the cells using Trizol and Chloroform was converted to cDNA using a Hi-Capacity cDNA kit (Applied Biosystems). RNA was diluted so that an equal amount of RNA from each sample could be added to each reverse transcriptase reaction to control for variations in concentration. qPCR was performed using SYBR Green, and run for ~40 cycles. Results were analyzed using ddCT to determine statistical significance. ddCT is a method that can be used to analyze the relative changes in gene expression by using a housekeeper gene and reference sample to normalize values[27].

Blood, Serum and Prostate Tissue Samples

Samples of prostate tissue, blood, and serum were collected from each of 46 patients who underwent radical prostatectomy surgeries. Samples were stored in liquid nitrogen.

Whole blood was used to analyze the percentage West African Ancestry of each of the patients, to verify self-reported race. 1 mL of each patient's blood was genotyped by Dr. Rick Kittle's laboratory, which used a SNP analysis to determine the ancestry of the patients.

Serum was used to determine the circulating levels of 25(OH)D3 and 1,25(OH)D3 for each of the patients. 500uL of serum was sent to Heartland Assays, LLC in Ames, Iowa, where concentrations were determined using LC-MS-MS.

Prostate tissue was used to assess prostatic levels of vitamin D, as well as gene expression. A small piece of prostatic tissue was sent to Heartland Assays, LLC along with the serum samples and the rest of the tissue was saved to use for Laser Capture Microdissection (LCM) for future studies of expression of vitamin D metabolism genes.

Results

Patient reported race and genotyped data

To determine whether percentage West African ancestry was related to vitamin D status, genotyping was done carried out by Dr. Rick Kittle's lab. Kittle's lab previously developed the screen for 106 SNPs that indicate West African ancestry that was used here. Genotyping results are presented in **Figure 2** below, which shows the percentage West African Ancestry for patients who self-reported being AA or EA. With the exception of one patient in each self-declared racial category, self-declared race was considered accurate. When a discrepancy existed between self-declared race and genotyping data, the genotype was used to assign which category a person should be analyzed under.

Patient serum vitamin D levels

Serum levels of $25(OH)D_3$ and D_2 as well as $1,25(OH)_2D_3$ and D2 were measured by Heartland Assays, Inc. by LC-MS-MS for each of the patients. In **Figures 3 and 4** the serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ In AA and EA men are compared. There was no significant difference in $1,25(OH)D$ levels based on race, but $25(OH)D$ levels were significantly ($p < 0.0001$) lower in AA patients than EA. $1,25(OH)_2D_3$ levels were much lower than $25(OH)D$ in both races.
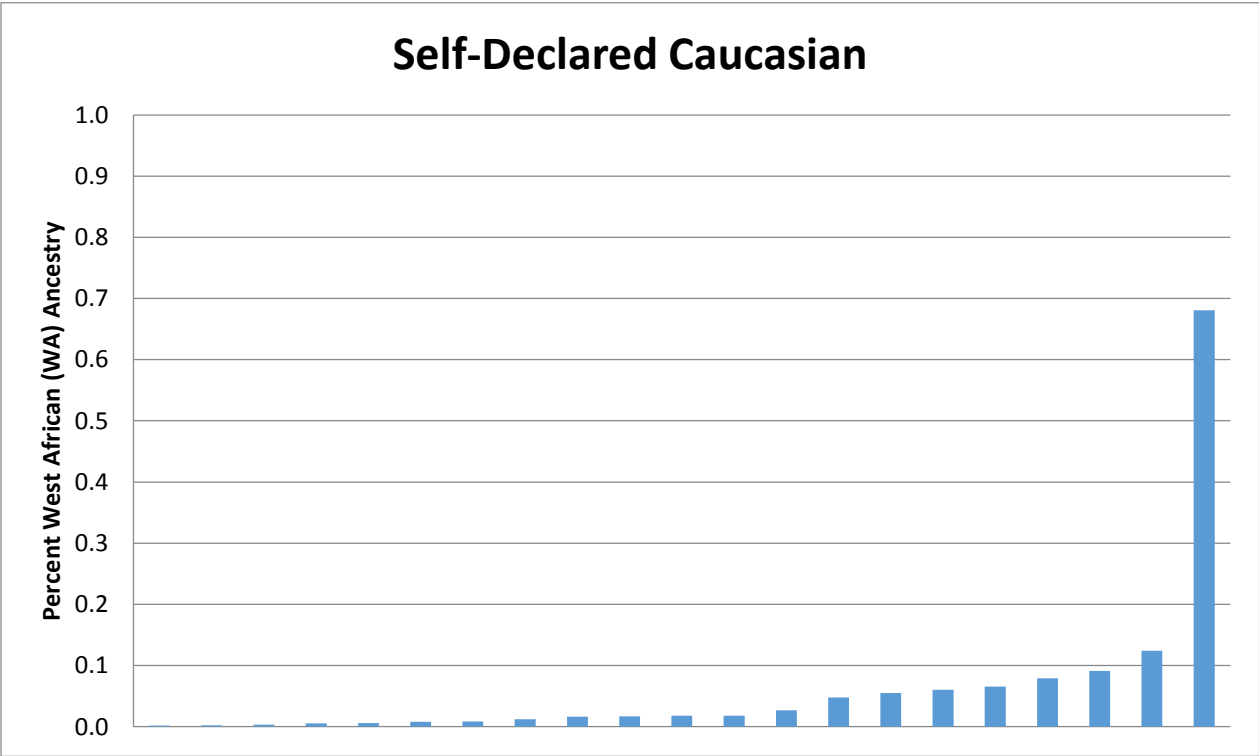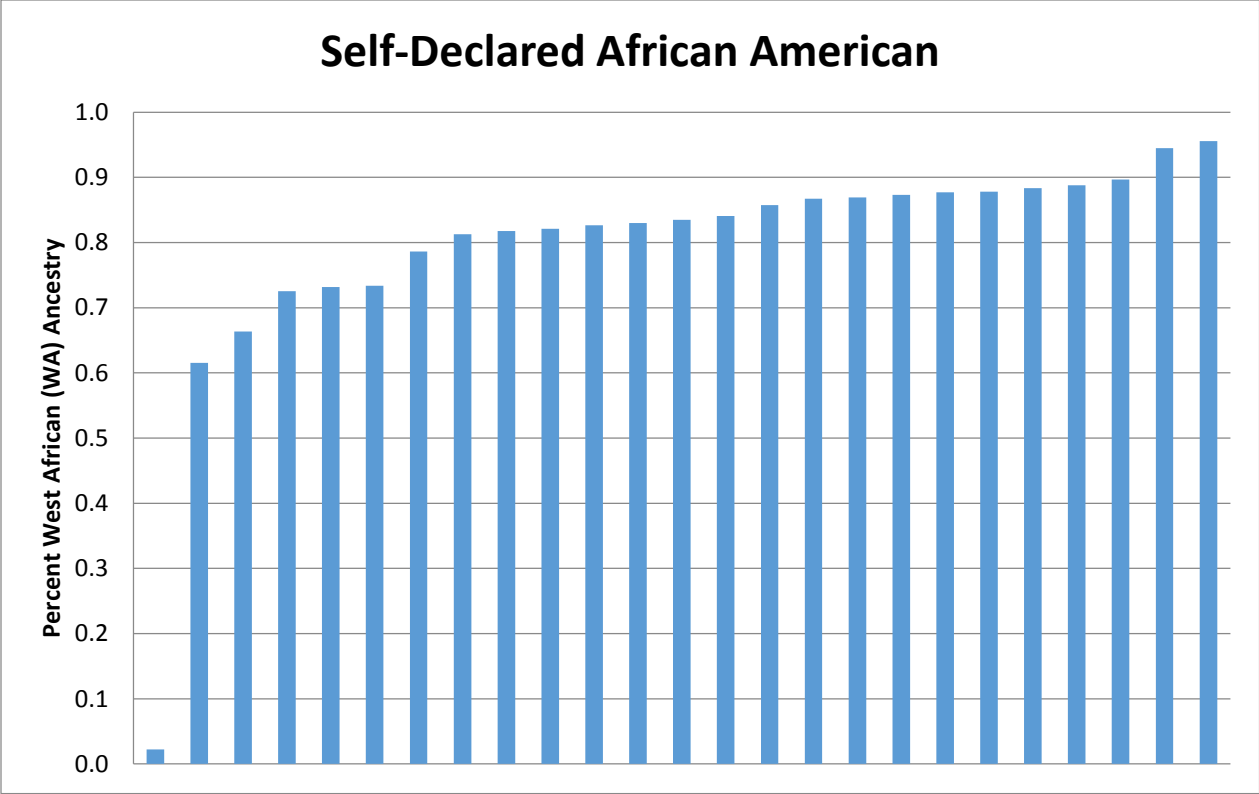
Figure 2: Percentage West African Ancestry of patients who self-declared as AA (above) and EA (below).

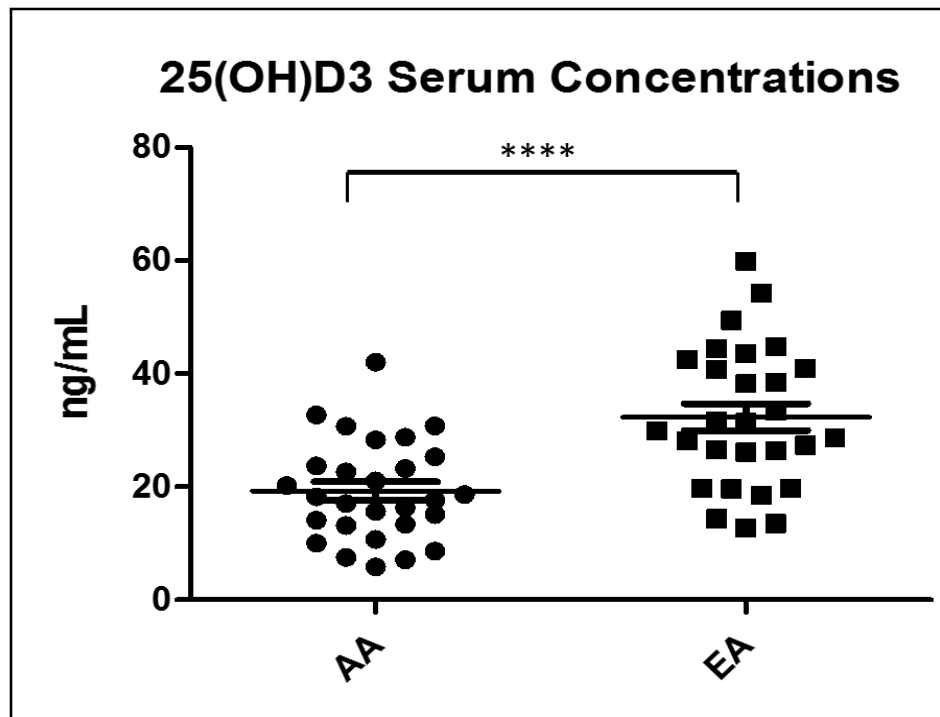**Figure 3:** 25(OH)D$_3$ levels in are significantly lower in AA men than their EA counterparts. Horizontal bars represent the mean concentration for each race, error bars represent standard deviation.
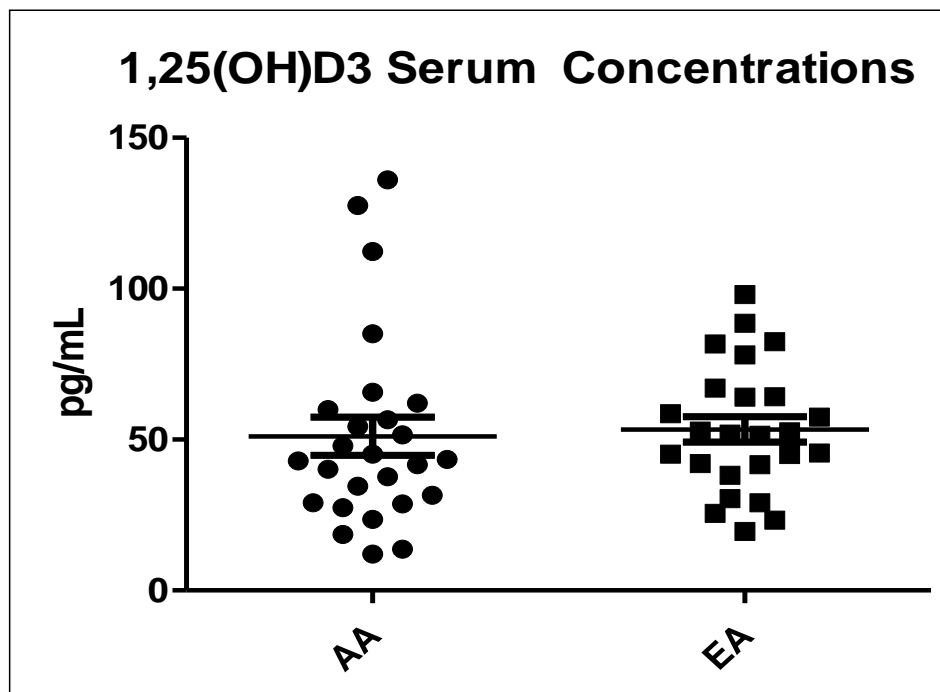

Figure 4: There is no significant difference in 1,25(OH)$_2$D$_3$ concentrations between AA and EA men. Horizontal bars represent the mean concentration for each race, error bars represent standard deviation.

The levels of CYP27B1 in primary human prostate stromal cell cultures derived from African American and European American patients

Primary human stromal cell cultures derived from the benign peripheral zone of the prostate (Spz cells) were used to measure and compare CYP27B1 gene expression in AA and EA men. Primary prostate cells maintain inter-patient heterogeneity in both genetic and epigenetic states of each patient[26]. The advantage of primary cells over immortalized cell lines is they are truly benign and normal without immortalization-induced changes and therefore give us a snapshot of what actually occurs in the cells of each of these patients.

CYP27B1 was measured by RT-qPCR in four Spz cultures, two AA (SPz-1 & Spz-2) and two EA (Spz-3 & Spz-4): one of the EA samples was lost after processing, as can be seen in **Figure 5**. CYP27B1 was detected in all of the Spz cultures, but the Spz cells from AA patients (SPz-1 & Spz-2) had lower expression of CYP27B1 than the single EA patient we have data for (**Figure 5**).

The levels of VDR in primary human prostate stromal cell cultures derived from African American and European American patients

VDR is present in almost all cells of the body[23], and thus is utilized to in most cells. The presence of VDR mRNA was confirmed in the Spz cells by RT-qPCR in four cultures, for two AA (SPz-1 & Spz-2) and two EA (Spz-3 & Spz-4). Data are presented in **Figure 5**.
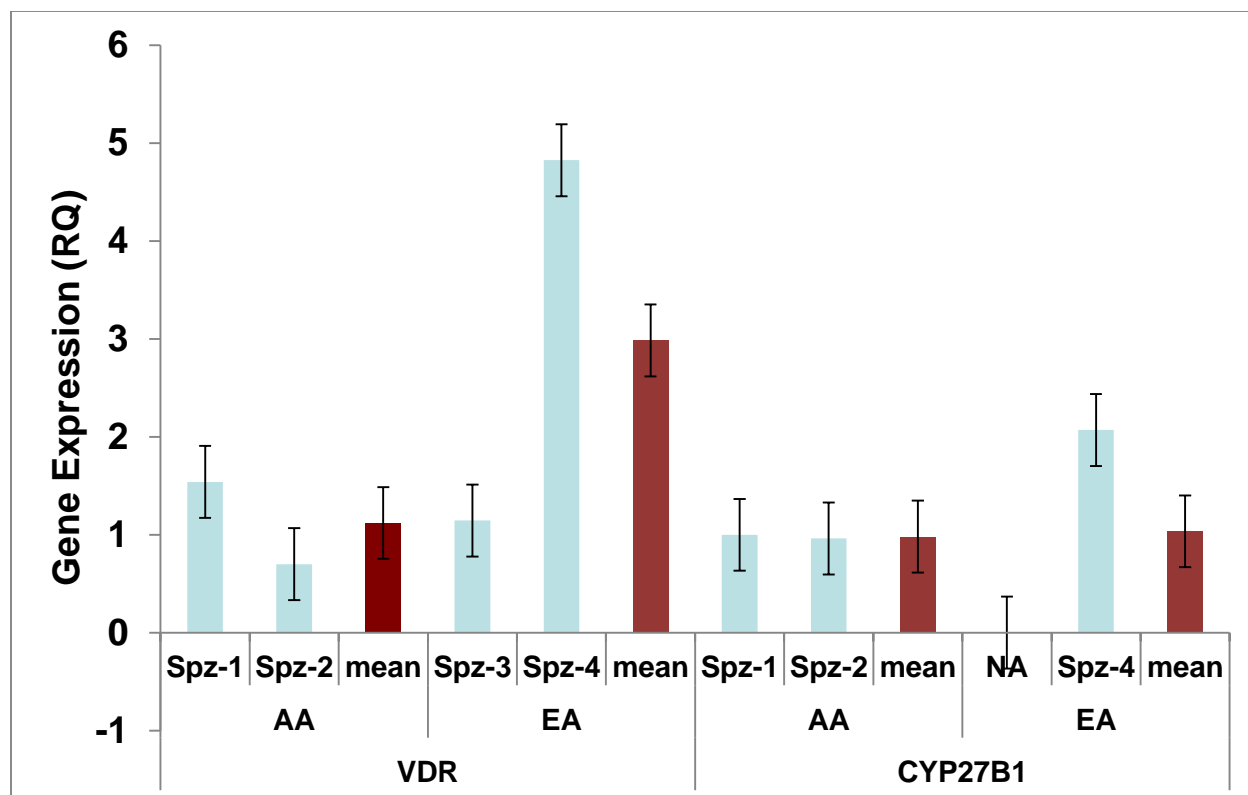
Figure 5: CYP27B1 and VDR were present in all Spz cells analyzed. N=4. Error bars on individual patient samples are standard deviation for technical replicates, error bars on means are the standard deviation for the entire group.

*NA: sample not available

The levels of CYP24A1 induction in primary human prostate stromal cell cultures derived from African American and European American patients

CYP24A1 mRNA was also measured by RT-qPCR in four Spz cultures, two AA (SPz-1 & Spz-2) and two EA (Spz-3 & Spz-4), after treatment with 50nM $1,25(OH)_2D_3$ or 50nM EtOH (control) for 16hrs (**Figure 6**). CYP24A1 enzymatically inactivates 25(OH) and 1,25(OH)D in the cell by hydroxylating C24[19]. The CYP24A1 promoter also contains multiple VDRE's and thus induction of CYP24A1 is a good *in vitro* readout of 1,25(OH)D activity.

In the absence of 1,25(OH)D, there is virtually no CYP24A1 present, therefore the RQ value which is normally used to represent gene expression becomes somewhat unreliable. 1,25(OH)D treatment caused greater CYP24A1 induction in cells derived from EA patients than AA patients, but the actual quantification given by the RQ is not necessarily representative of the fold difference between the samples. Therefore the results are presented as both raw dCT and RQ (**Figure 6**). The dCT value is the number of PCR cycles of replication needed to detect a signal, and therefore a higher dCT value indicates lower concentration of RNA in the starting material. Conversely, the RQ is normalized to the housekeeper genes and shows the relationship between genes in terms of quantity of starting material, with a higher RQ correlating to higher amount of RNA in the starting sample.

The results show a statistically significant ($p < 0.005$, dCT or $p<0.0001$, RQ) lower in induction of the expression of the vitamin D responsive gene, CYP24A1, in AA men compared to their EA counterparts. The mean dCT for AA men was 9.33 with a standard deviation of 0.86, compared to a mean of 5.47 for EA men with a 1.91 standard deviation. The mean RQ for CYP24A1 in AA men was 580.77 with a standard deviation of 317.05, versus a mean of 2,396.12 with a standard deviation of 2,255.71 for EA men.
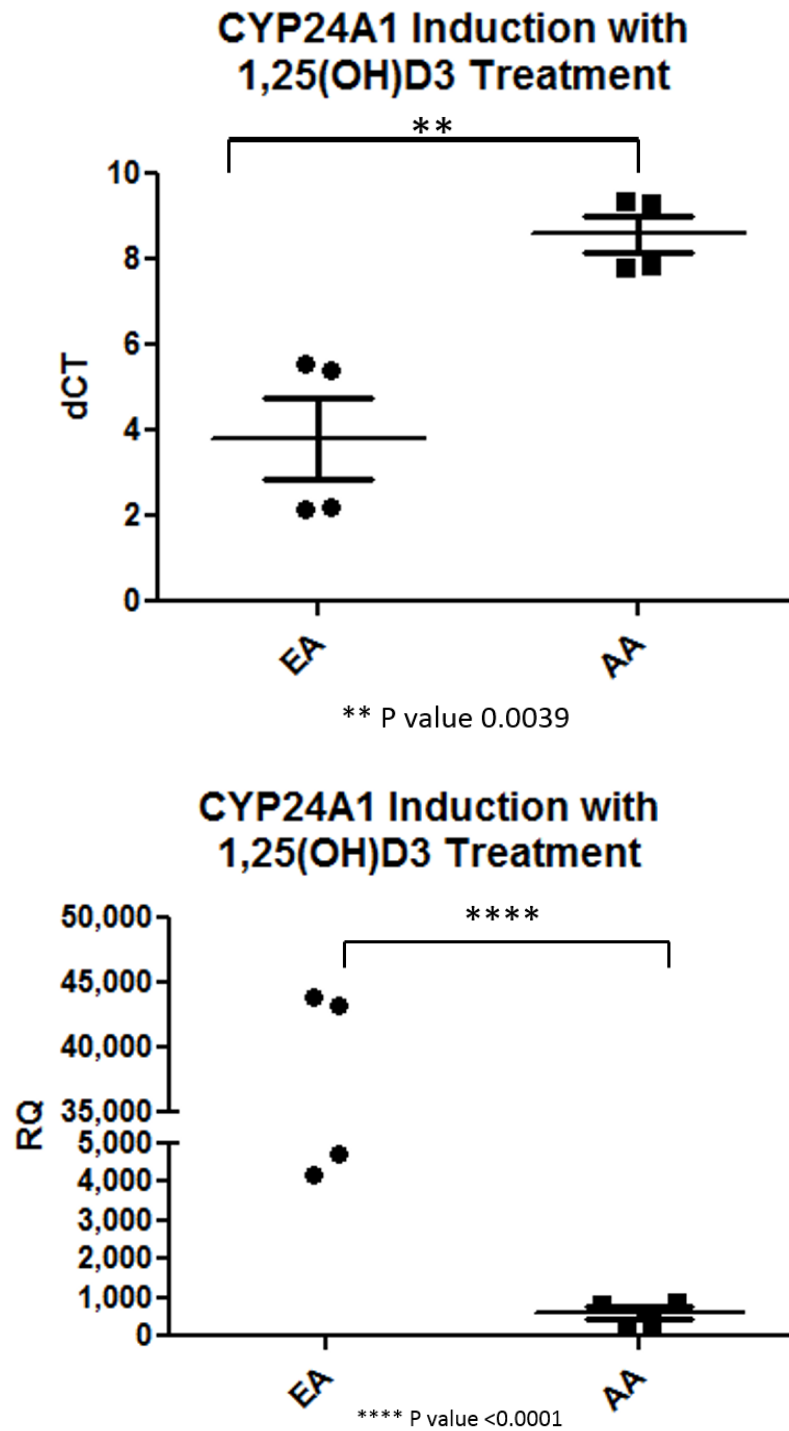
Figure 6: The dCT and RQ values for CYP24A1 induction in EA vs. AA Spz cells after treatment with 50nM $1,25(OH)_2D_3$ for 16 hours. N=4. Two technical replicates for each sample are shown as individual dots. Values were normalized to treatment with EtOH, and for RQ values, to the housekeeper GAPDH.

Stromal cell induced differentiation of primary prostate epithelial cells in co-culture

Moving away from looking at disparities, we next tested the effects of cross-talk between the stroma and epithelia of the prostate by using co-cultures. These co-cultures mimic the *in-vivo* microenvironment of the prostate and allow for reciprocal signaling between the cell types that results in differentiation of the PrE cells. PrE cells have a basal cell phenotype, but can be differentiated to luminal epithelial cells by co-culture with Spz cells. In order for differentiation to occur the PrE cells must be suspended in matrigel, which provides growth factors and allows the cells to be cultured in suspension. Over time, rare progenitor PrE cells in the culture will begin to form small 3D spheres, known as prostaspheres, that contain cells with both basal and luminal phenotypes[28]. We therefore used expression of cytokeratin 8 (CK8), a common marker for prostatic luminal epithelial cells[29], as a readout for differentiation. As time progresses, the prostaspheres become more differentiated and expression of CK8 increases. This is why the spheres were collected at both day 4 and days 10 or 21.

CK8 expression was measured by RT-qPCR and was induced in the epithelial prostaspheres when co-cultured with Spz cells for 4, 10, or 21 days. Co-cultures were treated with 50nM 1,25(OH)D, 10nM R1881 (synthetic androgen), a combination of the treatments, or 50nM EtOH control. **Figure 7** shows the induction of CK8 from two separate co-culture experiments, each with different patient derived PrE cells (PrE-1 & PrE-2) after 4 and 10/21 days of co-culture. The same stock of mixed Spz cells was used for both experiments, and was formed by combining Spz cells from four individuals. In both cases there was an induction of CK8 expression after co-culture with Spz cells, though there was no significant increase in CK8 expression with treatments of vitamin D.
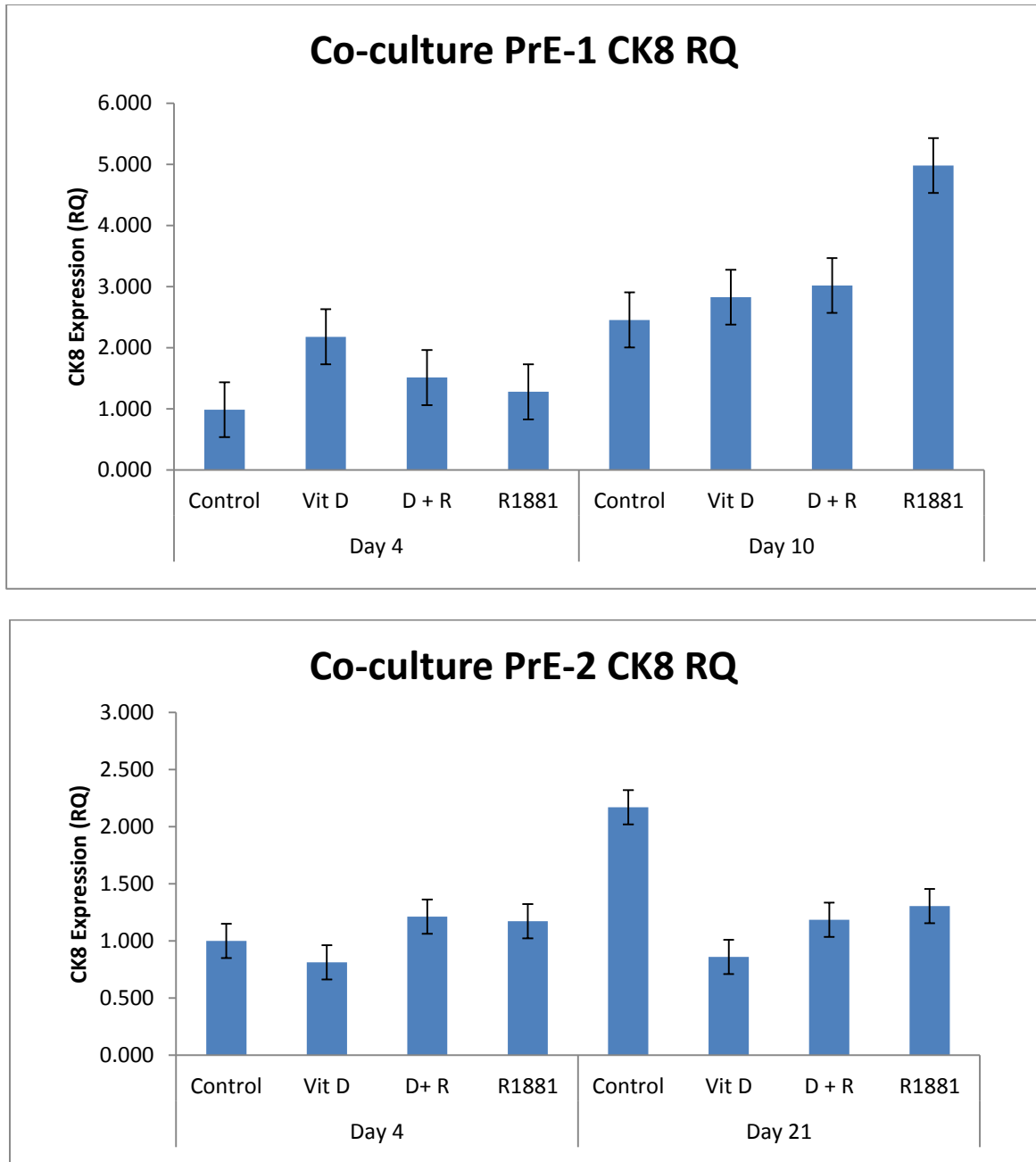
Figure 7: The luminal epithelial marker CK8 was induced in PrE cells when co-cultured with Spz cells. Each of the different graphs are results from different co-cultures with unique PrE's. Error bars are standard deviation from PCR technical replicates.

CHAPTER 4:

Discussion

The disparity of prostate cancer between AA and EA men is significant, and has remained even as the overall rates of prostate cancer have declined. AA men tend to present with more aggressive prostate cancer and younger ages, and are more likely to die from the disease compared to their EA counterparts[12]. While prostate cancer develops specifically from the prostatic epithelial cells, research has shown that the stromal cells and overall microenvironment of the prostate are crucial to cancer development[8,28]. Therefore studying stromal cells and the interaction between stromal and epithelial cells will provide insight into normal prostate biology as well as carcinogenesis.

In addition to more aggressive prostate cancer, AA men have lower levels of circulating 25(OH)D, which could be caused by a variety of factors including darker skin pigmentation that leads to decreased endogenous production of vitamin D. Vitamin D has been extensively studied and shown to have many important anti-cancer properties including reducing inflammation, arresting cell cycle, and increasing apoptosis and differentiation. Therefore, there is a distinct possibility that lower levels of this hormone could contribute to high incidence and aggressiveness of prostate cancer in AA men.

The research done for this thesis supports and expands upon these observations. AA patients who donated blood, prostate tissue, and serum had significantly lower serum levels of $25(OH)D_3$ (**Figure 3**) than EA men, but had equivalent 1,25D levels. This was expected, as serum 25(OH)D is used to determine a person's vitamin D status based on production in the body and dietary intake. 1,25(OH)D has a short half-life and is tightly regulated by PTH, calcium and phosphate, so it is therefore not indicative of vitamin D status[18]. Race in all patients was confirmed by genotyping (**Figure 2**) to eliminate variations due to the self-reported race of the patients and determine whether percentage West African ancestry was correlated with vitamin D status. We found little to no variation of vitamin D status based on percentage African ancestry, but AA men as a whole had significantly lower levels of circulating 25(OH)D. Our

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results are consistent with previously studied variations based on race and are therefore a suitable cohort to further investigate these disparities.

The disparity of lower levels of circulating 25(OH)D in AA men compared to EA men was as we expected, but may not be the whole story. Because the prostate is one of the few extra-renal sites of CYP27B1, circulating levels of 1,25(OH)D from the kidney are not the only source of active calcitriol in the cells. The prostate can uptake this circulating calcitriol, but can also produce its own 1,25(OH)D from circulating 25(OH)D. Therefore, we are interested in prostatic levels of 1,25(OH)D, and hypothesize that this may be a better indicator of a patients risk for prostate cancer than serum 25(OH)D.

To determine whether there are racial differences in the effect of vitamin D in the prostate, we examined the expression of vitamin D metabolism genes in prostate stroma cell cultures derived from EA and AA patients. We confirmed the presence of CYP27B1 and VDR mRNA in all of the Spz cells (**Figure 5**) but did not find significant differences between AA and EA derived cells.

However, when we examined CYP24A1 mRNA we found that induction of this vitamin D-responsive gene by 1,25(OH)D was lower in AA-Spz cells compared to EA derived cells (**Figure 6**). CYP24A1 is the enzyme that inactivates 1,25(OH)D and therefore lower levels of CYP24A1 would lead to more available vitamin D in the cell. However, CYP24A1 induction is also indicative of VDR activity. *In vivo,* cells would never be completely deficient of vitamin D as is the case in cellular *in vitro* experiments, and therefore CYP24A1 would never be completely absent from the cells. *In vitro*, due to the lack of any environmental vitamin D, levels of CYP24A1are extremely low or absent until 1,25(OH)D treatment, and then rapidly and robustly increase due to the activation of multiple VDREs in the promoter region. Therefore, rather than looking at the levels of CYP24A1 as evidence of the normal levels of the vitamin D metabolism genes in the prostate, these experiments focus on CYP24A1 for its purpose of readout of vitamin D activity. The reduced levels of CYP24A1 mRNA upon vitamin D treatment in AA-derived cells thus indicate that AA men may have decreased response to vitamin D in the prostate leading to a decreased chemopreventative effects of the hormone.

This strengthens the argument that prostate-specific vitamin D disparities may exist between AA and EA men and may be contributing to the poorer cancer outcomes, and indicates that stromal cells may specifically play an important role in vitamin D metabolism of the prostate. Therefore, to completely understand this disparity it will be necessary to measure the prostatic levels of 25(OH)D and 1,25(OH)D in addition to serum vitamin D.

Shifting gears away from health disparities, we also examined the ability of the 1,25(OH)D to alter epithelial cell differentiation *in vitro*. Previous studies have shown that 1,25(OH)D induces differentiation in prostate cells, which contributes to its possible chemopreventative or other anti-cancer effects[19]. To test the effects of cross-talk between the stroma and epithelium of the prostate, and the effects of vitamin D on the epithelium, we used a co-culture model in which stromal and epithelial cells were allowed to signal to one another for 4, 10, or 21 days with or without treatment of 1,25(OH)D and/or androgen (R1881). PrE cells, which are basal epithelial cells, differentiated into a more luminal phenotype after 10 and 21 days in co-culture with Spz cells. **Figure 7** shows the induction of the luminal cell marker CK8 in the PrE cells from the co-culture. Due to the pro-differentiation nature of vitamin D, we hypothesized that co-cultures with 1,25(OH)D treatment would show significantly more luminal differentiation than EtOH treated controls, and therefore higher levels of CK8 expression. Opposite to our hypothesis, exposure to 1,25D and or R1881 did not significantly alter this differentiation or CK8 expression.

More cell culture replicates are needed in future of this studies for a more definitive conclusion. Given the heterogeneity between patients reflected in PrE differentiation, a study with an N=2 is inadequate to detect relatively small changes, and more replicates will help us to elucidate the effects of 1,25D on PrE differentiation in co-culture. Finally, in addition to more co-culture experiments, more Spz cultures will be needed to more definitively examine the disparity of CYP24A1 induction in Spz cells following treatment with 1,25(OH)D. The current results are exciting, but more samples and technical replicates are needed add credibility and strength to this observation.

The work done for this thesis has reinforced previous findings about the disparities of prostate cancer and vitamin D status of AA and EA men. It has also possibly given insight into novel causes of these disparities, and should be used as a step towards looking specifically at prostatic levels and function of vitamin D as a possible indicator of cancer risk. With some more work, these findings will hopefully contribute to new methods of preventing and treating prostate cancer and reducing the racial disparities of this malignancy.

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APPENDICES

APPENDIX A

Prostate Tissue Processing

<u>Need</u>

- Antibiotic (100x)→ dilute to 1X in media
- Collagenase (note check units on vial)- at -20°C
- PREBM (complete media)
- HBS
- RPMI

<u>Prepare</u>

- HBS + 1X ABS
- RPMI+ 10% FBS+ collagenase (filter)
- PREBM + 1X ABS or MCDB-105+ 105+10% FBS+ 1X ABS (for ne

Collagenase (*check units on vial- units change with new order of collagenase)
Need 70 units/mL:
For 5 mL → 350 units
For 10 mL→ 700 units
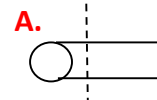For 15 mL → 1050 units
For 20 mL→ 1400 units

i.e.
193 units/mg=1050 units/ x
X=5.44 mg (0.00544 g in 15 mL of RMPI/FBS/ABS)
FILTER

**Tissue Processing for Histology analysis (by RRC Histo Core)
****<u>PLEASE GET A CONTAINER FROM ANDY IN THE CORE WITH 10%FORMALIN BEFORE</u>
<u>YOU START FOR THE MICROCASSETTES</u>

A.

1. Label blue microcassettes with **PENCIL** for each tissue core received (as in B; Patient initials, pz/spz from normal cores, ca/sca for cancer cores, Level#, orientation i.e. mid/left/right posterior/anterior).
2. Slice a very thin of tissue (cross section-as in A.)
3. Place in a blue microcassette in one of the six chambers (as in C.)
4. Submerge the microcassette in container with formalin.
5. Repeat for all the tissue cores received.
6. Next day replace the formalin with 70% EtOH
7. Take labeled tissue to RRC Histology core for processing EVERY TWO WEEKS.
8. Log into UICore and order the following:
   a. RHTIC / Histology - HE Staining (Quantity: number of cores being submitted)
   b. RHTIC / Histology - Paraffin Section - Unstained Slide (Quantity: number of cores being submitted)
   c. RHTIC / Histology - Tissue Processing and Embedding 1 + Samples (Quantity: number of cores being submitted)
   d. RHTIC / Histology - Tissue Processing and Embedding set up service run (Quantity: ONLY 1 each time placing order)
   e. After order is placed, send your email confirmation from UICore to Shuangping. Also provide her with a copy of the receipt stating that we are receiving a discount for the services. (NOTE: Do NOT use CC grant for histology core).

B.

C.

APPENDIX A

Tissue Processing for PrE and PrS cells

1. Wash tissue 3x with HBS/ABS solution
2. Pour tissue in HBS into a sterile glass dish
3. **Chop tissue using scalpel into small pieces (this is the step were you will take a section to keep for histology analysis by the RRC)
4. Using 10 mL pipette, transfer small pieces to fresh 15 mL tube
5. Repeat steps 2-4 for all tissues
6. Spin to pellet pieces
7. Aspirate supernatant, wash with HBS/ABS, spin, aspirate supernatant
8. Add filtered collagenase to each tube of tissue (5 mL/tube)
9. Let spin overnight in incubator
10. Rinse the plates, forceps and any tools used with bleach and autoclave if applicable.
11. Following day- spin, aspirate supernatant, wash 1x with HBS/ABS, spin, aspirate supernatant
12. Resuspend tissue in 10 mL of appropriate media (PREBM/ABS for PrE cell growth or MCDB-105/10%FBS/ABS for PrS growth)
13. Leave plate untouched in incubator for at least 7 days. On day 7, change media being very careful not to disrupt tissue that has stuck down. (you can remove a small amount of media and add fresh media to the rest)
14. Change media every few days and watch from PrE and PrS cells to grow out of tissue
15. When cells have grown out of tissue into patches, freeze down cells in aliquots for later use
16. Count 50,000 cells/vial in freezing media (70% FBS, 20% media, 10% DMSO)

APPENDIX B

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Determination Notice
Research Activity Does Not Involve "Human Subjects"

May 3, 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:

IMPORTANT NOTE: This activity is sponsored by the Department of Defense (DOD). As per DOD policy, OPRS must obtain written concurrence of this determination from the DOD Human Research Protection Officer (HRPO) prior to initiation of the activity.

PLEASE DO NOT INITIATE THIS ACTIVITY UNTIL YOU HAVE RECEIVED WRITTEN NOTIFICATION FROM OPRS THAT THE DOD HRPO HAS CONCURRED WITH THIS NOT HUMAN SUBJECT RESEARCH DETERMINATION.

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).

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 B

<div align="center">

Appendix A - Claim of Exemption From Review by the USAMRMC Office of Research Protections (ORP), Human Research Protection Office (HRPO)

</div>

PROTOCOL TITLE: Disparate Vitamin D3 activity in the prostate of men with African ancestry	
PRINCIPAL INVESTIGATOR'S NAME: Larisa Nonn	PROPOSAL NO: PC121923
INSTITUTION: University of Illinois at Chicago	

<div align="center">

COMPLETE THIS SECTION IF YOU ARE REQUESTING PERMISSION TO STUDY EXISTING DATA, DOCUMENTS, RECORDS, AND/OR BIOLOGICAL SPECIMENS (EXEMPTION UNDER CATEGORY 32 CFR 219/45 CFR 46 101.b.4). EACH QUESTION MUST BE COMPLETED.

</div>

	Yes	No
1. Will existing or archived data, documents, medical records, or database records be used? (Note: Data or biological specimens are considered to be "existing" or "archived" if all the data/biological specimens to be used for the research have been collected prior to the submission of this exemption application.)	X	
2. What is/are the (a) type and (b) source of the data/biological specimens? Provide detailed and specific information. Provide a copy of any surgical/donation consent forms used for obtaining data/biological specimens. Use an additional page if needed.		
Primary prostatic epithelial cell cultures- Existing cells will be used. The patient information was de-identified upon generation of the cell line. We have acess to treatment group race, disease stage, Gleason score and age (by year). Existing frozen prostate specimens will be used. The specimens are de-identified for all PHI and coded by study ID. We have access to treatment group race, disease stage, Gleason score and age (by year).		
3. Were data/biological specimens originally collected solely for research purposes? If yes is checked, please attach a copy of the IRB-approved Consent Form for the research under which the original data/biological specimens were collected.	Yes X	No

APPENDIX B

Appendix A - Claim of Exemption From Review by the USAMRMC Office of
Research Protections (ORP), Human Research Protection Office (HRPO)
(Continued)

	Yes	X No
4. Is the source of the data/biological specimens publicly available?		
5. How are the data/biological specimens identified when they are made available to you/your study team? Indicate by marking the appropriate box: a. [ ] Direct identifier (e.g., subject name, address, social security number, medical record number, etc.) b. [X] Indirect identifier (e.g., an assigned code which could be used by the investigator or the source providing the data/biological specimens to identify a subject such as a pathology tracking number or tracking code used by the source) c. [ ] No identifier (i.e., neither the researcher nor the source providing the data/biological specimens can identify a subject based upon information provided with the data/biological specimens) d. [ ] Other – please explain_____		
6. If 5.a or 5.b is checked above and you are requesting permission to study biological specimens, will the identifier(s) provided with the specimens be removed and destroyed upon receipt by your study team?	X Yes	No*
7. If 5.a or 5.b is checked above and you are requesting permission to study archived data, will you abstract and record any subject identifiers as a part of the data collection process?	Yes*	X No
8. Did the IRB of record determine that the research qualifies for exemption from the requirements of the regulations for the protections for human subjects? **(Provide a copy of the IRB of record's determination memo and/or OMB Form 0990-0263.)**	Yes X	No
*The research protocol does not qualify for exemption from the requirements at 32 CFR 219 (45 CFR 46).		

2

29 January 2007

APPENDIX B

Appendix A - Claim of Exemption From Review by the USAMRMC Office of Research Protections (ORP), Human Research Protection Office (HRPO) (Continued)

COMPLETE THIS SECTION IF YOU ARE REQUESTING EXEMPTION UNDER CATEGORIES 32 CFR 219/45 CFR 46.101.b.1 and/or 2

	Yes	No
1. Does the research involve children?	Yes	X No
2. Is the research conducted in established or commonly accepted educational settings?	Yes	X No
3. Does the research involves normal educational practices?	Yes	X No
4. Is this research on regular and/or special education instructional strategies? Describe	Yes	No X
5. Is this research on the effectiveness of or the comparison among instructional techniques, curricula, or classroom management methods? Describe	Yes	No X
6. Does the research involve any of the following? Educational tests (cognitive, diagnostic, aptitude, achievement) _____ Survey procedures _____ Interview procedures _____ Observation of public behavior _____ **Provide copies of all instruments.**	Yes	No X
7: Would any disclosure of the participants' responses outside the research reasonably place the participants at risk of criminal or civil liability or be damaging to the participants' financial standing, employability, or reputation?	Yes	X No
8. Will the information obtained be recorded in such a manner that participants can be identified directly or indirectly through identifiers linked to the participants?	Yes	X No

3

29 January 2007

APPENDIX B

Appendix A - Claim of Exemption From Review by the USAMRMC Office of
Research Protections (ORP), Human Research Protection Office (HRPO)
(Continued)

		X No
9. Does the research involve prisoners?	Yes	X No
10. Did the IRB of record determine that the research qualifies for exemption from the requirements of the regulations for the protections for human subjects? (**Provide a copy of the IRB of record's determination memo and/or OMB Form 0990-0263.**)	Yes X	No

Investigator's Statement

The undersigned certifies that the information provided in this document is complete and correct. Any modifications to this research that (a) change the research in a substantial way or (b) might change the basis for exemption will be provided to the IRB of record and the USAMRMC ORP HRPO for review to ensure that the exemption is still valid.

_____   9/12/2013
Principal Investigator's Signature   Date

4

29 January 2007

<p style="text-align:center;">**VITA**</p>

NAME: Rachael S. Farhat

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        Naoko Hiramatsu, Naomi Kiyono, Wendy White, Laura Liechty, Rachael Farhat, "Comparison of Dietary life focused on n-3/n-6PUFAs between Japanese students and American students", The 59th annual meeting of "The Japanese Society of Nutrition and Dietetics", Abstract No. O3-212-161, Nagoya Congress Center, Nagoya, Japan, Sept 12-14, 2012.

        Beyer A, Chung A, Farhat R, Omuron G. "Proposal to Improve Primary School Kitchens in Kamuli District, Uganda." Presented at Nutritional Sciences Council, Borlaug Student Poster Competition, Iowa State University College of Agriculture and Life Sciences 2009.

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EXPERIENCE   Nutrition Counselor
        Lactation Support Counselor

CERTIFICATIONS: Illinois Certified Lactation Support Counselor