PROTOCOL TITLE: Vitamin D supplementation in RNA-seq profiles of single core prostate biopsy samples, including diversity and stress determinants, among Veterans.

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1.0 Objectives / Specific Aims

Prostate cancer is the most prevalent non-cutaneous malignancy in men, responsible for approximately 30,000 deaths in 2013 (1). There are considerable and persistent racial disparities in prostate cancer outcomes. Prostate cancer disproportionately affects African American men in terms of incidence, morbidity, and mortality (1), even after adjustment for stage (2). African American men have a 2- to 3- times increased risk of developing prostate cancer and have a greater mortality rate compared to white men (2). Reduced access to health care services also contributes to racial disparities in prostate cancer outcomes, but even in equal access health care systems such as the Veterans Administration (VA), African American Veterans have higher serum prostate-specific antigen (PSA) values and higher-grade tumors than white Veterans (3, 4), even when presenting at the same stage of disease. Thus, access to health care is necessary but not sufficient for eliminating racial differences in prostate cancer outcomes. A better understanding of the biological mechanisms underlying these disparities is needed to develop strategies to overcome them.

Racial disparities in prostate cancer outcomes mirror racial differences in vitamin D levels (5). Several studies have shown that about 60% of African American men have sub-optimal levels of circulating 25(OH)D (6,7). For this reason, vitamin D_3 supplementation is likely to especially benefit these men. Vitamin D promotes the differentiation of prostate cancer cells and maintains the differentiated phenotype of prostate epithelial cells, raising the possibility that long-term vitamin D deficiency may contribute to the progression from subclinical prostate cancer to clinical disease (5), especially among African American men.

We propose to investigate: (a) the transcription and biological pathways that are especially relevant to prostate cancer disparities between African American and Caucasian men and (b) any significant differences in the molecular signature that exist in African American and Caucasian men in relation to their vitamin D levels.

The proposed studies will analyze transcriptional profiles and biological pathways in prostate tissue specimens obtained at time of prostate biopsy from these subjects, using bioinformatics analyses of RNA sequencing (RNA-seq) data. Implementation of the studies proposed in this application will allow us to test the central hypothesis that circulating levels of vitamin D significantly impact the transcriptional and biological profiles in prostate tissue samples from African American men.

To test our hypothesis we propose the following **Objectives:**

1. To utilize RNA-seq analyses to further demonstrate and identify a specific stress pattern signal that exists between African American and Caucasian men who are undergoing prostate biopsy. We have demonstrated initial findings that transcriptional profiles and biological pathways in the prostate are significantly different between African American and Caucasian subjects; the further elucidation as a result of these studies will allow us to identify the molecular underpinnings of health disparities in prostate cancer.

2. To identify changes in molecular signature that exist in African American and Caucasian men in relation to their vitamin D levels. We will assess ECM-receptor interaction, cell adhesion molecules (CAMs) and Pi3K-Akt signaling pathway according to serum levels as well as differences in those on supplementation.

3. Assess the above findings specifically as they relate to serum ancestry markers. We plan to assess the varying importance and relationship of specific markers of ancestry. Our population has significant proportions of pure African ancestry patients which will assist in this process.

2.0 Background

There are considerable and persistent racial disparities in prostate cancer outcomes. Prostate cancer disproportionately affects African American men in terms of incidence, morbidity, and mortality (1), even after adjustment for stage (2), as African American race is perhaps the most consistent risk factor for prostate cancer. Reduced access to health care services also contributes to racial disparities in prostate cancer outcomes, but even in equal access health care systems such as the Veterans Administration (VA), African American Veterans have higher serum PSA values and higher-grade tumors than white Veterans (3,4), even when presenting at the same stage of disease. Thus, access to health care is necessary but not sufficient for eliminating racial differences in prostate cancer outcomes. A better understanding of the biological mechanisms underlying these disparities is needed to develop strategies to overcome them. Vitamin D-mediated biological and biochemical actions are of great interest from the standpoint of racial disparities in prostate cancer outcomes because differences between African American and Caucasian men mirror racial differences in vitamin D levels (5).

Vitamin D and Prostate Cancer: Prostate cells express the vitamin D receptor (VDR), vitamin D-25-hydroxylase, 25-hydroxy-vitamin D-1-alpha-hydroxylase and the 25-hydroxyvitamin D-24-hydroxylase (8-13). Therefore, normal prostate cells can synthesize 25(OH)D (calcidiol) from vitamin D_3 (cholecalciferol), and $1,25(OH)_2$ D (calcitriol) from 25(OH)D (calcidiol) (14, 15). $1,25(OH)_2D$ is the hormonal, most potent form of vitamin D and in prostate cells it acts in a paracrine/autocrine fashion.

Several mechanisms of vitamin D-mediated anti-cancer action have been identified (16). Calcitriol induces cell cycle arrest through the expression of insulin growth factor binding protein-3 (IGFBP-3), which increases the levels of the cell-cycle inhibitor p21 in LNCaP prostate cancer cells (17); calcitriol also activates expression of IGFBP-3 by direct transcriptional stimulation through a vitamin D response element (VDRE) located in its promoter sequence (18). VDRE sequences have also been demonstrated in the promoter of the tumor growth factor beta (TGFβ)-2 gene (19), and treatment of PC-3 prostate cancer cells with TGFβ leads to enhanced expression of IGFBP-3 and subsequent growth arrest and apoptosis (20). Additional studies have suggested a link between chronic inflammation and prostate cancer (21). Vitamin D suppresses the expression of cyclooxygenase-II (COX-2), the key enzyme for the synthesis of prostaglandins, mediators of inflammation and thought to be important for cancer progression (22); COX-2 expression in biopsy cores and prostate cancer surgical specimen is an independent predictor of recurrence (23). Furthermore, there is considerable evidence that calcitriol inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling, and decreases the levels of the angiogenic and pro-inflammatory cytokine interleukin-8 (IL-8) in prostate cancer cells (24). NFκB is a transcription factor that plays a central role in the control of inflammation and is expressed at high levels in prostate cancers with high Gleason scores (25). This is only a very limited list of the many molecular pathways and mechanisms affected by vitamin D, as it is now well established that VDR may recognize cognate VDRE present within the regulatory sequences of hundreds of human genes, implicating vitamin D in a vast network of gene regulation, and underlying its broad physiological actions (26, 27). The recent emphasis on non-skeletal functions of vitamin D has to do with the realization that vitamin D deficiency has major implications for human health in general, and cancer biology in particular (32).

Hypovitaminosis D in African American Populations: Exposure of skin to sunlight in the ultraviolet B (UVB) range of the spectrum (290 to 315 nm) results in the photolytic conversion of 7-dehydrocholesterol to previtamin D_3, which is transformed to vitamin D_3 (cholecalciferol) by thermally induced isomerization (28, 29). Vitamin D_3 can be obtained from the diet; however, it is distributed very poorly in natural foodstuffs. Dark skin pigmentation, due to melanin, probably evolved in equatorial regions to protect individuals from skin cancers. Increased skin pigmentation, however, limits one's ability to produce vitamin D_3 (30). Vitamin D deficiency occurs when serum levels of 25(OH)D are at <50 nmol (<20 ng/mL); as a result, a majority of

African-Americans are vitamin D-deficient (4). We recently published the results of a prospective clinical study aimed at examining the effects of vitamin D_3 supplementation at 4,000 IU per day for two months in male subjects who had selected surgical removal of the prostate (prostatectomy) as a definitive treatment for their prostate cancer. According to current standard of care, a two-month interval between biopsy and prostatectomy is needed to resolve the inflammation due to the biopsy procedure. Moreover, the initial two months of vitamin D_3 supplementation register the fastest rise in serum levels of 25(OH)D, as reported by us (31,32) . This prostatectomy study was aimed at investigating the effects of vitamin D_3 supplementation (4000 IU per day for two months) on prostate tissue specimens obtained at surgery, by means of RNA-seq technologies, with special emphasis on differential gene expression in African American men compared to Caucasian men, and between supplemented and placebo groups (33). This paper is available in PDF format as Appendix 1.

We set Caucasian subjects (17 samples) as the control and African American subjects (10 samples) as the test. The results of our analyses showed that 3107 genes were differentially expressed using a stringent (highly significant) FDR<0.1, and 8237 genes were differentially expressed using a less stringent (still significant) FDR<0.4. Pathway analysis using the 3107 differentially expressed genes highlight major differences between the two groups, with African American showing higher expression of genes associated with immune response and inflammation. It must be noted that our observations are consistent with a previous report (34) based on the analysis of prostate tissue-derived RNA, performed using Affymetrix microarrays. This analysis revealed enhanced expression - in tissue samples from African American subjects - of biological pathways that are linked to autoimmune diseases, allergies, and inflammatory diseases. Even at this preliminary stage the results of our RNA-seq studies support the existence of considerable biological differences within the prostate between African American and Caucasian men and suggest that overexpression of genes linked to the inflammatory process may very well contribute to the increased severity and faster progression of prostate cancer in African American men, even at the early stage of disease (33).

Stress Response and Allostatic Load:

Allostatic load defines a set of clinical and biochemical parameters that can be easily measured in a basic clinical setting (35). These parameters can be analyzed to gauge the level of stress experienced by each subject. These values reflect chronic, steady state levels of stress as well as failure to shut-off responses to acute stressors. To the best of our knowledge, there is only one published report of a correlation between allostatic load in African American subjects and specific biomarkers (36). This report identified the dopamine receptor gene, *DRD4*, and the serotonin transporter gene, *SLC6A4*, as indicators of stress, because of a non-supportive family environment. Interestingly, both of these genes were very significantly up-regulated (at a FDR<0.1) in prostate tissue samples from African American men. These considerations suggest that the prostate appears to be, even at the molecular level, a “sentinel” organ for chronic stress.

The results of our RNA-seq analyses are consistent with our study objectives. These preliminary indications make us confident that the acquisition of additional RNA-seq data will allow us to strengthen the significance of our findings and help us identify specific pathways for possible targeted intervention.

Genomic technologies can be easily applied to the prostate tissue samples obtained from eligible subjects, undergoing prostate biopsy as part of their standard medical care. The results of our preliminary studies have already shown significant differences in the transcription profiles in prostate tissue samples between African American and Caucasian men. In addition, special emphasis will be given to RNA/seq analyses of Hypothalmic-Pituitary-Adrenal (HPA) -related transcription profiles, measurements of allostatic load and comparison of these profiles by race.

4.0 Study Endpoints

We will be investigating Veterans who are undergoing prostate biopsy. If the Veterans are eligible and seek active surveillance as their treatment modality, they will be offered an opportunity to receive 4,000 IU Vitamin D supplementation as per previous protocol. Upon repeat biopsy (standard of care), they will have repeat RNA-seq analyses which will serve as further internal control. We will be measuring serum prostate specific antigen (PSA) as per standard of care recommendations. A blood sample for ancestry markers will be collected at the conclusion of the study.

Primary clinical endpoint: We will be assessing for clinical activity based on PSA changes, and investigating whether or not vitamin D supplementation results in a 20% decrease from baseline PSA.

5.0 Inclusion and Exclusion Criteria/ Study Population

Males only, due to the limitation of the study to prostate tissue

Part 1

Inclusion Criteria

a) Age: 50 - 75 years of age
b) Scheduled to undergo a standard of care prostate biopsy because of an elevated PSA, an abnormal DRE (digital rectal exam) or other diagnostic procedure.
c) Permission for investigators to follow subject's post biopsy diagnosis, treatment decision and follow/up care (including subsequent prostate biopsies).

Exclusion criteria:

Inability or unwillingness of subject to give informed consent

Part 2

Inclusion Criteria

a) Prostate biopsy pathology report with diagnosis of prostate cancer
b) Treatment recommendation and/or subject decision of active surveillance
c) Agreement to take vitamin D_3 4000 IU daily for approximately one year
d) Standard of care repeat PSA at six months and surveillance (repeat) prostate biopsy after one year

Exclusion Criteria

a) Initial testing of prostate biopsy core sample indicates non-viable RNA for further sequencing.
b) Current vitamin D supplementation > 2000 IU daily
c) Inability or unwillingness to continue as a participant in the study

6.0 Number of Subjects

In an ongoing RNA-seq study of prostate tissue, 28 % of the enrolled subjects had pathology results of prostate cancer with the potential of active surveillance as the treatment option. Therefore, for this study, 250 African American and Caucasian males will enroll in Part 1 of the study with the goal of 60 African American and Caucasian males participating in Part 2. There may be less than 250 subjects in Part 1 if Part 2 accrues more quickly.

7.0 Setting

- Subject recruitment/enrollment will be conducted at the Ralph H. Johnson VA Medical Center's Urology Clinic and Research Offices/Clinics.
- The allostatic load blood analysis will be conducted by the Ralph H. Johnson Laboratory Services and MUSC's Laboratory Services
- The Vitamin D serum analysis will be conducted by Dr. Bruce Hollis' Laboratory at MUSC.

- RNA purification from biopsy tissue specimens will be performed at the Hollings Cancer Center Biorepository and Tissue Analysis Shared Resource. The RNA-seq will be performed by Gerard Hardiman, PhD Genomics Core Laboratory, Queens University Belfast, Ireland.

8.0 Recruitment Methods

- Potential subjects will be identified by the VAMC Urology Clinic schedule for prostate biopsies. A Recruitment letter from the Principal Investigator may be mailed to those potential subjects that may qualify for the study.
- Potential subjects may be identified by the Urology Provider when they have their preliminary Urology Clinic visit and the prostate biopsy is scheduled. Study introduction may be given to the potential subject at that time.
- If a progress note by the Urology Provider has been entered in the electronic record that the study has been introduced and then forwarded to the Research Coordinator, the Coordinator will then contact the potential subject before the biopsy date to answer any questions concerning the research study.
- The study may also be introduced to potential subjects by their Urology Provider just prior to the scheduled prostate biopsy. If the potential subject states he is interested in participating, he will be introduced to the Research Coordinator by the Urology Provider for further information and potential enrollment.
- The Research Coordinator(s) will meet the potential subject in the Urology Clinic or assigned research offices/space before their scheduled prostate biopsy to first obtain consent and then initiate study procedures.

9.0 Consent Process

Part 1

- Only patients who are not cognitively impaired will be asked to participate.
- When possible, the individual will be introduced to the study by their Urology Provider when the prostate biopsy is recommended and scheduled.
- The Health Care Provider will then indicate in the Progress Note that the patient has been introduced to the study and the Research Coordinator will receive a message to contact the patient.
- The patient will be contacted by the Research Coordinator to explain further the research requirements and answer any questions. The patient can then indicate whether he is willing to participate in the study.
- The study may also be introduced to potential subjects by their Urology Provider just prior to the scheduled prostate biopsy. If the potential subject states he is interested in participating, he will be introduced to the Research Coordinator by the Urology Provider for further information and potential enrollment.
- The consent process (time to read/review the consent and HIPAA and ask questions) will be conducted in a private exam room in the Urology Clinic or in assigned research offices/space.
- The consents will be signed only when the patient states that he understands the study procedures, has no questions, and is willing to participate.

Part 2

- Once a subject has (a) had a follow-up appointment with the Urology Provider to review the prostate biopsy pathology report, (b) received a diagnosis of prostate cancer, (c) reviewed the treatment options and (d) decided on active surveillance, the Research Coordinator will contact the subject for participation in Part 2. At that time, the subject will be asked if he wishes to continue with the study. If yes, Part 2 procedures will be initiated. If no, the subject will be released from further participation.

10.0 Study Design / Methods

Although some understanding of the race-specific and vitamin D-driven biochemical mechanisms and pathways affecting prostate cancer (37) have been demonstrated, the main objective of this proposal is to fill existing gaps in knowledge by identifying those mechanisms and pathways that are especially relevant to explain vitamin D effects on prostatic cells and tissues, as well as on prostate cancer disparities between African American and Caucasian men. The proposed studies will employ advanced technologies to analyze transcriptional profiles and biological pathways in prostate tissue specimens from male subjects undergoing prostate biopsy, and will involve studies of gene expression profiles, derived from RNA-seq data. Comparison of these transcriptional and biological profiles between African American and Caucasian subjects will allow us to identify the critical transcription profiles in the prostate affected by race. Additionally the potential variations related to vitamin D levels (before and after supplementation) as well as serum ancestry markers will be explored.

Objective 1. To utilize RNA-seq analyses to further demonstrate and identify a specific stress pattern signal that exists between black and white men who are undergoing prostate biopsy. We have demonstrated that transcriptional profiles and biological pathways in the prostate are significantly different between African American and Caucasian subjects. The results of these studies will allow us to further elucidate the molecular underpinnings of health disparities in prostate cancer, including identification of specific stress patterns. These studies will involve purification of ribonucleic acid (RNA) from prostate tissue specimens obtained at prostate biopsy, RNA sequencing (RNA/seq), analyses of transcription profiles, and comparison of these profiles across race by advanced bioinformatics tools.

Patient Cohort: As discussed in the Backround section, a substantial amount of information has already been collected through the pilot prostatectomy study as well as the initial aspects of this study. We have invested considerable resources to procure and analyze clinical samples obtained through our ongoing as well as previous research. This proposed study will leverage these investments and continue procuring and analyzing new samples that are annotated and linked with social determinants and allostatic load variables.

Male subjects (up to 250 in Part 1) will be enrolled in this study when they have their scheduled prostate biopsy. Pathology results at enrollment will be unknown. However, they will be followed to obtain data as to the biopsy results, treatment options, if recommended, for definitive treatment and/or management. For this project, in Part 2, sixty eligible subjects will be enrolled. Therefore, a total of 60 prostate tissue samples will be analyzed at baseline and again at study exit (repeat prostate biopsy). We anticipate that approximately 20 African American subjects will be enrolled in the study, since approximately 40% of the patients seen in the Urology Clinics of the Ralph H. Johnson VA Medical Center in Charleston are African American. As shown under Preliminary Studies, major differences in transcription within the prostate were observed between African American and Caucasian subjects enrolled in the pilot study (33).

Tissue Sample Procurement: The PI will oversee the procurement of prostate tissue core specimens. Specifically, he will orchestrate the single added core sample from a peripheral area of the prostate, during the enrolled subjects' prostate biopsy. This will ensure that the excision of tissue samples does not interfere with the diagnostic priorities.

RNA Purification, Sequencing, and Analysis of Transcription Profiles: This project will rely on several facilities. The Hollings Cancer Center Biorepository and Tissue Analysis Shared Resource will purify total RNA from each de-identified tissue sample using standard procedures. RNA integrity will be verified using RNA 6000 Nano Assay chips run in Agilent 2100 Bioanalyzer

(Agilent Technologies, Palo Alto, CA). High-quality RNA will be divided into two de-identified-coded samples and forwarded for storage to the Principal Investigator's Laboratory at the Charleston VAMC. Only one of the two split samples will be bulk shipped to Rick Kittles, PhD Genomics Core Laboratory at City of Hope Comprehensive Cancer Center in Duarte, CA. for RNA-sequencing. 100-200 ng of total RNA will be used to prepare RNA-Seq libraries using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) following the protocol described by the manufacturer. Paired end cluster generation will be performed on the cBot as described by the manufacturer (Illumina, San Diego, CA). Clustered RNA-seq libraries will be paired end sequenced with 250 cycles on a HiSeq2500 (Illumina, San Diego, CA). This new instrument acquired recently by MUSC generates up to 1 Tb per run, with data yields typically containing 80% greater than Q30, 2 x 100 bp read length. The "paired end" approach will be employed for the RNA-seq experiments, wherein a single long read is sequenced for 100 nucleotides at both ends. In addition to providing digital mRNA profiling, this strategy will permit tracking alternate splice junctions, insertions and deletions, and facilitate transcriptome assembly in these prostate cells and experimental groups.

Each sample will be sequenced to a depth of 100 million reads. Adapters will be trimmed by Trimmomatic (38). Alignment and annotation will be performed using DEseq2 (39), Edge R (40, 41) and the Tuxedo Suite comprising Bowtie2, Tophat2 and Cufflinks2 (42). As we are interested in applying RNA-seq as a discovery tool, the Tuxedo Suite will facilitate the identification of alternative splicing events, allele-specific expression, and rare and novel transcripts in these mRNA data sets.

Sample Size Estimation: The sample size of this study is 250 (Part 1) and 60 (Part 2) based on practical considerations and reasonable statistical power. Our inferences will be based on the determination of threshold on *p-values* to achieve a false discovery rate (FDR) of 10%. We anticipate that the large majority of genes will not be significant, but do not know the *p-value* threshold; we expect it to be between 0.001 and 0.01.

The primary endpoint is to estimate PSA reduction between pre and post treatment. We expect 20% PSA reduction post-treatment with the standard deviation of 40%. With 60 subjects , the 95% confidence interval for the estimated PSA reduction is 10% to 30%.

Data Analytic Approaches: Fold-change (FC) estimation and hypothesis testing for differential expression will be performed using the DESeq2 Bioconducter library (43, 44, 39). DESeq2 models read counts as arising from a negative binomial distribution using a generalized linear model (GLM) approach that facilitates effect estimation of experimental design factors. DESeq2 conducts gene-specific comparisons between groups based on model-based linear contrasts, with inference based on associated two-sided Wald tests. For each gene, DESeq2 reports estimated FC, a p-value, and a q-value (45-47) equivalent to the smallest false discovery rate (FDR) incurred when declaring that test significant. We will control the FDR at 10%, but will consider less conservative FDR thresholds (e.g. 15% or 20%) if the number of significant genes is too small (e.g. <20). We will construct volcano plots – scatterplots of – log10(p-value) versus log2(FC) – to identify genes strongly associated with race identification or circulating levels of Vitamin D with high statistical significance. Additionally, we will analyze read counts using edgeR (41) to confirm DESeq2 results. Finally, we will utilize Cufflinks to assemble the alignments into a parsimonious set of transcripts (42). This analysis will complement the approach described above and provide further validation of differentially expressed mRNAs.

Analysis of Cumulative Molecular Data: The cumulative data will be analyzed to identify race-associated differences between tissue samples from African American and Caucasian subjects. Using the list of differentially expressed genes from Objective 1, regression coefficients, t-statistics and statistical significance (*p-values*) will be saved and volcano plots will

be generated. Initially, unsupervised clustering (through hierarchal clustering) will be performed to identify sets of genes and proteins that tend to cluster by race and treatment as an exploratory approach. Furthermore, supervised machine learning techniques (random forests) will be used to identify genes/proteins that are associated with differential patterns by race to complement the analysis described above and allow us to interrogate pathways in a systematic way. These results can be combined with RNA-seq results using gene symbols, and additional enrichment analysis (e.g., KEGG, gene set enrichment analysis - GSEA) of each set separately, and combined. This will be accomplished through a combination of Ingenuity Pathway Analysis (Qiagen) and the ToppGene Suite (48).

Anticipated Results and Alternative Approaches: Prostate tissue specimens, collected prospectively, will be processed for RNA-seq. The methods to accomplish this are already established. The breadth of data to be generated will be large and unprecedented for Vitamin D and health disparity research studies. We have established data analysis methods, and these will continue to evolve as more samples are analyzed. We expect that there will be significant differences in gene expression by race as demonstrated by the results of our preliminary studies (33). Linking differences to specific transcription profiles, in order to identify molecular correlates of health disparities, is expected to require a merged analysis of all observed changes. This will no doubt require continued refinement of approaches to effectively interpret these data.

Objective 2. To identify changes in molecular signature that exist in African American and Caucasian men in relation to their vitamin D levels. This study will involve RNA/seq analyses of prostate tissue in African American and Caucasian Veterans. Previous studies have demonstrated varying molecular signatures that have existed both in relation to race and vitamin D levels. We will further explore this relationship to strengthen and refine the findings.

Precision medicine can play an important role in reducing racial disparities in morbidity and mortality among minority men because these efforts are designed to individualize health care based on biological, behavioral, and social factors that contribute to disease risks and enhance health outcomes. However, the development and implementation of approaches for precision medicine is still limited among minority men because empirical data are lacking on the ways in which risk factors and protective variables work on this underserved population that often relies for its health care needs more on small outpatient clinics than advanced teaching hospitals. This represents a significant gap that prevents this underserved population from taking advantage of what precision medicine can offer to minority men.

Allostatic Load Studies: These studies attempt to address this significant gap by proposing a research approach that allows for the extension of precision medicine benefits to an important segment of African American men: those diagnosed with prostate cancer. Stress response is often used to describe the body's attempt to adjust physically and psychologically to challenging conditions that are often perceived as adverse. The proposed studies will correlate relevant health parameters of African American subjects undergoing prostate biopsy with the molecular profiles obtained from prostate tissue samples. This objective is consistent with increasing evidence that pro-inflammatory mechanisms and the HPA axis are key mediators of the "allostatic load" and the results of our preliminary studies emphasize the transcriptional up-regulation of many genes associated with immune response and inflammation in prostate tissue samples of African American men.

Allostatic load parameters will be measured when subjects progress to Part 2 of the study. Allostatic load parameters selected for this study are the following:

- Systolic and diastolic blood pressure (indices of cardiovascular health)
- Waist-hip ratio (index of adipose tissue deposition associated with increased glucocorticoid activity)

- Serum high-density lipoproteins (HDL) lipid panel (index of atherosclerosis)
- Blood plasma levels of glycosylated hemoglobin (HbA1C, index of glucose metabolism over time)
- Serum dihydroepiandrosterone sulfate (DHEA-S) a functional HPA axis antagonist
- C-Reactive Protein and IL-6 (indices of inflammation)

Collections of overnight urinary output for measuring cortisol, epinephrine, and norepinephrine excretion levels will not be included in this study, since the outcome of the prostate biopsy would obviously interfere with the second collection.

For each of the chosen parameters, subjects will be classified into quartiles, based on the distribution of scores. Allostatic load will be measured by summing the number of parameters for which the subject will fall into the highest risk quartile, with the exception of HDL and DHEA-s values, for which the lowest quartile corresponds to the highest risk (35).

We anticipate that we will observe significant differences in allostatic load across races. We expect that these differences will correlate with differences in transcriptional profiles from prostate tissue sample from African American men compared to prostate tissue sample from Caucasian men.

Objective 3. To assess changes in RNA-seq profiles as they pertain to serum ancestry. Increasing evidence has shown the variability in genetic predisposition and permissiveness to a variety of illnesses and cancers. Our initial findings exhibit significant variations as they relate to race. Given the genetic heterogeneity that can exist in race, we seek to further explore these differences as they relate to genetic ancestry as determined through serum profiles. Our patient populations includes the Sea Island population which typically has higher percentages of pure African ancestry which can help to investigate these possibilities.

11.0 Study Related Procedures/Specimens and Risk Prevention

Part 1

1) The collection of an extra prostate tissue (punch) core from the peripheral area of the prostate
    - Will be collected during the Standard of Care prostate biopsy by the Urologist
    - Will be collected only with the subject's permission (signed Informed Consent)
    - Will not significantly create more risk to the subject than the standard 12 (punch) core collection incurred during the routine procedure
    - The extra collection will not interfere with the routine diagnostic process
    - Subjects will receive standard post-procedure instructions to minimize discomfort and risk as standard of care.
2) Electronic medical chart review will be performed by a Research Coordinator
    - To verify demographics: age, race, ethnicity
    - To verify pathology results from the prostate biopsy
    - To ensure that the subject receives follow-up care in the Urology Clinic by alerting Urology Clinic personnel if a subject does not attend scheduled follow-up appointments.
    - To follow subject's post-biopsy diagnosis, treatment decision and follow/up care (including potential collection of prostate tissue during subsequent prostate biopsies).
    - To determine if subject is eligible to proceed to Part 2 of the study
    - All data collected will be entered into the study database located on the VA secure server (V:) by a Research Coordinator and will be with the assigned codes.

Part 2

1) Blood for measuring serum concentration of 25(OH)D_3.
    - Obtained with other required bloodwork by an experienced phlebotomist or R.N. using sterile supplies and clean technique.

- Will be processed, labeled with an assigned code, and stored in -20º freezer located in the VA Mental Health Research Building – Lab DD159 for approximately 1-4 weeks before it is transported (refrigerated) by the Research Coordinator to Bruce Hollis' Laboratory at MUSC.
- Any remaining serum will be destroyed at the end of the study.
- Shared results will be with the assigned codes.

2) Blood for allostatic load (lipid panel, HgA1C, DHEA-S, C-Reactive Protein and IL-6)
   - Obtained before the biopsy with other study required bloodwork by an experienced phlebotomist or R.N. using sterile supplies and clean technique
   - Must be labeled with the subject's name and VA registration number, as per VA Laboratory Services regulations. Results will be processed by the VA Laboratory Services and entered into the VA CPRS (electronic record).
   - After analysis, the VA Laboratory will not store any remaining blood samples.
   - Results will be entered into the research study data base with the assigned code by a Research Coordinator.
   - Shared results will be with the assigned codes.
3) Allostatic load measurements: SBP/DBP (systolic and diastolic blood pressure), hart rate and waist/hip ratio are non-invasive measurements
   - SBP/DBP and heart rate will be measured in the Urology Clinic prior to the prostate biopsy by either the Research Coordinator or Urology Clinic staff, and recorded in the VA CPRS electronic record as per Urology Clinic protocol prior to this Standard of Care procedure.
   - The waist/hip ratio will be obtained using a designated tape measure by the Research Coordinator and recorded.
   - These data will be entered in the study spreadsheet with the assigned codes.
4) A survey (Social Determinants) will be given to each subject with an instruction sheet and stamped, addressed return envelope to be completed at home after their prostate biopsy.
   - The survey will be completed by subjects at home after their biopsy.
   - The survey will be identified by each subject's assigned code.
   - The survey will be mailed back to Dr. Chanita Hughes-Halbert, VA HEROIC Investigator, for analysis and storage.
   - Within 21 days if the survey has not been returned to the designated research staff, a research assistant will contact the subject to assess if they have completed the survey, or need additional assistance.
   - Subjects will also be given the option to have the survey administered by telephone by a trained research assistant if they are having issues with self-administering the survey.
   - Contact information for mailing the compensation gift card or verifying that the compensation has been received, will be destroyed once receipt of the gift card is documented.
   - At the end of the study, these surveys will be boxed, labelled and sent to storage per VA Office of Research and Development's (ORD) Records Control Schedule.
5) Subjects will begin vitamin D_3 supplementation of 4000 IU daily.
   - Previous studies have demonstrated no safety concern with this dose of vitamin D_3 supplementation.
   - According to the Endocrine Society and Institute of Medicine standards, a vitamin D blood level between 30-50 ng/mL is sufficient. Taking 4,000 IU of vitamin D_3 daily will not exceed that level in the blood. Therefore, this research study amount of 4,000 IU daily should not produce any risk.
   - Preliminary studies demonstrated evidence of decreased positive biopsy cores after one year of supplementation at 4000 IU daily

- RNA-seq analysis has demonstrated improvement in inflammatory profiles in prostate tissue.

6) Electronic medical chart review will be performed by a Research Coordinator
   - To verify demographics: age, race, ethnicity
   - To verify serum levels of PSA (prostate specific antigen) and pathology results from the prostate biopsies
   - To collect results of the study's allostatic load measurements and vitamin D serum levels
   - To ensure that the subject receives follow-up care in the Urology Clinic by scheduling the standard of care six month repeat PSA clinic visit and one year repeat PSA and prostate biopsy.
   - All data collected will be entered into the study database located on the VA secure server (V:) by a Research Coordinator and will be with the assigned codes.
7) Collection of Ancestry Markers serum sample at the final visit of the study
   - Obtained with other required bloodwork by an experienced phlebotomist or R.N. using sterile supplies and clean technique.
   - Will be labeled with an assigned code.
   - Will be stored in assigned research space in the VA Mental Health Research Building for batch shipment to City of Hope Comprehensive Cancer Center in Duarte, CA for analysis
   - Subjects will not receive results from this analysis
   - Any remaining serum will be destroyed at the end of the study.
   - Shared results will be with the assigned codes.

8) The collection of an extra prostate tissue (punch) core from the peripheral area of the prostate during the repeat prostate biopsy
   - Will be collected during the Standard of Care prostate biopsy by the Urologist
   - Will be collected only with the subject's permission (signed Informed Consent)
   - Will not significantly create more risk to the subject than the standard 12 (punch) core collection incurred during the routine procedure
   - The extra collection will not interfere with the routine diagnostic process
   - Subjects will receive standard post-procedure instructions to minimize discomfort and risk as standard of care.

12.0 Data Management

The study database spreadsheets

- The databases will be maintained in the VAMC Charleston secure (V:) drive and accessed only by the Research Coordinator(s) and the PI.
- All data entered will be with the assigned codes.
- All shared data will be with the assigned codes.

Sample Size Estimation: The sample size of this study is 250 (Part 1) and 60 (Part 2) based on practical considerations and reasonable statistical power. Our inferences will be based on the determination of threshold on *p-values* to achieve a false discovery rate (FDR) of 10%. We anticipate that the large majority of genes will not be significant, but do not know the *p-value* threshold; we expect it to be between 0.001 and 0.01. This sample size estimation is consistent with the data analytic approaches discussed under Study Design.

The primary endpoint is to estimate PSA reduction between pre and post treatment. We expect 20% PSA reduction posttreatment with the standard deviation of 40%. With 60 patients, the 95% confidence interval for the estimated PSA reduction is 10% to 30%.

Statistical Analysis
All statistical analyses will be performed using SAS v. 9.4 or R statistical analysis packages. The overall PSA reduction rate and the corresponding 95% confidence interval and percentage of each core from 12 cores will be estimated. Basic descriptive statistics will be estimated for demographic at pretreatment and clinical variables (blood pressure, weight, hips and waist circumference, vitamin D level, blood sugar, cholesterol, kidney function and stress hormones) at pre and post treatment.

13.0 Provisions to Monitor the Data to Ensure the Safety of Subjects

This study requires an initial visit (Part 1): consent process plus collection of an extra prostate biopsy tissue core for study analysis.
If the pathology report indicates prostate cancer and the subject's treatment decision is active surveillance, he may continue participation to Part 2. Data collected in this phase will be: demographics, blood pressure, heart rate, hip/waist ratio, allostatic load blood results, one survey, a repeat prostate biopsy core collection, vitamin D levels, PSAs, RNA-seq of prostate core samples, and serum ancestry markers.
All samples will be labeled with the assigned de-identified code (except for samples analyzed by the VAMC laboratory services which require name and last four of the social security number).VAMC laboratory results will be entered into the research database with the assigned code. All shared data will be under the assigned codes.

During each study visit, an adverse or unanticipated study-related event is unlikely, but may occur. Therefore, if an adverse event occurs, it will be reported as required to the Institutional IRB, VA R&D, and all other oversight committees and organizations. The Attending Urologist in the VA Urology Clinic at the time of the event will evaluate the subject and the event and schedule follow-up medical intervention as needed.

If, upon review of the study required blood results, an abnormal result is reported by one of the laboratories, the subject's primary health care provider will be notified via the VA CPRS electronic records system for evaluation, possible treatment and follow-up.

14.0 Withdrawal of Subjects

Participation in Part 1 of this study may be a single study visit, or if the subject agrees to another prostate core sample obtained as standard of care approximately 12 to 18 months after his initial prostate biopsy, participation will be two study visits. Participation in Part 2 will include an initial visit (when inclusion criteria are met), at six months for PSA and vitamin D level and a final visit for repeat prostate biopsy, allostatic load measurements, vitamin D level and ancestry marker. Subjects may withdraw their decision to participate at any time after signing the consent forms. This includes the use of any specimens that are not already exhausted by laboratory analysis.

15.0 Risks to Subjects

1. Venipuncture: the risks of drawing blood include temporary discomfort from the needle stick and bruising, fainting could occur.
2. Collection of an extra prostate tissue (punch) core from the peripheral area of the prostate will not significantly create more health risk to the subject than the standard 12 (punch) core collection incurred during the routine procedure. Prostate biopsies are done under local anesthesia, and the extra prostate biopsy may cause 7% more discomfort as a result. The collection from this area will not interfere with the Pathologist's analysis and diagnostic process.

3. Loss of Confidentiality of protected health information (unlikely side effect). A Master List with contact information is required as Good Clinical Practice for dissemination of new information and follow-up care. This Master List will be maintained by the Research Coordinator(s) on the VA secure (V:) drive. Only the Research Coordinator(s) and the PI will have access to this list. The Master List will also serve as the link in identifying each subject to his assigned code.
4. Taking vitamin D_3 supplementation at 4000 IU daily poses no risk to the subjects. This dose is well within the recommended doses by the Endocrinology Society and Institute of Medicine.
5. Some questions in the Social Determinants survey may make subjects uncomfortable or upset. Subjects will be instructed to skip any questions that they do not wish to answer.
6. Genetic Research. The RNA-sequencing will analyze transcriptional profiles and biological pathways on the molecular level in prostate tissue. It is not identifying specific genes and an individual's predisposition to a particular diagnosis or disease process. The ancestry markers will be analyzed for racial origin/significance only. No DNA analysis for disease identifiers will be performed.

16.0 Potential Benefits to Subjects or Others

A direct benefit may not be gained by the participants. However, if measuring gene expression in prostate tissue specimens provides molecular biomarker(s) for assessing the effects of circulating levels of vitamin D on cancer, it will be extremely important in determining treatment options for future cases of prostate cancer at time of diagnosis. Preliminary studies have demonstrated evidence of decreased positive biopsy cores after one year of supplementation at 4000 IU daily. Also, RNA-seq analysis has demonstrated improvement in inflammatory profiles in prostate tissue in men supplemented with vitamin D_3.

17.0 Sharing of Results with Subjects

- The allostatic load laboratory results analyzed in the VAMC laboratory will automatically be entered into the VA electronic record and can be viewed by each participant's Health Care Provider and shared as needed with the subject.
- Vitamin D serum levels (baseline, six months and exit) will be reported to the subject. The subject's Primary Health Care Provider will be notified for follow-up as needed at the end of Part 2.
- The blood pressure, heart rate and waist/hip measurements will be shared with each subject when they are obtained.
- The survey is self-completed and each subject will know the information his contains.
- The RNA-seq analyses will be published at the completion of the study. All results will remain de-identified.
- The ancestry markers will not be shared with the subjects.

18.0 Drugs – vitamin D supplementation

FDA IND# 77,839 was originally granted to Dr. Sebastiano Gattoni-Celli in 2007 for an open-label clinical study designed to assess the effect of vitamin D_3 on subjects with early-stage prostate cancer. This clinical trial will be conducted under the same IND, which has been transferred to the PI, Dr. Stephen Savage. The vitamin D_3 (cholecalciferol) is manufactured by JR Carlson Laboratories, Inc., 15 College Ave., Arlington Heights, IL 60004-1985. JR Carlson Laboratories will manufacture soft gel capsules containing 4000 IU of vitamin D_3 for the study. The study medication will be shipped to the research pharmacy of the Ralph H. Johnson VA Medical Center where it will be stored and dispensed as per standard procedure.

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Appendix 1

Flowchart of Study Procedures and Timeline

Procedure	Initial Prostate Biopsy Clinic Visit	Baseline	Six Months	Approximately 1 year	Comments
Part 1:					Standard of Care prostate biopsy
Consent process	X				
13th core prostate tissue sample	X			X	PSA is routinely determined before a prostate biopsy is scheduled
Part 2:					Subject has had follow-up appointment with Urology Provider and has decided on active surveillance as treatment option and meets inclusion criteria
Reaffirm Consent		X			
Allostatic load measurements[1]		X		X	
Allostatic load serum samples[2]		X		X	
Vitamin D level		X	X	X	
PSA			X	X	PSA not collected at baseline. PSA will be elevated due to the recent biopsy procedure.
Ancestry markers				X	
Dispense vitamin D_3 4000 IU bottle		X	X		
Collect vitamin D_3 bottle			X	X	
13th core prostate tissue sample				X	

[1] Blood pressure. heart rate, waist/hip ratio
[2] Lipid panel, HgbA1c, Comprehensive Metabolic Panel, CRP, IL-6 and DHEA-S