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14. ABSTRACT

We hypothesize that differences in the epigenomic landscape may interact with somatic genomic alterations and tumor microenvironment to drive racial disparities in prostate cancer outcomes and that epigenomic alterations may serve as prognostic molecular biomarkers in AA PCa. Here, we propose to conduct the largest study to comprehensively define the epigenomic landscape of PCa arising in AA men with long-term oncologic follow-up and/or tumor somatic sequencing and immune microenvironment characterization. This research will identify unique epigenomic drivers of aggressive biology and adverse PCa outcomes among AA patients, elucidating novel biological underpinnings of this critical health care disparity.

15. SUBJECT TERMS
Prostate cancer, heath disparities, methylation

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1. **INTRODUCTION:**

We hypothesize that differences in the epigenomic landscape may interact with somatic genomic alterations and tumor microenvironment to drive racial disparities in prostate cancer (PCa) outcomes and that epigenomic alterations may serve as prognostic molecular biomarkers in AA PCa. In **Aim 1**, we will identify differentially methylated CpG sites associated with genetic racial ancestry, oncologic outcomes, somatic genomic alterations and immune response and oncologic outcomes in a retrospective Johns Hopkins cohort of matched prostate tumors from 200 AA and 200 WH men at radical prostatectomy. Using the Infinium MethylationEPIC microarray platform to interrogate over 850K methylation sites per sample at single nucleotide resolution, we will identify differentially methylated CpG sites stratified by racial ancestry, clinical-pathologic parameters and oncologic outcomes (recurrence and metastasis) overall and within each group, focusing on the CpGs with concurrent transcriptional changes by completed microarray profiling (Affymetrix 1.0ST arrays). Then, we will integrate tumor methylation profiles with previously assessed tumor somatic genomic alterations and microenvironment immunophenotyping data to identify novel biomarkers of PCa outcome disparities**.** In **Aim 2**, we will validate epigenomic signatures associated with adverse oncologic outcomes in AA patients discovered in Aim 1 using the Baylor College of Medicine (BCM) retrospective cohort of 300 AA tumors at radical prostatectomy with long term follow-up. Race-specific methylation signatures from the JHU cohort will be validated by comparison of the BCM AA samples to historical WH control samples from TCGA (n=300) or the Moffitt Cancer Center (n=120) previously arrayed on Infinium 450K arrays. Finally, in **Aim 3**, we will validate epigenomic signatures associated with pathologic tumor aggression, somatic genomic alterations and immune microenvironment alterations in AA patients using the RESPOND cohort of 500 prospectively collected AA tumors from SEER cancer registry sites around the country. Her, we will perform and analyze whole-genome methylation profiling using the Infinium MethylationEPIC microarray platform to validate a signature of differentially methylated CpG sites associated with pathologic measures of tumor aggression (tumor grade and stage), tumor somatic genomic alterations, tumor immunophenotype and environmental stressors among AA patients.

2. **KEYWORDS:**

Prostate cancer, cancer health disparities, methylation, epigenomic

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

Please see SOW below with % completion annotated for each task

Research-Specific Tasks:

Specific Aim 1: Identify differentially methylated CpG sites associated with genetic racial ancestry, oncologic outcomes, somatic genomic alterations and immune response in a retrospective Johns Hopkins cohort of matched primary PCa from 200 AA and 200 WH men at radical prostatectomy.	**Timeline (Months)**	**% completed**
Major Task 1: Using previously collected data on the Infinium MethylationEPIC microarray platform to interrogate over 850K methylation sites per sample at single nucleotide resolution, we will identify differentially methylated CpG sites stratified by racial ancestry, clinical-pathologic parameters and oncologic outcomes (recurrence and metastasis) overall and within each group		
Subtask 1: Obtain HRPO Approval for study (Hopkins IRB approval is in	1-3	100%

place already)		
Subtask 2: Integrate tumor methylation profiles with previously assessed tumor somatic genomic alterations, microenvironment immunophenotyping data and genetic racial ancestry data to identify novel biomarkers of PCa outcome disparities**.**	3-24	70%
Subtask 3: Validate biomarkers by pyrosequencing/methylation specific PCR	24-36	0%
Specific Aim 2: Validate epigenomic signatures associated with racial ancestry and adverse oncologic outcomes in AA patients using the Baylor College of Medicine (BCM) retrospective cohort of 300 AA tumors at radical prostatectomy with long term follow-up.		
Major Task 2: Perform and analyze whole-genome methylation profiling using the Infinium MethylationEPIC microarray platform on BCM samples		
Subtask 1: Obtain HRPO Approval for study	1-3	100%
Subtask 2: Receive DNAs from BCM and run methylation arrays at NXTDx	6-18	0%
Subtask 3: Validate a signature of differentially methylated CpG sites associated with biochemical recurrence and metastasis among AA patients.	18-36	0%
Subtask 4: Validate a signature of differentially methylated CpG sites associated with racial ancestry by comparison to TCGA and Moffitt Cancer Center WH cohorts	18-36	0%
Specific Aim 3: Validate epigenomic signatures associated with pathologic tumor aggression, genetic racial ancestry, somatic genomic alterations and immune microenvironment alterations in AA patients using the RESPOND cohort of 400 <u>prospectively collected</u> AA tumors		
Major Task 3: Perform and analyze whole-genome methylation profiling using the Infinium MethylationEPIC microarray platform on RESPOND samples	25-36	0%
Subtask 1: Obtain HRPO Approval for studies	1-3	100%
Subtask 2: Receive DNAs from RESPOND and run methylation arrays at NXTDx	6-30	0%
Subtask 3: Validate a signature of differentially methylated CpG sites associated with clinical pathologic risk category among RESPOND AA patients.	30-36	0%
Subtask 4: Integrate methylome in RESPOND cohort with previously assessed tumor somatic genomic subtype, genetic racial ancestry and tumor immonophenotyping	30-36	0%
Milestone(s) Achieved: Validated prognostic signature for recurrence/metastasis in AA PCa	36	

- **What was accomplished under these goals?**

1) major activities: Most activities during this period were focused on Aim 1, examining the methylation data in the Johns Hopkins race-matched primary PCa cohort, and we additionally obtained IRB and HRPO approvals for all three Aims as planned. For the Johns Hopkins cohort, raw .idat methylation array files were obtained from Infinium MethylationEPIC Array (Illumina, Inc) from 320 prostate cancer and benign samples and de-identified clinical data from patients. Additional .idat files from 100 cases from Moffitt Cancer Center were also received. The data were subject to quality control checks to confirm the high-standard probe pairing during array experiments, including density, localization, and distribution of probes per sample. These analyses were performed by using the packages *minfi* and *shinyMethyl* in R. Data were subject to normalization through Subset-quantile Within Array Normalization (SWAN) through *minfi* in R. New quality control checks were performed to verify the distribution of probes across samples and sample types (tumor and benign). Individual probe beta values were obtained after SWAN normalization and ratio conversion using *ratioConvert*() function in *minfi*. Bbeta values were transformed into probe M values by using the following equation: *log2(beta/(1-beta)).* Methylation levels were compared across groups to determine clinical associations with differential methylation levels. First, methylation levels of known probes and genes were compared between benign and tumor samples. These known genes are frequently described in the literature as differentially methylated between benign and tumor samples in PCa. These genes are the following: *GSTP1*, *LTF*, *TIMP3*, and *SPARC*. A validation of this analysis was performed with samples from The Cancer Genome Atlas (TCGA) (**Figure 1**). After this quality control check, multivariable limma models were generated to compare tumors from African-American (AA) samples versus non-latino white samples (WH). The multivariable adjustments were performed employing *ERG* fusion status previously identified through immunohistochemistry. *ERG* fusions are strongly associated with distinct methylation levels in prostate cancer and are twice as common in WH as AA samples. In this manner, it is imperative to adjust the methylome comparisons between AA and EA with *ERG*. Moreover, the multivariable model was adjusted using standard prognostic variables in prostate cancer, such as Gleason score and age. Interaction models will be used to identify differentially methylated probes that are specific to race (AA vs. WH) and also for those specific for *ERG* (positive vs. negative). These models will be generated and analyzed by employing linear models with *limma*. Heatmaps and volcano plots will be generated for each comparison.

2) specific objectives: The objectives were to identify differentially methylated CpG sites associated with genetic racial ancestry, oncologic outcomes, somatic genomic alterations and immune response and oncologic outcomes in a retrospective Johns Hopkins cohort of matched

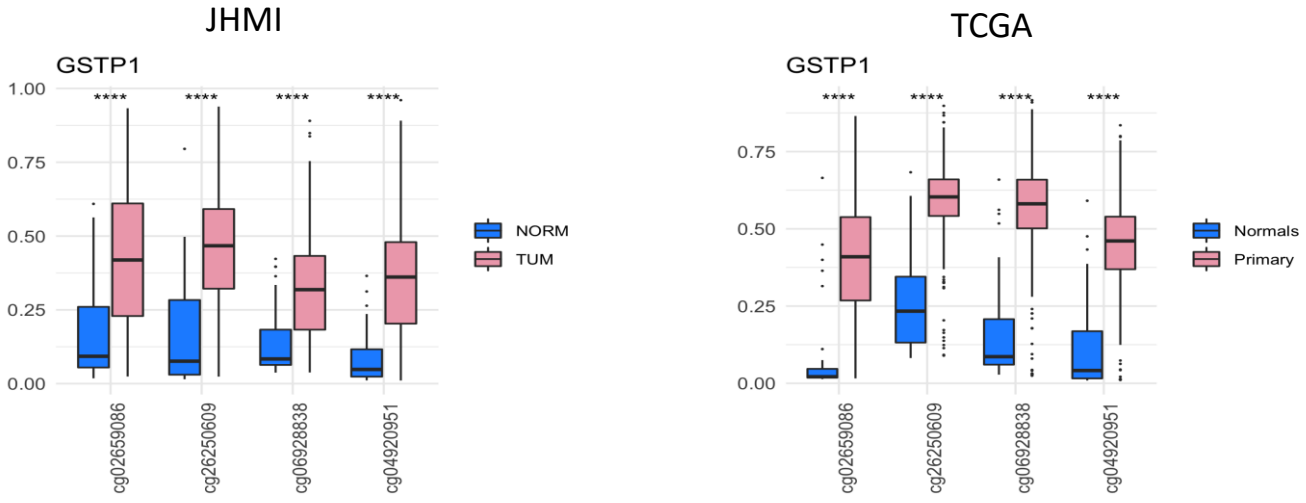

Figure 1: GSTP1 methylation in JHMI and TCGA cohorts confirms hypermethylation in tumor (tum) vs. benign (norm) samples in both cohorts as expected. While TCGA tum-norm differences are greater than in JHMI, this is expected given that TCGA samples were fresh frozen while JHMI samples were FFPE.

prostate tumors from 200 AA and 200 WH men at radical prostatectomy.

3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative) We are still refining the *limma* models as described above, however significant results include: a) finding that methylation data from the matched AA-WH cohort is of high quality for analysis; b) Discovery of ~240 probe sets that are differentially methylated in AA vs WH PCa in simple comparative analyses (**Figure 2**); c) Determination that *ERG* status is a confounding variable which must be accounted for in future *limma* models examining differences in methylation by race; d) additional modeling is currently in progress.

4) other achievements. To date, the only stated goal we have not met for the first year is the receipt of Baylor and RESPOND DNAs for methylation analysis. This delay was due to COVID19 as described in more detail below and we are hopeful that we can make up lost time during the second year of the award period.

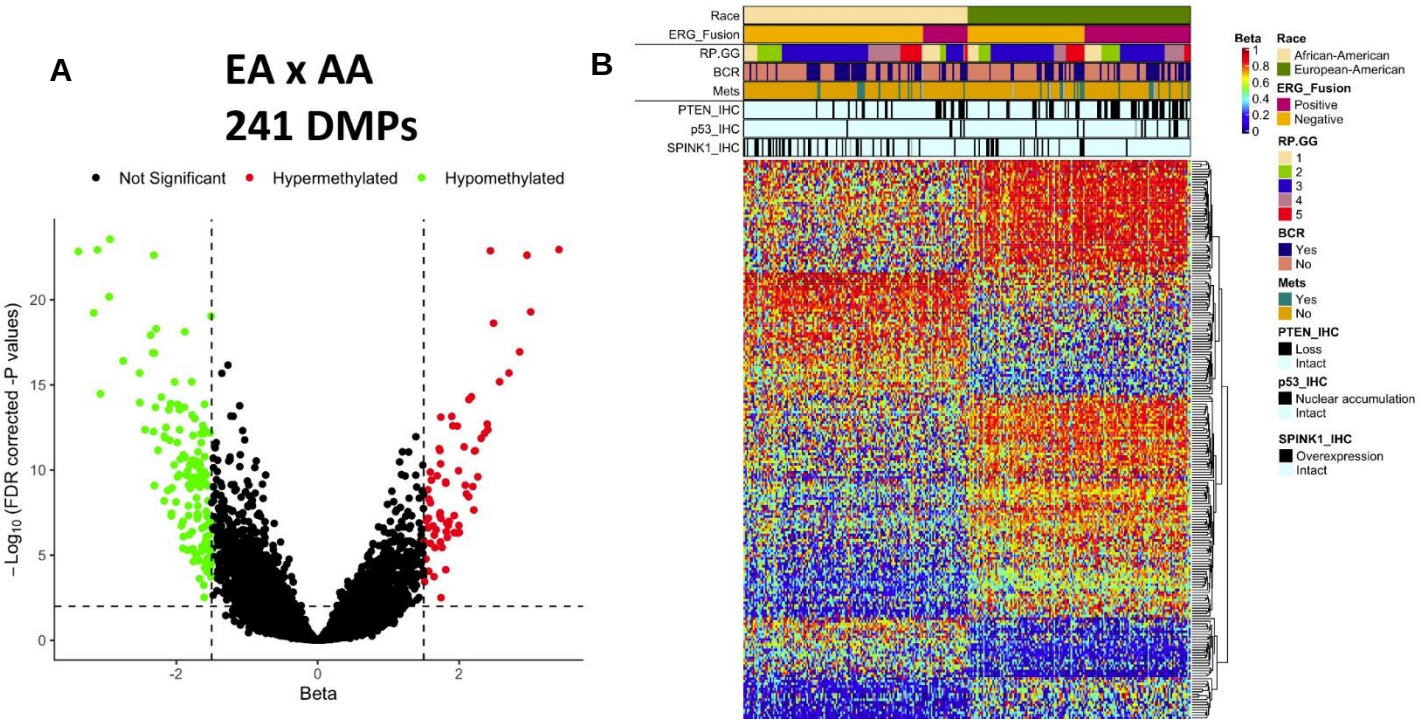

Figure 2: Initial limma models examining differentially methylated probes (DMPs) by racial ancestry in the JHU AA-WH primary PCa cohort. **(A)** Volcano plot illustrating DMPs by racial ancestry. **(B)** Heat map of differentially methylated probes by race, with annotation for molecular subtypes and clinical-pathologic variables.

- **What opportunities for training and professional development has the project provided?**
  - Nothing to report
- **How were the results disseminated to communities of interest?**
  - Nothing to report
- **What do you plan to do during the next reporting period to accomplish the goals?**

We will continue to refine our methylation models in the JHU cohort. Interaction models will be used to identify differentially methylated probes that are specific to race (AA vs. WH) and also for those specific for ERG (positive vs. negative). These models will be generated and analyzed by employing linear models with *limma*. Heatmaps and volcano plots will be generated for each comparison. After adjustment and contrast extraction using the above-mentioned variables (including race, tumor grade, molecular subtype, differentially methylated probes obtained by comparing AA and WH will be further investigated. Probe location in the genome will be annotated together with gene localization. The location of each differentially methylated probe from the

comparisons between AA and WH will be analyzed to distinguish which probes most significantly affect the expression of correspondent genes. Gene enrichment analysis will also be conducted with the differentially methylated genes to identify possible affected regulatory pathways. Gene and probe annotation will be performed in R using the package *minfi,* and gene enrichment analyses will be carried out with *TCGABiolinks* package in R. In TCGA cohort, the differentially methylated probes obtained from the analysis with the Johns Hopkins AA vs. WH comparison will be investigated through ELMER. ELMER integrates gene expression and methylation data to infer the regulatory element landscape and transcription factor network of tumors. Such analysis will shed light on putative markers that should be further validated in the Johns Hopkins cohorts through immunohistochemistry. After identifying differentially methylated probes, we will evaluate if their hypo- or hypermethylation status influences the outcome of patients (biochemical recurrence and metastasis as endpoints) through log-rank tests, Kaplan Meier curves, and uni- and multivariable Cox regression models. Other clinical variables will also be tested by using association tests (logistic regression and Fisher or Chi-square test). In addition, we hope to obtain DNA and perform methylation arrays in the Baylor and RESPOND samples over the next reporting period. These samples will serve as validation sets.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

   - **What was the impact on the development of the principal discipline(s) of the project?**

     - Once complete, the hope is that the work in this proposal will identify many of the molecular contributors to the disparity in PCa outcomes among AA patients, enabling the rational design of therapies targeted for this population of patients. Given that AA PCa shows fewer mutations and copy number alterations associated with adverse outcomes than seen in non-Latino White PCa (WH), current molecular biomarkers gleaned from studies of WH PCa are clearly inadequate and additional prognostic biomarkers are needed specifically in the AA population. In the short term, an immediate deliverable of this award is a highly validated epigenomic signature of tumor recurrence and metastasis among men with AA PCa.

   - **What was the impact on other disciplines?**

     - Nothing to report

   - **What was the impact on technology transfer?**

     - Nothing to report

   - **What was the impact on society beyond science and technology?**

     - Understanding the root causes (biological and social) of cancer health disparities will be a key stepping stone in rectifying the longstanding history of racial injustice in our country.

5. **CHANGES/PROBLEMS:**

   - **Changes in approach and reasons for change**

     - Nothing to report

   - **Actual or anticipated problems or delays and actions or plans to resolve them**

     - The COVID19 pandemic resulted in the shutdown of all research activities at Johns Hopkins in mid-March, 2020 and Baylor has been more recently affected by the second wave of infections. RESPOND sample acquisition has also be delayed due to recruiting difficulties. This has resulted in a delay in obtaining and processing the samples for Aims 2 and 3. However, we are hopeful that we can make up these delays in the next few months.

- **Changes that had a significant impact on expenditures**
    - The COVID19 pandemic resulted in delays as described above that have decreased expenditures on methylation profiling (these were the bulk of the costs in this project).
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - Nothing to report
- **Significant changes in use or care of human subjects**
    - Nothing to report
- **Significant changes in use or care of vertebrate animals.**
    - Nothing to report
- **Significant changes in use of biohazards and/or select agents**
    - Nothing to report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
    - Nothing to report
- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.
    - Nothing to report
- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.
    - Nothing to report
- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.
    - Nothing to report
- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- We have assembled a database of whole genome methylation profiling results for the race-matched Johns Hopkins cohort. This database, integrated with somatic genomic alterations and copy number alterations as well as clinical outcomes will be made available to public upon publication and provide a valuable resource and the first of its kind in prostate cancer.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

- *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Name:	*Tamara Lotan*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.20*
Contribution to Project:	*Dr. Lotan supervises all data collection and data analysis on the project*
Funding Support:	*Please see other support*

Name:	*Luigi Marchioni*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.20*
Contribution to Project:	*Dr. Marchionni supervises bioinformatics data analysis on the project*
Funding Support:	*Please see other support*

Name:	*Thiago Vidotto*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6.00*

Contribution to Project:	*Dr. Vidotto performs all of the bioinformatics data analysis on the project*
Funding Support:	*Please see other support*

Name:	*Daniela Salles*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2.40*
Contribution to Project:	*Dr. Salles assists with DNA extraction and methylation validation experiments*
Funding Support:	*Please see other support*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Dr. Lotan: changes since last report are as follows:

  National Institutes of Health #R01CA211695 – completed
  American Cancer Society #RSG-17-160-01-CSM – completed
  Prostate Cancer Foundation (Challenge Award) #18CHAL15 – completed
  Department of Defense - #W81-XWH-19-1-0292 – active
  Department of Defense - #W81-XWH-19-1-0686 – active
  Department of Defense - #W81-XWH-19-1-0781 – active

  Dr. Marchionni: changes since last report are as follows:

  National Institutes of Health #R01CA211695 – completed
  American Cancer Society #RSG-17-160-01-CSM – completed
  Department of Defense - #W81-XWH-19-1-0292 – active

- **What other organizations were involved as partners?**

  - **Organization Name: Baylor University School of Medicine**
  - **Location of Organization:** *Houston, TX*
    - **Other: Provides PCa samples for analysis**
  - **Organization Name: Moffitt Cancer Center**
  - **Location of Organization:** *Miami, FL*
    - **Other: Provides methylation data on PCa**
    -

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ebrap.org for each unique award.*

- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***