

Response to Reviewer Comments (Manuscript #2186476)

We thank the Reviewers and Editor for their thoughtful and constructive reviews. We appreciate that the reviewers noted the importance and significance of this study. Considering all reviewers' comments, we have revised the manuscript for greater clarity. We feel that these edits, along with rigorously addressing every comment, have greatly enhanced and improved the clarity of our manuscript. Each specific reviewer comment (**in bold**) is followed by our response below.

Reviewer 1

Major points

1. **Figure 2, graphs showing serum metabolite levels. The differences between groups are rather small and with overlapping error bars.**

We appreciate the reviewer's comments and have addressed this issue in the revised manuscript. We have categorized the statistical methods into sections for clear presentation (lines 134-179) and replaced the bar graphs with dot plots to show the distribution of serum metabolite levels (lines 320-328).

- a. **The first point is that it isn't clear what the error bars here represent; standard deviation? standard error? Second, I could not find detailed information about what statistical tests were performed; multiple Student's t-tests? Was correction for multiple comparisons applied? This should be detailed in the methods section, and both points should be very briefly mentioned in the figure caption. Without this information, the data is very difficult to interpret. Please revise.**
We agree with and thank the reviewer for this comment. We have described the statistical methods used, including univariate, multivariate, and survival analysis, in both the methods section and the figure and table legends where appropriate. The error bars represent the standard deviation (SD), and this has been described in the methods, and table legends. For the direct univariate comparisons in Figure 2, serum androgen metabolite levels were summarized using the mean, standard deviation, and median for each clinical variable (Supplementary Tables 5-10). To account for some observations "below the limit of quantification" (BLQ), the mean and standard deviation were estimated using a Tobit model (accounting for left censoring) and the maximum likelihood approach. The association between metabolite levels and each patient characteristic was assessed using a left-censored version of the log-rank test to account for BLQ observations. Figure 2 does not represent multiple comparisons. The correction for multiple comparisons is represented in Main Table 1. Furthermore, all the figures and table legends have been revised to reflect the statistical test performed (lines 134-179, 310-318, 320-328, 330-337, 338-345).
- b. **Values are aggregated and the averages for each category are represented as bars. Given the relatively small differences mentioned above, would it not be better to show the individual data points together with averaged values shown? This reviewer is concerned that these modest differences may be mostly due to one or a small number of individuals who have exceptionally high or low levels that 'distort' the averages shown to yield a presentable p-value, rather than revealing the existence of systematic differences as preferred by the authors. Please revise by**

including the individual data points together with the bars, perhaps also replacing the bar with more informative boxplots or violin plots.

We appreciate the reviewer's comment and have replaced the bar graphs in Figure 2 with dot plots to show individual patient data in the revised manuscript (lines 320-328). As the reviewer correctly points out, there is a range of values within each group, but the data is not distorted by one individual patient in the represented categories in panels A, B, and C. As seen in Figure 2, panel A and numbers in Supplementary Tables 5 mean ASD, DHEA, and hydroxypregnenolone levels are significantly higher in men younger than 55 years of age and with localized, therapy naïve prostate cancer. Similarly, means of cortisone-related metabolites are lower in the "Gleason score $\geq 4+3$ " and "persistent disease" groups. The mean, standard deviation, and min-max values are presented in Supplementary Tables 8 and 9. As the reviewer alluded to, there is a range of values within each group that is now represented as dot plots in Figure 2, panels B and C.

- 2. Table 1 (and Supplemental Tables S1-S14). The authors present the results of their multivariate analysis of serum androgens including a column of P-values. However, like discussed above, this reviewer is unable to find how these P-values were derived. Please revise.**

We agree with the reviewer and have clarified the description of the statistical analyses that were used. Therefore, we have revised the statistical analyses in the methods section and described them in new subsections to make it easier for the readership to find the relevant information (lines 134-179). Furthermore, each table has been revised to reflect the statistical method used for comparisons.

- 3. Supplementary Figure 2 and Discussion. The authors discuss exonic CYP11B SNPs and their "higher frequency" in AA compared with EA men. Please specify what the frequencies of these alleles are based on existing whole genome sequencing data (e.g., 1000 genomes project, or similar). A "very rare" variant could yet be said to have a higher frequency than an "extremely rare" variant, but the key point is whether this difference is likely to provide explanatory power vis-à-vis the authors' data. If it's something like 1% vs. 10%, then that would seem plausible. But if it's something like 0.00001% vs. 0.00002%, then it would seem implausible. Please elaborate.**

We are grateful to the reviewer for bringing up this important point for discussion. Upon analyses of the 1000 Genomes Project, we found that in the general population, the exonic CYP11B2 SNPs rs5313 (n=526 with C>T SNP, total n=1322) and rs4544 (n=594 with A>G SNP, total n=1322) are found in approximately 40-45% of people with African ancestry. However, in the cohort analyzed as part of this study, 60% of African American men with prostate cancer present with this SNP which is significantly higher than the general population ($p < 0.05$, Fischer exact test) (lines 246-253). No European American in our study presented with these exonic SNPs. Based on these data, we conclude that African Americans with prostate cancer have a higher frequency of exonic SNPs in the CYP11B gene. These data are now included in the revised manuscript.

- 4. Related to the above point, might cytochrome P450 variants not also (primarily?) affect chemotherapy cancer drug metabolism and/or selection of chemotherapy regimens, thereby**

confounding the authors' suggestion of an association with corticosterone-aldosterone conversion? Please discuss.

We thank the reviewer for this valuable comment. However, the cohort in this study consists of localized, treatment naïve prostate cancer patients. Therefore, these men would not have been treated with chemotherapy drugs that might potentially alter corticosterone-aldosterone conversion. We have added text to the manuscript to clarify this point (lines 251-253). We also investigated the presence of SNPs in other genes regulating androgen metabolism which may affect conversion between intermediate metabolites. Therefore, we analyzed if there are significant differences in the frequency of SNPs occurring in AA and EA men in other androgen metabolizing enzymes, including STAR, SRD5A1, SRD5A2, SRD5A3, AKR1C1, AKR1C2, AKR1C3, AKR1C4, AKR1D1, CYP11A1, CYP17A1, HSD3B1, HSD3B2, HSD17B6, HSD17B2, HSD17B3, UGT2B4, UGT2B7, UGT2B15, UGT2B17, UGT2B28, SULT2A1, HSD11B1, and HSD11B2. We did not find significant differences in SNP frequency between AA and EA men in this cohort.

5. Supplemental Table S2. Please specify which ancestry informative markers were used; there are widely different numbers and sets used by different colleagues.

We thank the reviewer for this comment. The AIMs panel consisted of 105 SNPs and were genotyped using the Sequenom MassARRAY genotyping platform with the methods described in detail in the following publication: Hooker S, Woods-Burnham L, Bathina M et al; Cancer Epidemiology Biomarkers Prevention, 2019; PMID: 30787054. This publication is now also included as a reference in the revised manuscript (line 79-81, reference 25).

Minor points

6. Figure 3. The authors sub-categorize metabolite levels into low (<50th percentile) and high (>50th percentile). What about the “50th” percentile, was it ignored? Was it counted twice? Or should one of the inequality signs be an “equal to or...” sign? Please clarify.

We thank the reviewer for this comment. The metabolite levels are categorized into low: $\leq$ median and high: $>$ median. The manuscript has been revised to reflect the accurate classification (lines 170-172).

7. Table and Figure captions throughout. Please re-define acronyms used throughout; figure captions should be interpretable by themselves without needing to search for acronyms in the main body.

We have revised all figure legends and avoided using acronyms throughout the manuscript.

Reviewer 2

1, It is interesting analysis for the biochemistry synthesis of androgens in AA and EA men, and this manuscript mainly focus the process that includes several intermediate metabolites. Perhaps, it is better to show a figure with these process or pathways and highlight your new findings.

We thank the reviewer for this valuable comment and have now included a graphical abstract to highlight the novel findings of our study. The metabolites outlined in blue are associated with

progression-free survival in a race-independent manner. The metabolites highlighted in blue significantly differ between African American and European American men. Additionally, Supplementary Tables S5-S10 show all the significant associations, as tested by univariate analysis, with clinical variables including age, PSA, and Gleason score.

2, Figure 5, why do you choose 55 year to separate men into two group? Do you consider other age such as 60, 70, 80? Similarly, How about other Gleason score?

We thank the reviewer for giving us the opportunity to clarify our approach to analyzing the data. We chose the 55 years of age or less category to focus a part of our analyses on clinically relevant early-onset prostate cancer, which is more common in African American men. The limited number of samples, when sub-categorized into smaller age groups, prevents us from deriving meaningful correlations between metabolite levels and multiple age groups. Our objective is to find molecular events that drive early-onset prostate cancer. It is also well-established that Gleason scores $\leq 3+4$ have a more favorable prognosis, since they belong to disease grades 1 and 2, whereas Gleason scores $\geq 4+3$ have a less favorable prognosis since they belong to disease grades 3, 4, and 5 (as described in American Cancer Society). Given the size of our cohort, we used these age and Gleason score categories to derive meaningful clinical correlations with metabolite levels. The manuscript has been revised to reflect this (lines 140-146)

3, It is the interesting finding that lower serum levels of cortisol and cortisone are indicators of aggressive disease in AA men. Perhaps, the expression of genes related to the cortisol and cortisone can be analyzed such as in TCGA database et al, which also can verify results of this manuscript.

We thank the reviewer for this interesting thought. Thus far, a single study investigated race-specific differences in the RNA expression of enzymes involved in androgen metabolism in prostate cancer utilizing the TCGA database. Liu W, Liu R and Wang L; Cancer Health Disparities, 2017; PMID: 30756097; show that specific genes encoding enzymes that regulate androgen metabolism, including AKR1C3, CYP17A1, HSD3B2, and SRD5A2, are differentially expressed between prostate tumors of African American and European American men. Cortisol production is regulated by CYP11B1, and cortisone production is regulated by HSD2B enzymes. Therefore, it is possible that the differences in gene expression of enzymes regulating cortisone and cortisol production are different in African American and European American men. We have added this information to the discussion section of the manuscript (lines 386-389).

4, As mentioned in the manuscript “to fully understand the relationship between serum androgens and androgens in prostatic tissues (healthy and diseased) direct comparisons from the same patients are needed.” I think that there were prostate cancer tissues with protein data such as western blot and Immunohistochemistry for many proteins. Therefore, it is better to check these papers and compare their data with this manuscript.

We thank the reviewer for this important suggestion. Several studies (listed at the end of the response) have shown that in the context of androgen deprivation therapies, the gene expression, and the frequency of SNPs of androgen metabolizing enzymes are altered in prostate tumors.

Only a few studies have determined the protein levels of genes involved in androgen metabolism. These studies used prostate cancer cell lines, cell line-derived xenografts, and a limited number of clinical samples which did not allow for comprehensive correlation analyses between tissue expression and serum levels. These studies, generally, did not focus on determining the race-specific differences in protein expression of androgen metabolizing enzymes. We have mentioned the need for such comparisons in the discussion (lines 386-398).

Select studies listed below (most recent first):

- i. Qin L, Chung Y, Berk M et al; Cancer Research; 2022; PMID: 35536859
- ii. Stucheli S, Araya S, Ercan C et al; Cancers; 2022; PMID: 35804847
- iii. Ko Hy, Berk M, Chung Y et al; Cell Reports; 2018; PMID: 29346776
- iv. Fiandalo M, Stocking J, Pop E et al; Oncotarget; 2018; PMID: 29541409
- v. Liu W, Liu R and Wang L; Cancer health disparities; 2017; PMID: 30756097
- vi. Ji Q, Chang L, Stanczyk F et al; Cancer Research; 2007; PMID: 17283174

5. This study reported “Serum levels of cortisol and cortisone are lower in AA men with prostate cancer. Combined these results suggest that lower serum levels of cortisol and cortisone are indicators of aggressive disease in AA men.” Could authors discuss the possible reasons of this finding?

The reviewer raises an important point. Liu W, Liu R and Wang L; Cancer health disparities; 2017; PMID: 30756097 show that genes encoding androgen metabolism enzymes, including AKR1C3, and SRD5A2, are differentially expressed between prostate tumors of African American and European American men. In the current study, we measure androgen metabolites in the serum of men diagnosed with localized, therapy naïve prostate cancer to provide a systemic view of androgen levels. Serum androgens can be produced by multiple cell types including the testis and adrenal gland. The adrenal gland is thought to be the primary source of androgens and related metabolites. Whether there are race-specific differences in gene expression of androgen metabolizing enzymes in the adrenal glands or the testis is unknown. It is possible that differences in the expression of enzymes that regulate androgen metabolism in the adrenal gland could be one of the reasons for altered androgen metabolite levels in the current study. We have mentioned this possibility in the discussion of the revised manuscript (lines 386-398).

6. This study showed interesting finding of the SNPs identified in CYP11B1 and CYP11B2 in AA and EA men. There are many serum androgen and related metabolites, and do you find other interesting SNPs related to other metabolites in AA and EA men, which may be interesting?

We thank the reviewer for this intriguing question. We investigated differences in the frequency of SNPs in AA and EA men in other androgen metabolizing enzymes, including STAR, SRD5A1, SRD5A2, SRD5A3, AKR1C1, AKR1C2, AKR1C3, AKR1C4, AKR1D1, CYP11A1, CYP17A1, HSD3B1, HSD3B2, HSD17B6, HSD17B2, HSD17B3, UGT2B4, UGT2B7, UGT2B15, UGT2B17, UGT2B28, SULT2A1, HSD11B1, and HSD11B2. However, we did not find significant differences in the

frequency of SNPs in these genes in African American and European American men with prostate cancer.