AWARD NUMBER: W81XWH-18-1-0173

TITLE: Proteomic-Based Biomarkers for Risk of Progression in Early Prostate Cancer

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REPORT DATE: November 2020

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution is unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
November 2020	Final	15Jul2018-14Jul2020

4. TITLE AND SUBTITLE

Proteomic-Based Biomarkers for Risk of Progression in Early Prostate Cancer

5a. CONTRACT NUMBER
W81XWH-18-1-0173

5b. GRANT NUMBER
PC171067

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Active surveillance is an increasingly utilized strategy for the management of newly diagnosed localized prostate cancer, ideally limiting morbidity associated with local treatment while safely treating men with aggressive disease who are at risk of progression. Identification of non-invasive biomarkers of disease progression would help improve the care of men by determining who can safely be watched on surveillance (and avoid life-altering radical treatment). I performed a comparative analysis using untargeted proteomic data from mass spectrometry performed on the plasma of 16 active surveillance patients with early progression and 16 with indolent disease, obtaining candidate circulating proteins for association with disease aggression. This report details my work verifying candidate markers using ELISA on baseline prostate cancer patient plasma, and expanding the work to include more patients and modalities. It also discusses the career development and training that I was able to complete thanks to this grant.

15. SUBJECT TERMS

Prostate cancer, proteomics, biomarker, active surveillance

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	26	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION:

Active surveillance is an increasingly utilized strategy for the management of newly diagnosed localized prostate cancer, ideally limiting morbidity associated with local treatment while safely treating men with aggressive disease who are at risk of progression. Identification of non-invasive biomarkers of disease progression would help improve the care of men by determining who can safely be watched on surveillance (and avoid life-altering radical treatment). I performed a comparative analysis using untargeted proteomic data from mass spectrometry performed on the plasma of 16 active surveillance patients with early progression and 16 with indolent disease, obtaining candidate circulating proteins for association with disease aggression. This ongoing study is working to verify and expand this analysis, seeking to identify circulating proteomic-based and related circulating metabolite markers associated with disease progression.

2. KEYWORDS:

Proteomic; prostate cancer; active surveillance; biomarkers; metabolomic

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major task 1: training and education development
Subtask 1: audit courses on courera.org. **STATUS: complete** (certificates in appendix)
Subtask 2: present at department meeting. **STATUS: complete**. Work presented at Division of Surgery grand rounds 5/8/19
Subtask 3: attend workshops related to grantsmanship. **STATUS: complete**. Attended full day RO1 grant writing workshop
Subtask 4: attend international scientific meeting. **STATUS: partially complete.** Presented related data at virtual EAU meeting and published manuscript
Major task 2: Candidate protein marker confirmation
Subtasks 1, 2: Obtain and annotate data, and optimize confirmatory ELISA. **STATUS: complete**
Subtask 3: Analyze protein expression data. **STATUS: complete**
Subtasks 4,5: Quantify plasma-based metabolites. **STATUS: complete**
Subtask 6: Analyze data. **STATUS: complete**
Subtask 7: Disseminate findings. **STATUS: ongoing.** Manuscript is being drafted and circulated for submission to peer review journal

What was accomplished under these goals?

The major activities for this reporting period surrounded training and education and deep investigation of biomarkers using ELISA. For each ELISA, a commercial kit was purchased and strips were verified with stock human plasma to ensure an appropriate dilution. Then, the dilution was performed using human samples of matched aggressive (early progression) and indolent (no progression over a number of years) baseline men from patients enrolled on active surveillance. ELISA was then performed as outlined in manufacturer instructions, and optical density measured in the laboratory. Results of ELISAs are found in the appendix. In short, multiple markers were pursued, and one (HMGB1) was expanded to the larger cohort and found to likely be associated with progression. We additionally evaluated metabolomic markers for association, and NMR based results are found in the appendix. Here, multiple lipid species and other metabolites were found to be associated with progression.

What opportunities for training and professional development has the project provided?

Through this award I have been able to successfully complete a number of training and development activities. As listed in the above "accomplishments" section, I successfully completed two online courses related to statistics and genomics. I was able to broaden my understanding regarding the breadth of genomic studies that are possible, and also gained knowledge about the use and deployment of statistics, particularly as they pertain to biomarker analysis. I was also able to attend a professional development activity related to competition for RO1 awards. Here, I learned about the different categories of applications and also individual applicants. I also received tips regarding the format, composition, and narrative approach of RO1 submission. These experiences will prove invaluable in my future role as an independent investigator.

How were the results disseminated to communities of interest?

A presentation relevant to the data discovered as part of this award was accepted and chosen to be among a select group of abstracts virtually presented at the international EAU 2020 virtual meeting. These data have also been accepted for publication in the journal *Cancer*. I have also presented these data to a divisional meeting. Finally, a manuscript describing these results is currently in preparation.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Not applicable (final reporting period)

4. **IMPACT:**

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

We have identified key blood-based markers that are associated with progression of disease in men with prostate cancer enrolled on active surveillance. We are currently expanding our work through national collaborations and funding applications to externally validate our findings, and we anticipate that these markers will act as novel, non-invasive means to monitor men on surveillance. They also have potential to guide non-invasive interventions that can be used to augment the care of men with localized prostate cancer.

What was the impact on other disciplines?

These findings have relevance in that they may help guide novel, non-invasive, diet-based interventions for use in men with prostate cancer. Preliminary data have been obtained through this award that metabolites, which may be responsive to diet, may be associated with progression on active surveillance. These findings serve as backing for ongoing studies and future funding proposals.

What was the impact on technology transfer?

Further work to externally validate findings that were discovered through this grant have been initiated, and there is a high likelihood that circulating markers of disease aggression will contribute to clinical management if they are successfully validated in external cohorts.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Note that prior to initiation of ELISA analyses, an error was noted in initial proteomic data extraction and normalization. Therefore, the analysis and normalization were repeated, leading to different targets of interest being investigated for verification using ELISA. Interestingly, multiple targets were mechanistically related to ongoing pathway analyses being completed by the research team through metabolomics-related investigations, and this actually led to our obtaining of approval to expand this grant to include metabolomic-based studies. While fewer proteomic targets were deemed to be of interest based on our specified analysis plan, we were able to expand our study to include interesting metabolite markers that are likely related to the narrative that was uncovered in part by this work.

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

In some cases, detection of ELISA optical density was not uniform. Some experiments were repeated, and after discussions with ELISA experts from Dr. Hanash's laboratory, we decided to initiate use of a DDM detergent to improve detection.

As detailed in the no-cost extension, fewer markers were deemed to be of interest than expected. We were able to pursue data on HMGB1 (detailed in appendix), and also to expand our analysis to include metabolite markers using multiple metabolomics platforms. These data will greatly enhance our discovery of non-invasive markers which will subsequently be pursued for external validation.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

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:

- **Publications, conference papers, and presentations**

Journal publications.

Gregg, J., Zhang, X., Chapin, B., Ward, J., Kim, J., Davis, J., Daniel, C., 2020. Adherence to the Mediterranean diet and grade group progression in localized prostate cancer: an active surveillance cohort. Cancer. (accepted). Federal support was acknowledged

Vykoukal, J., Fahrmann, J.F., Gregg, J.R., Tang, Z., Basourakos, S., Irajizad, E., Park, S., Yang, G., Creighton, C.J., Fleury, A., Mayo, J., Paulucci-Holthauzen, A., Dennison, J.B., Murage, E., Peterson, C.B., Davis, J.W., Kim, J., Hanash, S., Thompson, T.C., 2020. Caveolin-1-mediated sphingolipid oncometabolism underlies a metabolic vulnerability of prostate cancer. Nat. Commun. 11, 4279. (published). Federal support was acknowledged.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Material related to this grant was presented at the University of Texas MD Anderson Division of Surgery grand rounds on localized prostate cancer on 5/8/19.

Work related to this grant was also presented at the international EAU 2020 meeting in the Summer of 2020.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

No change, the only funded individual is the PI, Justin R. Gregg. Core activities in Dr. Thompson's laboratory and Dr. Hanash's laboratory were funded as part of this grant as outlined in the budget and were performed by multiple individuals.

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. Gregg was awarded a grant from the NIH through the MD Anderson Prostate SPORE. This resulted in 5% of his time being funded by this mechanism.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Appendix 1: Certificates of completion of Coursera courses

Appendix 2: Results from ELISA assays

Appendix 3: Results from Metabolomic assays

JOHNS HOPKINS UNIVERSITY

COURSE CERTIFICATE

04/06/2018

Justin Gregg

has successfully completed

Statistics for Genomic Data Science

an online non-credit course authorized by Johns Hopkins University and offered through Coursera

Jeffrey Leek, PhD
Department of Biostatistics
Johns Hopkins Bloomberg School of Public Health

Verify at coursera.org/verify/24LC63ZJUC67

Coursera has confirmed the identity of this individual and their participation in the course.

JOHNS HOPKINS UNIVERSITY

COURSE CERTIFICATE

05/19/2018

Justin Gregg

has successfully completed

Introduction to Genomic Technologies

an online non-credit course authorized by Johns Hopkins University and offered through Coursera

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Steven L. Salzberg, PhD
McKusick-Nathans Institute of Genetic Medicine
Johns Hopkins University

Jeffrey Leek, PhD
Department of Biostatistics
Johns Hopkins Bloomberg School of Public Health

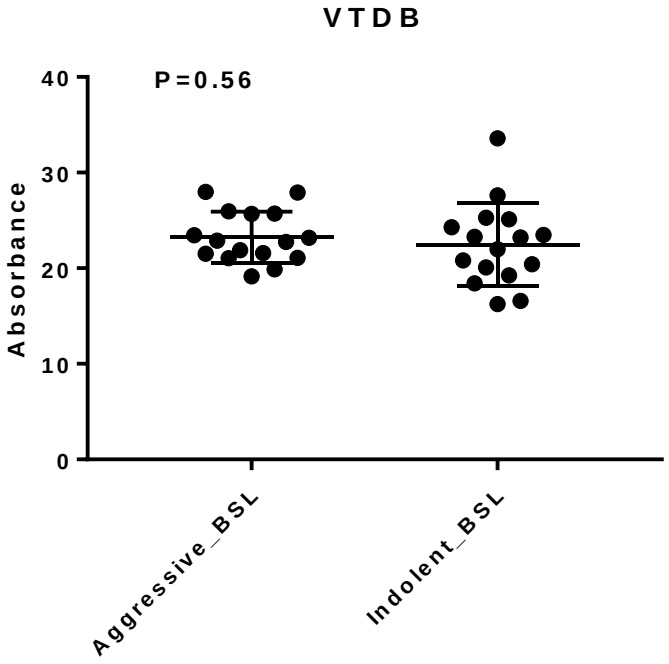
Verify at coursera.org/verify/H28WJ4SB5ZLA

Coursera has confirmed the identity of this individual and their participation in the course.

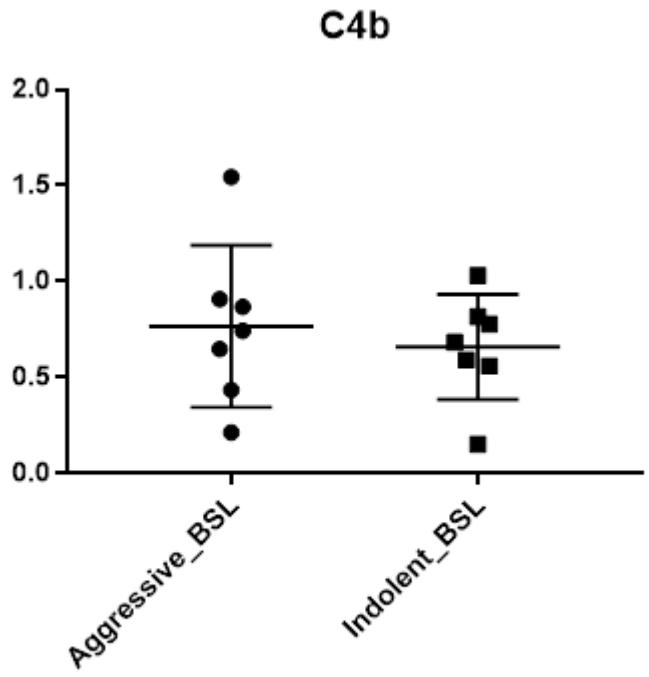
APPENDIX 2: Results of ELISA verification quantifying proteins of interest in baseline active surveillance patient plasma

I. Section 1: ELISA performed in initial group of 16 cases and 16 controls:

1. Vitamin D Binding Protein



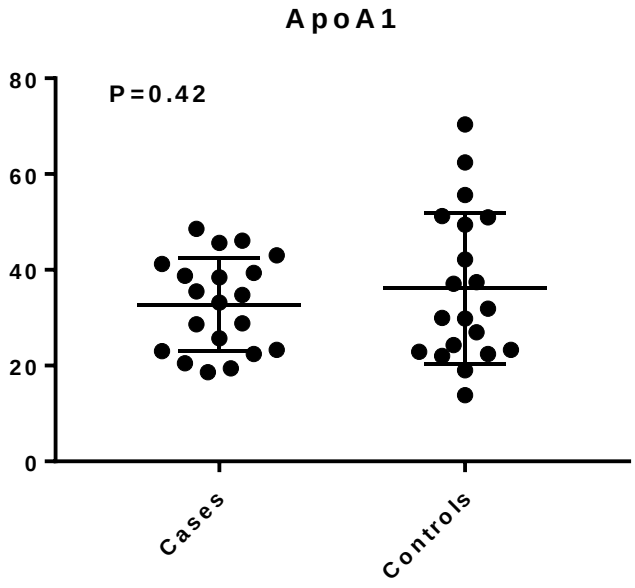
2. Complement factor C4b



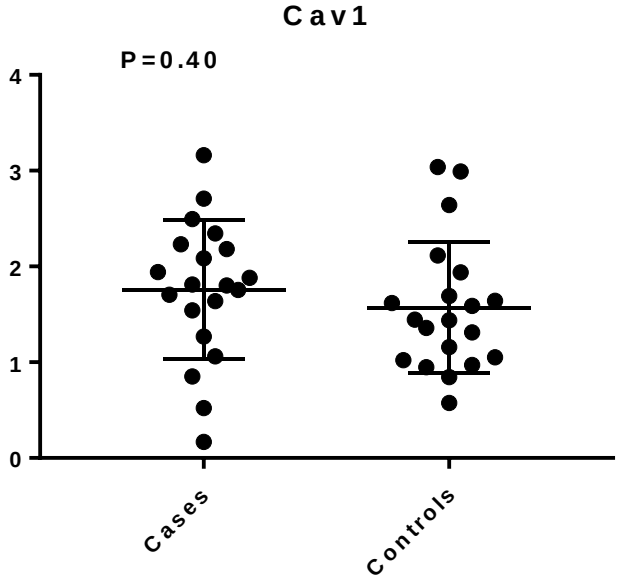
Note: These results may be limited, as dilution likely limited detection (many samples did not reach threshold for detection)

(note, the following ELISA were performed in larger cohort 20 cases and 20 controls)

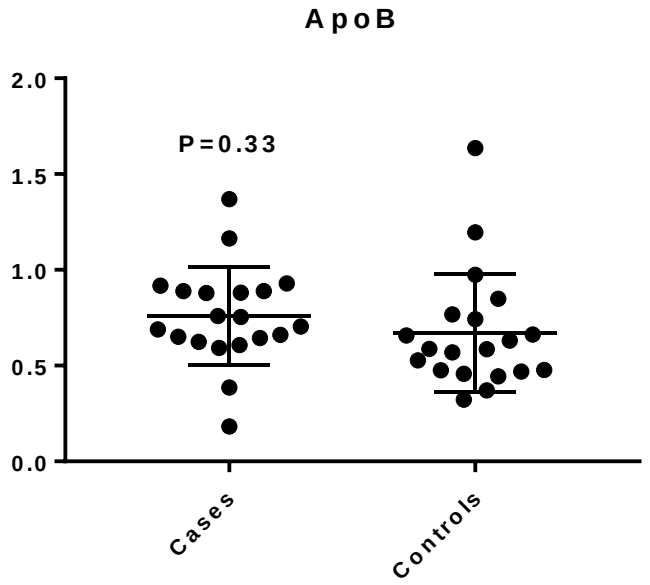
3. Apolipoprotein A1



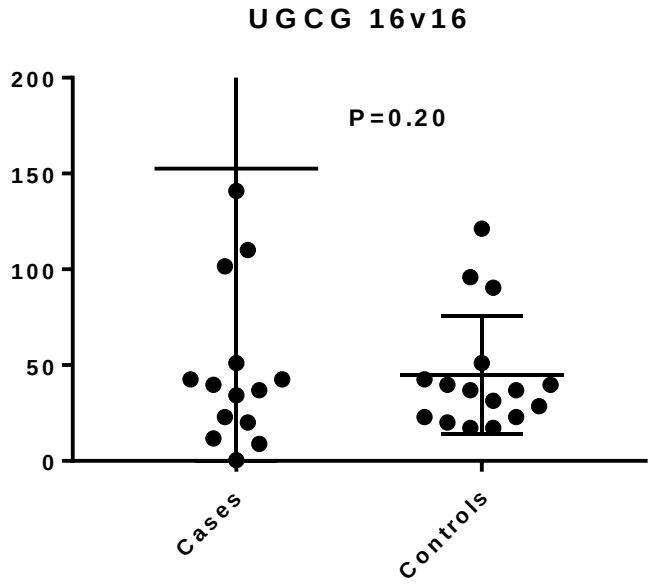
4. Caveolin-1



5. Apolipoprotein B

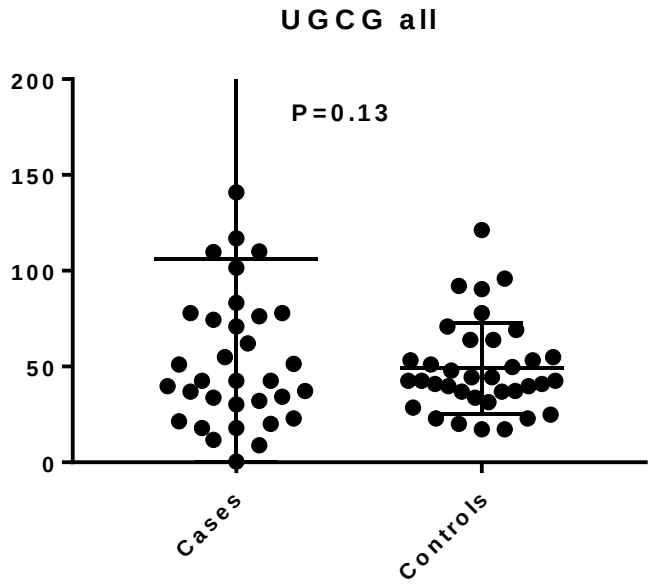


6. UDP-glucose ceramide glucosyltransferase



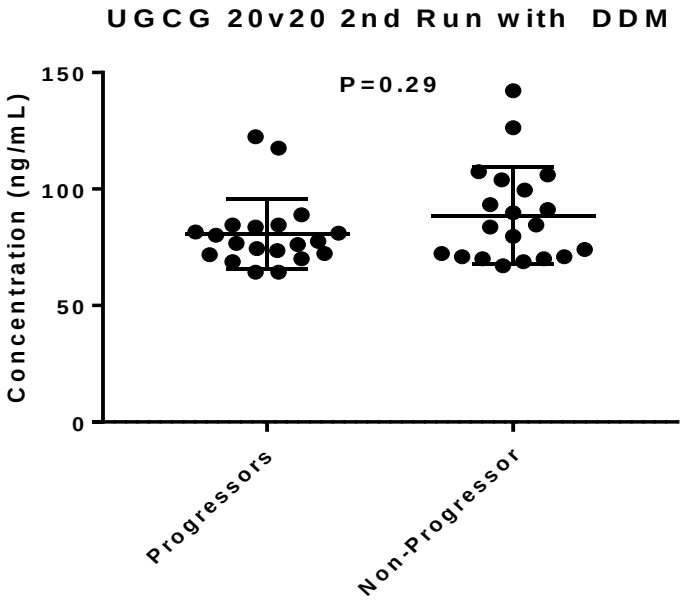
(Please note that results appeared promising and therefore were expanded to a larger cohort)

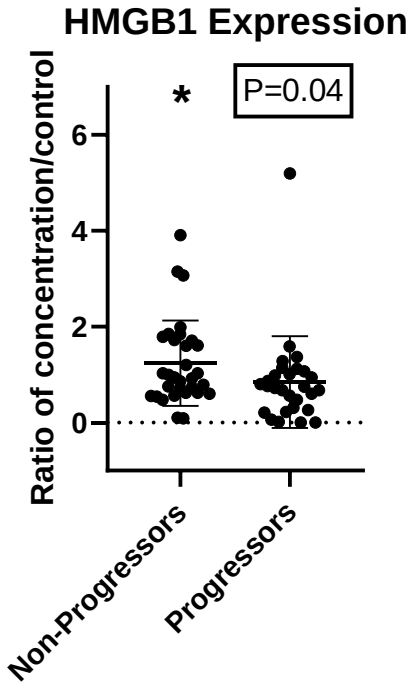
36 cases and 36 controls:



Note here that many cases were at the low end of detection. Therefore, after discussion with members of Dr. Hanash's team, a detergent DDM was used to hopefully improve detection of UGCG presence.

Results using the detergent are shown below:





Figure

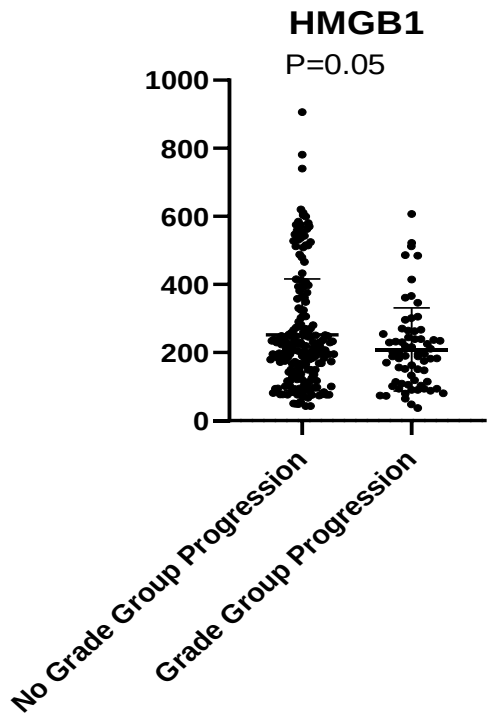
Note: results were promising and therefore discovery was expanded to include 59 total patients.

Interpretation: HMGB1 is a promising marker of progression in active surveillance, and was therefore expanded for assessment in a broader active surveillance population.

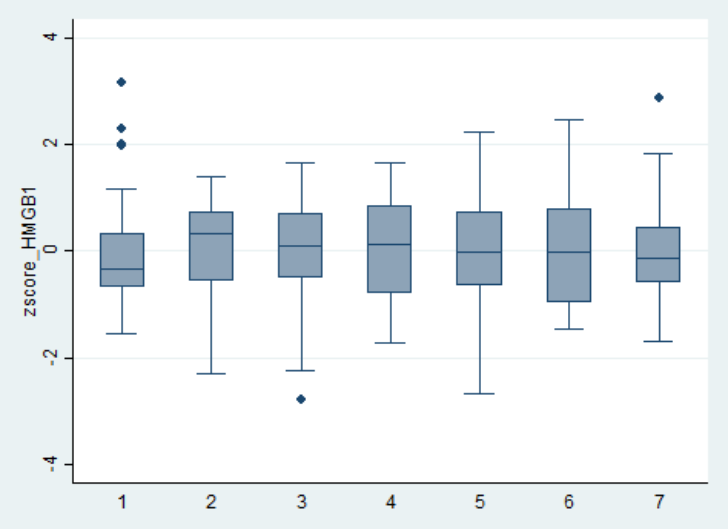
II. ELISA marker expansion

HMGB1 ELISA was successfully run on 259 baseline patient samples in 7 different batches. The following describes analytic techniques and also expansion of HMGB1 in the context of other research in a narrative fashion.

First, due to differences in assay between batches, normalization was undertaken using historical controls. Below are HMGB1 levels by progression status. ***Higher*** HMGB1 was associated with ***decreased*** risk of progression.



However, batch effect still remained. Therefore, because of this, a second normalization was done to mitigate batch effect: the Z score. Z score was calculated by subtracting the mean of a single batch then dividing by the SD of the batch. Note that zscore was effective at reducing batch effect as demonstrated in the following boxplot:

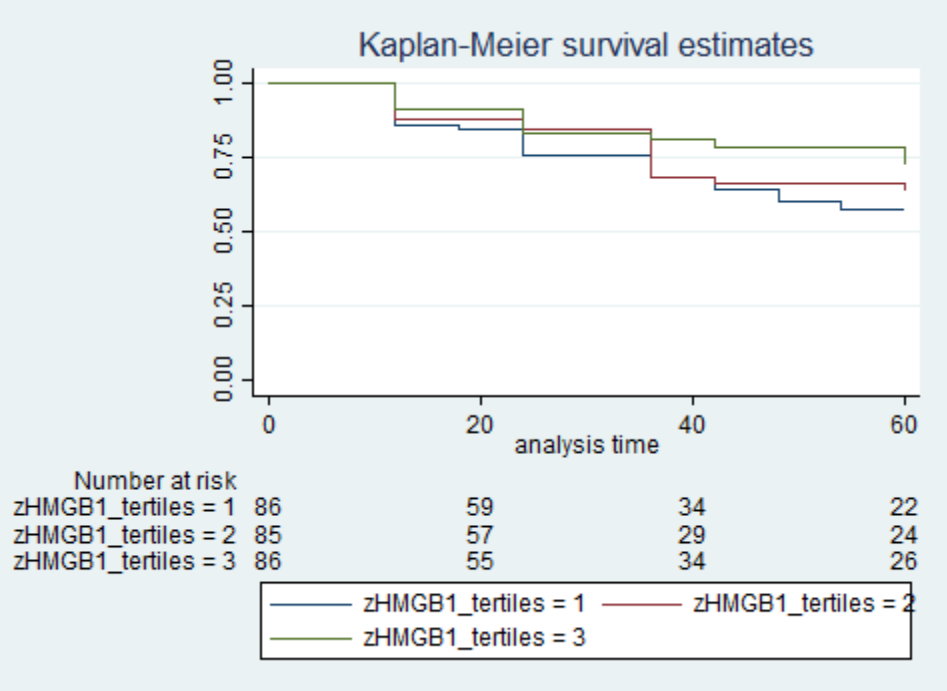


*x-axis shows each batch. Y axis shows zscore for the entire batch

Following this normalization, **HMGB1 was still higher in non-progressors, though P=0.18**

Next we evaluated HMGB1 in time to event analyses. Normalized HMGB1 levels were broken into tertiles and evaluated for association with progression using log rank test and the Kaplan Meier method.

Here is the Kaplan Meier survival curve after breaking into tertiles:



Logrank test P=0.28

Next, multivariable Cox proportional hazards models including clinically relevant variables (age, PSA and tumor volume) were used to assess risk for progression over time

And here is the Cox proportional hazards model by tertile:

_t	Haz. Ratio	Std. Err.	z	P>z	[95% Conf.	Interval]
zHMGB1_tertiles						
2	**.7368576**	**.2139779**	**-1.05**	**0.293**	**.4170625**	**1.301865**
3	**.6184305**	**.1905406**	**-1.56**	**0.119**	**.3380904**	**1.131225**
PSA	1.031023	.0298411	1.06	0.291	.9741632	1.091201
sumtumorlength	1.070876	.0279573	2.62	0.009	1.017459	1.127097

Age	1.02331	.0160384	1.47	0.142	.9923533	1.055232

*Ptrend = 0.11

Higher HMGB1 was associated with decreased risk of progression over time, though this observation was not significant.

Finally, we evaluated correlations between HMGB1 and emerging factors shown to be associated with localized prostate cancer aggression by our group: Cav-1-sphingolipid signature (as reported in recent Nature Communications paper, Vykoukal et al. 2020), Cav-1, peri-prostatic fat volume, and overall diet quality (using two variables: USDA guideline-based and Mediterranean diet). **Note that none correlated with HMGB1 level.**

However, when examining Mediterranean diet score, we noted that a **low** Mediterranean diet score and **low** HMGB1 level appeared to be strongly associated with worsened progression free survival.

Here is a joint effects analysis demonstrating this:

_t	Haz. Ratio	Std. Err.	z	P>z	[95% Conf.	Interval]
HMGB1_low_MDS_low_ref						
2	**.3471788**	.1549939	-2.37	**0.018**	.1447252	.8328413
3	**.3819583**	.1499959	-2.45	**0.014**	.1769078	.8246787
PSA	1.047161	.0568078	0.85	0.396	.9415346	1.164637
sumtumorlength	1.06287	.0352076	1.84	0.066	.9960564	1.134165
Age	1.037458	.0213986	1.78	0.075	.9963543	1.080258

*Note that low HMG1 and low diet score (by tertile) is the referent. The second group ("2") includes low HMGB1 middle diet and low HMGB1 high diet. The third group ("3") includes middle HMGB1 and middle diet, middle HMGB1 and high diet, high HMGB1 and low diet, and high HMGB1 and high diet scores.

Conclusion: While these data area limited by sample size, they offer preliminary evidence that decreased HMGB1 may be associated with worsened progression free survival and that this effect may be modifiable by diet.

Appendix 3: Metabolomic based data summary

Baseline samples from 233 patients in the active surveillance cohort (49 of whom had grade group progression during follow-up) were used in the analysis. Two hundred and forty-four metabolic traits ("metabolites") were quantified using a proton nuclear magnetic resonance (NMR) spectroscopy platform (Nightingale Health, Helsinki, Finland). Individual metabolites were evaluated for association with the presence of Gleason grade group progression using Wilcoxon rank sum statistics (**Table 1**). Individual metabolites significant at uncorrected P<0.1 were then included in a signature panel using a logistic regression model. Signature score was computed for each patient, and signature was then included in a multivariable Cox proportional hazards model (including measures of age, PSA and tumor volume) evaluating time to grade group progression. As expected, this model demonstrated that the metabolite signature was associated with increased risk of grade group progression over time (HR 2.21, 95% CI 1.44-3.40, P<0.01). Notably, due to the discovery nature of this analysis, multiple comparisons testing was not conducted, underscoring the importance of future external validation. As an alternative strategy, we performed the least absolute shrinkage and selection operator (LASSO) to estimate model coefficients for variable selection in a model evaluating GG progression (data not shown). LASSO is a non-standard estimator and, as such, does not provide an estimation of hazards ratios and standard errors. While the small size of our dataset precluded splitting into discovery and validation sets, use of an external validation set will enable determination of model accuracy, including standard estimators. Collaborative work using external cohorts and future funding applications have been initiated to accomplish these goals.

Metabolite	Median distribution increased or decreased in progressors	P Value
Omega 3 percentage	Increased	0.046
Unsaturation	Increased	0.051
L-VLDL-CE Percent	Decreased	0.064
L-VLDL-TG Percent	Increased	0.068
L-VLDL-FC Percent	Decreased	0.096
Creatinine	Increased	0.069
XL-VLDL-TG Percent	Increased	0.098

Table 1. Wilcoxon rank sum test results among metabolites associated with progression. Each metabolite associated with presence of Gleason grade group progression during follow-up. Omega 3 percentage: ratio of omega-3 fatty acids to total fatty acids; Unsaturation: degree of unsaturation among fatty acids; L-VLDL-CE percent: percentage of cholesterol esters in large very low density lipoprotein particles; L-VLDL-TG percent: percentage of triglycerides in large very low density lipoprotein particles; L-VLDL-FC percent: percentage of free cholesterol in large very low density lipoprotein particles; XL-VLDL-TG percent: percentage of triglycerides in extra large very low density lipoprotein particles