AWARD NUMBER: W81XWH-16-1-0382

TITLE: A Serum miR Signature Specific to Low-Risk Prostate Cancer

PRINCIPAL INVESTIGATOR: Larisa Nonn

CONTRACTING ORGANIZATION: University of Illinois at Chicago
College of Medicine, Department of Pathology
Chicago, IL

REPORT DATE: December 2020

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2020	Final	1Sep2016 - 31Aug 2020

4. TITLE AND SUBTITLE

A Serum miR Signature Specific to Low-Risk Prostate Cancer

5a. CONTRACT NUMBER
W81XWH-16-1-0382

5b. GRANT NUMBER
PC150495

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)

Larisa Nonn

E-Mail: lnonn@uic.edu

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

University of Illinois at Chicago
College of Medicine, Department of Pathology
840 S. Wood St
Chicago, IL 60612

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 **The goal of this new proposal is to develop a miR panel as a serum marker to identify biopsy-positive prostate cancer (PCa) patients with a low-risk of harboring aggressive disease. There are several useful pre-treatment risk calculators that use clinical parameters (age, biopsy grade, PSA). These calculators accurately identify high-risk patients defined by clinical parameters. However, there is uncertainty with low/intermediate risk patients with only Gleason grade 3 on biopsy and which of these men require curative treatment. To address this unmet need, we previously identified a serum microRNA (miR) signature that categorized, with extremely high accuracy, a subset of PCa patients with low-risk of harboring aggressive disease. miRs are stable biomarkers resistant to degradation. Our first study used a cohort designed to discover miRs that were differentially present in the pre-surgical sera from a unnatural cohort of 100 PCa patients with either low-grade (Gleason grade 3) or >50% high-grade (Gleason grade 4+5) disease. Using 14 miRs we created a combined "miR Score" which had clear threshold and a negative predictive value of 0.9 to predict the absence of high-grade PCa among the patients. A unique feature of our discovery study that provides confidence in the predictive ability of the miRs is that the entire radical prostatectomy specimen was step-wised sectioned to ensure absence of high-grade tumors in our low-risk group. As well, none of the PCa patients in our high-grade group, which had abundant Gleason 4+5 tumors, had high levels of the miRs in their serum. Thus were able to perfectly categorize any patient with high serum miR levels as low-grade and low-risk. The experiments in this study were designed to investigate the serum miRs not only as a biomarker, but also potentially as having biological function in PCa. This is distinct from our discovery study because all of the patients for this proposal will be of low to intermediate-risk. To query the above points this proposal includes two independent aims; the first based on clinical prediction and the second aim queries the origin of the serum miRs. Very little is known about how miRs get into serum (active or passive) and if they have endocrine activity.**

15. SUBJECT TERMS

Prostate Cancer, serum biomarker, microRNAs

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	14	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. INTRODUCTION:

The goal of this new proposal is to develop a miR panel as a serum marker to identify biopsy-positive prostate cancer (PCa) patients with a low-risk of harboring aggressive disease. There are several useful pre-treatment risk calculators that use clinical parameters (age, biopsy grade, PSA). These calculators accurately identify high-risk patients defined by clinical parameters. However, there is uncertainty with low/intermediate risk patients with only Gleason grade 3 on biopsy and which of these men require curative treatment. To address this unmet need, we previously identified a serum microRNA (miR) signature that categorized, with extremely high accuracy, a subset of PCa patients with low-risk of harboring aggressive disease. miRs are stable biomarkers resistant to degradation. Our first study used a cohort designed to discover miRs that were differentially present in the pre-surgical sera from a unnatural cohort of 100 PCa patients with either low-grade (Gleason grade 3) or >50% high-grade (Gleason grade 4+5) disease. Using 14 miRs we created a combined "miR Score" which had clear threshold and a **negative predictive value of 0.9** to predict the absence of high-grade PCa among the patients. A unique feature of our discovery study that provides confidence in the predictive ability of the miRs is that the entire radical prostatectomy specimen was step-wised sectioned to ensure absence of high-grade tumors in our low-risk group. As well, none of the PCa patients in our high-grade group, which had abundant Gleason 4+5 tumors, had high levels of the miRs in their serum. Thus were able to perfectly categorize any patient with high serum miR levels as low-grade and low-risk.

The experiments in this study were designed to investigate the serum miRs not only as a biomarker, but also potentially as having biological function in PCa. This is distinct from our discovery study because all of the patients for this proposal will be of low to intermediate-risk. To query the above points this proposal includes two independent aims; the first based on clinical prediction and the second aim queries the origin of the serum miRs. Very little is known about how miRs get into serum (active or passive) and if they have endocrine activity.

2. KEYWORDS: prostate cancer, serum biomarker, microRNAs

3. ACCOMPLISHMENTS:

3A. Major Goals:

Specific Aim 1: To establish the serum miR panel as a pre-treatment predictor of low-risk PCa in independent cohorts of radical prostatectomy patients.			Progress
Major Task 1: Acquire Sera and pathology from PCa patients			
Subtask 1: Obtain IRB approval for study	1-3	Dr. Nonn	100%
Subtask 2: Sera for use in this study will come from sera collected as part of Co-Investigators clinical trial (Fall of 2015 to 2018) N=300	1-24	Dr. Gann	100%
Subtask3: Identification of patients within the sera cohort who will be treated by radical prostatectomy (N=100)	1-24	Dr. Abern	100%
Subtask 4: Collect pathology findings from radical prostatectomy specimen (N=100)	1-24	Drs. Gann and Nonn	100%
Milestone(s) Achieved: Collection of pre-surgical sera cohort for RNA analysis	24		
Major Task 2: Measure miR panel in PCa patient sera			
Subtask 1: Isolate RNA (ongoing through this time frame)	6-24	Dr. Nonn	100%
Subtask 2: RT-qPCR analysis (ongoing through this time frame)	12-24	Dr. Nonn	100%
Milestone(s) Achieved: miR levels in the pre-surgical serum of PCa patients	30		
Major Task 3: Statistical analysis of serum miR panel to predict risk of favorable pathology			
ongoing through this time frame at 6 month intervals	18-36	Drs. Gann, Nonn and CCTS core	0%

Specific Aim 2: To examine prostatic origin and biological significance of the miRs in the serum panel.			
Major Task 1: Obtain sera and exosomes from PCa patient pre and post radical prostatectomy			
Subtask 1: Identify PCa patients with low-intermediate risk having radical prostatectomy at UIC (N=35*) *75% over estimate to ensure N=20 for study	1-12	Dr. Abern	100%
Subtask 2: Collect leftover fresh serum from those PCa patients pre- and post-surgical blood draws (N=35)	1-12	Drs. Nonn and Abern	100%
Subtask 3: Isolate exosomes from fresh serum(N=35)	1-12	Dr. Nonn	100%
Milestone(s) Achieved: Sera and exosomes for miR analysis	12		
Major Task 2: Identify prostatic miRs in PCa patient serum			
Subtask 1: RNA extraction from the serum and miR profiling by miRNome array - Updated method: small RNA sequencing	12-18	Dr. Nonn	100%
Subtask 2: statistical analysis to identify miRs differentially detected before and after prostatectomy	18-24	Dr. Nonn	100%
Milestone(s) Achieved: List of miRs in the serum of potential prostatic origin	24		
Major Task 3: Quantify miRs in PCa tissues			
Subtask 1: Create custom TMA of PCa tissues to examine miR expression by Gleason grade (N=60)	1-12	Dr. Nonn	100%
Subtask 2: in situ hybridization for miR detection in TMA (miRs identified in serum as likely prostatic origin)	24-30	Dr. Nonn	100%
Subtask 3: Quantitative analysis of ISH on TMA by Imaging Core (co-Investigator is the Director)	30-36	Dr. Gann	0%
Milestone(s) Achieved: Determine if miRs in the serum are of prostate origin and alter by Gleason grade	36		
Major Task 4: Quantify miRs in serum and prostate exosomes			
Subtask 1: Isolate exosomes from primary prostate epithelial and stromal cells collected from areas of Gleason 3 PCa (N=5 each)	12-18	Dr. Nonn	100%
Subtask 2:RT-qPCR analysis of prostatic miRs in exosomes Method was switched to RNAseq	18-24	Dr. Nonn	100%
Milestone(s) Achieved: Determine if miRs in the serum are from prostatic exosomes	24		

3B. What was accomplished under these goals?

Major Activities: All of the key activities were successfully accomplished with the exception of the final statistical analysis

Aim 1: In Y3 completed serum collection to validate the miR signature and in our Y4 NCE we completed all of the miR expression and clinical data collection (**Table 1**). Of the 210 sera we collected, 203 had usable RNA for miR expression analysis by RT-qPCR. For prognostic indicators, we have biopsy pathology for all 203 of those patients and Oncotype DX results from 42. Eight seven patients underwent radical prostatectomy and we have adverse pathology for all of those patients. Thus far, only 5 had had recurrence of disease after surgery. Of the 203 patients, 64% are African American, 20% are Caucasian non-hispanic, 14% are Hispanic and 2% are Asian, biracial or not disclosed. We additionally isolated extracellular vesicles from 141 patients. Note that we have updated our terminology to be consistent with the field which no longer differentiates exosomes from extracellular vesicles in sera.

Although the percentage of patients who opted for radical prostatectomy is less that we estimated, we have sufficient power to use the miR panel.

Table 1. Summary of Patient Samples and Clinical Data

Patients	Total collected	miR RT-qPCR	OncoTypeDX	Bpx Path	RP	RP Path	BCR
Whole Serum	210	203	63	203	87	87	5
EVs*	141	118	37	118	53	53	4

Bpx = Biopsy
RP =Radical Prostatectomy
BCR = Biochemical Recurrence (PSA rise after treatment)
EVs = extracellular vesicles isolated from sera
*collection included in this grant, but not analysis for full miR panel. However, subsequent funding by NCI for F30 will cover these analyses

The final miR panel included the 14 miRs from our original study as well as 42 other miRs we selected from the literature and our findings in Aim 2 below. The RT-PCR for these samples was delayed due the closure of the core facility during the pandemic, but was completed in December 2020. Of the miRs, 84% were detected in the majority of the patients. *Note: although not part of this DOD gratn, miR quantitation for all of the EV samples was also completed as part of the F30 funded for Morgan Zenner, demonstrating that the samples collected as part of this DOD were parlayed in to subsequent funding and projects.*

The final statistical analysis has just commenced in collaboration with Peter Gann, MD, ScD. We estimate completion in January 2021 and initial submission of the manuscript by April 2021.

MiR qPCR Panel Compilation

Serum Signature ***(Mihelich et. al)***

1. hsa-miR-100-5p
2. hsa-miR-103-3p
3. hsa-miR-106a-5p
4. hsa-miR-107
5. hsa-miR-130b-3p
6. hsa-miR-146a-5p
7. hsa-miR-223-3p
8. hsa-miR-24-3p
9. hsa-miR-26b-5p
10. hsa-miR-30c-5p
11. hsa-miR-451a
12. hsa-miR-874
13. hsa-miR-93-5p
14. hsa-let-7a-5p

NGS Data

15. hsa-miR-199a-3p
16. hsa-miR-199a-5p
17. hsa-miR-122-5p
18. hsa-miR-126-5p
19. hsa-miR-134-5p
20. hsa-miR-155-5p
21. hsa-miR-21-5p
22. hsa-miR-218-5p
23. hsa-miR-221-3p
24. hsa-miR-25-3p
25. hsa-miR-29b-3p
26. hsa-miR-29c-5p
27. hsa-miR-30a-5p
28. hsa-miR-374a-5p
29. hsa-miR-92a-3p
30. hsa-let-7b-5p
31. hsa-let-7i-5p

Literature Search

32. hsa-miR-1246
33. hsa-miR-141-3p
34. hsa-miR-146b-3p
35. hsa-miR-18b-5p
36. hsa-miR-193a-3p
37. hsa-miR-194-5p
38. hsa-miR-200a-3p
39. hsa-miR-200b-3p
40. hsa-miR-204-5p
41. hsa-miR-210-3p
42. hsa-miR-214-3p
43. hsa-miR-222-3p
44. hsa-miR-27a-3p
45. hsa-miR-320a
46. hsa-miR-330-3p
47. hsa-miR-335-5p
48. hsa-miR-345-5p
49. hsa-miR-363-3p
50. hsa-miR-375
51. hsa-miR-519c-5p
52. hsa-miR-574-3p
53. hsa-miR-708-5p
54. hsa-miR-9-3p

Aim 2: To examine if the prostate is contributing the serum microRNAs, we completed quantitation of the miR panel and full profiling of serum (before and after radical prostatectomy) and prostate derived exosomes.

In Y1 we optimized isolation of exosome showing these exosomes of the correct size of 50-100 nm as determined by the Nanocyte (**Figure 1A**) and transmission electron microscopy (TEM) (**Figure 1B**). Another peak is observed at 200 nm (**Figure 1A**), of unknown significance.

In Y2 we completed collection of matched pre-RP and post-RP serum and exosomes. We also did a preliminary screen to determine is serum storage conditions alter the exosome miR signature. We show that levels so the miRs was remarkably stable over time (**Figure 1C**). In Y2 we made the decision to revise the method small RNA sequencing, which is the gold standard and more robust that RT-qPCR. Small RNA sequencing was cost-prohibitive to put in the original proposal, but the cost has decreased substantially and is the preferred method.

To determine if the exosomes were coming from prostate cells, primary human prostate cell cultures were examined as a source for the microRNAs. In Y1 We determined that primary cells derived from the prostate stroma (PrS) and prostate epithelium (PrE) both secrete exosomes. (**Figure 2A**). In Y2 we showed that, interestingly, the PrE cells express miRs of the signature at higher levels compared to stroma despite producing similar number of exosomes as stromal cells overall (**Figure 2B**), supporting a model of selected loading of miRs into exosomes. In Y2 we determined that there is a notable difference in the size distribution of the exosomes of unknown significance (**Figure 2C-D**).

In Y3, we completed small RNA sequencing for the patient sera exosomes, PrE and PrS exosomes. We also had an additional data set from acquired from exosomes from patient tissue slices ex vivo to compare to the serum miRs. Analysis for overlapping miR shows eight miRs be prostate derived as they are reduced in sera after RP and highly expressed in prostate tissues (**Figure 3**).

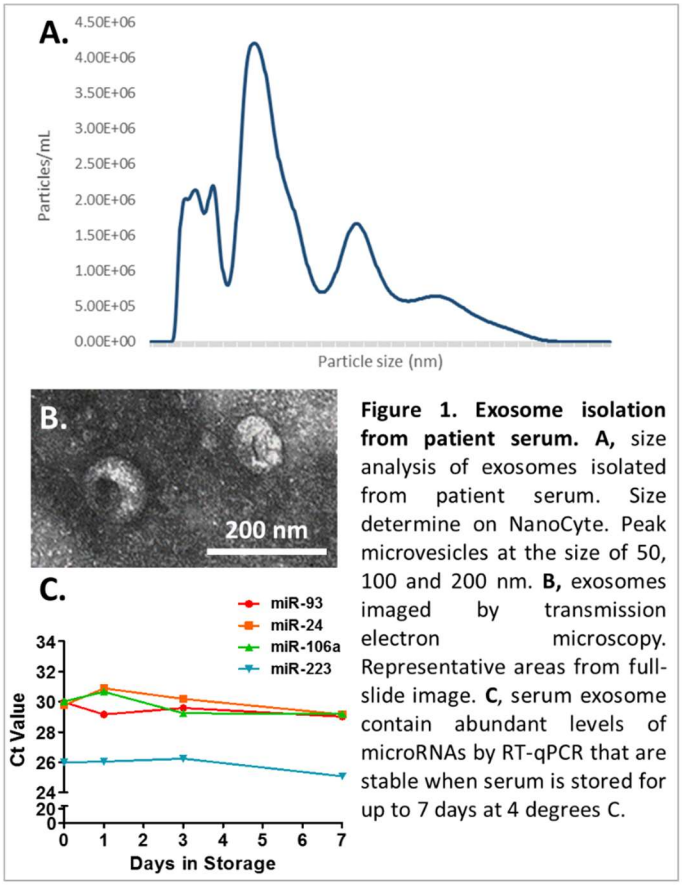

Figure 1. Exosome isolation from patient serum. A, size analysis of exosomes isolated from patient serum. Size determine on NanoCyte. Peak microvesicles at the size of 50, 100 and 200 nm. **B,** exosomes imaged by transmission electron microscopy. Representative areas from full-slide image. **C,** serum exosome contain abundant levels of microRNAs by RT-qPCR that are stable when serum is stored for up to 7 days at 4 degrees C.

Comparison to the patient-derived PrE and PrS exosomes to the patient sera suggests that some miRs may have origin to one cell type or another (**Figure 4A**). This analysis also allowed us to show that PrE and PrS cells selectively package some miRs over others into exosome (**Figure 3B**).

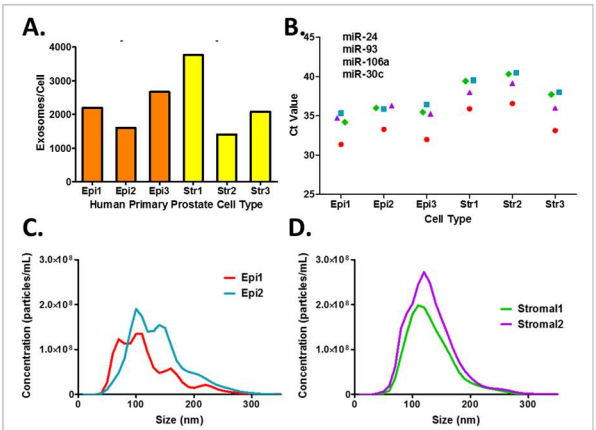

Figure 2. Characterization of exosomes from patient-derived primary prostate epithelial and stromal cells. A, quantitation of exosomes from primary prostate epithelial (PrE) and stroma (PrS) cells by anti-CD63 ELISA. PC3 prostate cancer cells included as positive control. **B,** exosomes from epithelial cells contain abundant levels of microRNAs by RT-qPCR. **C** and **D,** size analysis of exosomes from PrE and PrS cells by NanoCyte.

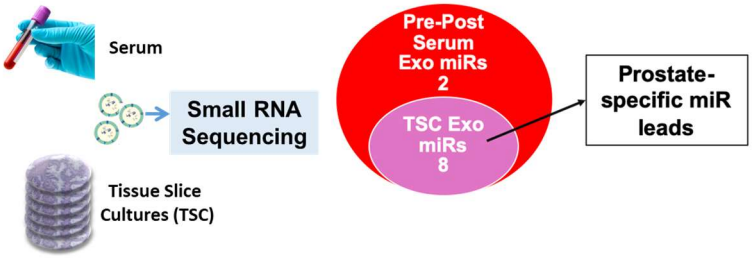

Figure 3. Prostate-specific miRs identified using pre- and post-radical prostatectomy sera and are robustly expressed in tissue slice exosomes. Prostate-specific miR leads were determined as those that were highly expressed in pre-RP serum exosomes but then decreased by ≥ 2-fold in the post-RP serum exosomes. To be prostate-specific, we further confirmed their expression in patient tissue slice cultures (TSC) exosomes by NGS.

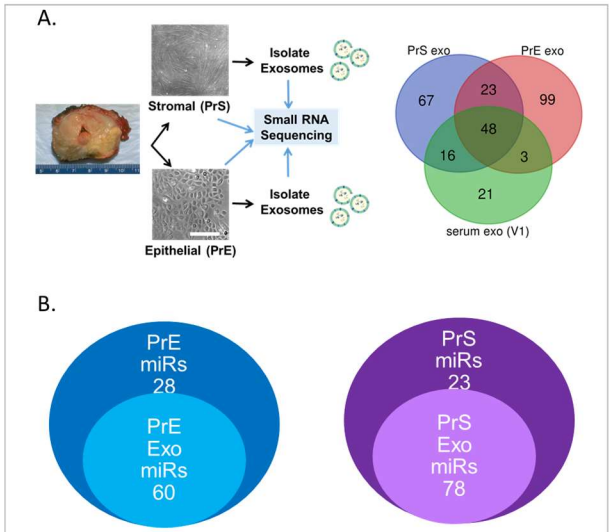

Figure 4. MiRs in serum exosomes also present in patient-derived primary prostate epithelial and stromal cells. A, comparison of miRs in PrE and PrS exosomes to serum exosomes identifies putative prostate derived miRs. **B,** exosomes from PrE and PrS cells have an enrichment of intracellular miRs.

A TMA of PCa and benign tissues was constructed and stained for miR-199a, which was the top prostate specific and stromal specific based on RNAseq data above. MiR-199a-3p was exclusively expressed in prostate stromal cells and EVS (**Figure 5A**). TMA created to examine tissue localization of miR-199a (**Figure 5B**). miR-199a-3p localization by in situ hybridization of benign and prostate tissue showed expression only in stroma and decreased expression in stroma surrounding PCa (**Figures 5C-D**). *Note: the funded F30 grant for Morgan Zenner expanded on this portion of the project to further examine stromal derived miRs.*

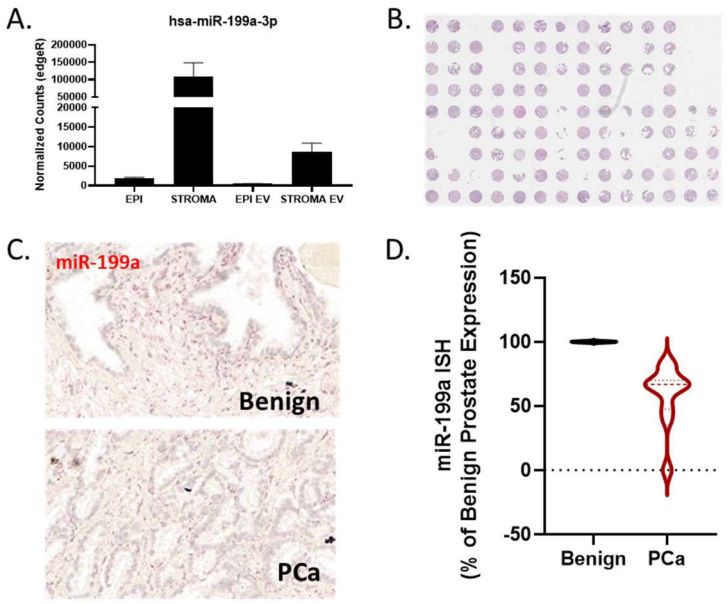

Figure 5. miR-199a-3p emerges as prostate specific miR exclusive to stroma. A, comparison of miR-199a-3p in epithelial (Epi) and stromal cells and exosomes by small RNA sequencing. **B,** creation of tissue microarray from PCa patients. N=32 benign and PCa, duplicate cores each. **C,** miR-199a-3p in situ hybridization (red) shows expression only in stroma and reduced in PCa from same patient. **D**, quantitation of miR-199a-3p in TMA by pathologist shows it is lower in stroma surrounding PCa.

3C. What opportunities for training and professional development has the project provided?

Morgan Zenner is the MD/PhD student working on this project. She is currently in her fourth year of graduate school (G4) having started in my lab summer of 2017. She completed her preliminary exam and advanced to candidacy in May of 2018 and was awared F30 fellowship in December 2020. Her F30 project directly grew from her findings about the extracellular vesicles in the primary cell cultures that are part of Aim 2 in this DOD project.

Morgan is part of the UIC Prostate Cancer Working Group with Drs Gann, Nonn and Abern and has gained experience with prostate cancer screening, diagnosis and treatments. She has also presented this DOD-funded work at several national conferences and was selected to give a talk at the annual GEMSSA Symposium on October 2 2018.

3D. How were the results disseminated to communities of interest?

Nothing to Report

3E. What do you plan to do during the next reporting period to accomplish the goals?

NA this is the final report

4. IMPACT:

4A. What was the impact on the development of the principal discipline(s) of the project?

We will be the first to report a prospective miR serum biomarker study in prostate cancer patients. In addition, our cohort is predominantly African American, a population with higher risk of aggressive PCa. Lastly, given the high number of patients who also OncoTypeDX scores, we will be able to compare the miRs head to head with the current standard of care.

4B. What was the impact on other disciplines?

The methodologies we developed for miR detection in situ will be useful to all miR researchers.

4C. What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

We may ultimately be able to improve prognostic stratification of PCa patients, thus spare unnecessary surgeries in patients who have a very low risk of harboring aggressive tumors.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change: The RT-qPCR based approach to profiling of serum exosomes for discovery in Aim 2 was changed to Illumina Platform small RNA sequencing. RNAseq is the gold standard and prices have come down to include in the budget. As well, RNAseq provides sequences for ALL small RNAs, allowing potential discovery of other prostate specific RNA species such as piRNAs and snoRNAs.

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

Problem 1: This study is ancillary to the DOD-funded ENACT study (PI: Peter Gann). There were initial delays in IRB approvals that have affected serum numbers for Aim 1. This issue was resolved late in in Y1 and reached target patient in early 2019.

Problem 2: The number of patients getting subsequent radical prostatectomies is less than expected. These issues impact Aim 2, for which we require pre- and post-surgical sera. Action: In Y1 we tied in to the UIC Prostate Cancer Blood Biorepository (PI: Larisa Nonn) in order to access serum from all UIC prostatectomy patients. This protocol is in place as of August 28, 2017 and we reached our target of pre-post 35 patients in May 2018. This protocol has also contributed 63 patients to the overall study.

Problem 3: The project was on track for completion in this final NCE year. However, after collection of all sera, the RNA isolation and final miR quantitation and analysis was delayed due to the covid-19 pandemic closure of the lab and core facilities. Although the NCE requested was sufficient to complete all of the data collection, the final statistics are still pending.

Action: I will share the final results and a presubmission version of the manuscript with the PO as soon as it is complete.

· **Changes that had a significant impact on expenditures**

The expenditures were below budget due to the initial delays in serum collection (described above) and a delay in hiring. The NCE requested was sufficient to cover the remaining studies.

· **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: Funded NCI F30 for Morgan Zenner, MD/PhD student

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Morgan Zenner*
Project Role:	*Key personnel, MD/PhD student*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Ms. Zenner started in the Nonn Lab in August 2017 and devoted 100% effort to this project.*
Funding Support:	*This DOD grant until Dec 2019, then NCI F30 grant*

Name:	*Rubin Sauer, MD*
Project Role:	*Key personnel, research coordinator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Sauer started on the project in fall of 2017. He recruits patients to the blood biorepository protocol, draws blood and does specimen processing. He devoted 25% effort to this project.*
Funding Support:	*This DOD grant*

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

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards: not applicable

Quad Charts: not applicable

9. APPENDICES: none