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

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> 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.					
<b>15. SUBJECT TERMS</b>  Prostate Cancer, serum biomarker, microRNAs					
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## 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:

<b>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.</b>			<b>Progress</b>
<b>Major Task 1: Acquire Sera and pathology from PCa patients</b>			
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%
<i>Milestone(s) Achieved: Collection of pre-surgical sera cohort for RNA analysis</i>	24		
<b>Major Task 2: Measure miR panel in PCa patient sera</b>			
Subtask 1: Isolate RNA (ongoing through this time frame)	6-24	Dr. Nonn	20%
Subtask 2: RT-qPCR analysis (ongoing through this time frame)	12-24	Dr. Nonn	0%
<i>Milestone(s) Achieved: miR levels in the pre-surgical serum of PCa patients</i>	30		
<b>Major Task 3: Statistical analysis of serum miR panel to predict risk of favorable pathology</b>			
ongoing through this time frame at 6 month intervals	18-36	Drs. Gann, Nonn and CCTS core	0%

<b>Specific Aim 2: To examine prostatic origin and biological significance of the miRs in the serum panel.</b>			
<b>Major Task 1: Obtain sera and exosomes from PCa patient pre and post radical prostatectomy</b>			
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%
<i>Milestone(s) Achieved: Sera and exosomes for miR analysis</i>	12		
<b>Major Task 2: Identify prostatic miRs in PCa patient serum</b>			
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%
<i>Milestone(s) Achieved: List of miRs in the serum of potential prostatic origin</i>	24		
<b>Major Task 3: Quantify miRs in PCa tissues</b>			
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 hybridation for miR detection in TMA (miRs identified in serum as likely prostatic origin)	24-30	Dr. Nonn	25%
Subtask 3: Quantitative analysis of ISH on TMA by Imaging Core (co-Investigator is the Director)	30-36	Dr. Gann	0%
<i>Milestone(s) Achieved: Determine if miRs in the serum are of prostate origin and alter by Gleason grade</i>	36		
<b>Major Task 4: Quantify miRs in serum and prostate exosomes</b>			
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	18-24	Dr. Nonn	100%
<i>Milestone(s) Achieved: Determine if miRs in the serum are from prostatic exosomes</i>	24		

### 3B. What was accomplished under these goals?

**Major Activities:** The key activities of Y2 span both Aim 1 and Aim 2.

**Aim 1:** We have completed serum collection to validate the miR signature. We obtained IRB approval from UIC, the UI Cancer Center and HRPO in Y1. Serum collection for Aim 1-Major Task is complete and we have serum from 210 patients (**Table 1**). Of those patients we have exosomes from 141 patients and Oncotype DX results from 147. Ninety one patients underwent radical prostatectomy and we have pathology. Although the percentage of patients who opted for radical prostatectomy is less than we estimated, we have sufficient power to use the miR panel.

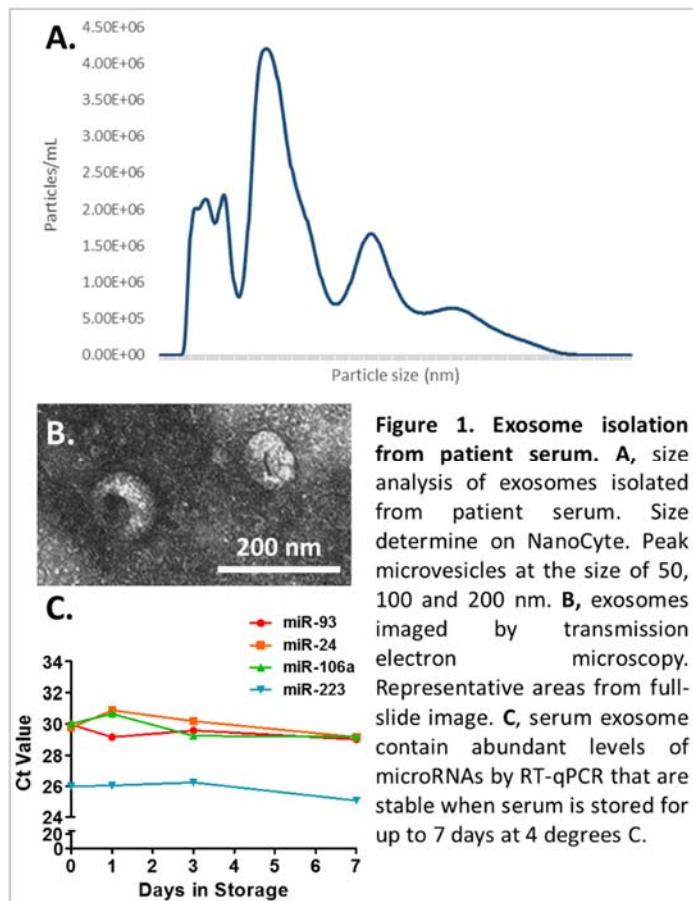
**Table 1. Patient Samples Collected Y1-Y3**

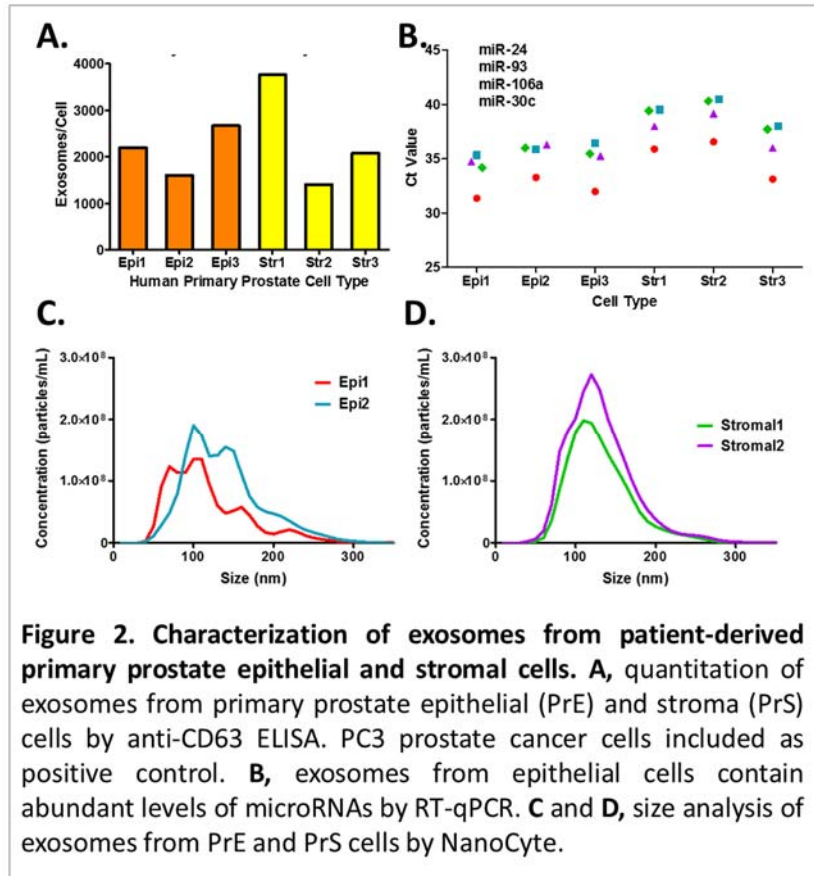
Dates	Serum	Exosomes	OncotypeDX	RP
7/28/2016-9/30/2019	210	141	147	81

**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 if 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 than RT-qPCR. Small RNA sequencing was





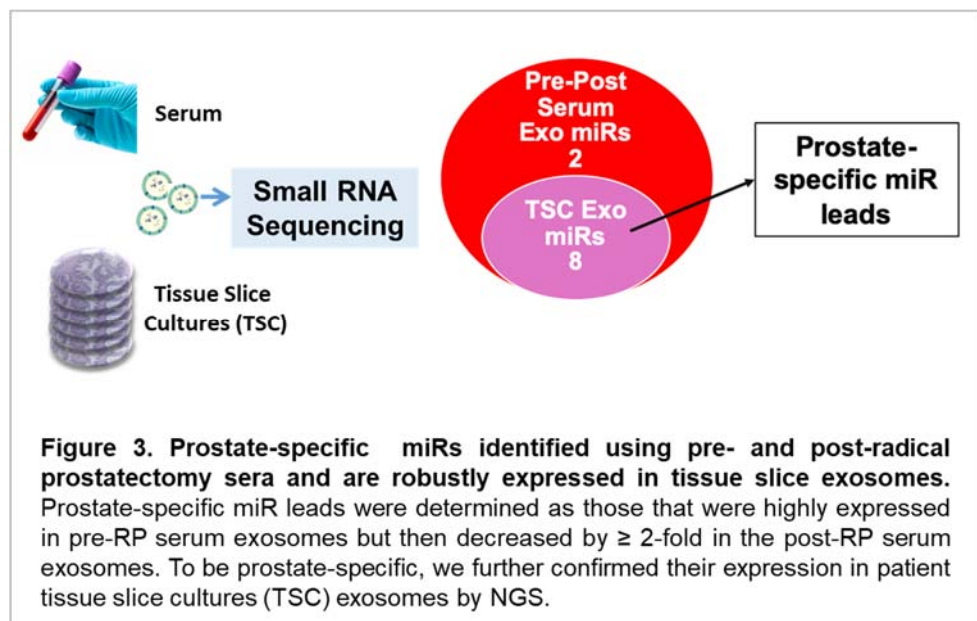
cost-prohibitive to put in the original proposal, but the cost has decreased substantially and is the preferred method.

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 show 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 smallRNA 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**).

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).

**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 third year of graduate school (G3) having started in my lab summer of 2017. She completed her preliminary exam and advanced to candidacy in May of 2018

and applied for an F30 fellowship in December 2019. She currently has a resubmission of that F30 being reviewed in November 2019. Her F30 project directly grew from her findings about the exosomes 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?**

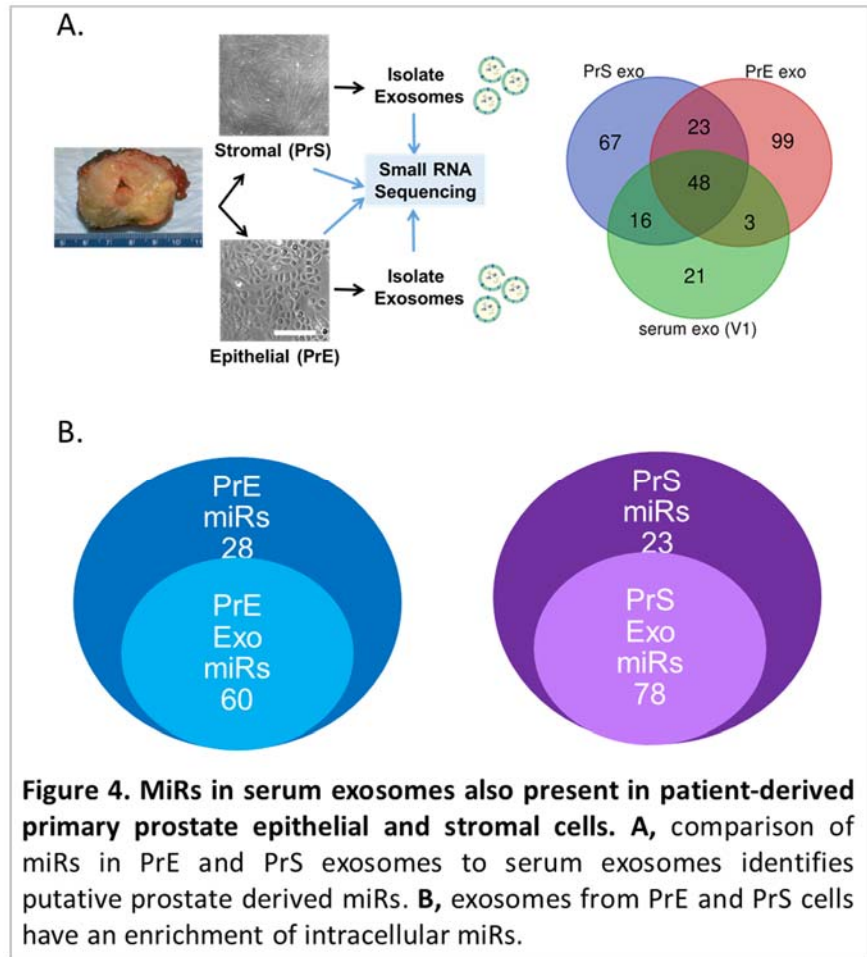
Nothing to Report

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

The project has been on track so far after delays in enrollment in Y1. Now that we have all sera, we just need to complete the final miR quantitation and analysis. The NCE requested is sufficient to cover the remaining studies.

**4. IMPACT:**

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



**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.

Nothing to Report

**4B. What was the impact on other disciplines?**

Nothing to Report

**4C. What was the impact on technology transfer?**

Nothing to Report

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

Nothing to Report

**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.

· **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 is 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:** Nothing to report.

**7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

**What individuals have worked on the project?**

Name:	<i>Morgan Zenner</i>
Project Role:	<i>Key personnel, MD/PhD student</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Ms. Zenner started in the Nonn Lab in August 2017 and devotes 100% effort to this project.</i>
Funding Support:	<i>This DOD grant</i>

Name:	<i>Rubin Sauer, MD</i>
Project Role:	<i>Key personnel, research coordinator</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>4</i>

Contribution to Project:	<i>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 devotes 25% effort to this project.</i>
Funding Support:	<i>This DOD grant</i>

**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