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

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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

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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:

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	60%
Subtask3: Identification of patients within the sera cohort who will be treated by radical prostatectomy (N=100)	1-24	Dr. Abern	ongoing
Subtask 4: Collect pathology findings from radical prostatectomy specimen (N=100)	1-24	Drs. Gann and Nonn	ongoing
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	20%
Subtask 2: RT-qPCR analysis (ongoing through this time frame)	12-24	Dr. Nonn	0%
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	ongoing
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 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%
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	18-24	Dr. Nonn	50%
Milestone(s) Achieved: Determine if miRs in the serum are from prostatic exosomes	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 are in the final stages of serum collection to validate the miR signature, which will subsequently be used to quantify the serum microRNA panel. We obtained IRB approval from UIC, the UI Cancer Center and HRPO in Y1. Serum collection for Aim 1-Major Task is ongoing and to date we have serum from 151 patients (**Table 1**) working to our goal of 200-300 patients. Of those patients we have exosomes from 115 patients and Oncotype DX results from 151. 42 patients underwent radical prostatectomy and we have both pathology and post-surgical serum.

Table 1. Patient Samples Collected Y1-Y2

Dates	Serum	Exosomes	OncoTypeDX	RP
7/28/2016-9/21/2018	189	115	151	42

Aim 2: To examine if the prostate is contributing the serum microRNAs, we will measure these microRNA in serum 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 have completed collection of matched pre-RP and post-RP serum and exosomes (**Table 1**). 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. In Y2 we completed the

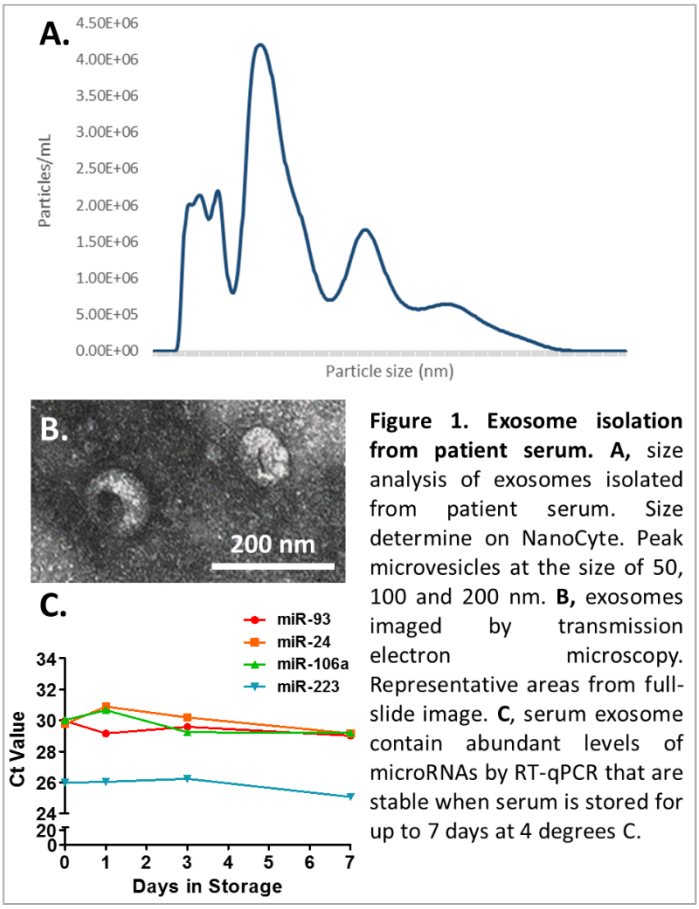

Figure 1. Exosome isolation from patient serum. A, size analysis of exosomes isolated from patient serum. Size determine on NanoCyte. Peak microvesicles at the size of 50, 100 and 200 nm. **B,** exosomes imaged by transmission electron microscopy. Representative areas from full-slide image. **C**, serum 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.

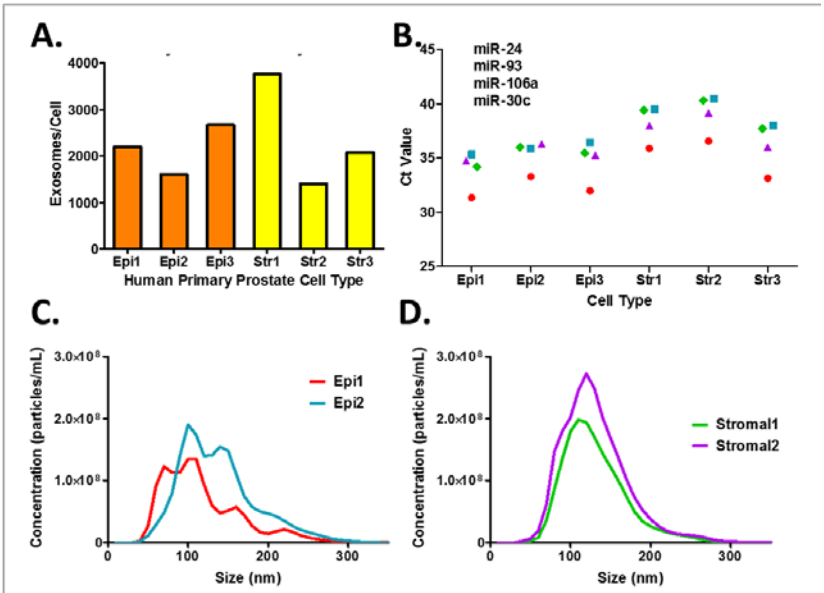

Figure 2. Characterization of exosomes from patient-derived primary prostate epithelial and stromal cells. A, quantitation of exosomes from primary prostate epithelial (PrE) and stroma (PrS) cells by anti-CD63 ELISA. PC3 prostate cancer cells included as positive control. **B,** exosomes from epithelial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

library prep for all samples and we await the sequencing results as I write this.

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**).

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 second year of graduate school (G2) having started in my lab summer of 2017. She completed her preliminary exam and advanced to candidacy in May of 2018 and will be applying for an F30 fellowship in December 2019. She has become 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 is on track so far after minor delays in enrollment in Y1. We anticipate the final results coming in quickly once we get the full patient cohort as all of the methods have been optimized.

4. IMPACT:

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

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 has been 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 we should reach target patient accrual by end of 2019 beginning of 2020.

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 42 patients to the overall study.

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

The expenditures are below budget due to the delays in serum collection (described above) and a delay in hiring. Ms. Morgan Zenner is a new MD/PhD student who started full-time in the laboratory of Dr. Larisa Nonn in August 2017. She had previously rotated in the lab in May 2017 and thus the position was reserved for her. Expenditures for Y2 are on track. There may be slight delay in analysis of the final cohort due to enrollment completion in spring 2020, but we estimate sufficient funds to request NCE if needed.

· **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:	*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 devotes 100% effort to this project.*
Funding Support:	*This DOD 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 devotes 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