AWARD NUMBER: W81XWH-21-1-0427

TITLE: Circulating Exosomal Protein Expression for Early Prediction of Platinum Resistance in High-Grade Serous Ovarian Cancer

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CONTRACTING ORGANIZATION: The Ohio State University, Columbus, OH

REPORT DATE: July 2022

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERE06/01/D
July 2022	Annual	15Jun2021-14Jun2022

4. TITLE AND SUBTITLE

Circulating Exosomal Protein Expression for Early Prediction of Platinum Resistance in High-Grade Serous Ovarian Cancer

5a. CONTRACT NUMBER
W81XWH-21-1-0427

5b. GRANT NUMBER
OC200475

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 Development 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

High-grade serous ovarian carcinoma (HGSOC), the most common epithelial ovarian cancer, is associated with a particularly poor prognosis as most patients are diagnosed at an advanced stage owing to a lack of early detection as well as due to the eventual development of platinum-resistant. Exosomes are an attractive source of biomarkers as they carry cargo (proteins, microRNAs, and lipids) from their cells of origin, are highly stable, and can be obtained from any biological fluid using non-invasive methods. We have identification differentially expressed and unique exosomal proteins (TMEM205, CD1B, ENPL, SA-A2, EP-CR and FAS) in platinum-resistant HGSOC, however it has not yet been proven or validated as a clinical tool. Thus, there is a critical need to establish novel methods for exosomal isolation and to validate their use in detecting potential biomarkers in HGSOC. This will pave the way for exploring the clinical implications that serum exosomal proteins could have as platinum-resistance markers, prognostic indicators and therapeutic targets.

15. SUBJECT TERMS

Platinum resistant, exosome, and Biomarkers

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Ovarian cancer continues to be the most lethal of all gynecological cancers in the United States (1, 2). High-grade serous ovarian carcinoma (HGSOC), the most common histologic subtype, is associated with a particularly poor prognosis as most patients are diagnosed at an advanced stage owing to the lack of screening methods for early detection. While initial response rates to chemotherapy are favorable, 25% of patients are resistance to first line chemotherapy (3-5). Furthermore, the majority of women with advanced stage HGSOC will recur and development of chemotherapy-resistance is inevitable. Patients with chemotherapy-resistant HGSOC (defined as progression-free interval less than six months, also known as "platinum-resistant") have a median survival of 7 to 12 months, and only 27% live longer than 12 months (3, 6, 7). In an effort to both identify and combat the eventual development of chemotherapy-resistance, it is important to evaluate potential predictive markers. Despite its high variability in expression, CA125 remains the primary clinical biomarker for HGSOC (8-11). However, it is not sensitive nor specific enough to detect early HGSOC or predict response to chemotherapy. The current study proposes to *identify expression of unique exosomal proteins* that may serve as biomarkers for detection of early-stage disease as well as predictive biomarkers for response to platinum-based chemotherapy. This would allow clinicians to not only identify patients earlier but also better tailor chemotherapy in order to improve treatment response and survival.

SPECIFIC AIMS:

Specific Aim 1: To identify the serum exosomal proteins that are differentially expressed in platinum-resistant HGSOC samples. Our *working hypothesis* is that unique serum exosomal proteins could be utilized as biomarkers for chemoresistance of HGSOC.

Specific Aim 2: To validate the clinical significance of patient serum exosomal protein expression as a biomarker for platinum-resistant HGSOC and correlate the protein expression with clinical outcomes (platinum-resistant disease, patient survival, and therapeutic responses) as compared with CA125. Our *working hypothesis* is that exosomal proteins can serve as sensitive and specific biomarkers that can provide early prediction of platinum resistance of HGSOC and therapeutic responses compared with CA125.

2. KEY WORDS

Ovarian Cancer

Platinum resistance

Exosome

Biomarkers

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goal of this study is to identify the novel exosomal proteins, as potential biomarkers for chemoresistance of high-grade serous ovarian cancer.

What was accomplished under these goals?

We have identified the significance of key findings in SA1

(i) Developed a MFD chip for exosome isolation in serum samples;
(ii) Exosomes are highly elevated in platinum resistant HGSOC serum samples;
(iii) Identified the exosomal proteins are highly elevated in chemoresistant HGSOC;
(iv) Standardization of the identified candidate exosomal proteins using ELISA

Aim 1: To identify the serum exosomes proteins that are differentially expressed in platinum-resistant HGSOC samples. Thus, the *objectives* of this aim are to identify the exosomal proteins that are unique to platinum resistance in HGSOC, and to validate the utility of our microfluidic device for exosome isolation. The microfluidic device will be used to identify serum exosomal proteins that are unique to platinum-resistant HGSOC compared to platinum-sensitive HGSOC and controls by LC-MS/MS.

1) Procurement, processing, and handling of human samples Prepare forms for approval of Animals use and protocols involved.
Prepare IRB forms for approval of human sample use and protocols involved.

Milestone # 1 human sample use approval (Year 1: month1-3): **Completed 100%.**

Approach 1. Developed a microfluidics device (MFD) for exosome isolation.

Milestone # 1. Developed Microfluidic chip standardization of the method and validation for exosome isolation (Year 1: 1 to 6 months). **Completed 100%**

Approach 2. MFD validation for exosome isolation and quantification using four different techniques:

Milestone # 2. Developed Microfluidic chip validation for exosome isolation using ultracentrifugation, NTA, dot blot and Western blot assay (Year 1: 3 to 6 months). **Completed 80%**

Approach 3a: Protein Extraction and Digestion for LC-MS/MS at OSU (PI): Candidate proteins were confirmed for HGSOC platinum resistant compared with platinum sensitive.

Approach 3b: Protein Extraction and Digestion for LC-MS/MS at Inova Schar Cancer Institute (Co:I): Candidate proteins will be confirmed for HGSOC platinum resistant compared with platinum sensitive.
Preparing the exosome samples for shipping to Inova Schar Cancer Institute.

Milestone # 3 Identifying the serum exosome proteins are differentially expressed in HGSOC platinum-resistant than sensitive by LC-MS/MS (Year 1: 6 - 9 months). **Completed 50%.**

Publication: **Draft 1 will preparing based on SA1 proposed study and being currently waiting the Co: I lab LC-MS/MS results.**

RESULTS

1. Development of microfluidics device (MFD) for the isolation of exosomes. In collaboration with Dr. Derek Hanford, Biomedical Engineering, The Ohio State University (see support letter), we have developed a unique microfluidic device (MFD) for exosome isolation (**Fig.1**), and our *objective* is to advance the use of this device for exosome isolation into clinical samples. Conventional methods of isolating exosomes in research laboratories are technically challenging, involve laborious ultracentrifugation, require a large sample volume, and are time consuming (39-41). Moreover, commercially available kits are costly and non-specific. Our study overcomes these drawbacks by using a novel, microfluidic-based approach, which allows for exosomal isolation from a small sample volume and provides a greater yield of high-quality exosomes compared to traditional techniques. Our novel MFD device isolates and releases intact and label-free exosomes (i.e., "Catch and Release" system; **Fig. 1B**), which allows for downstream processing without contamination. Specifically, our device combines surface antibody capture on a modified PDMS channel with our specific elution protocol that releases the exosomes from the antibodies with no residual components; thus, we are able to purify and isolate intact exosomes based on their surface markers with no interfering antibodies. This feature makes our device unique from the commercially available chips and gives it potential to be utilized as a rapid-screening tool for clinical use. We are currently using this approach to isolate exosomes from cell lines and clinical samples, which saves time and cost, and can be clinically translated with ease.

2. Microfluidics device (MFD) validation for the isolation of exosomes. Exosomes are nano-sized (30 -120 nm) vesicles released by a variety of cells and are generated within the endosomal system Exosomes are capable of reprograming normal and surrounding cancer cells to increase the expression of proteins associated with cell proliferation, survival, and chemotherapy-resistance in the tumor microenvironment (19-22). As noted above, exosomes have recently been found to serve as potential exporters of cytotoxic drugs from cancer cells (19, 23-25). This relationship between exosomes, cancer cells and chemotherapy suggests that the secretion of exosomes and exosomal proteins could be predictive biomarkers for HGSOC and chemotherapy-resistance. Our novel, microfluidic-based approach, allows for exosomal isolation from a small sample volume and provides a greater yield of high-quality exosomes compared to traditional techniques (**Fig. 2A**). Also, commercial kits are costly and non-specific. Using our novel microfluidics device (**Fig. 1**), we have isolated exosomes from both platinum-sensitive and resistant patient samples. Vesicle size was confirmed by nano tracking analysis (NTA) and transmission electron microscopy (TEM) and dot blot (**Fig. 2B - D**). We will address both technical and biological reproducibility of MFD capture of exosome isolaton, which carry oncogenic proteins and potential prognostic biomarkers, using different technologies and methods in different collaborators' by sharing the serum samples. Exosome isolation and purification was validated using a microfluidics device in PI and Dr. Hansford's laboratories (**Fig. 2E**) and were compared with other standard methods (ultracentrifugation and commercial kit, **Fig. 2A**). In addition, we identified important candidate proteins by LC-MS/MS (OSU Core lab) and will be validated these findings using a shot-gun proteomics at Inova Scar Cancer Institute (Co:I Dr. Maxwell).

3. Exosome secretion levels are high in chemoresistant HGSOC patient samples. Although there is evidence that exosome secretion is a key factor for platinum resistance (28) and our recent study showed that exosome secretion contributes to chemoresistance in cancer (REF). To determine if exosomes or exosomal proteins play a role in chemoresistance, we first compared the secretion of exosomes from chemoresistant to chemosensitive HGSOC patient serum samples. We have observed that the chemoresistant patient serum samples, had a 3-7-fold increase in exosome release when compared to chemosensitive serum samples, normalized to their cell counts (**Fig. 5A-C**). This suggests that increased exosomes release could contribute to chemoresistance and exosomal proteins could be used as a biomarker for HGSOC.

4. Identification of differentially-expressed proteins in serum exosomes from patients with platinum-resistant HGSOC. Exosomes isolated from the serum of patients with platinum-sensitive and -resistant HGSOC were analyzed using Liquid Chromotography-Mass Spectrometry (LC-MS/MS). We have identified a subset of proteins that are elevated in platinum-resistant HGSOC samples relative to platinum-sensitive and control samples. These candidate proteins are summarized in **Table 1** based on their differences in expression and fold change (FC); this is termed our discovery dataset. Identification of differentially expressed and unique exosomal TMEM205 (Transmembrane protein 205), CD1B (Transmembrane glycoprotein), Endoplasmin (ENPL), Endothelial Protein C-receptor (EPCR), Serum Amyloid-A2 (SA-A2) Ceruloplasmin (CP) and Fatty acid synthase (FAS) proteins has not been previously validated in HGSOC serum exosome samples. Previous reports, and our pilot studies, have shown that membrane proteins are amongst the first to sense any change in the event of pathological conditions (16, 42-44). The easy accessibility of membrane proteins renders them as perfect candidates for potential disease biomarkers with prognostic and/or therapeutic potential. In addition, compared to CA125, few exosomal proteins (TMEM205, ENPL, and CP) were significantly elevated in platinum resistant HGSOC serum exosomes; the expression of these proteins was confirmed by ELISA (**Fig. 4**). These data demonstrate that TMEM205, ENPL and CP may have greater expression in platinum resistant disease and may serve as better biomarkers than the current HGSOC biomarker, CA125.

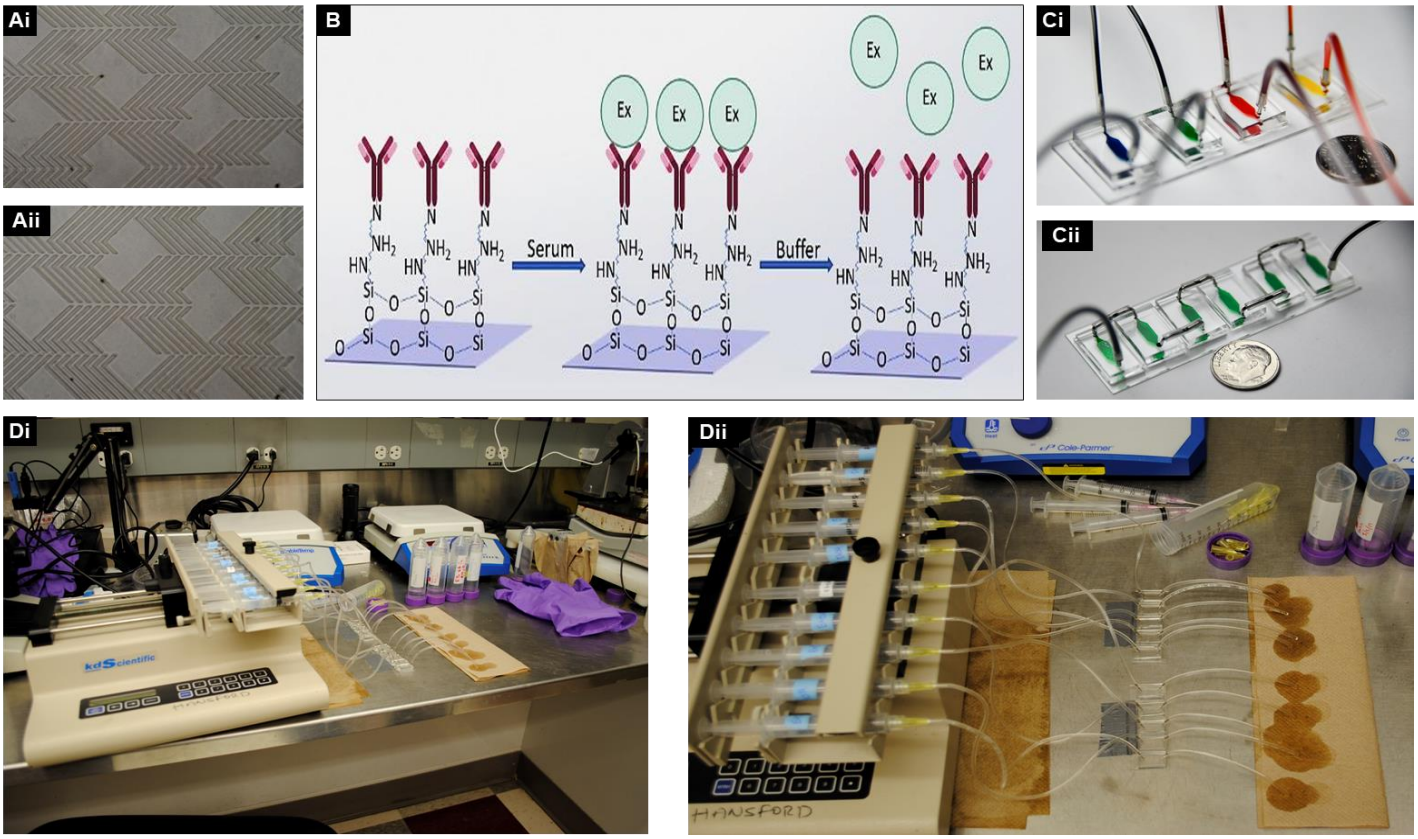

Figure 1. Microfluidic "catch and release" device. **Ai-ii)** First photolithography layer with base channel following exposure; Alt-herringbone layer after alignment and exposure and channel pattern following development of PDMS poured over pattern. **B)** Functionalization of PDMS surface with CD9 and CD63 antibody for exosome capture. **Ci & ii)** PDMS channels functionalized with CD9 antibodies in parallel and series. The flow is shown in different colors. **D**). Final set of MFD chip connected with power unit for exosomes capture and isolation.

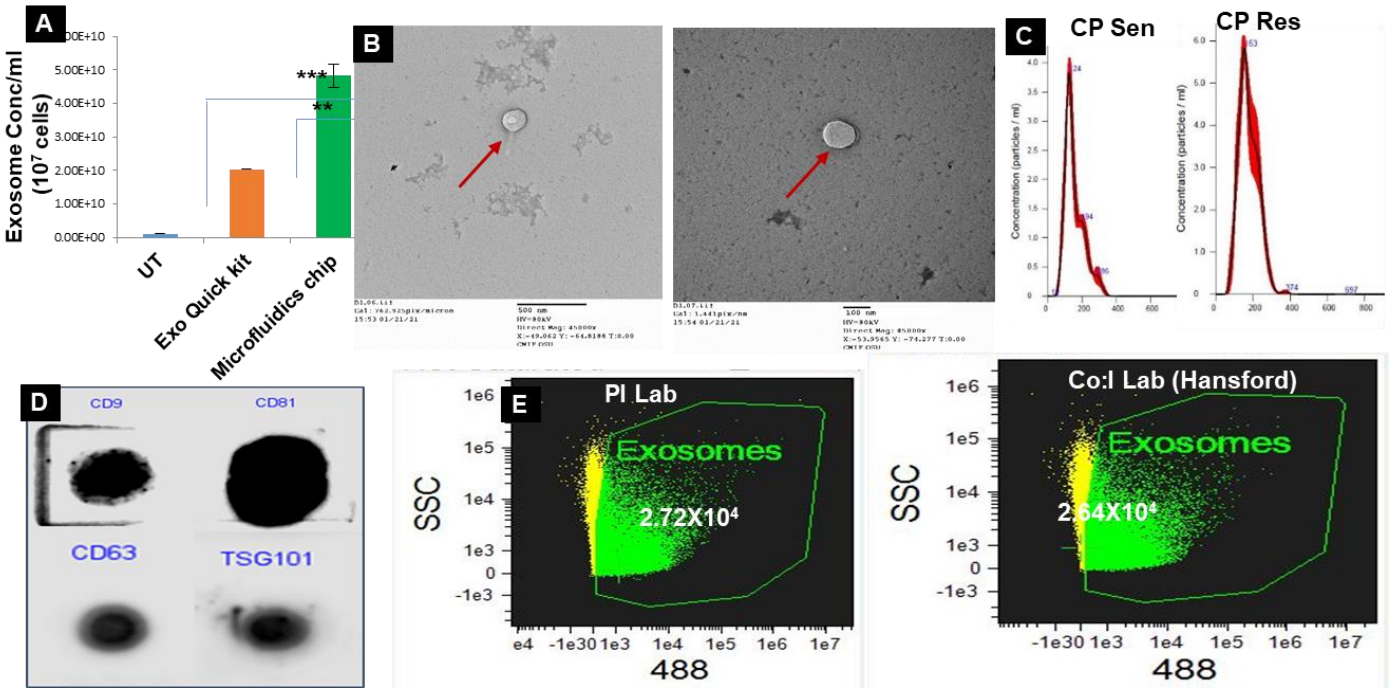

Figure 2. Exosome isolation by MFD and characterization. **A)** Higher exosome yield is obtained in the Microfluidics "catch & release" chip (MFD) compared with other conventional methods UT and commercial Kit (n=7 **p< 0.005, ***0.001). **B)** Morphological characterization and size measurement of exosomes (indicated by red arrows) by (**i**) Cryo and (**ii**) classical transmission electron microscope (TEM). **C)** NTA for exosome isolated by microfluidic chip in PI and Co-I labs showing reproducible results (n=5)**. D)** Dot-blot confirmation of CD9 and CD63 positive exosomes obtained by MFD. E**)** Quantification of Exosomes by image stream flow cytometry in HGSOC serum samples using microfluidic chip in PI and Co-I labs showing reproducible results (n=5).

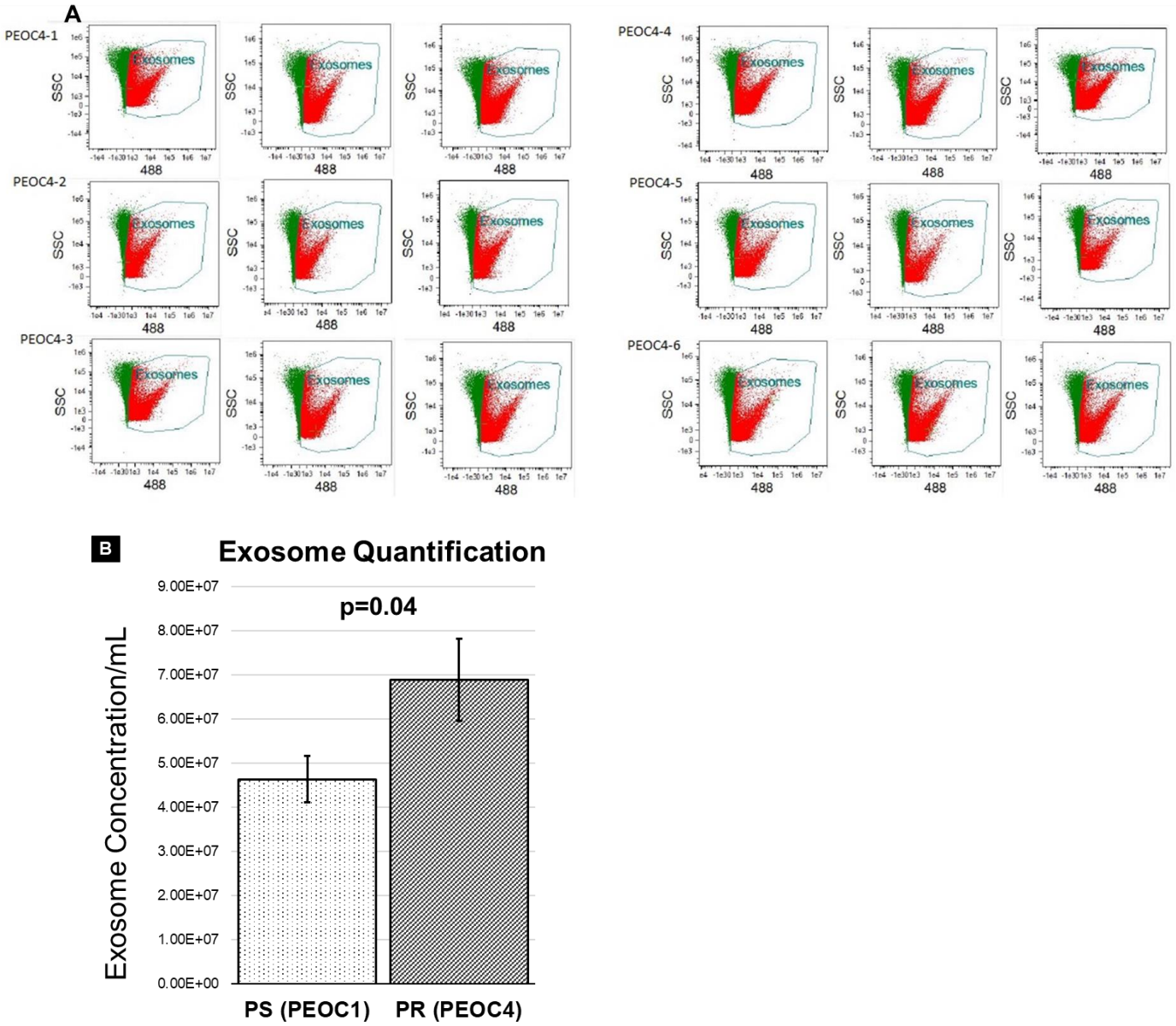

Figure 3. Exosome secretion in OC cells: **A**) Exosome secretion in HGSOC platinum resistant and sensitive serum samples as quantified by image stream flow-cytometry (ISF). **B)** Exosome secretion levels are higher in OC chemoresistant (PR) than chemosensitive (PS) (n=5, $p<0.04$).

Table 1. **Serum exosome proteins are differentially expressed in HGSOC platinum-resistant than sensitive by LC-MS/MS (n=5).**

Protein	Fold change	pValue
CD1B	11.2	0.005
FAS	5.6	0.0001
ENPL	5.3	0.01
TMEM205	4.7	0.001
SA-A2	4.6	0.05
EPCR	3.1	0.003
ANPEP	3.6	0.05
CTEP	2.5	NS
CFH	2.0	NS
CP	2.0	NS

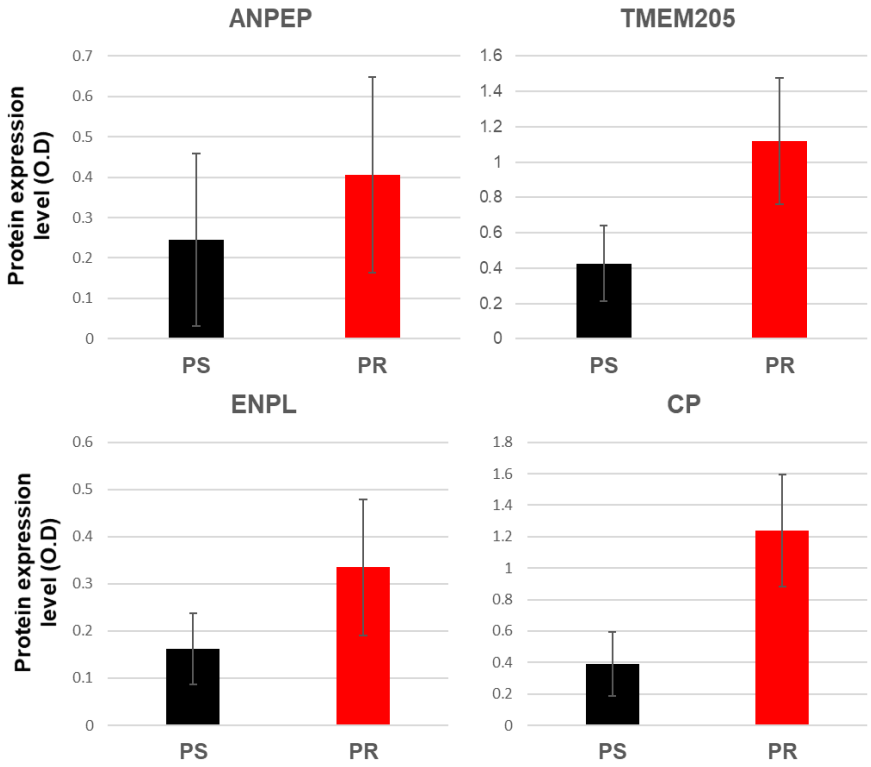

Fig. 4. Exosomal protein expression in HGSOC patient samples: TMEM205, ENPL and CP expression is highly elevated in platinum-resistant HGSHGSOC patient serum (n=9) compared to chemo-sensitive HGSOC serum by ELISA, results showing that TMEM205, ENPL and CP are significantly elevated in platinum-resistant HGSOC patient serum samples compared to chemo-sensitive and benign samples ($p<0.01$).

How were the results disseminated to communities of interest?

Nothing to Report

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

Currently we are finalizing the manuscript describing the data presented in the report and plan to submit it in by 2022.

For year 2, we plan to complete our proposed experiments from Aim 2, To validate the clinical significance of patient serum exosomal protein expression as a biomarker for platinum-resistant HGSOC and correlate the protein expression with clinical outcomes (platinum-resistant disease, patient survival, and therapeutic responses) as compared with CA125.

Initiate experiments from Aim 2 July.

4. IMPACT

1. Impact on the development of the principal discipline (ovarian cancer) of the project: this is the first proposed study aimed at determining serum exosomal protein expression (See Preliminary results Table 1) in HGSOC patient serum to be used as markers for platinum-resistance and therapeutic response. Our preliminary studies have shown that exosomal CD1B, TMEM205 and ENPL are more highly expressed in serum from patients with platinum-resistant HGSOC as compared to platinum-sensitive HGSOC, indicating that the identified exosomal proteins have potential to augment or replace CA125 as a cancer biomarker, especially with platinum resistance. Our research will yield a clinically relevant biomarker panel for the early detection and prediction of platinum resistance in HGSOC.

2. Impact on the development of other disciplines: Our study can have impact on all other solid tumors. By identifying the identify biomarkers which can more accurately predict HGSOC response to platinum-based chemotherapy.

3. Impact of the technology transfer: We have developed a novel microfluidics-based device for exosome isolation that is much quicker with greater purity and quality than standard laboratory techniques. With this microfluidics device, exosomes are isolated and released intact which allows for downstream processing without contamination. Specifically, our device combines surface antibody capture on a modified PDMS channel with our specific elution protocol that releases the exosomes from the antibodies with no residual components. Thus, we are able to purify and isolate intact exosomes based on their surface markers with no interfering antibodies. This feature makes our device unique from the prior developed chips and gives it the potential to be utilized as a rapid-screening tool for clinical samples, which will save time and cost, and can be used with clinical ease.

4. Impact on society beyond science and technology: nothing to report.

5. CHANGES & PROBLEMS

Changes: Nothing to report

Problems: We faced a problem with our developing MFD chip for exosome isolation using different HGSOC serum samples. It appeared that our chip yield exosome isolation is slow and low yeild. This significantly delayed our initial screening of exosome isolation and validation of the chip. We solved the problems within two months, modified the chip surface with our collaborator, and confirm the greater yield and quantification in different set of HGSOC patient samples.

- **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

- **Publications, conference papers, and presentations**
  Nothing to report

- **Journal publications.**
  Nothing to report

- **Books or other non-periodical, one-time publications**
  Nothing to report

- **Other publications, conference papers and presentations**
  Nothing to report

- **Website(s) or other Internet site(s)**
  Nothing to report

- **Technologies or techniques**
  We have developed a novel microfluidics based device to isolate intact exosomes with greater purity and quality in a shorter time that will allow for downstream processing. These factors are critical for moving forward in clinical translation and be directly applicable for exosome-based biomarker screening in patient serum samples.

- **Inventions, patent applications, and/or licenses**
  Our MFD chip, will filing a patent with OSU licenses

- **Other Products**
  Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project:

Name: Selvendiran Karuppaiyah, PhD
Project Role: PI
No Change

Name: Floor Backes, MD
Project Role: Co-I
No Change

Name: G. Larry Maxwell, MD
Project Role: Co-I
No Change

Name: Jing Zhao, PhD,
Project Role: Biostatistician
No Change

Name: Kalpana Deepa Priya Dorayappan
Project Role: Post Doc Fellow
No Change

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

Active Support Changes:

Selvendiran Karuppaiyah (PI)

Now Active / Awarded:
DOD FY20 Ovarian Cancer Research Program - Clinical Translational Research
Award W81XWH2110427 Total Costs: 06/15/2021 – 06/14/2023 3 calendar months

Jing Zhao (Biostatistician)

Active / Awarded:
DOD FY20 Ovarian Cancer Research Program - Clinical Translational Research
Award W81XWH2110427 Total Costs: 06/15/2021 – 06/14/2023 0.6 calendar months

Active / Awarded:
Role: **Biostatistician**
Nat In. Arthritis & Muscoskeletal & Skin
Title: Skeletal muscle in rheumatoid
arthritis K23AR068450 Total Costs: 09/012020 – 08/31/2021 2.4 calendar months

Active / Awarded:

Role: Biostatistician
National Institute of Neurological Disorders and Stroke
Title: Reducing infection susceptibility by immune function restoration in spinal cord injury
R01NS118200 Total Costs: 07/01/2020 – 06/30/2022 0.6 calendar months

Active / Awarded:
Role: Biostatistician
National Institute of Neurological Disorders and Stroke
Title: Implementation of machine learning workflows in primary brain tumor diagnostics R03NS116334 Total Costs: 06/01/2020 – 11/30/2021 0.6 calendar months

Active / Awarded:
Role: Biostatistician
NCI
Title: The translational regulation of pro-apoptotic genes
R01CA251753 Total Costs: 07/14/2020 – 06/30/2025 1.2 calendar months

Active / Awarded:
Role: Biostatistician
National Heart, Lung and Blood Institute
Title: ISGylation regulates lung endothelial inflammation
R01HL157164 Total Costs: 04/20/2021 – 03/31/2025 1.2 calendar months

Active / Awarded:
Role: Biostatistician
NCI
Title: Validating urine derived cancer cells (UDCC) – non-invasive and living liquid biopsies – in bladder cancer clinics
R33CA258016 Total Costs: 05/01/2021 – 04/30/2024 0.60 calendar months

What other organizations were involved as partners:

1. Additional OC platinum resistant and sensitive serum samples was provided by **Dr. Larry Maxwell, MD** at **Inova Schar Cancer Center** for evaluate the clinical significance of exosomal proteins expression as a marker of platinum resistance and survival in OC patient samples.

Nothing to report on any other personnel's and relationships.

8. Special Reporting Requirements

Nothing to report

9. APPENDICES