AWARD NUMBER: W81XWH-21-1-0427

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

PRINCIPAL INVESTIGATOR: Selvendiran Karuppaiyah, PhD

CONTRACTING ORGANIZATION: The Ohio State University, Columbus, OH

REPORT DATE: July 2023

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 2021	Annual	15Jun2022-14Jun2023

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)

Selvendiran Karuppaiyah, PhD

E-Mail: Selvendiran.karuppaiyah@osumc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

The Ohio State University
1960 Kenny Road
Columbus, Ohio 43210-1016

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, Ovarian cancer, exosome, EVs proteins, 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	22	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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

Biomarkers

Extracellular Vesicles (EVs)

Ovarian Cancer

Platinum resistance

EVs Proteins

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 SA2

(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 and Luminex assay.
(v) EVs proteins are elevated early cycle platinum treatment.

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-resistance disease, patient survival, and therapeutic responses) as compared to CA125. This aim, we will first test the identified candidate proteins in Table 1 as well as additional selected candidate proteins identified in SA1. This secondary analysis will include serum specimens from 100 patients with known platinum-resistant HGSOC and compare against 100 patients with known platinum-sensitive HGSOC as well as serum from 100 control. The same inclusion and exclusion criteria will be applied. To further validate our findings, in SA2b, we will be analyzing an additional 50 serum samples collected in the primary setting before receiving any chemotherapy (platinum status unknown) and re-collect serum from the same patients midway through their platinum-based chemotherapy regimen (after the 3rd or 4th cycle, n=50) and compare the top exosomal protein candidates in order to identify predictive biomarkers of platinum response

Approach 2.1. Exosome isolation, confirmation and quantification in serum samples.

Milestone # 1. Developed Microfluidic chip standardization of the method and validation for exosome isolation for Training and Validation Cohort samples (Year 2: 1 to 3 months). **Completed 100%**

Approach 2.2 & 2.3. Development of the training and validation sets:

Milestone # 2. Identified the top exosomal candidate proteins are clinically relevant biomarker panel for the early detection of platinum-resistant HGSOC disease, which will outperform CA125 (Year 2: 4 to 12 months). **Completed 90%.**

Specific Aim 2b: To determine the early predictive platinum-resistant biomarkers. Currently, there is no validated predictive biomarker for ovarian cancer, although there is an urgent need to identify patients who are unlikely to benefit from platinum therapy. Our preliminary study showed that compared to CA125, candidate exosomal proteins (ENPL, CD1B, TMEM205, SAA2, FAS and EP-CR)) were significantly elevated in platinum-resistant HGSOC serum exosomes with expression of these proteins confirmed by ELISA (Preliminary results **Table 1** and **Fig. 5 and 6).** In this final aim (SA2b), we will analyze the top candidate exosomal proteins as identified in our preliminary results (Table 1) and SA1, in order to confirm protein expression elevation early in first line platinum treatment. Based on pilot studies, we expect to find early expression of exosomal proteins that could be used as a predictive biomarker for platinum resistance.

Approach 2.4. Sample collection and experimental design: Approach 2.5. Exosome isolation and candidate proteins analysis: Exosome secretion levels will be isolated (MFD and confirmed by TEM and quantified by NTA. Approach 2.6. Candidate exosomal proteins will be analyzed by ELISA, PEA and Luminex.

Milestone # 3 Identifying the serum exosome proteins as early expression of that could be used as a predictive biomarker for platinum resistance.

(Year 2: 9-12 months). **Completed 50%. Currently this works continue to finish the remaining top candidate proteins within 6 to 9 months.**

Publication: **Draft 1 will preparing based on SA1 and SA2A proposed study and plan to submit this draft by October 2023.**

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. 1A-1 to AIII**), 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. 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. 1C-E**).

2. EVs proteins (TMEM205, CD1B and ENPL) expression in PS and PR-HGSOC tissues/serum. To determine if the elevated expression of EVs proteins from patient serum was due to platinum-resistance, we performed IHC, western blot and ELISA of human HGSOC tissues & serum samples. ENPL and TMEM205 are membrane proteins whose upregulation is associated with cisplatin-resistance in human epidermoid and breast cancer. Our preliminary studies indicate that ENPL, TMEM205 and associated membrane proteins, such as CD1B, is specifically expressed in platinum resistant HGSOC patient samples, as compared to platinum sensitive samples **(Fig. 2**). Since TMEM205, ENPL and CD1B expressions are elevated in platinum-resistant tissues and serum EVs samples, this demonstrates the potential as a biomarker for HGSOC platinum-resistance as well as a potential selective therapeutic target.

3. Determination of the sensitivity and specificity of EVs candidate proteins. Based on our ELISA data from a new set of patient samples obtained from Dr. Maxwell, Inova Schar Cancer Institute, we performed Receiver Operating Characteristic curve (ROC) analyses to determine the sensitivity and specificity of the candidate proteins that were identified (**Fig. 3)**. We assessed the diagnostic value of individual EVs proteins using ROC analysis (area under the curve, AUC). The expression of EVs proteins (CFH, CD1B, ENPL, FAS, EP-CR, TMEM and STAT3) had an AUC as high as 0.95, 0.92, 0.91, 0.80, 0.79 and 0.69 respectively. In comparison, CA125 has an AUC of 0.67. These data demonstrate that CFH, CD1B, ENPL, FAS and TMEM205 may serve as better biomarkers than the existing gold stander for HGSOC disease and treatment monitoring. Further, we have identified that combination of TMEM205 or STAT3 with CA125 showed increased sensitivity and specificity over individual expression in platinum-resistant serum EVs (AUC 0.89 or 0.86, data not shown here, due to space limitation). This proposed study will validate these findings using large cohort of samples.

4. **Early expression of EVs cargo proteins in HGSOC patients treated with platinum agents.** Our preliminary study showed that as opposed to Ca125, candidate EVs proteins (ENPL, CD1B, TMEM205, EP-CR) were significantly elevated in platinum-resistant HGSOC serum EVs with expression of these proteins confirmed by ELISA (**Fig. 4A).** Based on our preliminary studies, these candidate EV proteins could predict resistance to platinum treatment in HGSOC patients. HGSOC serum samples were obtained from the OSU Total Cancer Care (TCC) GYN/ONC tissue bank. These patients underwent platinum based. Serum samples were collected before initiation of chemotherapy or after the 3rd cycle of platinum treatment was completed (before 4th cycle started). EVs levels and EV candidate proteins were analyzed in a few selected patient serum samples. We found that EVs secretion and EV candidate cargo proteins expression (TMEM, CD1B, CFH,

ENPL and FAS) was highly elevated in HGSOC patients after the 3rd cycle of platinum treatment as compared to the same patient prior to platinum therapy (**Fig. 4B & C**). These results suggesting that early EVs candidate proteins expression could be predicative of platinum-resistant disease during first line therapy. Further, we have confirmed this patient samples study in *in vivo* orthotopic HGSOC mouse model treatment with carboplatin treatment for five cycles. We have observed that HGSOC mouse have a statistically significant increased EVs candidate cargo proteins expression in the first 2 cycles of platinum therapy but largely equalized by the third cycle (**Fig. 5**), suggesting EV cargo proteins (TMEM205, ENPL, STAT3, CD1B, Rab27a and CFH) predict early response of PR-HGSOC and possible up regulation of resistance mechanisms for HGSOC.

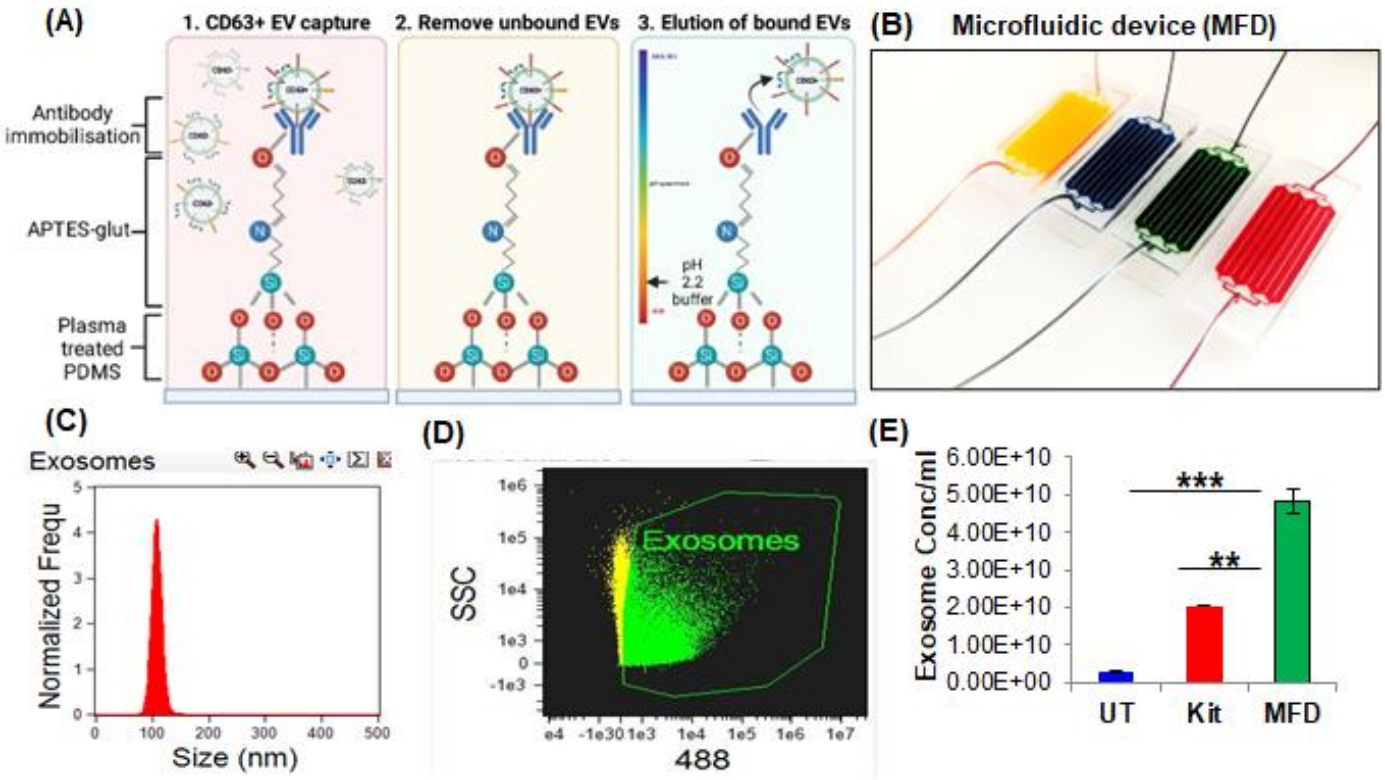

Figure 1. Microfluidic "catch and release" device (MFD). A) EVs capture and release system using antibody-functionalized microfluidic devices showing (A-1) covalent functionalization of PDMS surface chemistry with antibody to target EV antigens of interest and EV capture from biofluids, (A-2) removal of unbound EVs using PBS, (A-3) elution of selectively captured EVs using acidic buffer prior to immediate downstream neutralization. **B)** Image of microfluidic device (MFD). **C & D**) EVs isolation by MFD and confirmation of EVs size was limited by NTA and quantification EVs population by image stream flowcytometry (ISF) in the patient' serum samples. **E**) Quantity of EVs by MFD, Ultracentrifuge (UT) and Kit methods. Microfluidic device had greater yield than ultracentrifugation and commercial kits (n=7, ***p<0.0001 and **p<0.005, respectively)

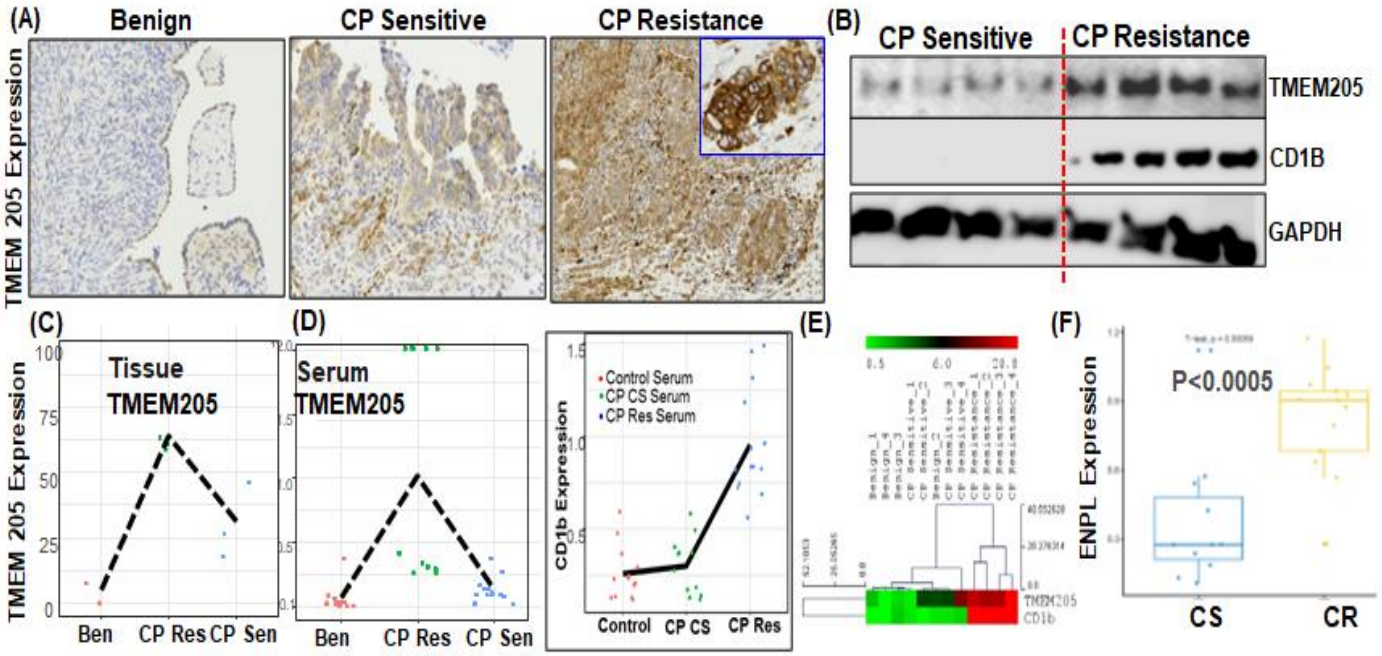

Fig. 2. TMEM205, CD1B and ENPL expression in HGSOC patient samples: A-C TMEM205 and CD1B expression is highly elevated in platinum-resistant HGSHGSOC patient tissues by IHC, Western blot and RT-PCR (n=4). **D & E**) EVs protein TMEM205 and CD1B (n=13) compared to platinum sensitive HGSOC and benign serum by ELISA and heat map analysis. **F**), ELISA results showing that ENPL is significantly elevated in platinum-resistant HGSOC patient serum samples compared to chemo-sensitive and benign samples (n=13, p<0.005).

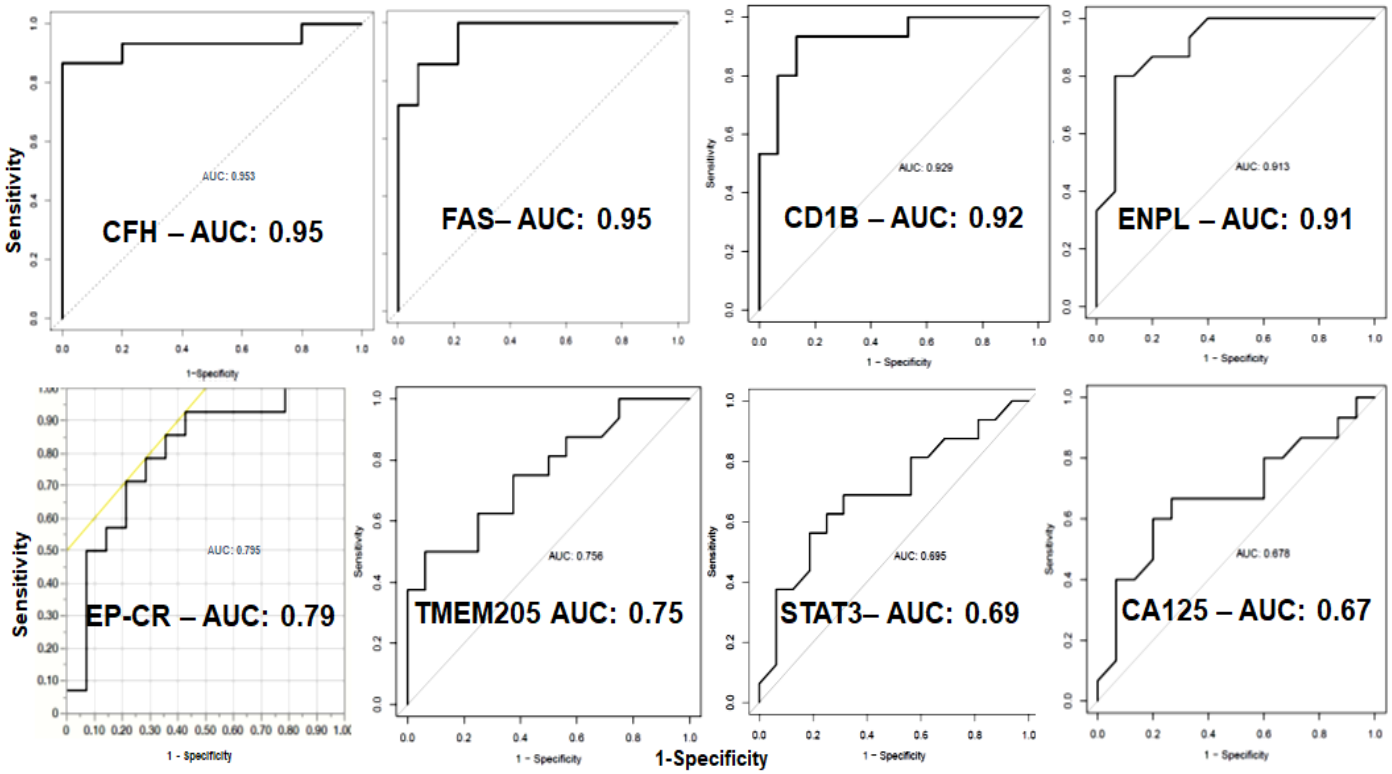

Fig.3. ROC curves of CA125, CFH, CD1B, ENPL, FAS, EP-CR, TMEM205 and STAT3 based on ELISA results from EVs isolated from platinum sensitive and resistant disease of HGSOC serum samples. Our candidate proteins had an AUC of greater than 0.69 to 0.95, compared to CA125 0.67 (aka MUC16) analyzed (n=16-23).

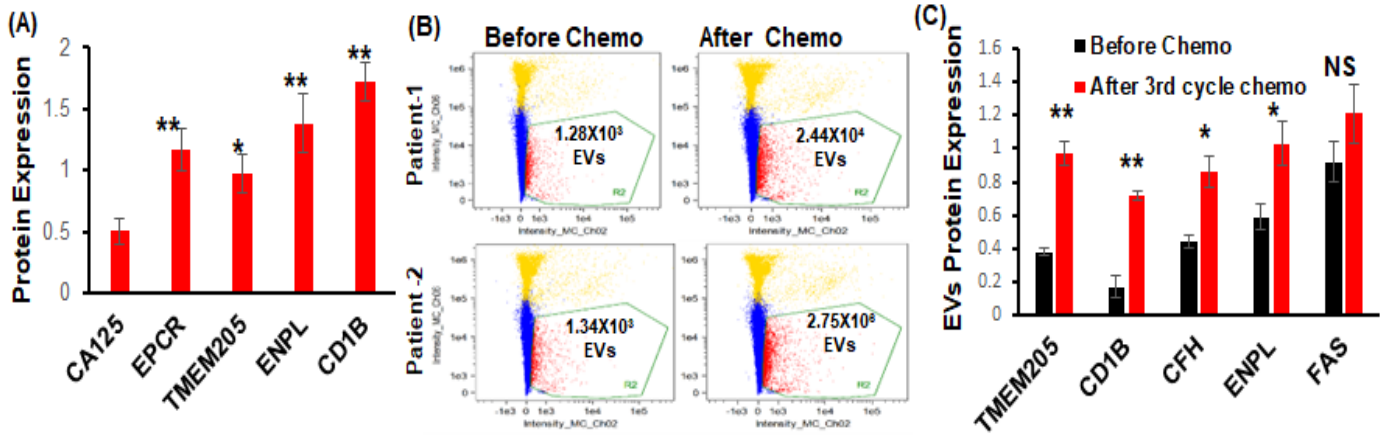

Fig. 4. A) EVs cargo protein EP-CR, TMEM205, ENPL and CD1B expression compared to CA125 (aka MUC16) analyzed by ELISA in a new set of platinum-resistant patient serum samples. All these EVs candidate proteins had on significant expression compared to CA125 (n=7, *p<0.1; **0.001). **B)** ISF for EVs concentration, we observed that EVs secretion is highly elevated in platinum-treatment patient after 3rd cycle compare with same patient in before platinum treatment. **C)** EVs cargo protein is significantly elevated in EVs after 3rd cycle treatment completed patient serum samples compare with before platinum treatment in same patients by ELISA (n=5, p<0.01).

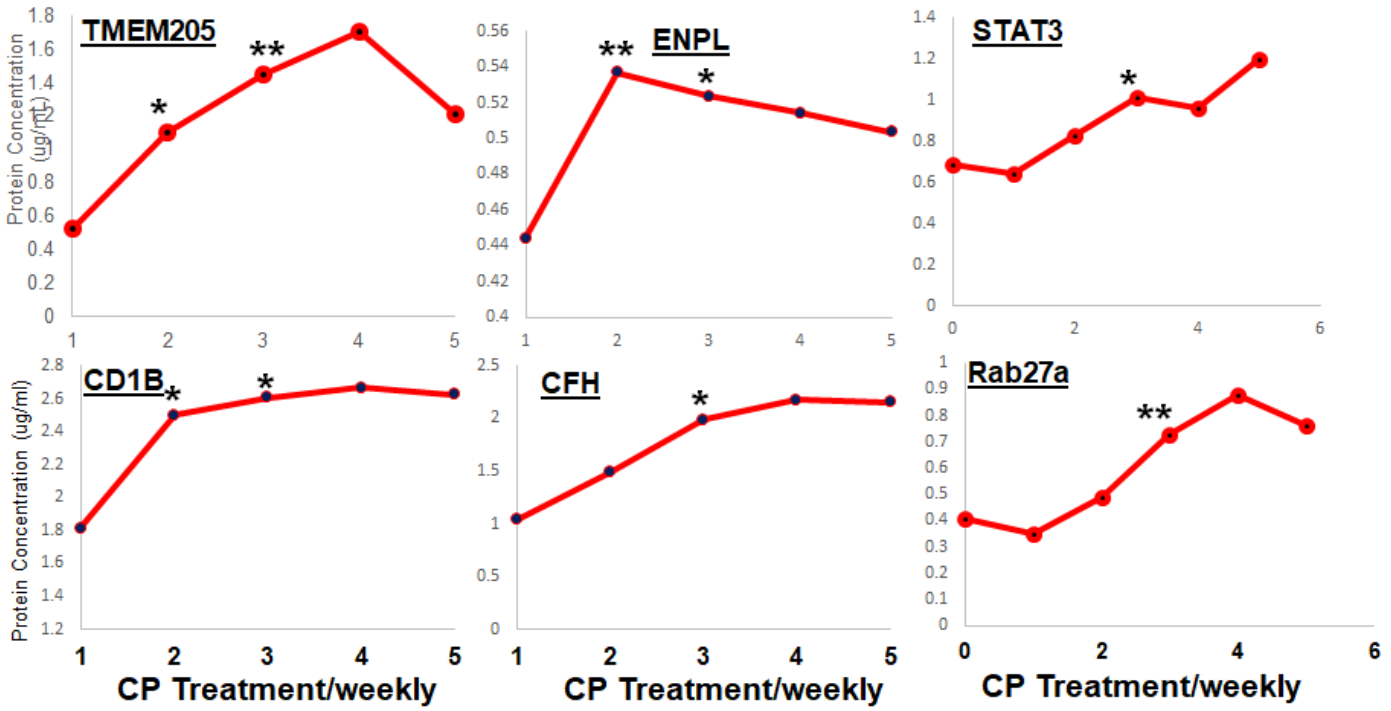

Fig. 5. Confirmation of EVs candidate proteins in orthotopic HGSOC mice: Injected the primary PR-HGSOC cells (ascites derived primary ovarian cancer cells) orthotopically (in the mice of ovarian bursa) and after tumor growth confirmed by MRI at 2nd weeks, treated with carboplatin (CP, 2mg/kg) treatments were delivered via intraperitoneal injection every week. serum samples were collected each cycle before next cycle treatment and EV proteins analyzed by ELISA (n=6; **p ≤ 0.005; *0.01 versus before CP or 1st cycle of CP treatment).

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 October end.

We have applied for No Cost Extension (NCE) to complete the SA2A and SA2B remaining couple of experiments, we plan to complete our proposed experiments from Aim 2B, **To determine the early predictive platinum-resistant biomarkers.**

Pending Experiments.

1. Developing new MFD Chip for exosome isolation for SA2B validation studies.

2. Validation studies of top candidate proteins (SA2B).

4. IMPACT

1. Impact on the development of the principal discipline (ovarian cancer) of the project. Although significant progress has been made in the treatment of HGSOC, the overall 5-year survival rate has had only modest improvement over the past 30 years. Resistance to chemotherapy almost inevitably occurs during the treatment of HGSOC, increasing the rate of disease recurrence and, ultimately, patient mortality. Patients with platinum-resistant HGSOC have a median survival of 7-12 months. The overall clinical response in patients with recurrent HGSOC is only 25-40%, with prognosis directly correlated to platinum sensitivity or resistance. To date, *no reliable predictive biomarker* has been recognized that is capable of identifying patients with pre-existing resistance to these agents. The most well-known biomarker for ovarian cancer is serum CA125, which is the gold standard for disease monitoring and chemotherapy response, however, its clinical application is significantly limited by both its sensitivity and specificity. Thus, *there is a need* to identify biomarkers which can more accurately predict HGSOC response to platinum-based chemotherapy.

2. Impact on the development of other disciplines: Our study can have impact on all other solid tumors. Identified 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. These exosome-derived proteins can be further confirmed in independent patient cohorts consisting of a large number of platinum-sensitive and -resistant serum samples, as proposed in this study. Our research will yield a clinically relevant biomarker panel for the early detection and prediction of platinum resistance in HGSOC and other solid tumors

3. Impact of the technology transfer: Translational Technology - Microfluidics device: 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.

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

5. CHANGES & PROBLEMS

Changes: Nothing to report

Problems: Approximately 3 months prior, we faced a problem with ELISA assay to not detect couple of exosomal proteins (FAS and CD1B) in platinum-resistant or sensitive HGSOC samples. We solved the problems within a month, selected candidate proteins using different company antibody and techniques such as Lumines assay.

- **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**
  Vincent Wagner, Kalpana Deepa Priya Dorayappan, Molly Morton, Takahiko Sakaue, Larry J. Maxwell, Floor Backes, David O'Malley, David E. Cohn[1] and Karuppaiyah Selvendiran. **Circulating exosomal protein expression as a biomarker of platinum-resistance in high grade serous ovarian cancer. Preparation for** Journal of Extracellular Vesicles 2023).
- **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**
  Nothing to report
- **Other Products**
  Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project:

Name: Selvendiran Karuppaiyah
Project Role: PI
No Change

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

Name: Jing Zhao
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 ENPL 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