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TITLE: Endoplasmin: A Novel Therapeutic Target and Potential Marker of Chemoresistance

PRINCIPAL INVESTIGATOR: Selvendiran Karuppaiyah, PhD

CONTRACTING ORGANIZATION: The Ohio State University

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Selvendiran Karuppaiyah, PhD

E-Mail: Selvendiran.karuppaiyah@osumc.edu

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The Ohio State University
1960 Kenny Road
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14. ABSTRACT

Our proposed study is to identify endoplasmin protein expression as a predictive marker associated with OC chemoresistance and identification of these pathways as potential therapeutic targets. Utilizing an endoplasmin small molecule inhibitor we plan to assess chemotherapy treatment using in vitro and in vivo studies with mice models. Our proposed research has the potential to identify novel the mechanisms of endoplasmin-mediated chemoresistance and targeting ENPL might serve to revert tumors to a chemosensitive state on a cellular level, therefore, improving treatment effect and prognosis/overall survival.

15. SUBJECT TERMS

Cancer Biology, Target Therapy and Biomarkers

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TABLE OF CONTENTS

1. INTRODUCTION

Mortality rates from ovarian cancer (OC) are the highest among all gynecologic cancers and current therapies to treat the disease have failed to improve survival rates. Thus, there is a *critical need* to identify novel targets for alternative therapies for OC to improve outcomes. To effectively treat OC, it is important to: (i) Identify predictive markers associated with chemoresistance and (ii) elucidate the pathways involved in intrinsic chemoresistance to identify novel therapeutic targets. With these targets objectives in mind, we propose to identify the association of endoplasmin (ENPL) with platinum-resistant OC. ENPL is a member of a family of adenosine triphosphate (ATP)-metabolizing molecular chaperones that have roles in stabilizing and folding other proteins localized to melanosomes and the endoplasmic reticulum (ER). While ENPL expression has been demonstrated to play a critical role in immune modulation, cancer, and chemoresistance, the exact molecular pathways by which it acts remain unknown. Our current preliminary study demonstrated that ENPL expression in the membrane fraction is highly elevated in platinum resistant OC cell lines and patient samples. The hypothesize of our proposed study is that overexpression of endoplasmin contributes to chemo-resistance and that targeting endoplasmin using novel small molecule compounds will facilitate more successful treatment of the disease. To test this hypotheses, the following specific aims are proposed:

Specific Aim 1: To identify the role of ENPL expression in chemosresistance and progression OC. Our *working hypothesis is that* OC cells expressing ENPL expel carboplatin through exosome secretion and that OC cells with high levels of ENPL will be resistant to platinum treatment.

Specific Aim 2: To evaluate the effects of inhibiting ENPL on the efficacy of carboplatin treatment using *in vitro and in vivo* mouse models. Our *working hypothesis* is that inhibiting ENPL by small molecule inhibitors (DAP-1 or DAP-2) will significantly increase the efficacy of carboplatin (CP).

2. KEY WORDS

Ovarian Cancer

Platinum resistance

Endoplasmin

Exosomes

Biomarker

Small molecule inhibitor (DAP-1)

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goal of this study is to evaluate a novel protein, Endoplasmin, as a potential therapeutic target in chemoresistant ovarian cancer

What was accomplished under these goals?

We have identified the significance of key findings in SA1

(i) ENPL plays a key role in chemo-resistant;
(ii) Developed a MFD chip for exosome isolation in cancer cells;
(iii) Exosomes are highly elevated in platinum resistant OC cells;
(iv) ENPL expression as a marker of platinum resistance.
(v) ENPL inhibitor suppress tumor growth.

Specific Aim 1: To identify the role of endoplasmin (ENPL) expression in chemosresistance and progression OC.
1a) Prepare forms for approval of Animals use and protocols involved.
Milestone # 1 Animal use approval (Year 1: month1-2): **Completed 100%.**

1b) 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. To determine the role of ENPL in OC cell survival, proliferation, and drug resistance.

Milestone # 1. Confirm and correlate the ENPL expression to Platinum resistance by ENPL knockdown (KD) or overexpression (OE) in chemo resistant cells and sensitive ovarian cancer cell lines (Year 2: 1 to 3 months). **Completed 100%**

Approach 2. To determine the mechanism of OC chemoresistance by ENPL. **Approach 1**: Assessment of cells' sensitivity to carboplatin expressing wild type, knock down and over expression ENPL or MRP2 in OC sensitive or resistant cell lines.

Milestone # 2. Depletion of ENPL in WT chemoresistant cells impacts the MRP2 expression, analyze the carboplatin sensitivity in either ENPL or MRP KD cells (Year 2: 3 to 4 months). **Completed 100%**

Approach 3 & **4.** To determine if ENPL regulates exosome secretion through MRP2 in OC cells.

Milestone # 3 Confirm the role of ENPL-mediated exosome secretion through MRP2 in OC and further identify ENPL, carried in exosomes, can confer cisplatin resistance to a cisplatin sensitive background (Year 2: 4 to 5 months). **Completed 75%**

Approach 4. To evaluate the clinical significance of ENPL expression as a marker of platinum resistance and survival in OC patient samples.

Milestone # 4. This study will confirm the prognostic value of ENPL as a biomarker for chemoresistance, disease progression, and prediction of potential treatment response (Year 1: months 7 to 12). **Completed 100%.**

Milestone # 5. Evaluate the efficacy of ENPL inhibitor in combination with carboplatin in three different mice modes including immunocompetent and PDX mice model; the synergistic effects of ENPL inhibitor and CP

evaluate vivo mice studies based on the ENPL inhibition, suppression of OC tumor growth and apoptotic proteins expression in mice tissues (Year 2: months 8 to 12). **Completed 70%.**

Approach: Determine the treatment efficacy of DAP1 and Platinum based drugs alone and in combination on primary ovarian cancer cells isolated from patient ascites or tissues.

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

We have training for Gynecology Oncology Clinical fellows in this proposed project in vitro study.

Publication: **Draft 1 is preparing based on SA1 and SA2 proposed study and being currently finalizing the results.**

RESULTS

1. Increased ENPL and exosome secretion and lysosomal phenotype in OC platinum resistant cells: OC cell exosomes were isolated using a microfluidics (MFD) chip and confirmed by TEM. Since OC is hypoxic in nature (32), the effects of oxygen tension on the release of exosomes from OC platinum reistant cells was assessed by incubating cells for 48h (in exosome-free culture medium) in normoxia (20% O_2) and hypoxia (1% O_2). Exosome release from different cell lines ranged from a 2-8fold increase in the hypoxic environment, as compared to normoxia (**Fig. 1A**). We next focused on investigating the changes in lysosomes, as they are important organelles involved in the exosome trafficking pathway. Lysosomal membrane docking and peripheral distribution was observed using the lyso-tracker red dye in hypoxic OV8 or TR127 cells (**Fig. 1B**). Additionally, the co-localization of lysosome associated membrane protein-1 (LAMP1) with lysosomes, stained with lyso-tracker red and visualized by fluorescence microscopy, confirmed the peripheral distribution of the lysosomes in cells isolated from OC patient ascites (**Fig. 1C**). These results demonstrate that hypoxic tumor environments play a role in exosome secretion.

2. **Exosomal ENPL and its target proteins are highly elevated in hypoxic OC cells:** To determine if exosomal proteins play a role in OC progression and platinum resiatnce, we first compared the secretion of exosomal proteins from normoxic vs. hypoxic OC cell exosomes. The exosomes were isolated from normoxic (NExo) and hypoxic (HExo) OC cells by MFD and quantified ISF (**Fig. 2**). NExo and HExo were analyzed for their protein profile using Liquid Chromatography Mass Spectrometry (LC-MS/MS). We have identified that ENPL and its associated proteins are highly expressed in hypoxic exosomes. Potential candidate proteins are summarized in **Table 1** based on the statistical significance and fold change.

3. ENPL knockdown or inhibition by ENPL inhibitor, restores the CP sensitivity in OC *in vivo* mice model. TR-127 ENPL KD cells ($1x10^6$) were injected into the ovarian bursa (orthotopic model) of immunocompromised mice, as described previously (58, 59). We observed a significant reduction of OC progression in the orthotopic mouse model treated with CP in ENPL KD cells transplanted mice compared to TR-127 WT cells treated with CP (2mg/kg) for 5 weeks (**Fig.3**). Another model included intraperitoneal (IP) injection of TR-127 cells into nude mice. These mice had significantly greater tumor growth and more numerous metastatic nodules in the mesentery, diaphragm, and pelvis than mice injected with TR-127 cells that had been treated with ENPL or exosome inhibitor either alone or combination with CP. When tumors were evident (based on MRI) the mice were assigned to one of five treatment groups: CP (Weekly), ENPL (Weekly). Tumor nodules were significantly reduced in the ENPL pre-treatment + CP group, compared to those treated with CP alone (**Fig.3A & B**), without affecting body weight. Collectively, these data demonstrate that ENPL inhibitors, increased CP accumulation and specifically cytotoxic toward OC cells, resulting decreased tumor growth. This study is ongoing, we are currently evaluating ENPL and its target protein expression in mice tissues.

4. ENPL expression is highly elevated in chemoresistant OC. Membrane proteins are amongst the first members to sense any change in the event of pathological conditions. This easy accessibility renders them as perfect candidates for potential disease biomarkers with both diagnostic and prognostic potential. ENPL is one such membrane protein whose upregulation is associated with platinum resistance. Our *working hypothesis* is that the presence of ENPL in serum exosomes can serve as biomarkers for this resistance. To determine whether ENPL is most strongly associated with chemoresistance, we will evaluate the differential expression of ENPL in serum samples derived from OC patients using an ELISA in human OC serum samples were performed which revealed highly elevated ENPL expression in the chemo-resistant OC patient samples but absent or lower ENPL expression in the chemosensitive and control serum samples (**Fig. 9A & B**). ENPL expression levels are 8-25 fold higher in chemo-resistant OC serum samples when compared with sensitive samples. This indicates that

ENPL expression is very specific to OC chemoresistant tissues and serum compared with benign or chemosensitive tissues and serum. We further validated this expression in a different set of benign, OC patients samples and demonstrated a high expression of ENPL and CD1B when analyzed using ELISA (**Fig. 10A & B**). This supports its potential as a biomarker as well as a therapeutic target.

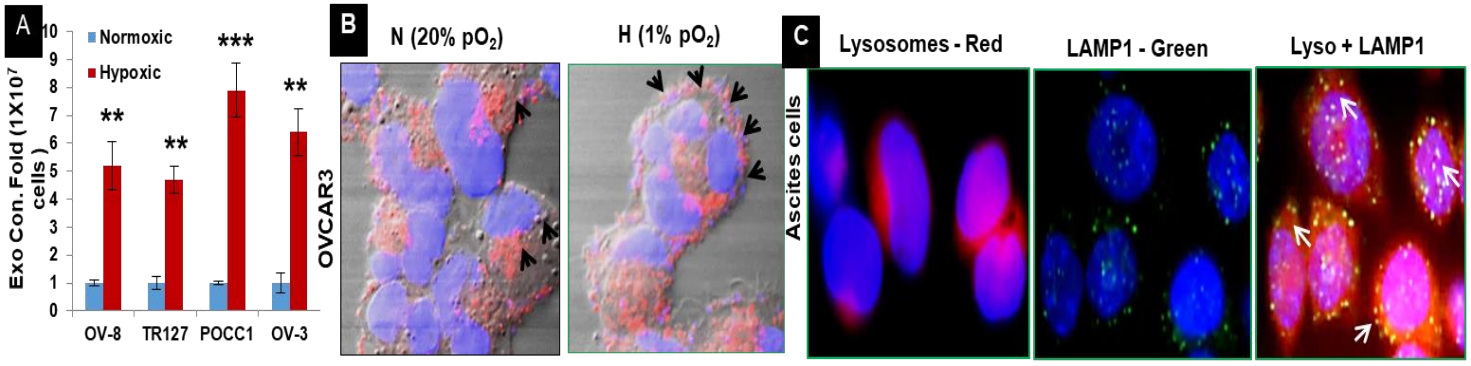


Fig. 1. **Exosome secretion and lysosome phenotype in hypoxic OC: A)** Fold change in the exosome concentration isolated from different OC cell lines (n=3±SD; p-value **0.005 or ***0.0001). **B)** The lysosomes visualized by confocal microscopy in OV cells show a perinuclear localization in normoxia and a peripheral orientation favoring docking to the plasma membrane; thus confirming a more secretory phenotype (arrow indicates). **C)** The peripheral lysosome (red) co-localization with LAMP-1 (green) was confirmed in OC ascites cells.

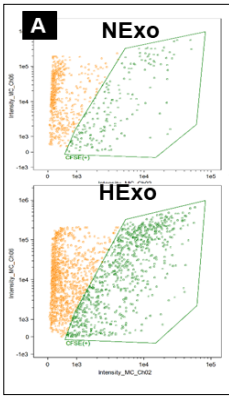
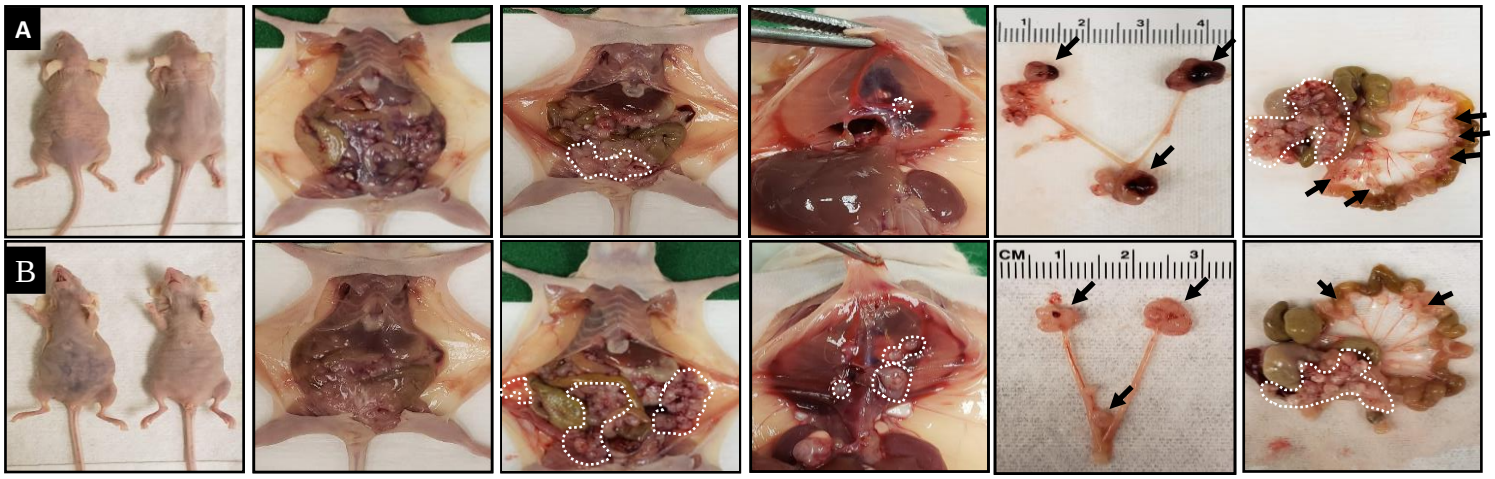


Table 1

No	Gene Symbol	Fold mean diff. NExo vs HExo	p. mean diff. NExo vs HExo
1	ENPL	9.03	0.001
2	MMP-7	8.75	0.0002
3	VEGF-A	6.53	0.004
4	STAT3	6.17	0.001
5	FAS	5.18	0.003

Fig. 2. A. OV4 cells cultured (normoxic or hypoxic) for 48 hours, exosomes quantified by image stream flow cytometry (ISF), exosomes count was NExo 5.53 x 10^7/ml and HExo 2.34x10^8/ml**. Table 1.** Exosomes proteins profile analyzed by LC-MS/MS, high mass accuracy MS determination the full scan was performed at FT mode with resolution set at a 120,000. ENPL and its associated protein 5 to 9-fold time elevated in hypoxic exosomes (n=4).



ENPL inhibitor increases CP sensitivity in OC peritoneal mice. A TR-172 cells (10^{-5}) injected i.p and 50uL of purified from normoxic and hypoxic cultured cells exosomes containing ENPL (100 µg) were i.p. injected into mice twice a week for 3 weeks. The exosome treatments were started on day 3 after tumor cell injection so that sufficient time was given for the attachment of the cancer cells to the inner side of the abdominal wall and the surfaces of the intraperitoneal organs. Aggressive metastasis growth and the number of metastatic nodules was significantly higher (circle indicated) in mice injected with hypxic exosomes containing ENPL. **B**) Mice treated with ENPL inhibitor (5mg/kg, i.p), CP (2mg/kg, i.p), ENPL inhibitor in combination with CP showed significant anti-tumor efficacy compared to CP treatment alone. (Study is ongoing to complete the ENPL and its target protein expression in mice tissues)

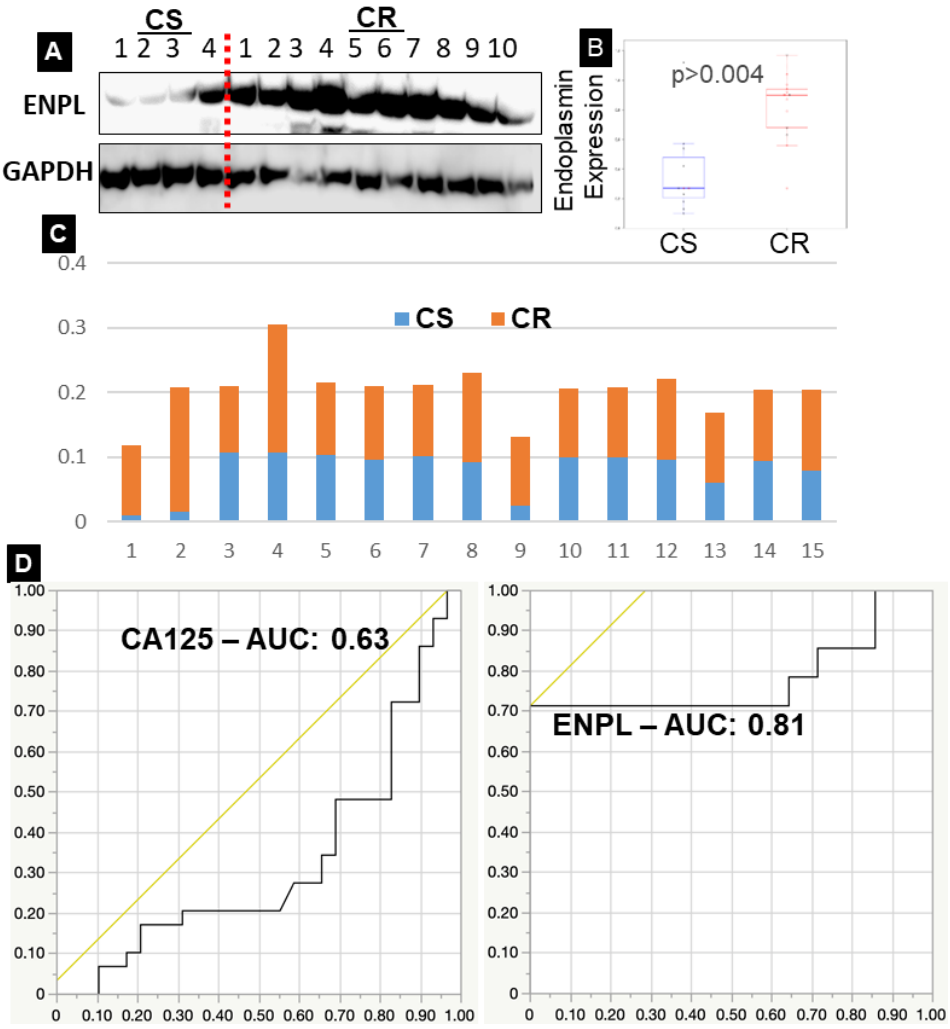


Figure 4. ENPL expression in OC patients: A & B) ENPL expression is highly elevated in OC platinum resistant patient serum or tissues than platinum sensitive (n=10 for WB; n=4 for IHC); **C)** High ENPL in OC patient serum samples than OC platinum sensitive serum exosomes samples (n-18); **D)** ROC curves of CA125, and ENPL based on ELISA results from exosomes isolated from platinum sensitive and resistant disease of OC serum samples. ENPL had an AUC of greater than 0.81, compared to CA125 0.63 (aka MUC16) analyzed (n=16).

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, waiting for final in vivo data completion. Plan to submit it in November end.

4. IMPACT

1. Impact on the development of the principal discipline (ovarian cancer) of the project: Identifying the role of endoplasmin in the development of platinum-resistance of OC: Our preliminary results showing that ENPL could play a role in in promoting OC progression and chemoresistance through the exosome secretion payways. Based on these findings, we will evaluate novel mechanisms linking ENPL with exosomal secretion, including, how ENPL expression plays a role in exosome release and contributes to OC chemoresistance through the MRP2 activation in OC in SA1 and SA2. This finding will potentially lead to the identification of novel biomarkers and therapeutic targets for chemoresistant OC.

2. Impact on the development of other disciplines: Our study can have impact on all other solid tumors. By identifying the role of ENPL and combining the approaches of blocking exosome secretion with cisplatin treatment approaches, the outcome of combination thearpy can me enhanced and made for successful for the patient.

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: We faced a problem with our ENPL antibody issue in patient serum sample study by ELISA and few research supplies were delay due to COVID. This significantly delayed our in vivo study experiments. We solved the problems within couple of months, purchased new antibody in different company, working good in patient samples serum and tissue.

- **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**
  Role of ENPL mediated platinum resistant in ovarian cancer. Dorayappan KDP, Saini U, Colin H, Takahiko S, Smith BQ, Lightfood M, Flannery M, Hays J, Hansford D, O'Malley D, Cohn DE, **Selvendiran K**. *Manuscript under preparation for Molecular cancer research 2022.*

- **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: David Cohn
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