**AWARD NUMBER:** W81XWH-21-1-0378

**TITLE: Targeting Cholesterol Metabolism in Platinum-Resistant Ovarian Cancer**

**PRINCIPAL INVESTIGATOR: Daniela Matei, MD**

**RECIPIENT: Northwestern University, Evanston, IL**

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Fort Detrick, Maryland 21702-5012

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Daniela Matei

E-Mail:

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The purpose of the project is to analyze cholesterol content and uptake in platinum resistant ovarian cancer and clear cell carcinoma and target this pathway by using HDL nanoparticles. The hypothesis is that platinum resistant ovarian cancer cells and tumors harbor unique metabolic features which render them dependent on cholesterol uptake and high intracellular cholesterol, due to increased capacity to withstand oxidative stress, and are susceptible to ferroptosis.

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## 1. INTRODUCTION:

The purpose of the project is to analyze cholesterol content and uptake in platinum resistant ovarian cancer and clear cell carcinoma and target this pathway by using HDL nanoparticles. The hypothesis is that platinum resistant ovarian cancer cells and tumors harbor unique metabolic features which render them dependent on cholesterol uptake and high intracellular cholesterol, due to increased capacity to withstand oxidative stress, and are susceptible to ferroptosis.

## 2. KEYWORDS:

Ovarian cancer, GPX4, cholesterol, nanoparticles, platinum resistance, ferroptosis, onco-metabolic switch

## 3. ACCOMPLISHMENTS:

### What were the major goals of the project?

*SA1:* **Test the hypothesis that GPX4 and cholesterol metabolism regulate platinum resistance in ovarian cancer cell lines and in xenografts**

To investigate if platinum resistant (Pt-R) ovarian cancer (OC) cells are more dependent on cholesterol metabolism for survival, we first measured total intracellular cholesterol level, including cholesteryl esters and free cholesterol and in platinum sensitive and resistant OC cells by using the Amplex™ Red cholesterol assay. Our results indicate that total intracellular cholesterol accumulation in platinum resistant (Pt-R) OVCAR4 and OVCAR5 cells was much higher compared to parental cells (Figure 1A-B, $p<0.05$). Next, intracellular cholesterol accumulation was imaged and further quantified by hyperspectral stimulated Raman scattering (SRS) imaging (Figure 1 C-D; in collaboration with Dr. Cheng from Boston University). SRS is a novel label-free chemical imaging technique that detects the intrinsic chemical bond vibrations, including C-H bond vibration associated with cholesterol structure. SRS microscopy indicated that cholesterol accumulation was enriched in OVCAR5 Pt-R OC cells than in OVCAR5 wild type (WT) cells, further supporting cholesterol accumulation in chemo-resistant OC cells. As the enrichment in cholesterol can be contributed by either upregulation of intrinsic *de novo* cholesterol synthesis or enhanced extrinsic cholesterol import, we next checked expression levels of genes related to *de no* synthesis pathways at transcription levels by performed RNA sequencing on OVCAR5 platinum sensitive and resistant OC cells. GSEA results showed that *Hallmark cholesterol homeostasis, Reactome cholesterol biosynthesis* and *Reactome regulation of cholesterol biosynthesis by SREBP* were enriched in OVCAR5 WT compared to Pt-R cells (Figure 1E, $p=0$). In addition, compared to immortalized fallopian tube epithelial cells (FT-190) (1), two of key rate-limited enzymes in de novo cholesterol synthesis pathway, 3-Hydroxy-3-Methylglutaryl-CoA

Synthase 1 (HMGCS1), and squalene epoxidase (SQLE) expression weas reduced in the platinum resistant OVCAR5 cells (Figure 1F, p<0.05), confirming that cholesterol *de novo* synthesis is blunted in OC cells with acquired chemoresistance. We next explored the possibility that cholesterol import may be increased in these resistant cells. Intriguingly, the expression of the high density lipoprotein (HDL) receptor, Scavenger Receptor Class B Type I (SR-BI), which facilitates the uptake of cholesterol esters from circulating lipoproteins (2), was increased in OVCAR5, OVCAR4 Pt-R cells compared to their respective parental cells, normal endometrial cells (NoEM) (3) and FT-190 (1) (Figure 1G). These results were further confirmed in HGSOC primary cells derived from tumor collected after neo-adjuvant chemotherapy. SR-BI was highly expressed in Pt-R HGSOC cells compared to FT190 and NoEM cells (Figure 1H), demonstrating that platinum resistant OC cells may be more dependent on SR-B1 mediated cholesterol import for survival.

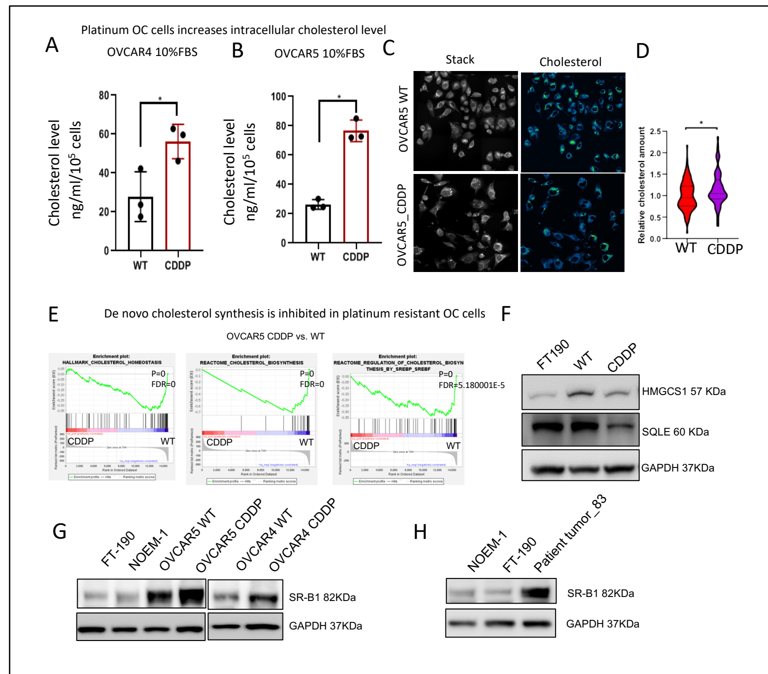


**Figure 1. Platinum resistant OC cells are more dependent on intracellular cholesterol accumulation for survival.** (A). Intracellular total cholesterol level (cholesteryl esters and free cholesterol) level (ng/ml/$10^5$) cells in OVCAR4 and (B) OVCAR5 wild type (WT) and cisplatin resistant (CDDP) variants cells. The average mean of cholesterol was measured by the Amplex™ Red cholesterol assay. Mean values of 3 biological replicates ± SD are shown (*, P<0.05; **P<0.01; ***. P<0.001). (C) Representative raw hyperspectral SRS images and SRS images of cholesterol level in OVCAR5 WT and CDDP OC cells. (D) Quantification of SRS cholesterol signal intensity from OVCAR5 WT and CDDP OC cells (n = 8-10). (E) Gene set enrichment (GSEA) plot depicting the enrichment of genes upregulated in *cholesterol homeostasis, biosynthesis, and regulation of cholesterol biosynthesis by SREBP* in the OVCAR5 CDDP cells compared to WT cells (FDR<0.05). (F) HMGCS1, SQLE and GAPDH protein level in FT-190, OVCRA5 WT and CDDP cells. (G) SRB1, and GAPDH protein level in FT-190, OVCRA5/4 WT and OVCRA5/4 CDDP cells (n=3). (H) SRB1, and GAPDH protein level in FT-190, NOEM, Pt-R HGSOC patient tumors derived cells.

**GPX4 blockade re-sensitizes OC cells to chemotherapy by targeting SRB1 mediated cholesterol import and intracellular accumulation.** We next investigated whether GPX4 blockade resensitized platinum resistant OC cells to chemotherapy. We first inhibited GPX4 expression by genetic knocking down (KD) GPX4 expression in OVACR5 and OVCAR4 Pt-R cells by stable transfection with shRNA. We achieved efficient GPX4 knock down in OVACR5 and OVCAR4 Pt-R cells (Fig. 2A and 2C). GPX4 knock down in Pt-R cells decreased $IC_{50}$ to CDDP by ~ 2-fold (Figure 2B and 2D, p<0.05).

Next, we treated cells with HDL nanoparticle (NP), which block SR-B1 mediated cholesterol import. Not surprising, combining HDL NP with platinum (carboplatin) not only decreased $IC_{50}$ of OVCAR5 Pt-R cells to platinum treatment (36.27nM vs. 58.51), but also showed synergistic effects on Pt-R OC cells survival (ED50, CI= 0.969). To further investigate if GPX4 blockade re-sensitization of OC cells to chemotherapy was associated with changes in cholesterol import or intracellular cholesterol accumulation, we measured total cholesterol levels in OVCAR5 cells in which GPX4 was KD and found significantly decreased cholesterol accumulation compared to control cells (Figure 2G, p<0.05). We confirmed these findings by using SRS imaging which also

detected lower intracellular cholesterol in OVCAR5 transduced with shGPX4 compared to cells transduced with control vector (Figure 2 H-I). We next performed RNA Sequencing to identify pathways disrupted by GPX4 knock down. We identified HDL-mediated lipid transport as one of the top enriched pathways associated with OVCAR5 Pt-R cells in which GPX4 was KD vs. control cells (Figure 2J, FDR<0.05). In addition, KD GPX4 in OVCAR5 WT and Pt-R cells (Figure 2K-L) inhibited SR-B1 expression, further supporting that cholesterol import is affected in Pt-R OC cells in which GPX4 was knocked down. Future mechanistic studies in Year 2 will try to precisely define how this phenomenon takes place.

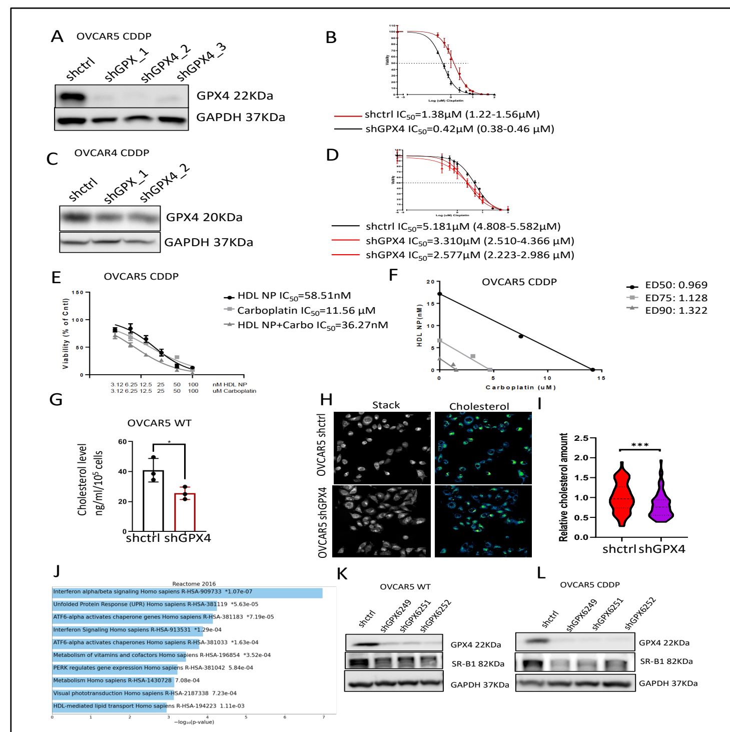

**Figure 2. GPX4 blockade resensitizes OC cells to chemotherapy by targeting SRB1 mediated cholesterol import and intracellular accumulation.** (A) GPX4 and GAPDH protein expression in OVCAR5 CDDP cells transfected with control (ctrl) or GPX4 shRNA. (B) Cell survival curves of OVCAR5 CDDP cells transfected with shctrl and shGPX4 in response to CDDP. $IC_{50}$ values are shown. (C) GPX4 and GAPDH protein expression in OVCAR4 CDDP cells transfected with control or GPX4 shRNA. (D) Cell survival curves of OVCAR5 CDDP cells transfected with shctrl and shGPX4 in response to CDDP. IC50 values are shown. (E) Cell survival curves of OVCAR5 CDDP cells treated with HDL NP, carboplatin alone or in combination. $IC_{50}$ values of each treatment are shown. (F) The Combination Index (CI) number of OVCAR5 CDDP cells treated with HDL NP combined with carboplatin. (G) Intracellular total cholesterol level (ng/ml/$10^5$) cells in OVCAR5 cells transfected with shctrl and shGPX4 vectors. (H) Representative raw hyperspectral SRS images and SRS images of cholesterol in OVCAR5 cells transfected shctrl and shGPX4 vectors. (I) Quantification of SRS cholesterol signal intensity from OVCAR5 shctrl and shGPX4 cells (n = 8-10). (J) Reactome analysis of top molecular pathways are enriched in the downregulated DEGs of OVCAR5_CDDP cells transfected shGPX4 vs. shctrl cells and discovered by Enrichr (FDR<0.05, n=3). (K) SR-B1, GPX4 and GAPDH protein level in OVCAR5 WT, (L) OVCAR5 CDDP OC cells transfected with shctrl and shGPX4 vectors. Mean values of 3 biological replicates ± SD are shown (*, P<0.05; **P<0.01; ***. P<0.001).

## SA2: Measure effects of HDL-NPs on OC cells in vitro and in vivo

We started treatment with HDL NP of parental and Pt-R OC cells. We observed that Pt-R cells were more sensitive to NP compared to parental cells (Figure 3A; IC50; WT: 4.48 Pt-R 1.93 nM). Interestingly treatment with HDL NP caused decrease in intracellular cholesterol in Pt-R OC cells (Figure 2B). Ferroptosis was measured by using C11 Bodipy staining and flow cytometry. Treatment with HDL NPs induced increased oxidized lipids (corresponding to ferroptosis) in both parental and Pt-R OC cells (Figure 3 C-D). Additionally, treatment with HDL NP induced decreased GPX4 in both parental and Pt-R OC cells (Figure 4 E-F). These data support the hypothesis that HDL NPs inhibit OC cell growth and promote cell death through ferroptosis which is linked to decrease uptake of cholesterol. The effects are more pronounced in Pt-R cells. Similar results were obtained in Pt-R OVCAR 4 cells (not shown).

Lastly, we injected Pt-R OVCAR 5 cells in female nude mice. Mice were treated with HDL NP daily 5 days per week. At end of the treatment, mice were sacrificed and tumors collected. The total number of tumors (Figure 4A) and the total tumor weight (Figure 4B) were significantly reduced by HDL-NP vs control treatment. Tumors were dissociated into single cells and oxidized lipids were measured by C11-BODIPY flow cytometry. Increased levels of oxidized lipids were

observed in tumors treated with HDL-NP compared to controls (Figure 4C), confirming the *in vitro* findings.

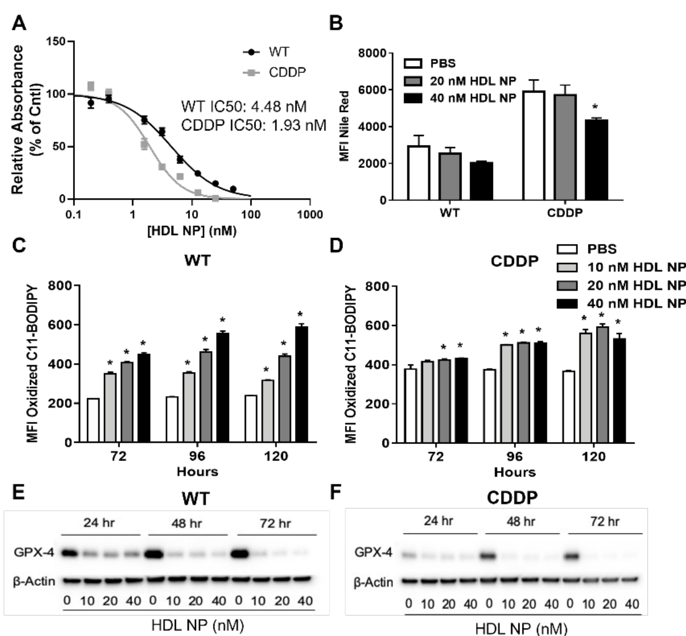


**Figure 3: OVCAR5 CDDP cells are more sensitive to ferroptotic cell death through cholesterol depletion by HDL NPs. A.** OVCAR5 WT and CDDP cells treated with HDL NP for 120 hours. (IC50; WT: 4.48 Pt-R (CDDP)1.93 nM,). **B.** OVCAR5 WT and CDDP cells treated with HDL NP for 24 hours and cholesterol content of cells measured by Nile Red flow cytometry. OVCAR5 WT (**C**) and CDDP (**D**) cells treated with HDL NP for 72, 96, or 120 hours and oxidized lipids measured by C11-BODIPY flow cytometry. OVCAR5 WT (**E**) and CDDP (**F**) treated with HDL NP for 24, 48, or 72 hours and GPX4 protein expression measured by western blot. Student t-test * $p < 0.05$.

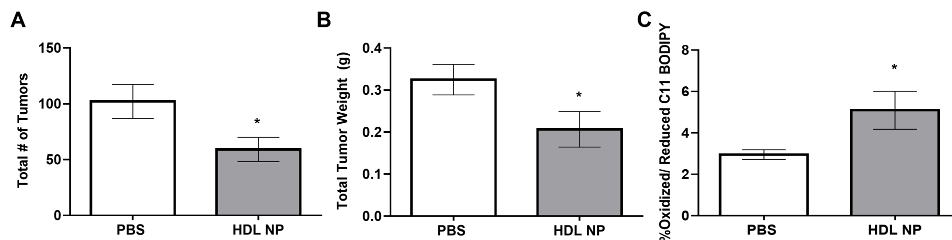


**Figure 4: HDL NPs inhibit tumor growth through ferroptosis.** OVCAR5 CDDP cells were injected IP into athymic nude mice and mice were treated 5 times a week (M-F) IP with 200 µL of 1 µM HDL NP. At end of the treatment, mice were sacrificed and tumors collected. The total number of tumors (**A**) and the total tumor weight (**B**) were recorded. **C.** Tumors were dissociated into single cells and oxidized lipids measured by C11-BODIPY flow cytometry. Student t-test * $p < 0.05$.

Studies using clear cell OC models will be pursued in Year 2.

A summary of tasks completed and ongoing experiments is included bellow.

| Specific Aim 1 | Timeline months | Site 1 |
|---|---|---|
| **Major Task 1: Test the hypothesis that GPX4 and cholesterol metabolism regulate platinum resistance in ovarian cancer cell lines and in xenografts** | 1-18 | |
| **Subtask 1:** Knock down and overexpress GPX4 in OC cells | 1-3 | completed |
| **Subtask 2:** Effects of GPX4 on cell survival and response to platinum | 4-6 | completed |
| **Subtask 3**: Effects of GPX4 on ferroptosis | 4-6 | completed |
| **Subtask 4:** Effects of GPX4 on cholesterol metabolism | 6-12 | completed |
| **Subtask 5:** Effects of GPX4 knockdown on response to platinum in vivo<br>A total of 144 nude mice will be used for these experiments<br>2 cell lines (OVCAR5-Resistant and ES2), 4 conditions—9 mice per condition (control shRNA, shRNA GPX4, carboplatin, PBS); 2 models (sq and ip). | 12-18 | Pending |
| **Subtask 5:** Effects of GPX4 knockdown on ferroptosis and cholesterol metabolism in vivo | 12-18 | Pending |
| **Subtask 5**: Data analysis | 15-18 | Pending |
| **Milestone(s) Achieved:**<br>1) Determine the role of GPX4 on response to platinum<br>2) Determine the link between GPX4 and cholesterol metabolism<br>3) Complete 1 manuscript<br>4) Present data at AACR and DOD meeting | 18 | Ongoing |
| **Specific Aim 2** | **Timeline** | **Site 1** |
| **Major Task 2: Measure effects of HDL-NPs on OC cells in vitro** | **1-12** | |
| **Subtask 6:** HDL NP synthesis | 1-3<br>18-21 | Completed |
| **Subtask 7:** HDL-NPs effects on OC cells—cell survival and ferroptosis assays | 3-6 | Completed |
| **Subtask 7:** HDL-NPs effects on OC cells—Cholesterol metabolism | 6-9 | ongoing |
| **Subtask 8**: Data analysis | 9-12 | ongoing |
| **Specific Aim 3** | | |
| **Major Task 3: Measure effects of HDL-NPs on OC cells in vivo** | **18-24** | |
| **Subtask 9:** Measure tumor growth in xenograft model derived from platinum resistant OC cells treated with HDL NPs<br>The total number of animals will be 108 nude mice.<br>3 cell lines (OVCAR5-Resistant and OVCAR5-Sesntive and ES2, 4 conditions—9 mice per condition (PBS control, human cholesterol-rich HDL, PEG NPs, and HDL NPs) | 18-21 | Part of this is completed |

| | | |
|---|---|---|
| **Subtask 10:** Measure tumor growth in platinum resistant PDX treated with HDL NPs<br>The total number of NSG mice will be 80 mice<br>2 PDX models-4 conditions—9 mice per condition (PBS control, human cholesterol-rich HDL, PEG NPs, and HDL NPs) plus host mice to expand the PDX prior to treatment (at least 4 per PDX model) | 18-21 | pending |
| **Subtask 11:** Measure ferroptosis and apoptosis in xenografts and PDX treated with HDL NPs vs. control | 21-24 | Part is completed |
| **Subtask 12:** Measure effects of HDL NPs on cholesterol stores in vivo | 24-30 | pending |
| **Subtask 13:** Effects of HDL-NP on normal tissue | 24-30 | pending |
| **Subtask 16:** Metabolomic analyses of xenografts | 30-33 | pending |
| **Subtask 17:** Data integration and analysis | 33-36 | pending |
| **Milestone(s) Achieved:**<br>6) Elucidate effects of HDL-NPs in ovarian tumors<br>7) Complete and submit second manuscript<br>8) Present data at AACR and DOD meeting | 24-36 | ongoing |

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

Ellie Siu, undergraduate student, summer 2022
Yinu Wang, postdoctoral fellow, 2021-2022
Andres Valdivia, postdoctoral fellow, 2021-2022

**How were the results disseminated to communities of interest?**

Results were not yet presented. We are working on a manuscript.

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

We are continuing in vivo testing of HDL-NP particles in platinum resistant xenografts, in clear cell xenografts, and in PDX models. We are also continuing analyses of tumors treated with HDL-NP—ferroptosis and cholesterol content. We are still elucidating the mechanism linking the oxidative state of the cell regulated by GPX4 to the dependence on cholesterol and fatty acids.

4. **IMPACT:**

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

Nothing to report

**What was the impact on other disciplines?**

Nothing to report

**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**

No significant changes in approach have occurred. One small modification will be replacing the ES2 clear cell line for in vivo study with another clear cell line TOV21G because the ES2 cell line grows too aggressively in mice and effects of treatment are challenging to observe (not sufficient time). We have also added cholesterol measurements by using SRS imaging, through an outside collaboration, to enhance the significance and novelty of the findings. No extra costs are incurred by the project.

**Actual or anticipated problems or delays and actions or plans to resolve them**

No issues to report, progress of the project is on track.

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

There is a slight lag in expenditures, as personnel recruitment was delayed.

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

**Significant changes in use or care of human subjects**

None

**Significant changes in use or care of vertebrate animals**

None

**Significant changes in use of biohazards and/or select agents**

None

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

**Journal publications.**

Nothing to report

**Books or other non-periodical, one-time publications.**

Nothing to report

**Other publications, conference papers and presentations**.

A manuscript is in preparation.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **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: Daniela Matei
Project Role: PD/PI
Nearest person month worked: 1
Contribution: oversees research activities, organizes monthly meeting with co-Is, meets individually with co-Is at least quarterly, meets with postdoctoral fellow weekly, reviews results, organizes plan for analyses

Name: Colby Thaxton
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution: responsible for completion of Aim 2 and 3, oversees onescientist, reviews results, organizes plan for analyses

Name: Horacio Cardenas
Project Role: Co-Investigator
Nearest person month worked: 2
Contribution: animal experiments and in vivo treatments

Name: Xiaolei Situ
Project Role: Research Technician
Nearest person month worked: 6
Contribution: cholesterol measurements in cells, effects of GPX4 on chemosensitivity, data analysis

Name: Andrea Calvert
Project Role: Co-Investigator
Nearest person month worked: 9
Contribution: in vitro treatment with HDL NP, assessment of ferroptosis, flow cytometry, data analysis

Name: Andres Valdivia
Project Role: Co-I
Nearest person month worked: 3
Contribution: Cholesterol analysis, co-cultures with adipocytes, chemosensitivity

**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 |
| --- |

**SPECIAL REPORTING REQUIREMENTS**

**COLLABORATIVE AWARDS:**

**QUAD CHARTS:**

**APPENDICES:**