Award Number: W81XWH-21-1-0476

TITLE: Differential ADAR1 Dependency in Breast Cancer Reveals Therapeutic Opportunities Through Regulation of MDM2 and Ferroptosis

PRINCIPAL INVESTIGATOR: Jason D. Weber, PhD

CONTRACTING ORGANIZATION: Washington University, St. Louis, MO

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 COVERED
July 2023	Annual	01Jul2022-30Jun2023

4. TITLE AND SUBTITLE

Differential ADAR1 Dependency in Breast Cancer Reveals Therapeutic Opportunities Through Regulation of MDM2 and Ferroptosis

5a. CONTRACT NUMBER
W81XWH-21-1-0476

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jason D. Weber, PhD

E-Mail: jweber@wustl.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Washington University
One Brookings Drive
Campus Box 1054
St. Louis, MO 63130-4862

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

We aim to examine hypotheses that the ADAR1-MDM2 signaling axis could **1)** mediate TNBC-associated ADAR1-dependency through ferroptotic cell-death; **2)** induce MDM2-addiction and sensitize breast cancer cells to MDM2-inhibition therapy, **depending on breast cancer subtypes**. To maximize the potential clinical utility and translational impact, we will incorporate high-throughput drug screening to identify drug candidates, from FDA-approved drug libraries, to provide proof-of-concept of ferroptosis-based therapeutic strategies against breast cancer. We hope to achieve both the **short-term-goal** of re-purposing existing drugs to demonstrate and amplify the clinical effect, and the **long-term-goal** of establishing a sustainable research program to highlight an innovative strategy against hard-to-treat breast cancers. In this second year, we have shown that ADAR1 protects TNBC from ferroptosis through regulating lipid remodeling and altering abundance of PUFA. We have discovered that ADAR1 loss sensitizes TNBC to ferroptosis and MDM2 is a potential contributor to ADAR1-regulated ferroptosis sensitizing.

15. SUBJECT TERMS
ADAR1, triple-negative breast cancer, MDM2, ferroptosis, drug screening, drug repurposing

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	10	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. INTRODUCTION

We recently demonstrated that the expression of an RNA-editing enzyme, ADAR1, is essential for TNBC tumorigenesis. This TNBC-centric phenomenon suggests distinct mechanisms separating the biology and vulnerability between TNBC and non-TNBC. To identify genetic factors involved in this pathway through RNA sequencing, we recognized MDM2 as the most prominently overexpressed gene upon ADAR1 loss, in both TNBC and non-TNBC cell lines. In this proposal, we provide rationales, supported by preliminary data, to investigate potential roles of ADAR1-repressed MDM2 expression in the survival of breast cancer cells. We aim to examine hypotheses that the ADAR1-MDM2 signaling axis could **1)** mediate TNBC-associated ADAR1-dependency through ferroptotic cell-death; **2)** induce MDM2-addiction and sensitize breast cancer cells to MDM2-inhibition therapy, **depending on breast cancer subtypes**. To maximize the potential clinical utility and translational impact, we will incorporate high-throughput drug screening to identify drug candidates, from FDA-approved drug libraries, to provide proof-of-concept of ferroptosis-based therapeutic strategies against breast cancer. We hope to achieve both the **short-term-goal** of re-purposing existing drugs to demonstrate and amplify the clinical effect, and the **long-term-goal** of establishing a sustainable research program to highlight an innovative strategy against hard-to-treat breast cancers.

2. KEYWORDS

ADAR1, triple-negative breast cancer, MDM2, ferroptosis, drug screening, drug repurposing

3. ACCOMPLISHMENTS

Major Goals of the Project

There was one major goal for the second year of the grant proposal: determine if TNBC-associated ADAR1-dependency can be attributed to MDM2-mediated ferroptosis.

Goals Accomplished

Specific Aim 1: To investigate if ADAR1 loss sensitizes TNBC cells to MDM2-mediated ferroptosis

***Major Task 1*.** To determine if TNBC-associated ADAR1-dependency can be attributed to MDM2-mediated ferroptosis (Months 1-18):

Subtask 4: To test if MDM2-mediated ferroptosis contributes to TNBC-associated ADAR1-depednency (Months 12-18).

***Major Task 2*.** Repurpose existing drugs that are capable of 1) inducing ferroptosis or 2) sensitizing TNBC cells to ferroptosis inducer RSL3 (Months 6-24):

Subtask 1: High-throughput drug screening to identify drug candidates activating ferroptotic pathway in TNBC (Months 6-12).

Subtask 2: High-throughput drug screening to identify drug candidates synergizing with ferroptosis inducer in TNBC (Months 12-18).

Subtask 3: In vitro assay to verify screen results; Counter-screen to validate efficacy and specificity of drugs identified in task 2.1 and 2.2 (Months 18-24)

Specific Aim 2: To investigate if ADAR1 loss sensitizes non-TNBC BC cells to MDM2 inhibition.

***Major Task 1*.** To determine if inhibitions of ADAR1 and MDM2 provide synthetic lethality in non-TNBC cells (Months 12-24):

Subtask 1: To determine if reduction of ADAR1 and MDM2 provide synthetic lethality in non-TNBC cells (Months 12-16).

Subtask 2: To determine if pharmacological inhibitions of ADAR1 and MDM2 provide synthetic lethality in non-TNBC cells (Months 16-20).

Subtask 3: To determine if ADAR1 inhibition sensitizes non-TNBC cells to inhibition of MDM2 in vivo (Months 20-24)

During the first year of this research project, we have demonstrated that **1)** ADAR1 protects TNBC from iron-dependent metabolic cell death, ferroptosis; **2)** ADAR1 loss sensitizes TNBC to ferroptosis and results in significant lipid remodeling; and **3)** MDM2 is a potential contributor to ADAR1-regulated ferroptosis sensitization. To further determine if MDM2 contributes to ADAR1-mediated protection from ferroptosis and identify the underlying mechanism, we first wanted to

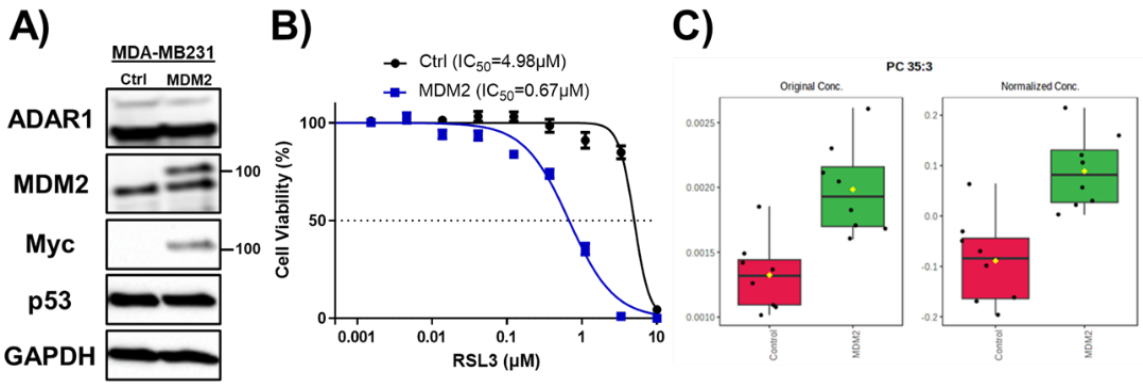

Figure 1. MDM2 overexpression sensitizes MDA-MB231 cells to RSL3 treatment. **A)** Overexpression of MDM2 in MDA-MB231 cells shown by western blot analysis. Myc antibody detects MDM2 due to the presence of a myc-tag. **B)** MDM2-overexpressing MDA-MB231 cells are more sensitive to RSL3 treatment. **C)** The level of PC (35:3) is induced in MDM2-overexpressing MDA-MB231 cells, measured by single-reaction monitoring-based liquid chromatography coupled to mass spectrometry (LC-MS).

determine if overexpression of MDM2 is sufficient to sensitize TNBC cells to ferroptosis and contributes to shADAR1-induced lipid remodeling. We found that MDA-MB231 cells overexpressing MDM2 to similar level compared to shADAR1-treated (ADAR1-Deficient) cells are more sensitive to treatment of ferroptosis inducer RSL3 (**Fig. 1A-B**). This result suggests that MDM2 induction upon ADAR1 loss contributes to elevated sensitivity to ferroptosis. Moreover, using single-reaction monitoring-based liquid chromatography coupled to mass spectrometry (LC-MS) to measure intracellular lipid contents, we obtained preliminary results that MDM2 overexpression increased the abundance of certain polyunsaturated fatty acid (PUFA) species, such as PC (35:3) shown in **Fig. 1C**.

To determine if we can rescue shADAR-mediated sensitization to ferroptosis by modulating MDM2 expression or function, we will first reduce the MDM2 induction in ADAR1-Deficient cells by treating ADAR1-Deficient MDA-MB231 cells with a MDM2 Proteolysis targeting chimeric (PROTAC) inhibitor, MD224 (**Fig. 2A**). We were able to demonstrate that MD224 effectively

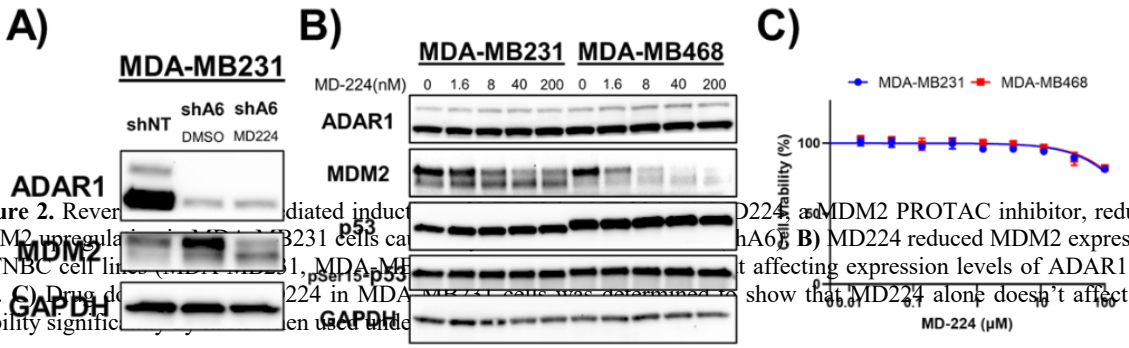

Figure 2. Rever diated induct D224, a MDM2 PROTAC inhibitor, reduced MDM2 upregula B231 cells ca hA6). **B)** MD224 reduced MDM2 expression in TNBC cell lin 1, MDA-MB t affecting expression levels of ADAR1 and p53. **C)** Drug d 224 in MDA MB231 cells was determined to show that MD224 alone doesn't affect cell viability signific en used alone

reduces MDM2 expression at nano-molar (nM) concentration range without affecting cell viability significantly (**Fig. 2B-C**). We will use MD224 to reverse the MDM2 induction in ADAR1-Deficient cells to determine if it is sufficient to reverse the sensitization phenotype.

In our Specific Aim 2, we proposed to determine if ADAR1 loss sensitizes non-TNBC BC cells to MDM2 inhibition. So far, we have shown that unlike TNBC, ADAR1 loss does not sensitize non-TNBC cells that we have tested to ferroptosis inducer RSL3 (**Fig. 3A**). It indicates that shADAR1-mediated sensitization to ferroptosis is a TNBC-specific phenomenon. Our focus, therefore, will continue to be on verifying and understanding mechanisms underlying the synthetic lethality phenotype we previously observed by combining knockdowns of ADAR1 and MDM2 (**Fig. 3B**). Our proposal planned to determine if pharmacological inhibitions of ADAR1 and MDM2 provide synthetic lethality in non-TNBC cells both in vitro and in vivo. Our recent findings, however, called into question whether the ADAR1-targeting drug we proposed to use (8-Azaadenosine) is specific enough to address this question adequately (Cottrell KA *et al. Cancer Research Communications* (2021)1(2): 56–64). Therefore, we will investigate this potential synthetic lethality in non-TNBC instead by combining recently discovered ADAR1 inhibitors, such as Fludarabine-Cl, and MDM2 inhibitors, such as MD224, SP-141 or Idasanutlin, to evaluate their collective effects on tumorigenesis of non-TNBC cell lines.

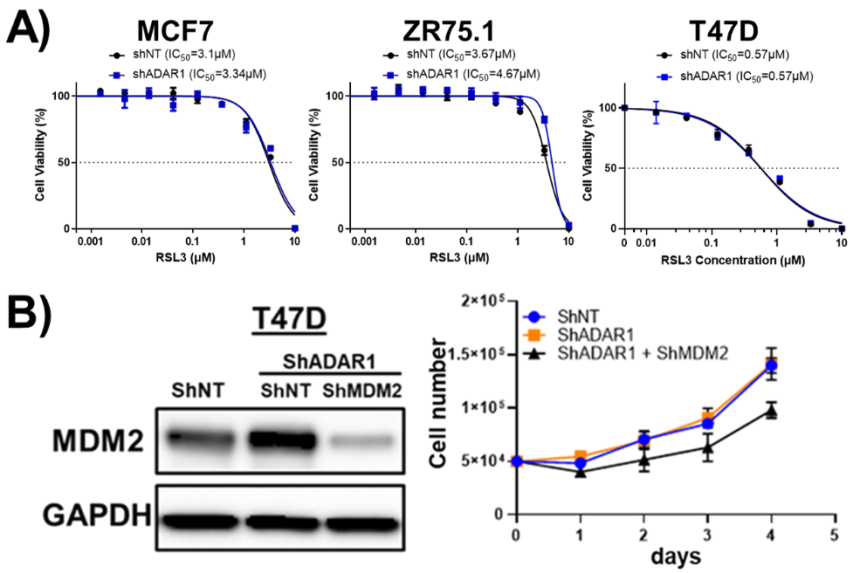

Figure 3. ADAR1 loss does not sensitize non-TNBC cells to ferroptosis. **A)** Treatment of shADAR1 in non-TNBC cells did not sensitize them to ferroptosis inducer RSL3. Black dots/trendline represent ADAR1-Intact (shNT) cells; Blue dots/trendline represent ADAR1-Deficient (shADAR1) cells. IC50, half maximal inhibitory concentration. **B)** Left: Treatment of shMDM2 in ADAR1-Deficient T47D cells reduced shADAR1-induced expression of MDM2. Right: Cell growth analysis revealed that ADAR1 loss alone did not affect T47D proliferation, while knockdown of both ADAR1 and MDM2 resulted in reduced proliferation of T47D cells.

In our Specific Aim 1, *Major Task 2,* we proposed to repurpose existing drugs that are capable of **1)** inducing ferroptosis or **2)** sensitizing TNBC cells to ferroptosis inducer RSL3. With our new understanding that ADAR1 loss sensitizes TNBC to ferroptosis, plus rapid discovery of novel/existing drugs possessing ferroptosis-inducing activity, we decided to pivot our approach to identify additional drug candidates that synergize with ADAR1 loss to induce ferroptosis in TNBC. To accomplish this, we subjected ADAR1-Intact and ADAR1-Deficient MDA-MB231 cells to a high-throughput screening analysis with a ferroptosis-focus library (MedChemExpress) consisting of more than 600 novel and clinically available compounds that have been suggested, in literature, capable of regulating ferroptosis sensitivity in cancer cells. CellTiterGlo cell viability assay was conducted to identify drug candidates whose efficacy is dictated by the presence or absence of ADAR1 in MDA-MB231 cells (**Fig. 4A**). Out of more than 600 compounds, including novel, preclinical and clinically available drugs, 8 were identified to

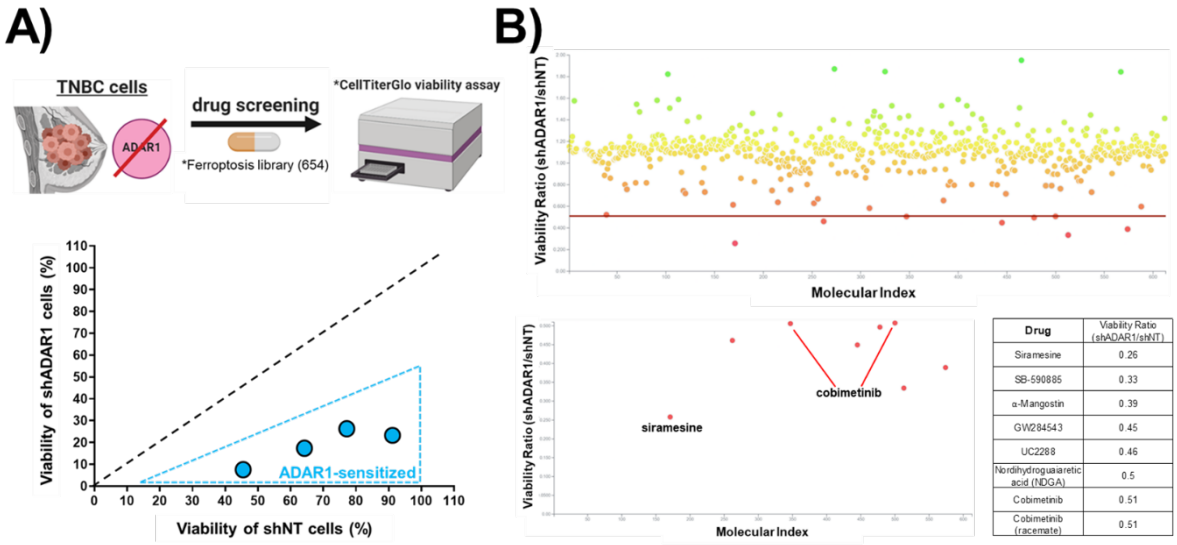

Figure 4. Identification of drug candidates synergizing with ADAR1 loss to suppress TNBC tumorigenesis. **A)** Top: Design of a high-throughput screen by subjecting ADAR1-Intact and ADAR1-Deficient TNBC cells to a ferroptosis-focused library. Bottom: Illustration of plotting viability of ADAR1-Deficient (shADAR1) and -Intact (shNT) cells to identify drugs sensitized by ADAR1 loss. **B)** Top: Comparing viability ratio between ADAR1-Deficient and -Intact cells (shADAR1/shNT) to identify drugs sensitized by ADAR1 loss. Bottom: Top 8 hits from the screen with the viability ratio listed on the right.

target ADAR1-Deficient MDA-MB231 cells with > 2X sensitivity compared to ADAR1-Intact cells (**Fig. 4B**). Hits identified from this screen include siramesine, a sigma-2 receptor agonist previously shown to regulate iron metabolism, and nordihydroguaiaretic acid (NDGA), a inhibitor of 5-lipoxygenase (5LOX) that metabolize PUFA into other biologically active products. Interestingly, 2 out of the 8 hits point to cobimetinib, an inhibitor of MAPK signaling pathway that has been used to treat aggressive melanoma in the clinic (brand name Cotellic).

In vitro drug response curves will be determined to confirm the screening results. We also plan to test the effects of these hits combining with ADAR1 reduction *in vivo* to evaluate the feasibility of using these combinatorial strategies for treatment of TNBC. As cobimetinib has been included in multiple clinical trials for to treat breast cancer, including TNBC (NCT02322814), either in a monotherapy setting or in combination with other drugs, our study could uncover yet another novel strategy to suppress TNBC tumorigenesis.

KEY RESEARCH ACCOMPLISHMENTS

- ADAR1 protects TNBC from iron-dependent metabolic cell death, ferroptosis, through regulating lipid remodeling.
- MDM2 is a potential contributor to ADAR1-regulated ferroptosis, and MDM2 overexpression mimics shADAR1-mediated sensitization to ferroptosis.
- From a high-throughput screen using a ferroptosis-focused library, multiple drug candidates were identified to synergize with ADAR1 inhibition to suppress TNBC.

MAJOR/SUB TASKS

- **Completed**: Specific Aim 1 – Major Task 2 - Subtask 1 and 2
- **Partially completed**: Specific Aim 1 – Major Task 1 – Subtask 4; Specific Aim 1 – Major Task 2 – Subtask 3; Specific Aim 2 – Major Task 1 – Subtask 1-3.

REPORTABLE OUTCOMES

None

CONCLUSIONS

We demonstrated that ADAR1 protects TNBC from ferroptosis through regulating lipid remodeling and altering abundance of PUFA. A high-throughput screening using a ferroptosis-focused library identified 8 drug candidates, including an anti-cancer medication cobimetinib (Cotellic), synergizing with ADAR1 loss to suppress tumorigenesis of MDA-MB231 TNBC cells. In vitro and in vivo experiments will be performed to further evaluate these results to assess the feasibility of these strategies in treatment of TNBC. Ongoing investigation is aiming to study the roles of MDM2, as well as other targets, and related mechanisms underlying ADAR1-regulated ferroptosis sensitivity in TNBC.

Training Opportunities

Nothing to Report

Results Disseminated to the Community

I participated this past year in disseminating our initial findings to three independent groups of large donors to the American Cancer Society. These donors visited my laboratory at Washington University where I discussed the research in this grant proposal and how our results were moving the field of breast cancer research forward. We engaged in a question-and-answer session where the donors queried me on the clinical impact of this work. I anticipate doing this laboratory tour again next year and have already been asked by the American Cancer Society to do so. I also was the keynote speaker at the American Cancer Society CEOs Against Cancer event.

Plans for Next Reporting Period
In the third year, we will focus on finishing the experiments in aims 1 and 2.

4. IMPACT
Impact on Principal Discipline
Our current work will be incredibly impactful for those studying breast cancer aggression in vitro and in vivo. We have uncovered a novel pathway underlying the ability of breast cancer epithelial cells to proliferate at a high rate and readily form transformed colonies in soft agar. These are all hallmarks of aggressive tumors. In year 3, we will move these findings into a more relevant in vivo model system, hoping to underscore the importance of ADAR1 and ferroptosis in breast tumor aggressiveness and metastasis.

Impact on Other Disciplines
Nothing to Report

Impact on Technology Transfer
Nothing to Report

Impact on Society
We have disseminated the data and ideals from this grant proposal to several groups in the St. Louis community including the American Cancer Society and Bridget's Brigade for breast cancer. They were encouraged by our progress and excited about the future clinical impact our work might provide.

5. CHANGES/PROBLEMS
Changes in Approach
Nothing to Report

Anticipated Problems or Delays
Nothing to Report

Changes in Human, Animal Biohazards and/or Selective Agents
Nothing to Report

6. PRODUCTS
Publications, Conference Papers and Presentations
Nothing to Report

Internet Sites
Nothing to Report

Technologies or Techniques
Nothing to Report

Inventions, Patents and/or Licenses
Nothing to Report

7. PARTICIPANTS
Individuals That Have Worked on Project

Name:	Jason D. Weber
Project Role:	PI
Nearest person month worked:	0.6
Contribution to Project:	Dr. Weber served as the mentor for Dr. Kung in planning all experiments and overseeing the final data analysis.
Funding Support:	NIH R01CA190986, NIHR01CA174743, W81XWH-15-1-0528

Name:	Maxene Ilagen
Project Role:	Co-Investigator
Nearest person month worked:	0.6
Contribution to Project:	Ms. Ilagen will provide expertise and consultation in assay development for drug screenings and access to drug libraries.

Name:	Che-Pei Kung
Project Role:	Co-Investigator and Staff Scientist
Nearest person month worked:	12
Contribution to Project:	Dr. Kung will perform all of the experiments described in the proposal.
Funding Support:	None

Name:	Miriam Sindelar
Project Role:	Staff Scientist
Nearest person month worked:	0.6
Contribution to Project:	Ms. Sindelar will perform all of the free fatty acid measurementds and quantification using LC/MS.
Funding Support:	None

Changes in Active Other Support for PD/PI
Nothing to Report

Other Organizations Involved as Partners
Nothing to Report