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14. ABSTRACT

Several reports from human studies and animal models of breast carcinogenesis have established that puberty is a critical window for the increased susceptibility to mammary cancer. We reasoned that field carcinogenesis or cancerization effect (FCE) could play a critical role in increasing the risk of mammary cancer at puberty. Based on our preliminary studies, we proposed cancer like mitochondrial bioenergetic tissue fields as a mechanism of mammary carcinogenesis. To establish this phenomenon, we treated the animals with NAD+ precursor, which could reverse cancer-like mitochondrial bioenergetics. If our hypothesis is true, then treatment with NAD+ precursor should prevent mammary cancer. Our results show that pre-pubertal treatment of NAD+ significantly inhibited mammary cancer development in classic breast carcinogenesis model. This suggests that cancer-like mitochondrial bioenergetics play a key role in mammary carcinogenesis.

15. SUBJECT TERMS
None listed.

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TABLE OF CONTENTS

1. INTRODUCTION:

At present it is not clear what makes the breast susceptible to cancer development. However, different breast developmental stages that are vulnerable to the onset of cancer have been already established. The most vulnerable stages are fetal, pubertal, postpartum involution (retrograde change that result in normal size after delivery), and perimenopause (a stage before menopause). This high risk/high gain proposal is aimed at identifying the mechanism for increased susceptibility to breast cancer risk at pubertal stage of breast development.

Puberty is the most important stage that have been studied in detail to establish the relationship between timing of exposure to cancer causing agents (carcinogens) and risk of breast cancer development. For example, as in the case of atomic bomb explosions in Hiroshima and Nagasaki and Chernobyl tragedy, women exposed to radiation as teenagers (pubertal) were more susceptible to breast cancer later in life than those who are exposed as adults. Similarly, pubertal exposures to DDT, alcohol and cigarette also leads to greater breast cancer risk. In support of these observations in humans, animal models of breast cancer also established that the risk is set by the timing of exposure to carcinogens, especially at puberty. However, the mechanism for such an increased susceptibility is not well understood.

2. KEYWORDS:

Mitochondria, Bioenergetics, Mitochondrial oncobioenergetics, breast cancer, mammary carcinogenesis, cancer prevention.

3. ACCOMPLISHMENTS:

i. What were the major goals of the project?

Aim-1: Define the mitochondrial bioenergetic functional status of mammary tissues at different stages of development. We will investigate the MOBs in MG tissues and stabilization of HIF-1α and its target genes in mammary epithelium of rodents at different stages of development.

Aim-2. Determine the role of mitochondrial oncobioenergetics on mammary carcinogenesis. Wild-type, conditional HIF-1α knockout, and HIF-1α overexpressing female mice will be exposed to 7,12-dimethylbenz(a) anthracene (DMBA). We will determine the extent to which it can induce tumors in these animals. Further, we will therapeutically elevate NAD+ levels in the MG to enhance oxidative metabolism and will test whether it could prevent mammary carcinogenesis in rat model of BC.

ii. What was accomplished under these goals?

a. Major Task 1: Oncobioenergetics in the mammary gland of rats and mice

Mitochondrial oncobioenergetics

Methods: Mammary glands of Sprague-Dawley rats (3/group) was analyzed at 3 weeks(pre-puberty), ~26 days (day of vaginal opening), 7 weeks (mid-puberty), and 10 weeks (completion of puberty). Mitochondrial oncobioenergetics was measured in mammary gland precision-cut tissue slices using Seahorse XF analyzer technology (**Figure-1**).

We used the state-of-the-art Seahorse Extracellular Flux (XF) 24 Analyzer (Seahorse Bioscience, Inc, North Billerica, MA, USA), to measure the oxygen consumption rate (OCR), an indicator of mitochondrial respiration, and the extracellular acidification rate (ECAR), an indicator of glycolysis, in real-time in mammary gland tissue slices. Several measures of mitochondrial respiration, including

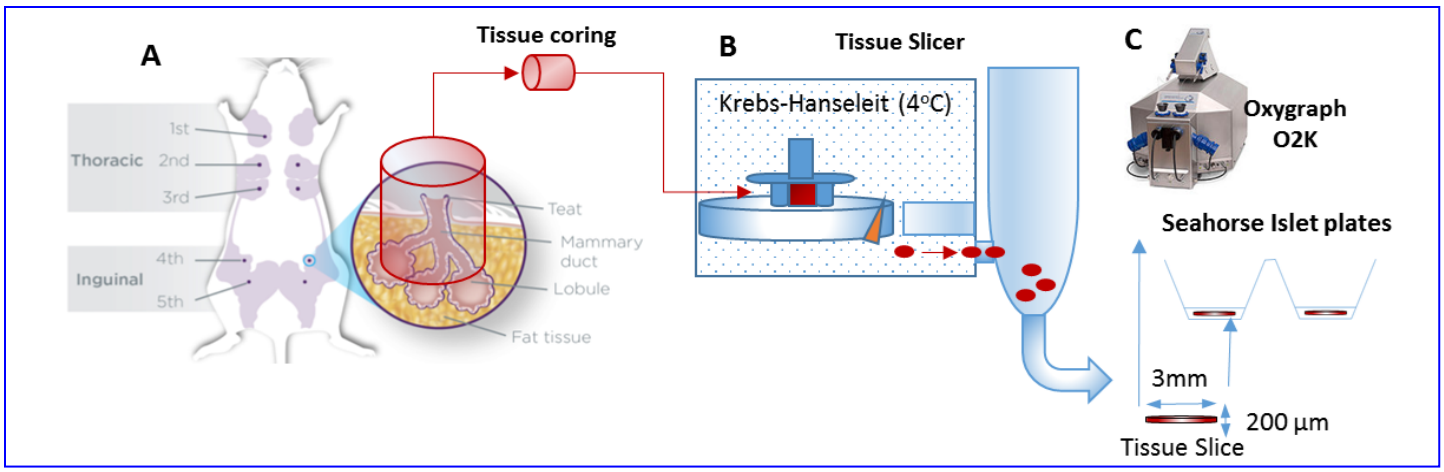

Figure 1: Tissue slicing workflow. (A) animal dissection and tissue coring; (B) precision cut tissue slicing.

basal respiration, ATP-linked respiration, proton leak respiration and reserve capacity, were derived by the sequential addition of pharmacological agents to the respiring cells, as diagramed in **Figure 2**. For each parameter, 3-10 repeated rates of oxygen consumption are made over several periods of time. First, baseline cellular oxygen consumption is measured, from which basal respiration is derived by subtracting non-mitochondrial respiration. Next oligomycin, an inhibitor of complex V, is added, and the resulting OCR is used to derive ATP-linked respiration (by subtracting the oligomycin rate from baseline cellular OCR) and proton leak respiration (by subtracting non-mitochondrial respiration from the oligomycin rate). Next carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP), a protonophore, is added to collapse the inner membrane gradient, driving the electron transport chain (ETC) to function to its maximal rate, and maximal respiratory capacity is derived by subtracting non-mitochondrial respiration from the FCCP OCR. Lastly, antimycin A, a complex III inhibitor, and rotenone, a complex I inhibitor, are added to shut down ETC function, revealing the non- mitochondrial respiration. The mitochondrial reserve capacity is calculated by subtracting basal respiration from maximal respiratory

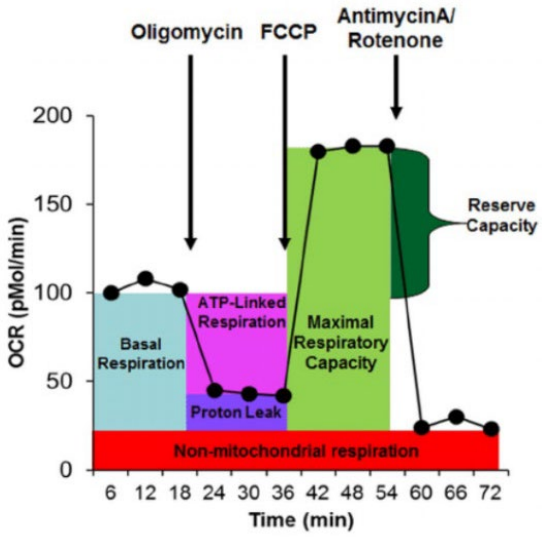

Figure 2: The Seahorse assay. Oxygen consumption rate (OCR) is measured before and after the addition of inhibitors to derive several parameters of mitochondrial respiration. Initially, baseline cellular OCR is measured, from which basal respiration can be derived by subtracting non-mitochondrial respiration. Next oligomycin, a complex V inhibitor, is added and the resulting OCR is used to derive ATP-linked respiration (by subtracting the oligomycin rate from baseline cellular OCR) and proton leak respiration (by subtracting non-mitochondrial respiration from the oligomycin rate). Next carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP), a protonophore, is added to collapse the inner membrane gradient, allowing the ETC to function at its maximal rate, and maximal respiratory capacity is derived by subtracting non-mitochondrial respiration from the FCCP rate. Lastly, antimycin A and rotenone, inhibitors of complex III and I, are added to shut down ETC function, revealing the non-mitochondrial respiration. Mitochondrial reserve capacity is calculated by subtracting basal respiration from maximal respiratory capacity.

capacity.

ECAR (**Figure 3**) is primarily a measure of lactate production and can be equated to the glycolytic rate (i.e., glycolysis), and ECAR is measured simultaneously with OCR in the Seahorse assay. Basal ECAR refers to the ECAR measured before the injection of oligomycin. Glycolytic reserve capacity is calculated by subtracting the basal ECAR from the oligomycin-induced ECAR.

Statistics: The differences will be analyzed for significant changes between groups using ANOVA or students t-test. Differences in tumor multiplicity will be performed using non-parametric methods, the Mann-Whitney U test.

Specific objectives: Our hypothesis is that the increased vulnerability of pubertal mammary gland to carcinogenesis is due to mitochondrial oncobioenergetic (MOB; cancer-like bioenergetic) tissue field. Therefore, we proposed that there will be a shift in the bioenergetic profile of mammary tissue at the time of puberty towards glycolysis that would favor carcinogenesis process.

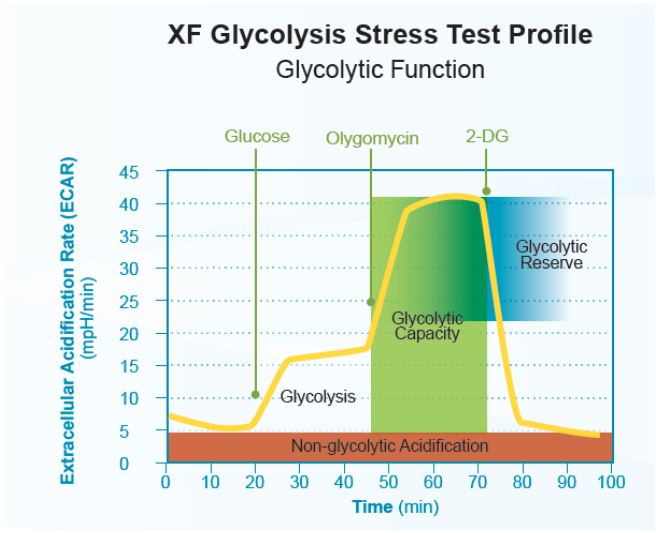

Figure 3: The glycolysis stress test is based on extracellular acidification rate (ECAR) and measures three key parameters of glycolytic function including glycolysis, glycolytic capacity and glycolytic reserve. Arrows mark the addition of Glucose (initiates glycolytic respiration), oligomycin (inhibitor of mitochondrial respiration and enhances glycolysis), antimycin A, and 2 deoxyglucose (inhibits glycolysis).

Significant results or key outcomes: Firstly, we determined the mitochondrial bioenergetics of the tissue slices. After anesthetizing the animal, abdominal skin was cut open and cylindrical mammary tissue cores around the teats of the 4th pair of abdominal mammary gland was prepared using coring press (**Figure 1**). Slices were obtained from same tissue depths below the teats in each group. The bioenergetic data obtained from tissue slices were normalized to mammary gland protein determined by commercially available protein estimation kits.

Mitochondrial oncobioenergetics of mammary gland slices from rats: To assess comprehensively the mitochondrial oncobioenergetic parameters of mammary tissues, oxygen consumption rate (OCR) was measured using high throughput respirometry. We performed a "mitochondrial stress test" (MiST) using modulators of mitochondrial function such as oligomycin, FCCP and antimycin A, which allows interrogation of certain components of mitochondrial oncobioenergetic function. The standard assay medium contained 5.0 mM glucose and 4.0mM glutamine as energy substrates.

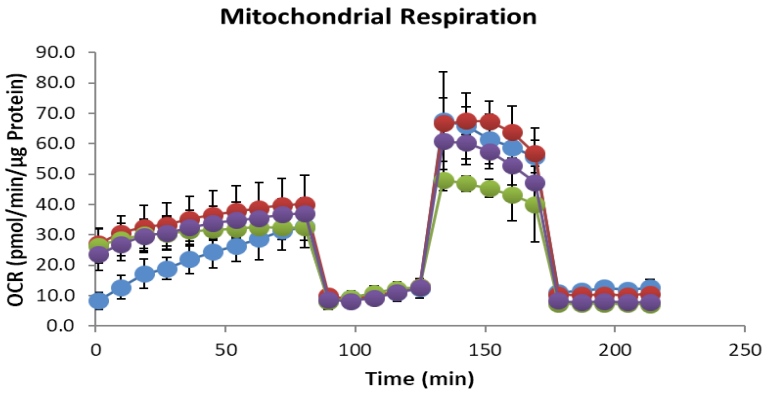

Figure 4 Mitochondrial oncobioenergetics of mammary tissues.

Control; Day 21; Day 50; 10 weeks

Under standard assay media

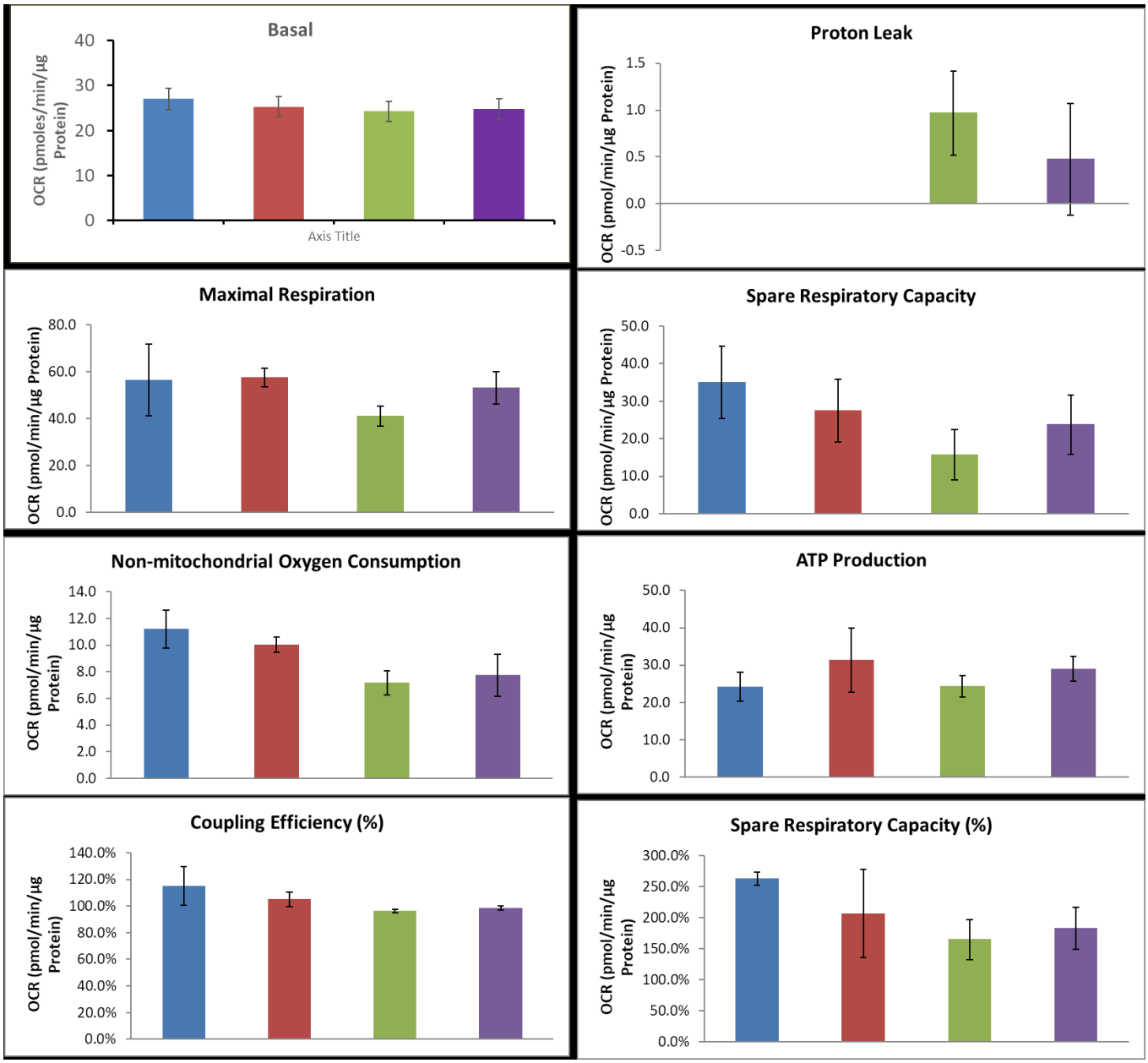

Figure 5: After three baseline OCR measurements in an assay medium (DMEM containing 10 mM glucose, 4 mM glutamine at pH 7.4 without bicarbonate), Oligomycin (2.0 µM), FCCP (1.0 µM), and antimycin A (10 µM) were injected sequentially with OCR and ECAR measurements recorded after each injection. The following mitochondrial respiratory functional characteristics were elucidated from the OCR trace: basal OCR (OCR in the absence of any mitochondrial inhibitors), ATP-dependent OCR (OCR necessary to synthesize ATP), reserve capacity (difference between the maximal and basal OCR), which is an estimate of the potential bioenergetic reserve the cell can call upon in times of stress, non-mitochondrial (OCR after the addition of Antimycin A) and proton leak. Basal=OCR without inhibitors-Antimycin inhibited OCR; Maximal=OCR due to FCCP-Antimycin inhibited OCR; Reserve Capacity=Maximal-Basal; Proton Leak=Oligomycin Inhibited OCR-Antimycin inhibited OCR; Non-mitochondrial = Antimycin Inhibited OCR; ●● Control; ●●Day 21; ●●Day 50; ●●10 weeks

conditions, OCR trace normalized to protein concentration has clearly demonstrated no difference in OCR in different age groups when interrogated with modulators of mitochondrial function as shown in **Figure 4** except spare respiratory capacity or reserve capacity (for details about reserve capacity see Figure 2 caption). Most notable difference among the various mitochondrial oncobioenergetic parameters in these tissue slices standard assay media conditions is the spare reserve capacity

(**Figure-5**). Spare reserve capacity is the difference between maximal and basal respiration, which is an estimate of the potential bioenergetic reserve the cell can call upon at times of stress. When cells are subjected to stress, this mitochondrial reserve capacity is available to serve the increased energy demands

Glycolysis:

Warburg effect is a phenomenon in tissues or cells characterized by their glycolytic phenotype even in the presence of ample amount of oxygen (O_2). Comprehensive analysis of glycolytic parameters using rat mammary tissues are still elusive. To analyze the glycolytic profile in detail, tissue slices were subjected to glycolytic stress test (GlyST). By GlyST, four major glycolytic parameters could be analyzed from the ECAR trace as shown in the **Figure 6**. When normalized to baseline (ECAR in the absence of glucose and represented as percentage), there was no significant change in any of the parameters of glycolysis (**Figure 7**).

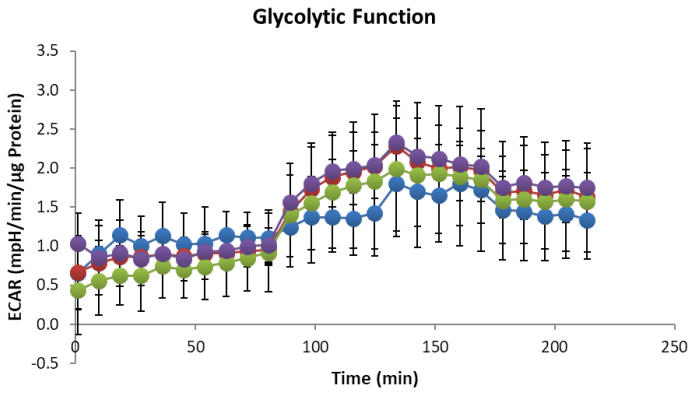

Figure 6: Glycolytic stress test; Three initial measurements were done in glucose free assay media (glutamine alone), which is followed by injection of glucose (10 mM), oligomycin (1.0 µM) and 2DG (100 mM) were added successively and ECAR was measured. Glycolytic parameters such as glycolytic rate (ECAR in the presence of glucose), glycolytic capacity (maximum attainable glycolysis), and glycolytic reserve (difference between glycolytic capacity and glycolytic reserve) were determined from the ECAR trace. ●● Control; ●●Day 21; ●●Day 50; ●●10 weeks

b. Major Task 2: Determine the expression of HIF-1α target and TCA cycle genes, activity of selected TCA cycle enzymes and its abundance.

Methods: The animals were left untreated or treated with nicotinamide riboside (NR; 400 mg compound/kg animal weight per day) starting from day 21 to day 49. The animals were sacrificed on

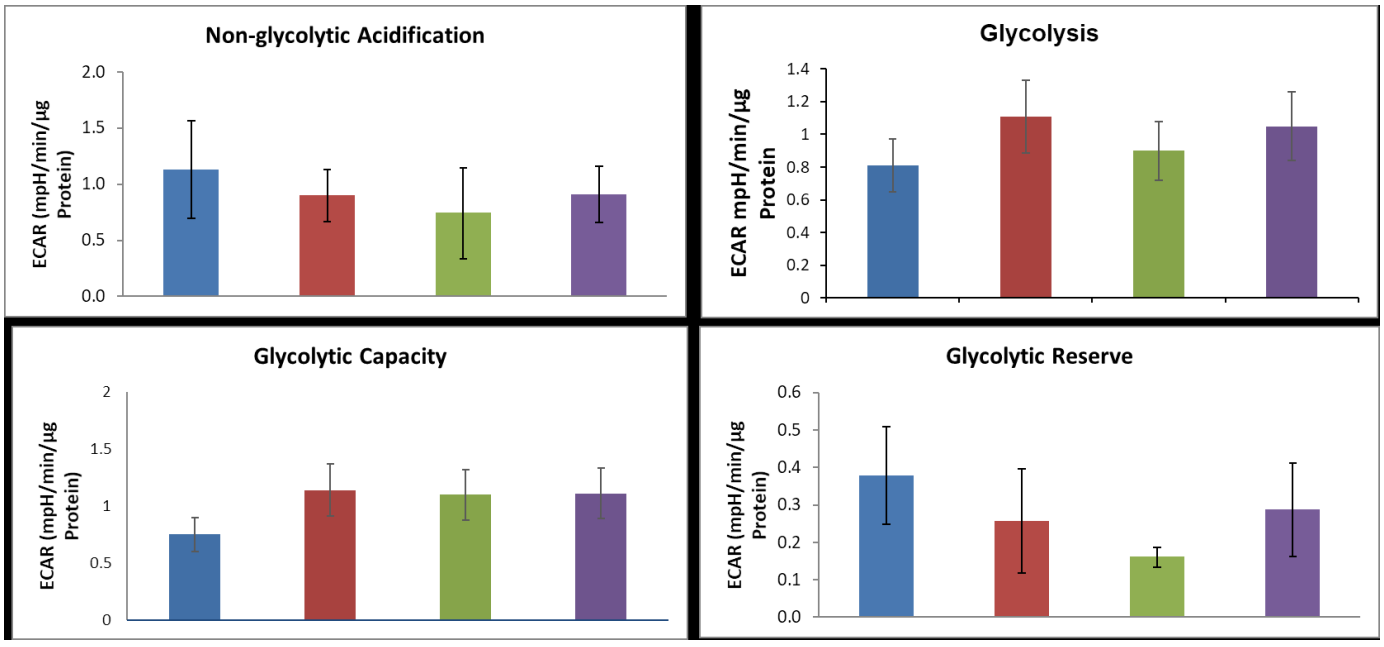

Figure 7: Glycolytic parameters. ●● Control; ●●Day 21; ●●Day 50; ●●10 weeks.

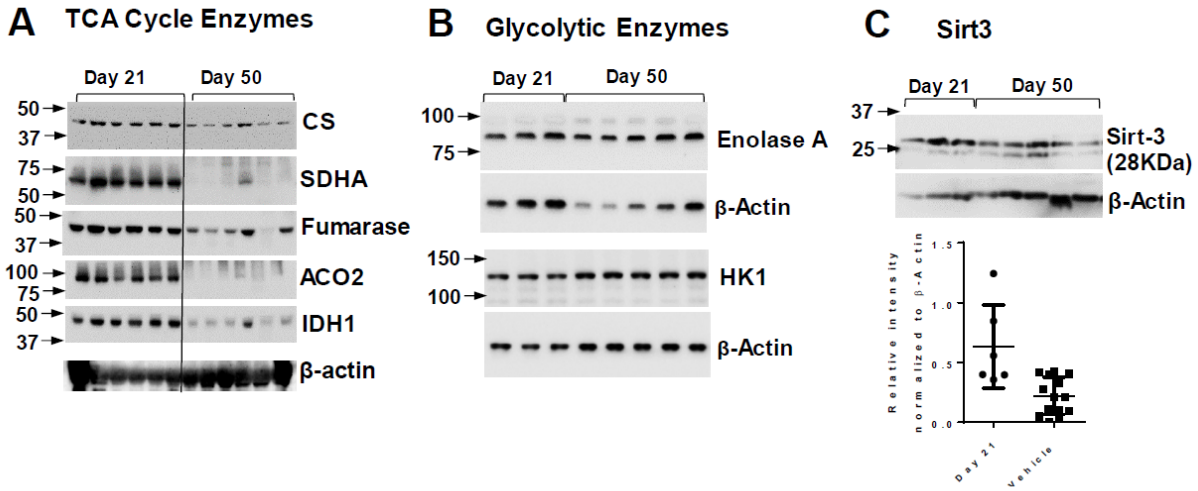

Figure-8: Enzyme levels in the mammary gland of rats at puberty compared to pre-puberty. (A) Levels of TCA cycle enzymes in rat pubertal and pre-pubertal mammary gland. CS - Citrate synthase; SDHA – Succinate dehydrogenase A; ACO2 - Aconitase 2; IDH1 – Isocitrate dehudrogenase. Levels of glycolytic enzymes in rat pubertal and pre-pubertal mammary gland HK1 – Hexokinase 1. (C) Sirt3 levels in the pubertal mammary glands of rats

day 21 and day 50, and mammary glands were collected. It represents tissue from pre-pubertal, and pubertal animals treated with and without NR. Each group had 6 animals each.

***Significant results or key outcomes*:** Firstly (**Figure 8**), we determined the levels of TCA cycle and glycolytic enzymes in the mammary glands of rats at pre-puberty and puberty. TCA cycle enzymes was found to be downregulated at puberty (on day 50). All the tested enzymes such as Citrate synthase, Succinate dehydrogenase A, Fumarase, Aconitase 2, isocitrate dehydrogenase etc. are found to be downregulated at puberty compared to pre-pubertal period. However, there was no significant reduction glycolytic enzymes such as enolase, hexokinase etc. More importantly, there was a significant down-regulation of Sirt3 enzyme levels at puberty. This suggests that at puberty there was a significant reduction in TCA cycle enzymes without any change in glycolytic enzymes, which is probably mediated

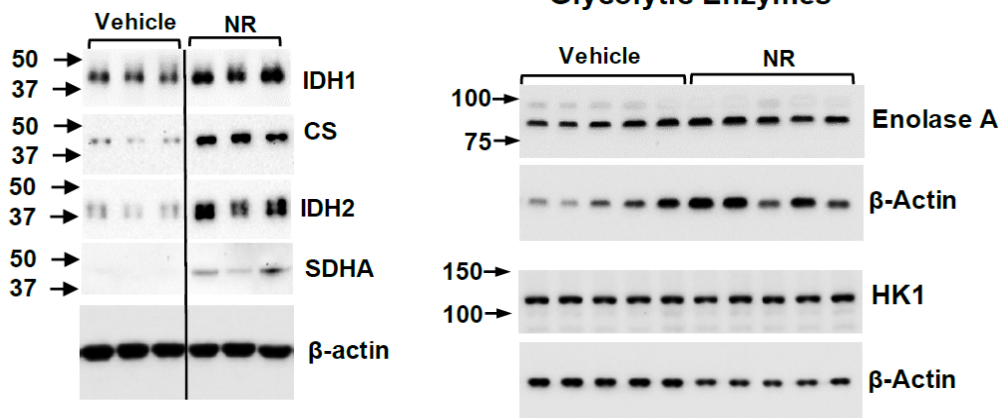

Figure-9: Enzyme levels in the mammary gland of rats at puberty treated with and without nicotinamide riboside

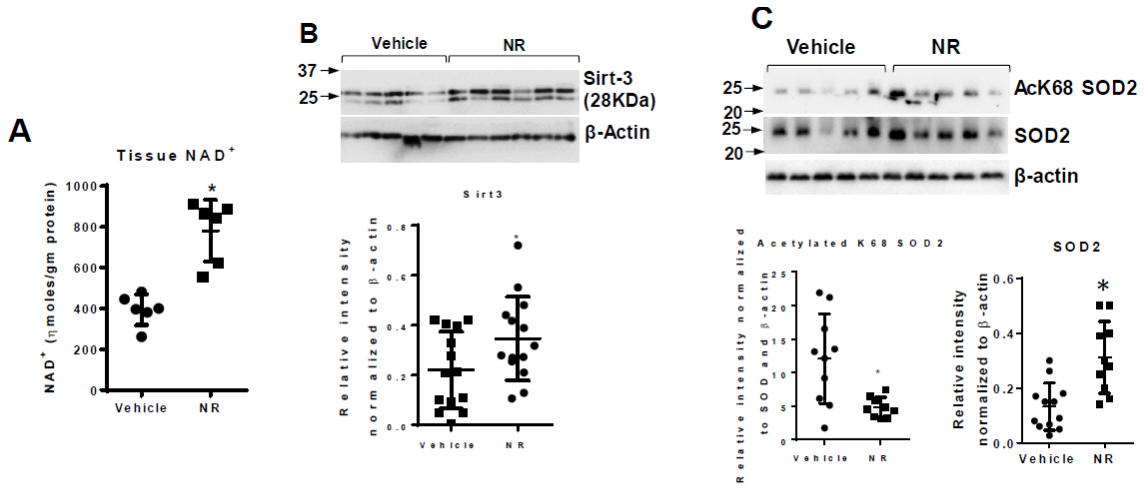

Figure-10: Tissue NAD+ upregulates the activity of Sirt3 in the rat mammary gland. (A) Effect of treatment of NR on the mammary tissue NAD+ levels; (B) Effect of NR on Sirt3 levels in the mammary tissue (C) Effect of NR treatment on SOD acetylation in the rat mammary gland at puberty.

by the downregulation of Sirt3. Sirt3 plays a significant role in regulating TCA cycle enzymes levels.

Secondly (**Figure-9**), mice treated with synthetically derived nicotinamide riboside (NR; at a dose of 400 mg compound/kg animal weight per day), showed an increase of NAD+ levels in tissues. Treatment with NR from day 21 to day 49 elevated Citrate Synthase (CS), DLST, isocitrate dehydrogenase 1 & 2, succinate dehydrogenase compared to day 21 or pre-puberty. However, there was no significant change in glycolytic enzymes.

More interestingly, there was a down regulation of Sirt3 in the rat mammary gland that regulates the expression of the mammary gland TCA cycle enzymes (**Figure-10**). Consistent with increases in tissue NAD+ levels, SIRT3 appeared to be upregulated, as measured by SOD2 acetylation levels. In these studies, nicotinamide riboside was also shown to have a greater ability to increase NAD+ level. Recent data indicate that mitochondrial NAD+ enables cells to resist genotoxic stress. Increased SIRT3 activity stimulates reactive oxygen species and detoxifying enzymes such as superoxide dismutase 2. These data suggest that nicotinamide

Table-1: Mouse Genotypes, Background Strains, Crosses, and the Resulting Offspring.

Breeding Pairs		Offspring
Genotype 1	Genotype 2	
F1 Generation		
WT (C57Bl/6)	WT (C57Bl/6)	WT (C57Bl/6)
MMTV-*Cre* (C57Bl/6)	WT (C57Bl/6)	MMTV-*Cre*
Rosa26R (C57Bl/6)	MMTV-*Cre* (C57Bl/6)	Rosa26R-MMTV-*Cre* Rosa26R
HIF-1α$^{+/+}$ (C57Bl/6); (Jackson Labs)	MMTV-*Cre* (C57Bl/6)	HIF-1α$^{+/wt}$- MMTV-*Cre* HIF-1α$^{+/wt}$,
HIF-1α$^{fl/fl}$ (C57Bl/6); (Jackson Labs	MMTV-*Cre* (C57Bl/6)	HIF-1α$^{fl/wt}$-MMTV-*Cre* HIF-1α$^{fl/wt}$
F2 Generation overexpressing (+/+) and knockouts (fl/fl)		
HIF-1α$^{+/wt}$-MMTV-*Cre*	HIF-1α$^{+/+}$	HIF-1α$^{+/wt}$ -MMTV-*Cre* HIF-1α$^{+/wt}$ HIF-1α$^{+/+}$ -MMTV-*Cre* HIF-1α$^{+/+}$
HIF-1α$^{fl/fl}$-MMTV-*Cre*	HIF-1α$^{fl/fl}$	HIF-1α$^{fl/wt}$ -MMTV-*Cre* HIF-1α$^{fl/wt}$ HIF-1α$^{fl/fl}$ -MMTV-*Cre* HIF-1α$^{fl/fl}$

Highlighted in blue are controls and in red are mammary gland specific overexpressed and KO. Background strains in parenthesis.

riboside stimulation of mitochondrial NAD+ could provide several potential benefits in disease states in which cell death and reactive oxygen detoxification are abnormal.

Conclusion: Our studies demonstrated that at day 50 there is a drastic decrease in the levels of Krebs cycle enzymes in the mammary gland of rats compared to mammary gland at day 21. Simultaneously, there was no change in glycolytic enzyme levels between these groups. More interestingly, there was a significant decrease in the levels of Sirt3, which is in concordance with the reduced expression of Krebs cycle enzymes in the mammary gland. On the other hand, administration of NAD significantly upregulated NAD levels in the mammary gland. This resulted in the increased expression of Sirt3 which is reflected in the levels of SOD2 in the mammary gland

c. Major Task 3: Breeding genetically modified mouse.

Generation of conditional knockout mice*:*

In this specific aim, we have investigated whether cancer-like mitochondrial bioenergetics is permissive to the onset of mammary cancer. HIF-1α directly regulates mitochondrial function and glycolysis. We could alter the oncobioenergetics by conditionally silencing or overexpressing HIF-1α in the mammary gland and could be compared with wild type. Therefore, we performed classical DMBA-induced mammary carcinogenesis studies using mammary gland-specific HIF-1α knockout (OXOHOS overwhelms glycolysis), overexpressed (glycolysis overwhelms OXPHOS/cancer-like) and its corresponding control mice. A comparison of the extent to which DMBA could induce mammary cancer among these groups of animals would reveal the role of mitochondrial oncobioenergetics on the onset of mammary cancer.

HIF-1α+/+ (overexpressing (OE)) and HIF-1αfl/fl (knockout (KO)) mice was bred with MMTV-Cre for MG specific HIF-1α OE and KO mice respectively until these animals were homozygous knockout or overexpressed for HIF-1α (**Table-1**). The genotype was determined by qRT-PCR using specific primers designed and performed by Transnetyx Inc, Cordova, TN 38016. Appropriate controls were included to rule out the possible influence of genetic manipulations on mammary carcinogenesis. Studies have shown that MG-specific loss or overexpression of HIF-1α does not affect MG vascular density or develop spontaneous tumors and therefore, generation of models of HIF-1α is feasible.

d. Major Task 5: To test the hypothesis that elevating oxidative metabolism would inhibit oncobioenergetics at puberty and prevent mammary carcinogenesis.

Subtask 1: ***Optimize the dose of NR/activity of NR on Mammary Glands***

Dose of NR has been established in rats/mice/humans recently (Trammell *et al.*, 2016) Therefore, as reported, we selected 300 mg/kg body weight for our studies.

Subtask 2: Mammary carcinogenesis in Sprague-Dawley rats will be performed.

Methods*:* Chemical-induced rat mammary cancer provides an experimental system for better understanding the molecular alterations and pathogenesis associated with human BC. At 50days postpartum (puberty), groups of rats (no treatment, Nicotinamide riboside (NR) alone, DMBA alone, and NR + DMBA [25 animal/group]) was gavaged with 60 mg DMBA/kg bodyweight, a dose sufficient to cause 100% tumor incidence in the DMBA group over the course of the study. NR treated groups will be fed with NR orally gavaged from day 21 to day 50 (pre-pubertal exposure only; 300 mg/kg body weight).

Rats were palpated every week starting two weeks after DMBA administration to record the presence, location, size, and date of detection for all tumors. Animals will be sacrificed when the tumor diameter reaches one inch, animals become moribund, or rats reach 22 weeks post-DMBA treatment. Endpoint of carcinogenesis was assessed by determining the number of animals with tumors (tumor incidence), number of tumors/animal (tumor multiplicity), time to tumor appearance (latency) and tumor volume.

Statistics: Differences in tumor multiplicity will be performed using non-parametric methods, the Mann-Whitney U test. The $\chi 2$ test will be used to compare tumor incidence between groups. Latency to tumor appearance or survival time will be performed using Kaplan-Meier survival analysis.

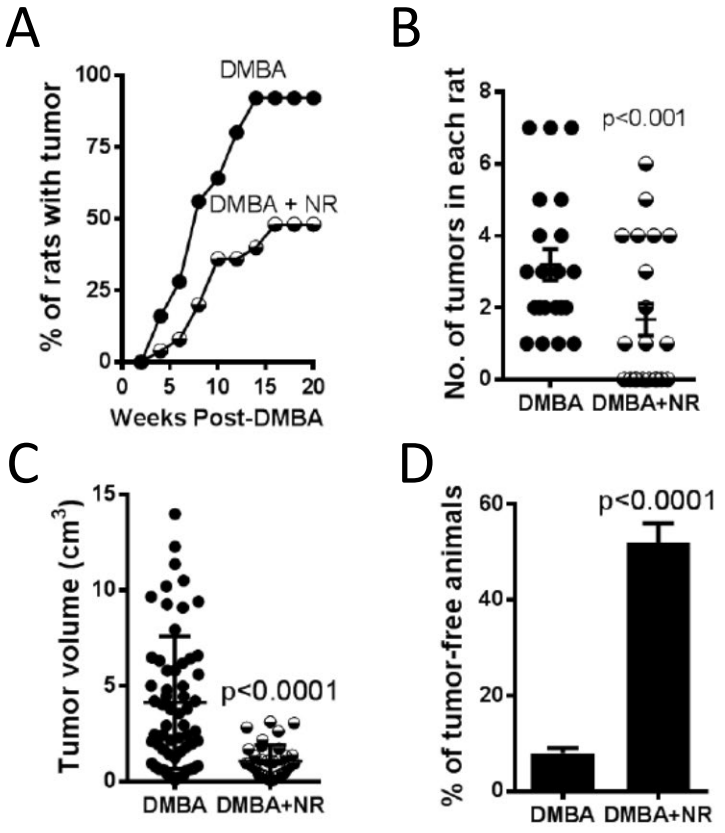

Figure-11: Effect of elevation of NAD+ at puberty in the mammary gland on mammary carcinogenesis.

Specific objectives*:* Our hypothesis is that the increased vulnerability of pubertal mammary gland to carcinogenesis is due to mitochondrial oncobioenergetic (MOB; cancer-like bioenergetic) tissue field. Therefore, we proposed that altering the MOB field of carcinogenesis would significantly affect the carcinogenesis process. So, we therapeutically changed the bioenergetics in the mammary gland by elevating NAD+ using NR (elevation of oxidative phosphorylation) and its effect on chemical-induced mammary carcinogenesis was determined in rats exposed to DMBA.

Significant results or key outcomes: Our results showed a significant inhibition on the DMBA-induced mammary tumor incidence in rats exposed to NR as shown in the ***figure-11***. By week 10 post DMBA, more than 50% of the animals developed at least one tumor in the control animals not treated with NR and by 15 weeks 92% animals developed tumors. However, pre-pubertal treatment of animals with NR significantly reduced the number of animals developed tumors 30% and 48% respectively at week 10 and 15 (***Figure-11A***). Thus 52% of the animals did not develop any tumors at all (***Figure-11D***). Most remarkable effect is the tumor growth which is represented as tumor volume. All the animals were sacrificed at the end of the experiment and all the tumors were collected and measured the tumor volume using digital calipers. Our data showed that pubertal treatment with NR has significantly inhibited the growth of mammary tumor (***Figure-11C***). Moreover, average number of tumors developed in animals were also significantly reduced by prepubertal administration of NR (***Figure-11B***) suggesting that modulation of mitochondrial oncobioenergetics can influence the mammary tumor development significantly.

Conclusion: Our studies demonstrated that as the mitochondrial bioenergetics changes with the administration of NR significantly altered the mammary carcinogenesis process in the rats. These results are in concordance with our original hypothesis that onset of cancer involves a tissue field with

favorable mitochondrial oncobioenergetics.

DESCRIPTION OF WHAT WAS PERFORMED IN THE FINAL YEAR OF THE PERIOD OF PERFORMANCE (May 2021 – December 2021)

e. Major Task 4: Mammary carcinogenesis studies in genetically modified mice

Carcinogenesis experiments:
The animals (5-17/group) will be exposed to dimethylbenzanthracene (DMBA) orally by gavage (1mg/animal/day/week) starting from 8th week for seven weeks (Trammell et al., 2016). The dose and duration of DMBA administration was selected based on previous studies. Mice will be followed for tumor appearance by weekly palpation for a year starting from the 5th week after the start of DMBA.

Endpoint of carcinogenesis was assessed by determining the number of animals with tumors (tumor incidence), number of tumors/animal (tumor multiplicity), time to tumor appearance (latency) and tumor volume. We will compare the endpoints between KO and OE mice within and between the groups exposed to DMBA.

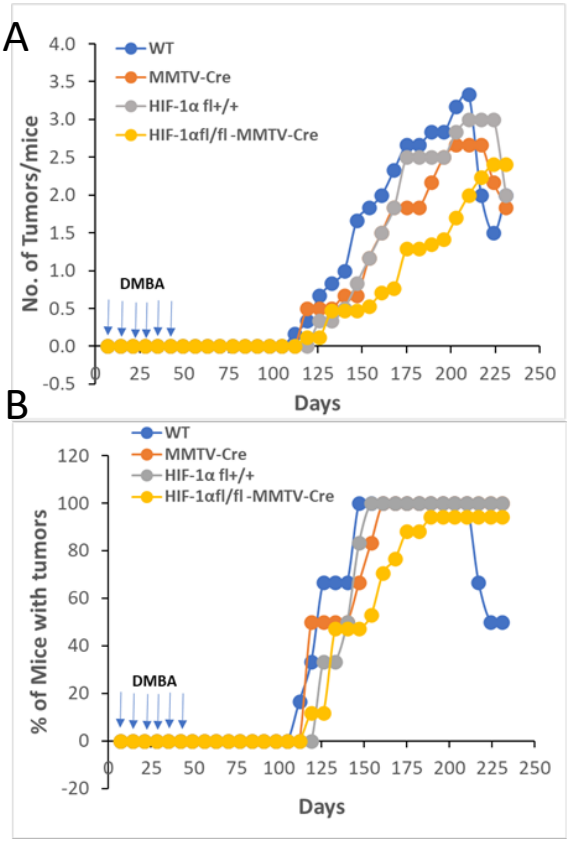

Figure-12: Mammary carcinogenesis studies in genetically modified mice.

Results of Carcinogenesis experiments with genetically modified animals:

More recent Carcinogenesis experiments done in HIF-1α KO mice produced an early reduction in the number of tumors formed compared to its control mice such as WT, MMTV-Cre, HIF-1α fl+/+ mice. However, it did not produce any drastic change in the tumor formation later in HIF-1α fl/fl-MMTV-Cre animals. A significantly reduced tumor development in HIF-1α fl/fl-MMTV-Cre animals was observed from day 160 -175 days compared to its controls (**Figure-12A**). Similarly, percentage of mouse with tumor was also comparatively reduced in HIF-1α fl/fl-MMTV-Cre animals as mentioned above (**Figure-12B**).

Conclusion: The study using HIF-1α KO mice demonstrated an inhibition of tumor formation during the carcinogenic process especially at day 175. However, it did not show much difference in either early or later part of the whole carcinogenesis experiment compared to its control mice.

Other achievements. Include a discussion of stated goals not met: The above results are strongly indicative of the role of mitochondrial oncobioenergetic tissue fields of carcinogenesis in mammary cancer development. We did this as our first experiment because, the bioassay is quick, less expensive and a positive result of this experiment would be a strong indication in support our hypothesis. Now we are confident that rest of the experiments that follow will also support our hypothesis.

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

"Nothing to Report."

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?

4. IMPACT:

These studies will not have any significant impact in the future studies.

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

These studies will not have any significant impact on future studies.

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:

"Nothing to Report,"

a) Changes in approach and reasons for change
b) Actual or anticipated problems or delays and actions or plans to resolve them
c) Changes that had a significant impact on expenditures
d) Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
e) Significant changes in use or care of human subjects
f) Significant changes in use or care of vertebrate animals.
g) Significant changes in use of biohazards and/or select agents

6. PRODUCTS:

"Nothing to Report."

- Publications, conference papers, and presentations
  Report only the major publication(s) resulting from the work under this award.

- Journal publications.
- Books or other non-periodical, one-time publications.
- Other publications, conference papers, and presentations.
- Website(s) or other Internet site(s)
- Technologies or techniques
- Inventions, patent applications, and/or licenses
- Other Products

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?
- 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."

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**
- **QUAD CHARTS:** APPENDICES:

9. APPENDIX

References

Trammell, S.A., Schmidt, M.S., Weidemann, B.J., Redpath, P., Jaksch, F., Dellinger, R.W., Li, Z., Abel, E.D., Migaud, M.E., and Brenner, C. (2016). Nicotinamide riboside is uniquely and orally bioavailable in mice and humans. Nat Commun *7*, 12948. 10.1038/ncomms12948.