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TITLE: Targeting Fatty Acid Synthase: A mechanism-guided approach to develop a novel therapeutic intervention for drug-resistant breast cancer

PRINCIPAL INVESTIGATOR: **Ruth Lupu, PhD**

CONTRACTING ORGANIZATION: Mayo Clinic, Rochester, MN

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Mayo Clinic
200 First St, SW
Rochester, MN, 55905

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Resistance to trastuzumab and HER2-directed therapy remains an unmet clinical need for patients with HER2+ breast cancer, and currently there are no FDA-approved drugs that can reverse resistance to trastuzumab or other HER2-directed therapies. Our preliminary data show that Fatty Acid Synthase (FASN) plays a major role in the maintenance of an aggressive breast cancer phenotype, and that FASN inhibition reduces tumor growth and augments the cytotoxicity of trastuzumab and paclitaxel. In this proposal we will evaluate TVB-2640, a FASN inhibitor that targets cancer metabolism and inhibits breast cancer growth. We will conduct a phase II trial of TVB-2640 in combination with paclitaxel and trastuzumab in patients with metastatic breast cancer who have disease resistant to trastuzumab. We will evaluate the safety and clinical efficacy of TVB-2640, as well as the value of serum and tissue FASN as novel biomarkers of response in HER2+ breast cancer

15. SUBJECT TERMS
Breast cancer, Trastuzumab, Paclitaxel, HER2, Fatty Acid Synthase (FASN), TVB-3199, Cancer metabolism Drug resistance Apoptosis Biomarkers

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1.**INTRODUCTION:**

The development of HER2-targeted therapies has altered the natural course of HER2+ metastatic breast cancer (MBC) with a more favorable trajectory. The monoclonal HER2-directed antibody, trastuzumab (Trz), in combination with taxane-based chemotherapy such as paclitaxel (PXL) has an established clinical benefit for the treatment of HER2+ MBC. However, resistance inevitably ensues even for those with initial response, and novel approaches to overcome Trz-resistance remain an unmet clinical need. ***No FDA-approved drug that reverse resistance to trastuzumab (Trz) or other HER2-directed therapies*** **are currently available.**

Our preliminary data show that Fatty Acid Synthase (FASN) plays a major role in the maintenance of an aggressive BC phenotype. FASN inhibition interferes with BC tumor growth and augments the cytotoxicity of Trz and PXL, indicating that its inhibition has a chemo-sensitizing effect in BC. Most importantly, this is also true *in vivo* as FASN inhibition reduces tumor volume and synergizes with Trz in Trz-resistant, HER2+ BC xenograft models. ***Extending upon our prior studies of FASN and its role in tumor progression and response to therapy, we aim to develop novel, rationally-designed therapeutic approaches for BC.***

In this proposal we will evaluate a potentially revolutionary BC therapy, TVB-2640, that targets cancer metabolism and inhibits BC growth in part through induction of cellular apoptosis. Resistance to standard therapies further stimulates BC progression, and our preclinical work suggests TVB-2640 can overcome Trz- and PXL-resistance in HER2+ BC models. ***We will conduct a phase II trial of TVB-2640 in combination with PXL and Trz in patients with breast cancer who have disease resistant to Trz. We will evaluate the clinical efficacy of TVB-2640, as well as the value of serum and tissue FASN as novel biomarkers of response in HER2+ BC.***

2. **KEYWORDS:**

Breast cancer
Trastuzumab
Paclitaxel
HER2
Fatty Acid Synthase (FASN)
TVB-2640
Cancer metabolism
Drug resistance
Clinical Trial
Biomarkers
Sphingolipids
S1P- sphingosine-1-phosphate

3. ACCOMPLISHMENTS:

3.1. What were the major goals of the project?

Specific Aim 1: ***To assess the clinical activity of a novel FASN inhibitor, TVB-2640, in combination with paclitaxel and trastuzumab in a phase II clinical trial of patients with HER2+ metastatic breast cancer resistant to taxane and HER2-directed therapy.***

Specific Aim 2: ***To examine the clinical value of serum and tissue FASN expression as a novel theranostic marker in HER2+ breast cancer.***

Aim 1 and 2 are under the direction of Dr. Tufia Haddad. Please see separate annual progress report for details related to Specific Aims 1 & 2.

Specific Aim 3: ***To determine the mechanistic link between FASN inhibition-induced Bcl-2 pro-apoptotic BH3-only proteins and develop preclinical models in PDX mice based on targeting FASN and Bcl-2.***

- **Major Task 8: Mechanism of apoptotic synergy between FASN inhibition and PXL**
  Milestone in progress: Determine the effect of FASN inhibition on lipid composition of the mitochondrial membrane (In different models of FASN expression in breast cancer)
  Study completed to be reported

- **Major Task 9: Linking FASN inhibition to increased ROS production**
  Milestone in progress: Completed

- **Major Task 10: Preclinical assessment of the FASN inhibitor TVB-3166 (the form of TVB-2640 for animal use) in combination with ABT263**
  Milestone in progress: Study is partially completed.

3.2: What accomplished under these goals?

- **Major Task 8: Mechanism of apoptotic synergy between FASN inhibition and PXL**
  - Determine the effect of FASN inhibition on lipid composition of the mitochondrial membrane (In different models of FASN expression in breast cancer)

- **Major Task 10: Preclinical assessment of the FASN inhibitor TVB-3166 (the form of TVB-2640 for animal use) in combination with ABT263**
  - Continues preclinical studies *in vivo* studies to assess TVB3166+ ABT199 and TVB3166+ ABT263
  - Histopathological assessment of Tumor derived from the *in vivo* studies

REPRESENTATIVE RESULTS

Task 8: Subaim: Determine the effect of FASN inhibition on lipid composition of the mitochondrial membrane (In different models of FASN expression in breast cancer)

FASN regulates sphingolipid metabolism in HER2 overexpressing breast cancer cells: We determine the effect of FASN inhibition on lipid composition of the mitochondrial membrane using TBV3166 lipid profile of breast cancer cells. Isolated mitochondrial membranes pre- and post-treatment were analyzed for lipid composition for the inner mitochondrial membranes, as opposed to the untreated control membranes. After a thorough analysis, we concluded that one specific sphingolipid, S1P-Sphingolipid 1-phosphate was substantially regulated by inhibition of FASN in breast cancer cells expressing high levels of FASN. Fig. 1 shows two BC cell lines, BT-474 and MCF-7/HER2-18, in both cases TVB3166 significantly block the

synthesis of S1P. BT-474 and MCF-7/HER2-18 cells express high levels of FASN, HER2 and ER. No change in S1P levels was seen, when MCF-7/neo cells were tested. MCF-7/neo cells are ER+ but do not express detectable levels of either HER2 or FASN. Isolated mitochondrial membranes after treatment with TVB3166 were analyzed for S1P levels.

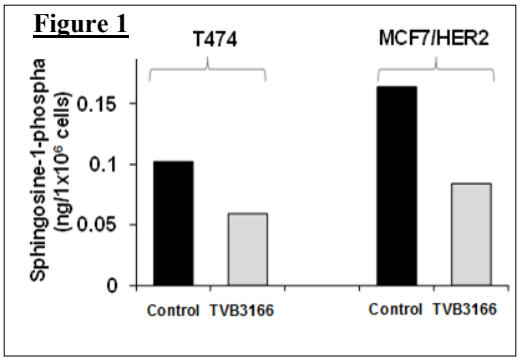


Figure 1: FASN expression and activity controls sphingosine metabolism via regulation of S1P synthesis. Cells were tested to determine whether pharmacological inhibition of FASN is associated with S1P synthesis. S1P synthesis was measured in BT4-474 and MCF-HER2 cells treated with TVB3166 (200 nM). Isolated mitochondrial membranes were analyzed for S1P levels by the Metabolomic Facility.

FASN regulates sphingolipid metabolism in Aggressive breast cancer cells: To explore the link between FASN and sphingolipid metabolism, we used a FASN-knockdown model, previously developed in our laboratory. This model consists of tumorigenic cell lines, MCF-10A-CAID and MCF-10A-CAIA, derived from non-tumorigenic MCF-10A cells. MCF-10A-CAID and MCF-10A-CAIA cells express higher levels of FASN than MCF-10A derived cells. We showed that, while MCF-10A-CAID-shFASN and MCF-10A-CAIA-sh-FASN lost their tumorigenic potential when implanted in athymic nude mice, their control scramble-sh-control maintained tumorigenic capacity in the laboratory, which indicates that FASN was a key regulator of tumor growth. We next measured total ceramide, sphingosine, and S1P FASN knockdown in BC cell lines. According to the ceramide-sphingosine-S1P rheostat model both ceramide and sphingosine are apoptotic, whereas S1P enhances cell survival. Thus, we measured total ceramide, sphingosine, and S1P in FASN-knockdown MCF-10A-CAID and MCF-10A-CAIA cells. A slight increase in total ceramide and sphingosine was associated with knockdown of FASN (data not shown). Conversely, a significant decrease in S1P levels was seen in the shRNA-FASN cells (Fig 2A), ***indicating a link between FASN and S1P synthesis***. We then assessed whether pharmacological inhibition of FASN would also reduce the synthesis of S1P, thus, MCF-10A, MCF-10A-CAID and MCF-10A-CAIA cells were treated with TVB3664 (a preclinical FASNi) (200 nM) and S1P levels were measured (Fig 3B). Inhibition of FASN resulted in a significant decrease in S1P synthesis in the FASN expressing cells (Fig 2B), ***which infers that pharmacological inhibition of FASN also abrogates S1P synthesis.***

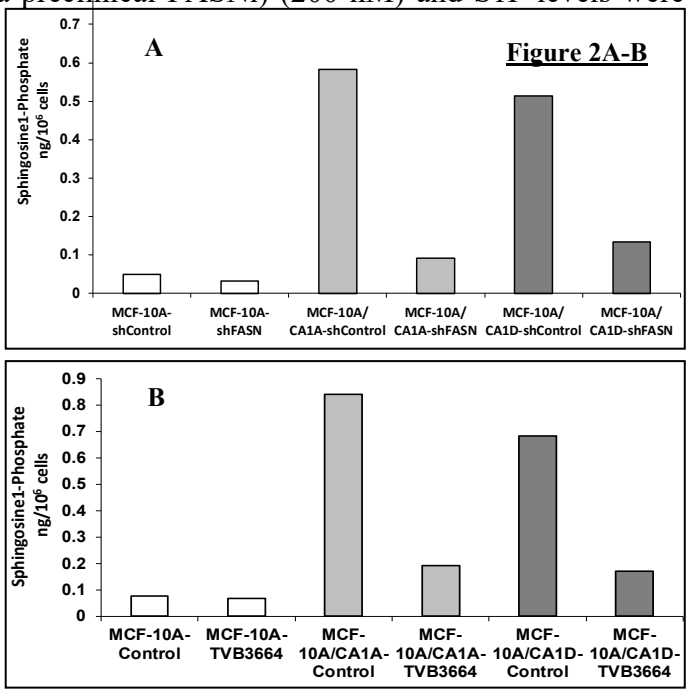


Figure 2A-B: FASN expression and activity controls sphingosine metabolism via regulation of S1P synthesis. Cells were tested to determine whether genetic or pharmacological inhibition of FASN is associated with S1P synthesis. **(A)** S1P synthesis was measured in FASN-knockdown MCF-10A, MCF-10A/CAID and MCF-10A/CAIA cells and compared with sh-Control (scramble-shRNA) cells. **(B)** S1P synthesis was measured in MCF-10A, MCF-10A/CAID and MCF-10A/CAIA cells treated with vehicle (control) or with TVB3664 (200 nM). Isolated mitochondrial membranes were analyzed for S1P levels.

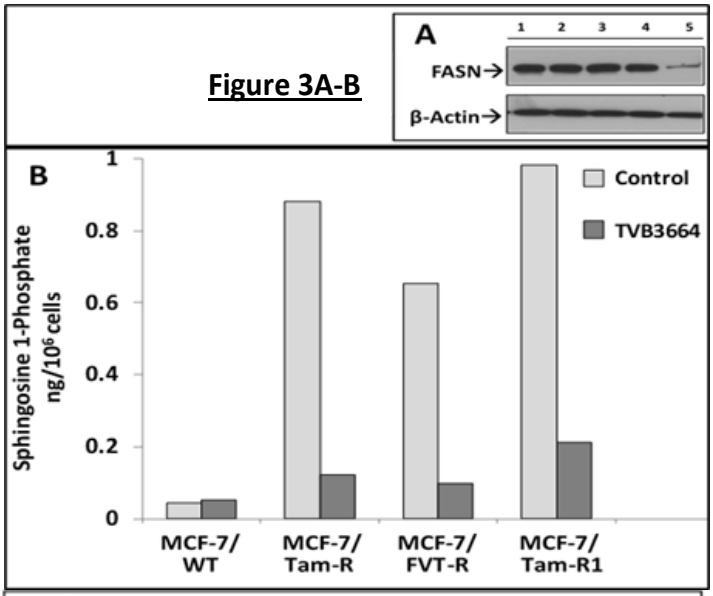
FASN regulates sphingolipid metabolism in endocrine -resistant breast cancer cells: To explore the link between FASN, sphingolipid metabolism and endocrine resistance in HR+ BC, we assessed the effect of the preclinical FASNi, TVB3664 on S1P levels in HR+ BCC. The studies were performed with MCF-7/WT, MCF-7/Tam-R, MCF-7/FVT-R, and MCF-7/Tam-R1 cells (Table 1). We measured levels of FASN expression in the

cells and showed that, while the Tam and FVT sensitive MCF-7/WT cells express low levels of FASN, the **Tam-R and FVT-R MCF-7/Tam-R, MCF-7/FVT-R, and MCF-7/Tam-R1 cells express high levels of FASN** (Fig 3A). These is consistent with our data showing that FASN is involved in the acquisition of Tam resistance. The results in Fig. 3, together with the increased FASN expression in the more aggressive cells, we wondered whether there is also a link between FASN and S1P in the setting of acquired endocrine resistance. Thus, MCF-7/Tam-R, MCF-7/FVT-R, and MCF-7/Tam-R1 cells were treated in the presence or absence of FASNi, TVB3664 (200 nM). While inhibition of FASN significantly decreased S1P in Tam and FVT resistant cell lines, little or no effect was noted in endocrine sensitive cells (Fig. 3B). ***These results are indicative of a link between endocrine resistance and FASN mediated regulation of S1P.*** Given the results in Fig. 3, together with the increased FASN expression in the more aggressive cells, we wondered whether there is also a link between FASN and S1P in the setting of acquired endocrine resistance. Thus, MCF-7/Tam-R, MCF-7/FVT-R, and MCF-7/Tam-R1 cells were treated in the presence or absence of FASNi, TVB3664 (200 nM). While inhibition of FASN significantly decreased S1P in Tam and FVT resistant cell lines, little or no effect was noted in endocrine sensitive cells (Fig. 3B). ***These results are indicative of a link between endocrine resistance and FASN mediated regulation of S1P.***

Table 1: Description of the cell lines used for the studies

Cells/Hormone Response	E_2 - Dependent	E_2 - Independent	E_2 - Responsive	Tam-S	Tam-R	FVT-S	FVT-R	FVT & Tam Cross-Resistant
MCF-7/WT	✓	NO	NO	✓	NO	✓	NO	NO
MCF-7/Tam-R	NO	✓	✓	NO	✓	✓	NO	NO
MCF-7/FVT-R	NO	✓	✓	NO	✓	NO	✓	✓
MCF-7/Tam-R1	✓	NO	NO	NO	✓	✓	NO	NO

Figure 3A-B: Hormone-Resistant BCC express high levels of FASN and FASNi reduces S1P synthesis:(A) MCF-7/TAM-R, MCF-7/FVT-R and MCF-7/TAM-R1 cells were treated with vehicle or with TVB3664, and S1P synthesis was measured. MCF-7/TAM-R, and MCF-7/FVT-R cells expressed high levels of FASN, HER2 and ER. No change in S1P levels was seen in MCF-7/neo cells (data not shown). **(B)** shows an immunoblot for FASN expression in HR and sensitive BCC, protein was electrophoresed, transferred, and probed for FASN. β-actin was used as a control. MCF-7/TAM-R (1), and MCF-7/FVT-R (2) express high levels of FASN, whereas MCF-7/WT (3), MCF-7/S (4) and MCF-10A (5) cell lines express low levels of FASN. Isolated mitochondrial membranes after treatment with TVB3166 (200mM) were analyzed for S1P levels as per Fig 2.



Concomitant inhibition of FASN and S1P induces maximal ROS production: Our recent published data reveal that inhibition of FASN induces apoptosis through mitochondrial damage, which is mediated by increase of ROS and upregulation of pro-apoptotic proteins, in particular the BH3-only protein. Here we show that inhibition of the FASN/S1P axis with the preclinical FASNi TVB-3664 plus the S1P receptor modulator FTY-720 results in an increase in ROS production, significantly exceeding that seen with each drug separately (Fig. 4). These data suggests that both pathways are necessary for max ROS production.

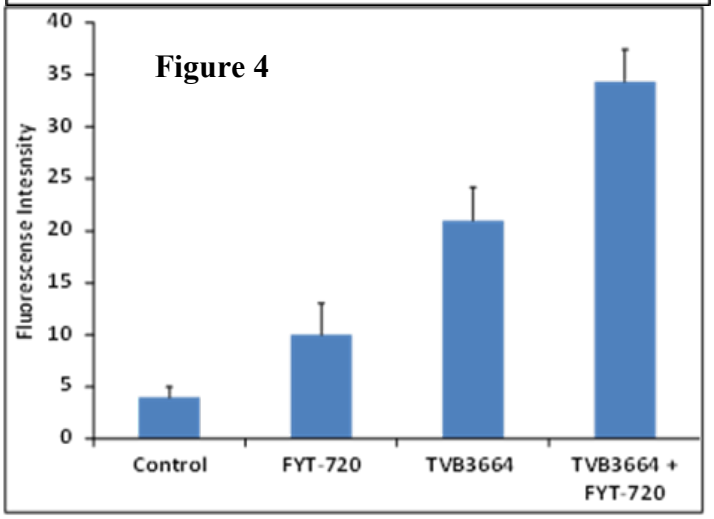


Figure 4: Reactive Oxygen Species (ROS) were determined in BCC. Cells were treated with TVB3664 (200 nM) and FYT-720 (2.5 mM). ROS was assessed using the DCFDA / H2DCFDA - Cellular ROS Assay Kit (Abcam). The kit uses the cell permeant reagent 2',7' –dichlorofluorescin diacetate (DCFDA, also known as H2DCFDA, DCFHDA, and DCFH) to quantitatively assess ROS in live cell samples.

Task 10: Subaim: Continues preclinical studies *in vivo* studies to assess TVB3166+ ABT199 and TVB3166 + ABT263:

- ***The studies were completed using ABT-263 and TVB3664.***

A clinical grade FASNi enhances sensitivity to navitoclax/ABT-263 and venetoclax/ABT-199 *in vivo*: We finally sought to determine the efficacy of combining navitoclax/ABT-263 or venetoclax/ABT-199 with TVB-3664 against BT-474 human breast cancer xenografts in nude mice. BH3 mimetics and TVB-3664 were administered by oral gavage to mimic human oral drug administration. Both navitoclax/ABT-263 and venetoclax/ABT-199 failed to elicit any tumor growth delay of BT-474 xenograft tumors; notably, single agent TVB-3664 was notably efficacious in producing a tumor response (44% tumor growth inhibition) (Fig. 5). The completely lack of anti-tumor efficacy of navitoclax/ABT-263 and venetoclax/ABT-199 as single agents was fully circumvented when FASN activity was pharmacologically targeted in BT-474 tumor xenografts; thus, when administered in combination with the FASNi TVB-3664, navitoclax/ABT-263 and venetoclax/ABT-199 caused strong tumor growth inhibition (80% and 78%, respectively; Fig. 5). Combination therapy appeared to be well-tolerated, with mice maintaining normal body weight.

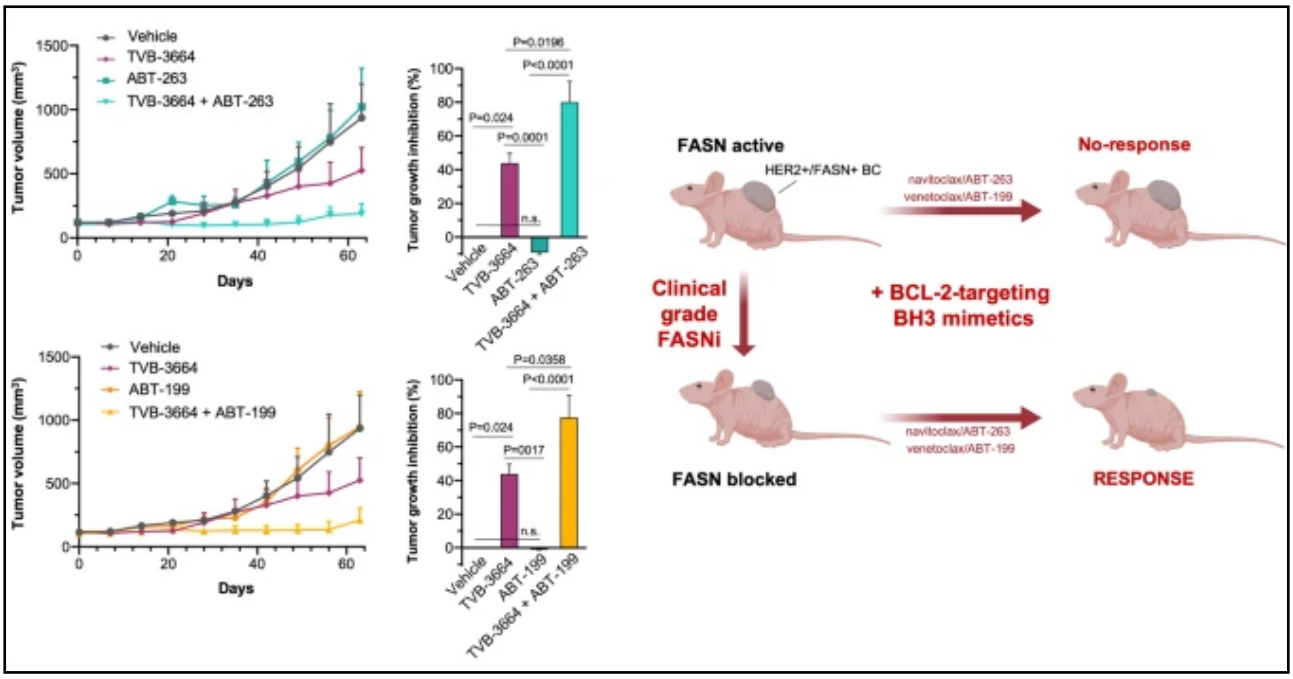


Figure 5: FASN inhibition sensitizes human breast tumor xenografts to BCL-2-targeting BH3 mimetics *Left*. Growth of BT-474 xenograft tumors in athymic female mice treated with BH3 mimetics navitoclax/ABT-263 (*top*) and venetoclax/ABT-199 (*bottom*) in the absence or presence of the FASNi TVB-3664. The maximum length for each treatment was 63 days. Results are presented as the mean tumor volume ± S.D. ($n = 10$ mice/experimental group). Tumor growth inhibition (TGI) was calculated as the percentage of tumor growth, relative to tumor size at the start of treatment, in drug-treated groups compared to vehicles-treated group. *Right*. *In vivo* findings from the HER2 + /FASN-overexpressing breast cancer model BT-474 uncovers a novel FASN-dependent mitochondrial priming that links *de novo* FA biosynthesis to the intrinsic apoptotic threshold in breast cancer cells. The discovery that FASN-inhibited cancer cells exist in an apoptosis-prone state highly sensitive to BCL-2-targeting BH3 mimetics might warrant clinical exploration in patients with HER2 + /FASN-addicted breast carcinomas (see the discussion section). FASN inhibition increases mitochondrial priming and enhances breast cancer cell sensitivity to BCL2-targeting BH3 mimetics: a working model.

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

4. **IMPACT:**

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

Nothing to report at this time

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

Nothing to report at this time

- **What was the impact on technology transfer?**

Nothing to report at this time

- **What was the impact on society beyond science and technology?**
  Nothing to report at this time

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**
  Nothing to report at this time
- **Actual or anticipated problems or delays and actions/plans to resolve them**

Tasks related to tissue and serum specimens will be delayed due to delay in the clinical trial (Explained in Dr. Tufia Haddad's progress report)

- **Changes that had a significant impact on expenditures**
  Nothing to report at this time
- **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**

  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

5. **PRODUCTS:**
Publications, conference papers, and presentations

Journal publications

1. Schroeder B, Vander Steen T, Espinoza I, Venkatapoorna Cmk, Hu Z, Silva Fm, Regan K, Cuyàs E, Meng Xw, Verdura S, Arbusà A, Schneider Pa, Flatten Ks, Kemble G, Montero J, Kaufmann Sh, Menendez Ja, **Lupu R.** Fatty Acid Synthase (FASN) Regulates The Mitochondrial Priming Of Cancer Cells. Cell Death Dis. 2021 OCT 21;12(11):977. DOI: 10.1038/S41419-021-04262-X.PMID: 34675185

2. Menendez JA, Papadimitropoulou A, Vander Steen T, Cuyàs E, Oza-Gajera BP, Verdura S, Espinoza I, Vellon L, Mehmi I, **Lupu R.** Fatty Acid Synthase Confers Tamoxifen Resistance to ER+/HER2+ Breast Cancer.. Cancers (Basel). 2021 Mar 6;13(5):1132. Doi: 10.3390/Cancers13051132.

3. Menendez JA, Peirce SK, Papadimitropoulou A, Cuyàs E, Steen TV, Verdura S, Vellon L, Chen WY, **Lupu R.** Progesterone receptor isoform-dependent cross-talk between prolactin and fatty acid synthase in breast cancer. Aging (Albany NY). 2020 Dec 10;12(24):24671-24692. doi: 10.18632/aging.202289. Epub 2020 Dec 10.PMID: 33335078.

4. Papadimitropoulou A, Vellon L, Atlas E, Steen TV, Cuyàs E, Verdura S, Espinoza I, Menendez JA, **Lupu R.** Heregulin Drives Endocrine Resistance by Altering IL-8 Expression in ER-Positive Breast Cancer. Int J Mol Sci. 2020 Oct 19;21(20):7737. doi: 10.3390/ijms21207737.PMID: 33086721

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

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Ruth Lupu*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	0000-0001-8226-3581
Nearest person month worked:	1.8
Contribution to Project:	Authored the Translational research and contributed all the preliminary data for the research proposal except the clinical trial data. Led training and logistics review for the laboratory study personnel; facilitated contract completion with 3V Biosciences; active oversight the research and the collaborative studies
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No Change

What other organizations were involved as partners?

We continue collaboration with 3V-Biosciences, Inc. (Renamed SAGIMET Inc.)

Organization Name: SAGIMET, Inc.
Location of Organization: 3715 Haven Ave. Suite 220, Menlo Park, CA 94025

Partner's contribution to the project: 3V Biosciences is providing the investigational agent, TVB-3166, TVB3446, TVB-2640, and the company will oversee serum FASN and tissue pAKT and pS6 correlative studies

Financial support: Financial support from 3V Biosciences is not provided to Mayo Clinic, Dr. Haddad, or the clinical trial participant's

In-kind support:
Not applicable
Facilities:
Not applicable

Collaboration: Scientists from 3V Biosciences will
- Review study safety data and assist with safety monitoring
- Participate in data interpretation, as appropriate

Personnel exchanges:
Not applicable

Other:
Not applicable

Submitted Proposal:
Department of Defense BCRP Breakthrough Award - Funding Level 3 - Partnering PI Option
BC210816P1: Title: "Targeting Fatty Acid Synthase and the Sphingosine Phosphate Kinase 1/Sphingosine 1-Phosphate Axis in Hormone Receptor-Positive Metastatic Breast Cancer"
Funding Period: 04-30-20122– 03-29-2026

Pending Proposals:

RO1-NIH/NCI- To be submitted February 5th, 2022
Title: Co-Targeting Fatty Acid Synthase (FASN) and Sphingosine Phosphate Kinase 1 (SphK1)/Sphingosine 1-Phosphate (S1P) Axis in Hormone Receptor Positive (HR+)/Hormone-Resistant Metastatic Breast Cancer
Funding Period: 10/31/2022 – 09/30/2027

RO1-NIH/NCI- To be submitted February 5th, 2022
Title: "Targeting Fatty Acid Synthase: A Mechanism-Guided Approach to Target Pancreatic Adenocarcinoma and the Tumor Microenvironment"
Funding Period: 10/31/2022 – 09/30/2027

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS;

Dr. Ruth Lupu, PhD. Principal Investigator (PI)

Dr. Haddad is the Partnering PI.

QUAD CHARTS:

Nothing to report

APPENDICES:

No Appendices