AWARD NUMBER: W81XWH-19-1-0556

TITLE: MYCN Reprograms Neuroblastoma Metabolism

PRINCIPAL INVESTIGATOR: Eveline Barbieri, MD PhD

CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX

REPORT DATE: October 2021

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2021	Annual	01Sep2020-31Aug2021

4. TITLE AND SUBTITLE
MYCN Reprograms Neuroblastoma Metabolism

5a. CONTRACT NUMBER
CA180461

5b. GRANT NUMBER
W81XWH-19-1-0556

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Eveline Barbieri, Ling Tao, Mirthala Moreno Smith, Cristian Coarfa.

Email: exbarbie@txch.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel 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

Despite current aggressive regimens, the majority of patients with MYCN amplification die due to drug-resistant disease, and further intensification of chemotherapy will not significantly improve this outcome. We propose an entirely novel strategy to oppose MYCN oncogenic function in NB: by blocking the metabolic reprogramming driven by MYCN. Our guiding hypothesis is that lipid metabolism is required for NB tumorigenesis. We have shown that lipid metabolism is a selective metabolic dependency of MYCN-induced tumors. Targeting MYCN-driven lipogenesis effectively blocks in vivo tumor growth in multiple models of NB and sensitize NB tumors to conventional chemotherapy.

15. SUBJECT TERMS Neuroblastoma, MYCN, fatty acid metabolism, targeted therapies.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	12	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Our long-term goal is to elucidate the MYCN-dependent pathways that will serve as targets for NB therapy. Toward this goal, our overall objective in this application is to determine **how MYCN rewires lipid metabolism to support tumor growth**. We hypothesize that: a) lipid metabolism is required for NB tumorigenesis, and b) targeting MYCN-driven lipogenesis will effectively block NB tumor growth. In this proposal we will: **1)** Determine how MYCN reprograms lipid metabolism in NB, and **2)** Elucidate the anti-tumor activity of targeting MYCN-driven lipogenesis. These studies will reveal insights into critical molecular and metabolic alterations, which will provide novel, and more sensitive targets that could be deployed with currently available therapies to treat this highly aggressive disease.

2. KEYWORDS:

Neuroblastoma (NB)
V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN)
Lipid metabolism
Fatty acids (FA)
Tumorigenesis
Targeted therapies
Fatty Acid Transport Protein 2 (FATP2)
MYCN-amplified (MNA)
MYCN non-amplified (non-MNA)

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine how MYCN reprograms lipid metabolism in neuroblastoma.
1.1 Metabolic *in vitro* characterization upon changes in MYCN expression.
1.2 *In vitro* effects of genetic interference and pharmacological inhibition of lipogenesis.
1.3 Elucidate how MYCN alters *in vivo* lipid metabolism and tumor growth.

Specific Aim 2: Elucidate the anti-tumor activity of targeting MYCN-driven lipogenesis.
2.1 *In vivo* anti-tumor activity of targeting lipogenesis via single agent FASN and FATP2 inhibitors.
2.2. *In vivo* anti-tumor activity of targeting fatty acid synthesis and uptake.
2.3 Determine how inhibition of lipogenesis alters *in vivo* chemo-sensitivity.

What was accomplished under these goals?

Specific Aim 1: Determine how MYCN reprograms lipid metabolism in neuroblastoma.

1. Metabolic *in vitro* characterization upon changes in MYCN expression

Since our last report, we have validated that FA uptake is necessary for MYCN-amplified (MNA) cell survival, as deprivation of exogenous FAs blocks cell proliferation ($p<0.01$) and induces apoptotic cell death in multiple models of MYCN-amplified and MYCN-induced cells (**Figure 1**). By contrast MYCN non-amplified (non-MNA) and not-induced cells are not as sensitive to lipid deprivation.

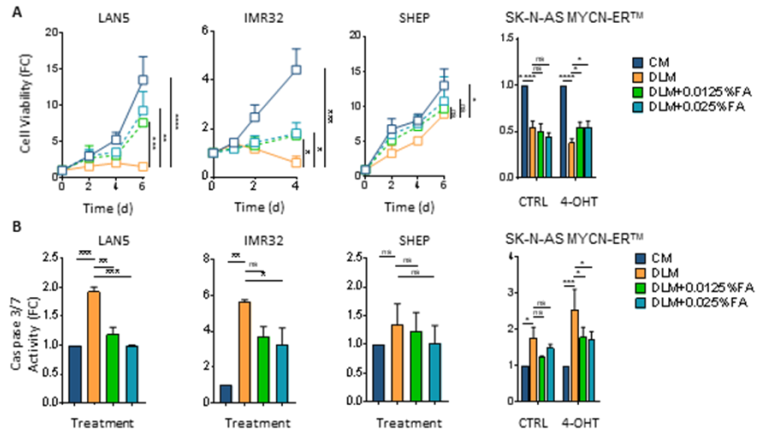

Figure 1. A. Cell viability in complete and delipidized media. MNA: LAN5 (0–6 day) and IMR32 (0–4 day); non-MNA: SHEP (0–6 day); SK-N-AS MYCN-ERTM (−/+ 5 nM 4-OHT at day 6). Mean ± SD (n = 2–4); two-way ANOVA with Dunnett's multiple comparisons test. **B.** Caspase 3/7 activity in complete and delipidized media. MNA: LAN5 and IMR32; non-MNA: SHEP (all at day 4); SK-N-AS MYCN-ERTM (−/+ 5 nM 4-OHT at day 6). Mean ± SD (n = 2); one-way or two-way ANOVA with Dunnett's multiple comparisons test. FC = fold change; CM = complete media; DLM = delipidized media; ns = no significance; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2. *In vitro* effects of genetic interference and pharmacological inhibition of lipogenesis

Since our last report, to assess the impact of FA synthesis and uptake on cell survival, we evaluated NB cell viability (MNA: LAN5 and IMR32; non-MNA: SH-SY5Y and SHEP) and normal cells (ARPE-19, C2C12, and HS-5) after treatment with two FA synthesis inhibitors (A939572 and Orlistat) and two FA uptake inhibitors (CB16.2 and CB5) (**Figure 2A**). NB cells were more sensitive to inhibition of FA uptake (MNA IC50: 0.5–3.8 μM; non-MNA IC50: 1.3–7.5 μM) than to inhibition of FA synthesis (MNA IC50: 6.9–41.8 μM; non-MNA IC50: 7.4–73.1 μM), suggesting that NB cells actively use exogenous FA for survival. Importantly, neither A939572 nor CB5 exerted cytotoxic effects against normal cells, with CB5 being less toxic. Conversely, CB16.2 was toxic to all tested normal cells, despite having the highest efficacy in NB cells. Importantly, genetic depletion of FATP2 effectively reduces FA uptake (not shown), and impairs cell growth ($p<0.0001$) as well as colony forming ability ($p < 0.05$, **Figure 2B**), suggesting that SLC27A2 is required for cell growth.

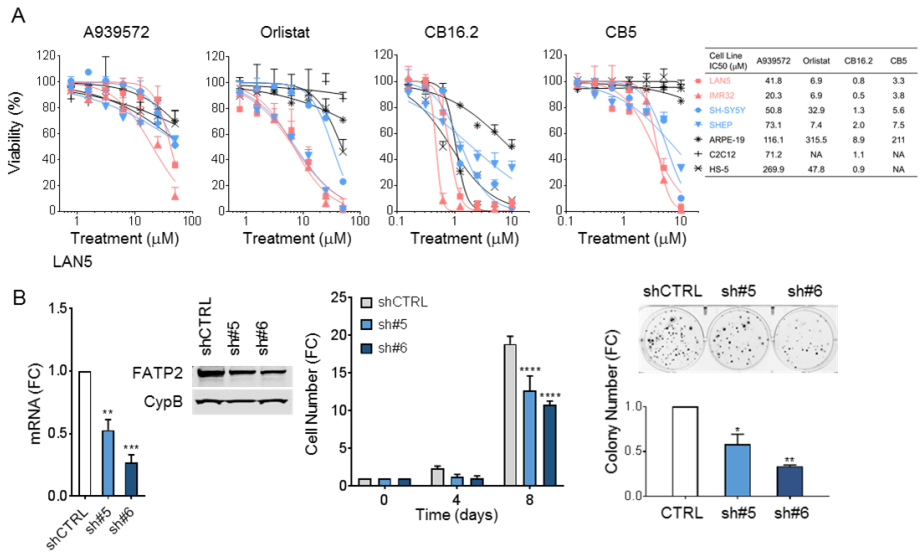

Figure 2. A. Viability of NB and normal cells upon treatment of FA synthesis inhibitors (A939572, orlistat) and FA uptake inhibitors (CB16.2 and CB5). Mean ± SD (n=2). **B.** Left: mRNA and protein expression of FATP2 in LAN5 cells. Two shRNA GIPZ vectors were tested and GIPZ empty vector was used as control. Mean ± SEM (n=3). Middle: cell growth in LAN5 shCTRL and shFATP2 cells. Fold change in cell number from day 0 is shown. Mean ± SD (n=3). Right: clonogenic assay in LAN5 shCTRL and shFATP2 cells. Data are mean ± SD (n = 2).

Moreover, MYCN enhances SCD1 activity and A939572 suppresses SCD1 activity ($p < 0.0001$) and de novo FA synthesis ($p < 0.05$) in MNA cells (data not shown). However, SCD1 inhibition was not effective in inhibiting cell growth and stimulated a compensatory and dose-dependent FA import from the media ($p < 0.05$) (**Figure 3**). Thus, we hypothesized that exogenous FAs uptake reduces cell sensitivity to FA synthesis inhibition.

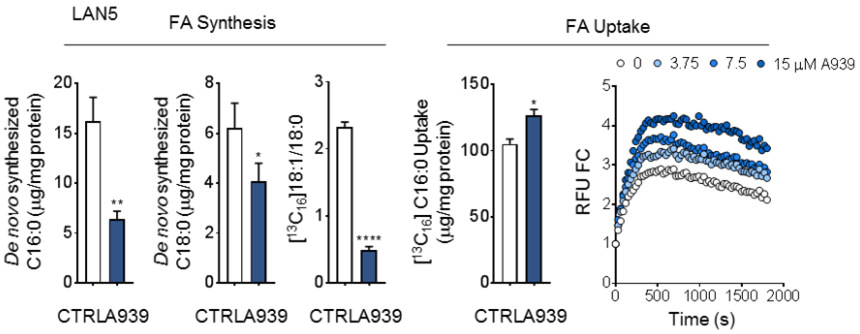

Figure 3. The effect of A939572 on FA synthesis is validated by stable isotope tracing in LAN5 cells with and without A939572 (25 µM for 48 h). Mean ± SD (n =3). The effect of A939572 on compensatory FA uptake is evaluated by both tracing (−/+ 25 µM A939572 for 48 h) and real-time FA uptake assay (0–15 µM A939572 for 15 min). Tracing: mean ± SD (n=3).

3. Elucidate how MYCN alters *in vivo* lipid metabolism and tumor growth

We have not yet elucidate how MYCN alters *in vivo* lipid metabolism. This objective was started due to the suspension of animal work caused by Covid-19. We will be working on this sub-aim in this upcoming year.

Specific Aim 2: Elucidate the anti-tumor activity of targeting MYCN-driven lipogenesis.

4. *In vivo* anti-tumor activity of targeting lipogenesis via single agent FASN and FATP2 inhibitors

In our previous report we have compared the *in vivo* anti-tumor activity of the FASN inhibitor (orlistat) and the FATP2 inhibitor (CB5) in MYCN-amplified LAN5-derived xenografts, and we have demonstrated that both these agents are able to significantly reduce tumor growth in this model of NB. Because of our preliminary data showing compensatory uptake of FAs upon inhibition of lipogenesis and the selective dependency of NB cells to exogenous FAs, we validated the anti-tumor activity of CB5 as single agent and genetic depletion of FATP2 in multiple MYCN-driven NB models and assessed their role on *in vivo* lipid metabolism. Genetic depletion of SLC27A2/FATP2 effectively reduced tumor growth (MRI imaging, p=0.048), tumor weights (p=0.012), and intratumoral neutral lipids (Oil Red O staining, p=0.003) (**Figure 4A**). To identify the specific lipid categories altered by FATP2, we then performed lipidomic analysis in shCTRL (n=8) and shFATP2 (n=8) tumors. The majority of DGs and TGs were significantly downregulated upon genetic depletion of FATP2 (42% of total downregulated lipids, FDR < 0.25, absolute FC > 2), confirming that inhibition of FATP2 blocks glycerolipid accumulation (**Figure 4B**). Moreover, to evaluate the efficacy of blocking FA transport in the presence of an intact immune microenvironment, we generated a TH-MYCN+/+-derived orthotopic syngeneic model by implanting a TH-MYCN+/+ tumor into the renal capsule of wild-type immunocompetent 129x1/svj mice. After two weeks, these mice were treated with either CTRL (vehicle) or CB5 single agent (30 mg/kg, b.i.d.) for two weeks. CB5 treatment blocked tumor growth ($p < 0.0001$) without apparent toxicity, suggesting that targeting FA uptake effectively suppresses tumor growth in the context of an intact immune microenvironment (**Figure 4C**).

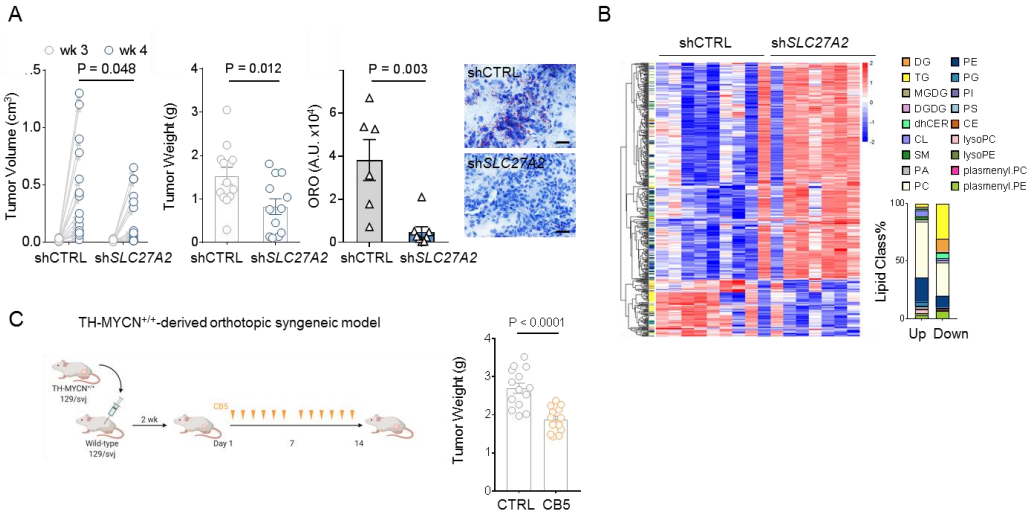

Figure 4. A. Anti-tumor effect of silencing SLC27A2/FATP2. LAN5 shCTRL and shSLC27A2 cells were implanted in the renal capsule of NCr nude mice. Mice were imaged by MRI at week 3 and 4 post-implantation for monitoring changes in tumor volume (CTRL=15; CB5=14). Tumors were harvested and weighed at week 5. Mean ± SEM (CTRL=13, CB5=12). Intratumoral lipids were determined by Oil Red O staining. Mean ± SEM (CTRL= 6, CB5=8). **B.** Lipidomics profiling of shCTRL (n=8) and shSLC27A2 (n=8) tumors. Significantly altered lipids (FDR < 0.25, absolute FC > 2) were selected for heatmap (color is scaled by z-score: red = upregulated; blue = downregulated) and lipid class% presentation. **C.** Anti-tumor effect of CB5 in TH-MYCN+/+-derived orthotopic syngeneic model. Two weeks after implantation, mice were treated with CTRL (vehicle) or CB5 (30 mg/kg, b.i.d., 6 days/week) for two weeks. Tumors were harvested and weighed at day 14. Mean ± SEM (CTRL=14, CB5=13); two-sided unpaired Mann-Whitney test.

5. *In vivo* anti-tumor activity of targeting fatty acid synthesis and uptake

We have not started testing the anti-tumor activity of combined FASN and FATP2 inhibition in MYCN cell-derived xenografts and PDXs. This goal was delayed due to the suspension of animal work caused by Covid-19. In addition, based on our preliminary data, we prioritized *in vivo* testing of single agent CB5.

6. Determine how inhibition of lipogenesis alters *in vivo* chemo-sensitivity

Because the CB5+VP16 combination had promising *in vitro* synergistic effects (see previous report), we assessed the anti-tumor activity of CB5+VP16 in a MNA LAN5-derived orthotopic mouse model. After two weeks of combination therapy (CB5: 15 mg/kg, b.i.d., 6 days/week; VP16: 8 mg/kg, 3 days/week), tumor weights were significantly lower than with single drug therapies ($p < 0.05$). In addition, no body weight loss or clinical signs of toxicity were observed. These data suggest that blocking FA transport effectively enhances tumor responses to VP16 (**Figure 5A**). To then determine the long-term effects of this combination therapy on animal survival, we used our MNA patient-derived orthotopic xenograft model. Mice were subjected to single or combination therapy (CB5: 15 mg/kg, b.i.d., 6 days/week; VP16: 6 mg/kg, 3 days/week) for five weeks. Animals treated with CB5+VP16 combination therapy survived significantly longer than those receiving single therapies (**Figure 5B**). All treatments did not cause significant body weight loss or clinical signs of toxicity. Collectively, our data suggest that blocking MYCN-induced metabolic reprogramming enhances the anti-tumor effects of conventional chemotherapy.

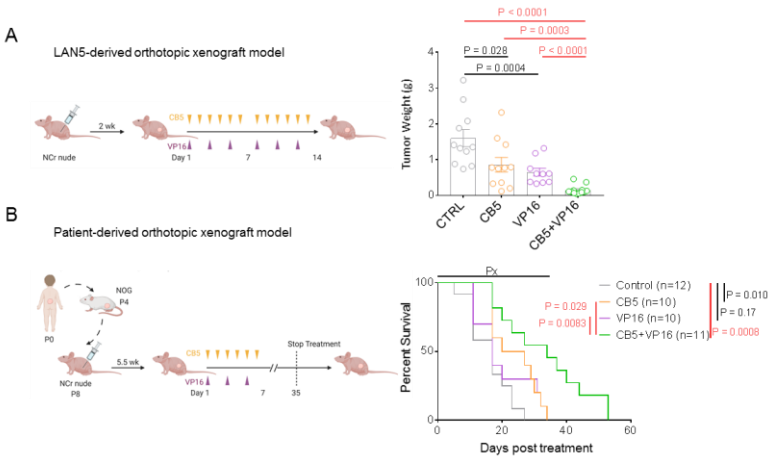

Figure 5. A. Anti-tumor activity of CB5+VP16 combination therapy in LAN5-derived orthotopic xenografts. Two weeks after implantation, mice were treated with CTRL (vehicle), CB5 (15 mg/kg, b.i.d. 6 days/week), or VP16 (8 mg/kg daily, 3 days/week) for two weeks. Mean ± SEM; two-sided unpaired Mann-Whitney test. **B.** Survival analysis in patient-derived orthotopic xenografts. Treatment groups: CTRL (vehicle), CB5 (15 mg/kg, b.i.d. 6 days/week), VP16 (6 mg/kg daily, 3 days/week), or CB5+VP16 for five weeks. Survival plotted as Kaplan-Meier curve and analyzed by log-rank test.

What opportunities for training and professional development has the project provided?
Ling Tao Ph.D., post-doctoral associate (100% effort supported by this grant), has learned and refined new lab skills necessary for this project, including stable isotope tracing, FA profiling, lipidomics, and MYCN-induced in vivo models. These skills will expand her expertise both in the field of molecular biology and cancer metabolism, and will enable her to identify distinct metabolic phenotypes and novel MYCN targets to pursue in her future research efforts. Throughout the project, she has also developed critical skills that will help foster her academic career. Towards this goal, she is planning to apply to faculty positions in spring 2022. She has trained numerous junior members of the lab, including SMART undergraduate students, PhD students, visiting postdoctoral fellows, and clinical fellows. She presents her work at the Texas Children's Hospital (TCH) Neuroblastoma Work in Progress meetings every 6 months, the TCH Research Symposium annually, and national/international conferences, including keystone symposiums, and AACR and ANR (Advances in Neuroblastoma Research) meetings.

How were the results disseminated to communities of interest?
This work was presented as oral communication at the international conference Advances in Neuroblastoma Research (ANR) 2021.
This work is also currently under revision in *Nature Communications* 2021.

What do you plan to do during the next reporting period to accomplish the goals?
As part of aim1, we will elucidate how genetic depletion of MYCN alters *in vivo* lipid metabolism and tumor growth.
As part of aim2, we will determine how FATP2 inhibition in combination with *de novo* synthesis inhibitors alter NB *in vivo* sensitivity to therapy.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
MYCN amplification occurs in half of high-risk NB patients and strongly correlates with disease progression and treatment failure. Hence, there is an unmet need to identify novel MYCN-dependent pathways and develop effective therapies for high-risk patients. The MYC oncogene is well documented as a master regulator of cell metabolism to support tumor growth. However, how

MYCN reprograms NB tumor metabolism and its impact on tumor growth remain elusive. The overall goal of our study was to identify novel MYCN-driven metabolic alterations that contribute to NB oncogenesis. **By selectively targeting specific metabolic dependencies, we will be able to identify innovative and effective therapeutic approaches for high-risk disease**.
Our data reveal a novel metabolic dependency of MYCN-amplified tumors: MYCN activates lipid metabolism and specifically drives fatty acids uptake to support tumor growth. Pharmacological inhibition of fatty acids uptake effectively blocks tumor growth and sensitizes NB cells to conventional therapy.

What was the impact on other disciplines?
Our rationale for the proposed research is that it will reveal novel pathways and modes of regulation that will provide us with new and more sensitive therapeutic targets for MYCN amplified NBs. This will also provide a novel and important approach to intervention for the many human cancers that utilize MYC for oncogenesis. More broadly, we expect that the proposed research will provide new insight into the regulation of energy metabolism in cancer progression, with implications for metabolic syndromes and other human diseases.

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
This approach is exciting because it elucidates novel MYCN-dependent pathways that will serve as targets for NB treatment. Finding novel effective targeted therapies that could be safely included in current regiments for relapse disease has enormous clinical implications.

5. CHANGES/PROBLEMS:

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report.

Changes that had a significant impact on expenditures
Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

Significant changes in use or care of human subjects
Not applicable.

Significant changes in use or care of vertebrate animals
Nothing to Report.

Significant changes in use of biohazards and/or select agents
Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  - This work was presented at the virtual 2021 ANR, Advances in Neuroblastoma Research (ANR) webinar January 25- 26- 27, 2021; https://www.anr2021.org. Oral communication: Targeting Fatty Acid Transport in MYCN-amplified Neuroblastoma. Ling Tao, Myrthala Moreno-Smith, Giorgio Milazzo, Mahmoud A. Mohammad, Nagireddy Putluri, Giovanni Perini, Cristian Coarfa, and Eveline Barbieri.

  - This work is currently under revision in *Nature Communications 2021*. MYCN-driven Fatty Acid Uptake is a Novel Metabolic Vulnerability in Neuroblastoma. Ling Tao, Mahmoud A. Mohammad, Giorgio Milazzo, Myrthala Moreno-Smith, Tajhal Patel, Barry Zorman, Andrew Badachhape, Blanca E. Hernandez, Amber B. Wolf, Jennifer H. Forster, Pavel Sumazin, John Hicks, Ketan B. Ghaghada, Nagireddy Putluri, Giovanni Perini, Cristian Coarfa, and Eveline Barbieri.

  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.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Eveline Barbieri, MD PhD, PI – no changes

Ling Tao, PhD, Postdoctoral Associate – no changes

Mirthala Moreno Smith, PhD, Research Associate – no changes

Nagireddy Putluri, PhD, Co-Investigator – no changes

Sanjeev A. Vasudevan, MD, Co-Investigator – no changes

Cristian Coarfa, PhD, Co-Investigator – no changes

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:
Nothing to Report.

QUAD CHARTS
Nothing to Report.

APPENDICES:
Nothing to Report.