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

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8. PERFORMING ORGANIZATION REPORT

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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. Based on our data and the recent literature, our guiding hypotheses are that: a) lipid metabolism is required for NB tumorigenesis, and b) targeting MYCN-driven lipogenesis will effectively block NB tumor growth. We have demonstrated that lipid metabolism is a selective metabolic dependency of MYCN-driven tumors. MYCN drives both fatty acid (FA) synthesis and FA uptake to maintain NB cell survival. Targeting FA uptake effectively blocks NB in vivo tumor growth.

15. SUBJECT TERMS Neuroblastoma, MYCN, fatty acid metabolism, targeted therapies.					
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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)

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.

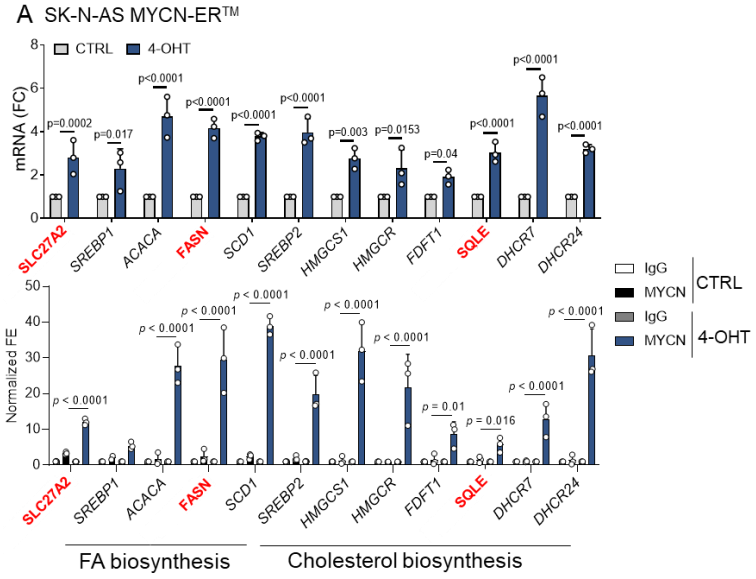
What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

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

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

1.1 Sub-aim 1.1 was completed and results are summarized in our recent publication:
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**. *Nature Communications* 2022, https://doi.org/10.1038/s41467-022-31331-2.

In addition, to investigate how MYCN regulates lipid metabolism we performed MYCN ChIP and q-PCR analyses in the SKNAS MYCN-ER system ± 4-OHT, where upon 4-OHT MYCN translocates to the nucleus and activates transcription. We found that MYCN directly binds to lipogenic loci involved in *de novo* lipogenesis (ACACA, FASN, SCD1), and cholesterol (not shown) and triglycerides (TG) synthesis (DGAT1 and DGAT2) upregulating their expression. This leads to cell neutral lipid accumulation (**Fig. 1A**). We have shown that MYCN directly regulates the expression of factors that drive lipid synthesis, including FASN (FA biosynthesis), SQLE (cholesterol biosynthesis) and FATP2 (FA uptake). NB are highly dependent on these lipid nodes and the high expression of these enzymes predicts NB clinical outcomes. The mRNA levels of these enzymes progressively increase during spontaneous tumor development (TH-MYCN GEMM, not shown) and strongly associate with MYCN amplification (**Fig. 1B**).



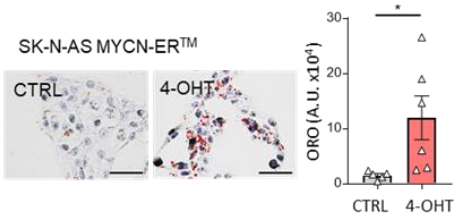


Fig. 1A. MYCN directly activates the transcription of key FA and cholesterol biosynthesis enzymes. Top: FA transporter *SLC27A2*, and FA and cholesterol biosynthesis enzymes mRNA expression in SK-N-AS MYCN-ER™ cells (± 4-OHT to induce MYCN transcriptional activity). Mean±SEM (n=3). FC= Fold Change. Middle: MYCN ChIP-qPCR assays in SK-N-AS MYCN-ER™ cells (± 4-OHT). Mean±SD (n=3). FE= Fold Enrichment. Bottom: Oil Red O (ORO) staining (neutral lipids) in SK-N-AS MYCN-ER™ cells (± 4-OHT) (n=6).

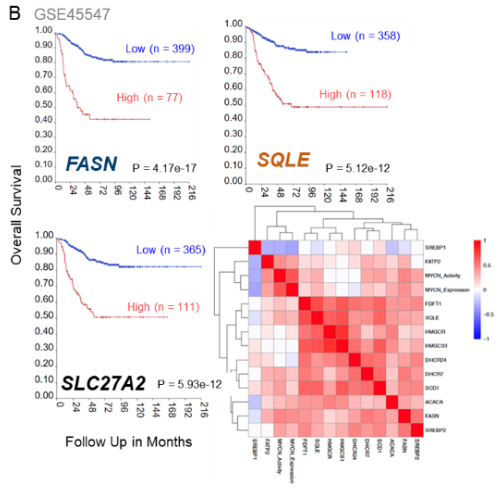


Fig. 1B. High expression of lipogenic enzymes predicts poor clinical outcome and associates with MYCN activity/expression. Survival analyses (OS) in NB cohort GSE45547 (n=649) for patients with high or low *FASN*, *SQLE*, and *SLC27A2* expression. Bottom right: correlation matrix of FA and cholesterol gene expression and MYCN expression/activity. $P < 0.05$; Red = positive; blue = negative.

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

To assess the impact of fatty acid (FA) synthesis and uptake on cell survival, we evaluated NB cell viability (MYCN-amplified, MNA, and non MYCN-amplified, non-MNA) and normal cells after treatment with two FA synthesis inhibitors (A939572, an SCD1 inhibitor and orlistat, an FASN inhibitor) and two FA uptake inhibitors (CB16.2 and CB5, which target FATP2). NB cells were more sensitive to FA uptake inhibition (MNA IC50: 0.5–7.9 µM; non-MNA IC50: 1.4–10.4 µM) than to FA synthesis inhibition (MNA IC50: 7.6–60.7 µM; non-MNA IC50: 9.2–77.5 µM), suggesting that **NB cells actively use exogenous FA for survival**. Moreover, NB cells were highly dependent on exogenous FAs and deprivation of FAs induced cell growth arrest. CB16.2 was toxic to all tested normal cells, despite having the highest efficacy in NB cells. Conversely, CB5 was highly effective in NB cells and did not elicit cytotoxicity against normal cells (**Fig. 2**). MYCN enhances SCD1 activity, whereas A939572 suppresses SCD1 activity ($p<0.0001$) and *de novo* FA synthesis ($p<0.05$) in MNA cells. However, SCD1 inhibition did not effectively inhibit cell growth and stimulated compensatory dose-dependent FA import from the media, suggesting that **exogenous FA uptake may reduce cell sensitivity to FA synthesis inhibition.** To test this hypothesis, we evaluated the viability of MNA cells in complete and delipidized media with and without A939572. The removal of exogenous lipids significantly enhanced the cytotoxicity of A939572, which was partially rescued by FAs supplementation. Pharmacological inhibition of FA uptake via CB5 also enhanced the cytotoxic effects of A939572 and increased cell apoptosis ($p<0.05$). These results suggest that NB cells import exogenous FAs as a compensatory mechanism to evade FA synthesis inhibition. We are currently targeting in the lab FASN with a highly potent, specific, and reversible inhibitor, TVB-2640, which is currently under several Phase I/II clinical trials for solid tumors (NCT03808558, NCT03179904, and NCT03032484). Preliminary data from patients with breast cancer suggest prolonged disease control when given with cytotoxic chemotherapy. Compared with previous FASN inhibitors (orlistat), TVB-2640 inhibits the ketoacylreductase (KR) enzymatic activity of the FASN enzyme with a great safety profile due to the lack of indirect activation of CPT1 in peripheral tissue.

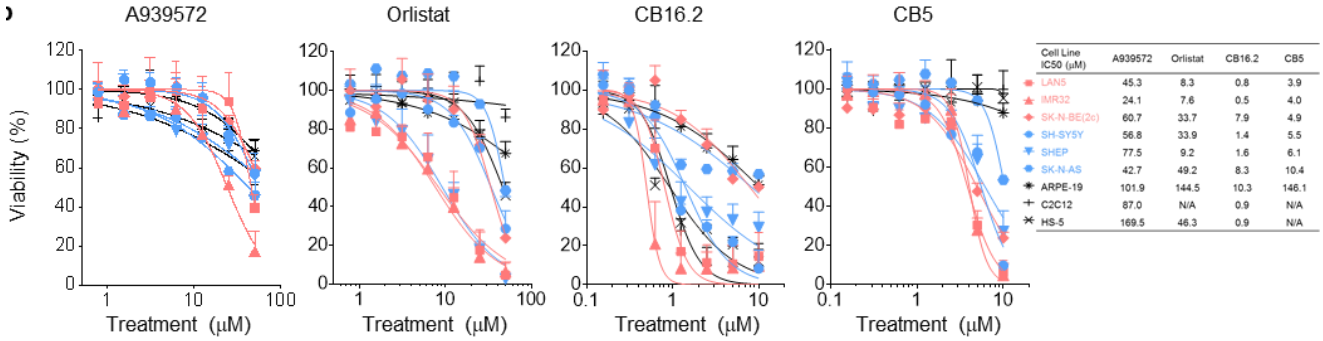


Fig. 2: Viability of NB and normal cells upon 72 h treatment with FA synthesis inhibitors (A939572, orlistat) and FA uptake inhibitors (CB16.2 and CB5). IC50 calculated in GraphPad Prism (7.01). Mean±SD (n=3).

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

Recently, our laboratory through integrated analyses of tumor metabolome, isotope-traced metabolic pathways, and gene expression profiling in primary human NB tumors and cell lines has shown that MYCN induces distinct changes in lipid metabolism to drive NB oncogenesis. Using different MYCN-driven *in vitro* and *in vivo* models of NB and multiple integrated metabolic approaches, we have comprehensively characterized the metabolic landscape of NB cell lines and

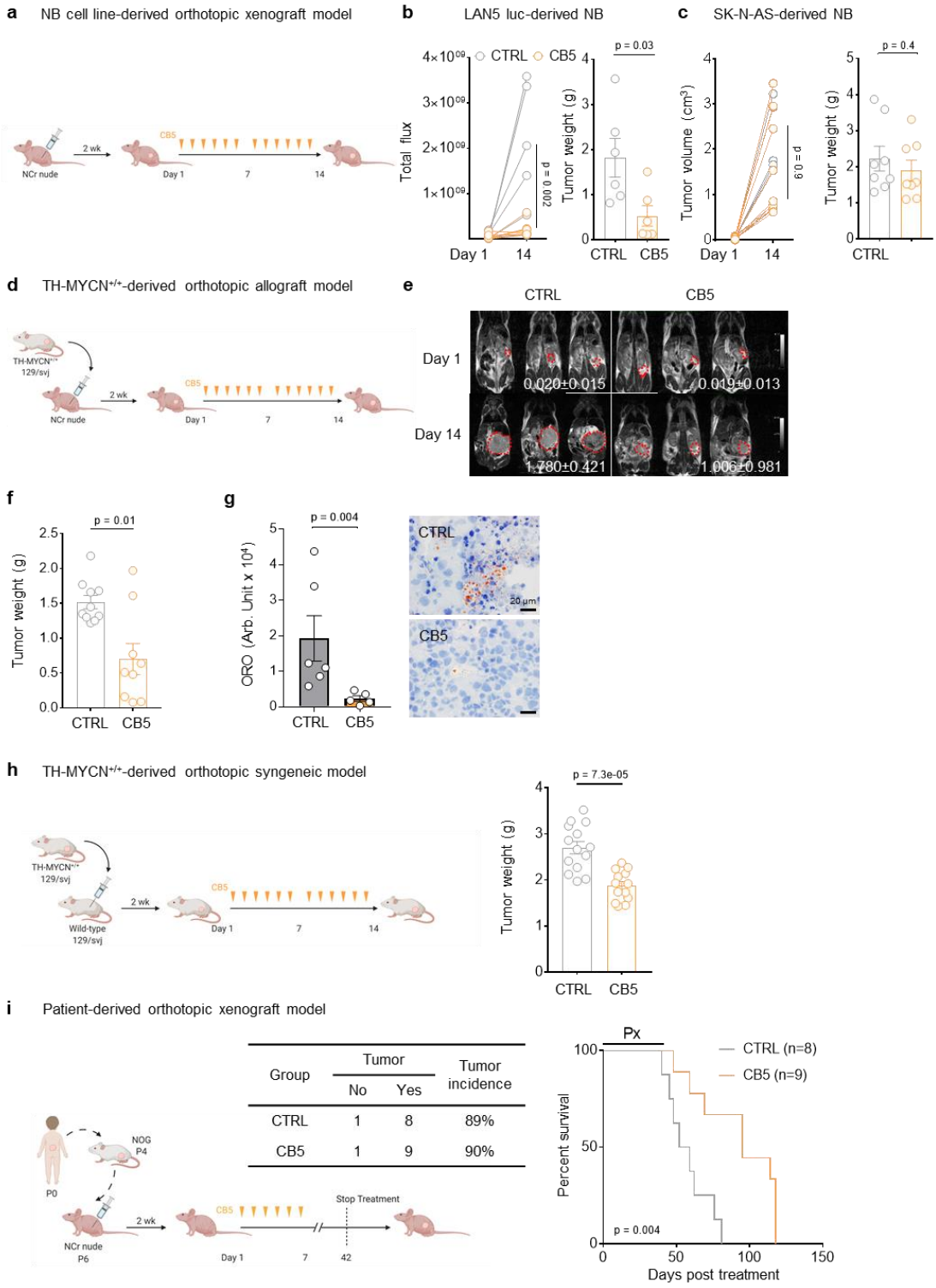
tumors derived from both TH-MYCN GEMM and MNA patients, and found that MYCN promotes the abundance of FAs required for glycerolipid accumulation. We then correlated lipid species with the expression of lipid metabolism-related genes and found that **MYCN profoundly alters distinct glycerolipid classes, including diacylglycerides (DGs) and triacylglycerides (TGs)** (Nature Comm. 2022). We have also established an *in vivo* ShMYCN mouse model, which showed reduced growth upon doxycycline treatment. We are currently developing in the lab a genetically engineered mouse model (GEMM) with Cre-conditional induction of MYCN in dopamine β-hydroxylase-expressing cells (LSL-MYCN), which in will be of help in confirming these lipogenic features in MYCN-driven NB. These results are summarized in our recent publication **Nature Comm. 2022**, https://doi.org/10.1038/s41467-022-31331-2.

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

To determine the contribution of FA transport to tumor growth, we evaluated the anti-tumor activity of CB5 in multiple preclinical NB models. We orthotopically implanted MNA LAN5 cells and non-MNA SK-N-AS cells into the renal capsule of NCr nude mice. After tumor engraftment, mice were randomly assigned to CTRL (vehicle) or CB5 [25 mg/kg, twice a day (b.i.d.)] treatment groups. CB5 significantly inhibited the growth of LAN5 xenografts as measured by luciferase activity ($p=0.002$) and tumor weights ($p=0.03$, **Fig. 3B**). However, CB5 did not reduce tumor volumes and weights of SK-N-AS xenografts (**Fig. 3C**), suggesting that CB5 preferentially inhibits MNA tumors. Notably, CB5 treatment did not cause toxicity assessed by mouse general clinical conditions and weight changes during treatment. The TH-MYCN transgenic model is an aggressive MYCN-induced *de novo* NB model. To assess the anti-cancer activity of CB5, we generated an orthotopic allograft model of NB by implanting a TH-MYCN+/+ tumor into NCr nude mice (**Fig. 3D**). Tumors developed after 2 weeks, at which time mice were randomly assigned to CTRL (vehicle) or CB5 (25 mg/kg, b.i.d.) treatment groups. MRI was performed on treatment days 1 and 14 to monitor tumor growth. CB5 significantly reduced tumor volumes ($p=0.006$, **Fig. 3E**) and weights ($p=0.01$, **Fig. 3F**) in this model, and no signs of toxicity were observed during the study. Moreover, mice responsive to CB5 treatment showed lower neutral lipid levels than CTRL mice ($p=0.004$, **Fig. 3G**), suggesting that **CB5 blocks lipid accumulation *in vivo***. This is likely due to the CB5-mediated inhibition of MYCN and MYCN-targeted FA synthesis and transport protein expression (data not shown).

To evaluate the efficacy of blocking FA transport in the presence of an intact TIME, we generated a mouse-derived allograft model by implanting a TH-MYCN+/+ tumor into the renal capsule of WT 129x1/svj mice. After 2 weeks, mice were treated with either CTRL (vehicle) or CB5 (30 mg/kg, b.i.d. i.p.) for 2 weeks. CB5 treatment blocked tumor growth without apparent toxicity (**Fig. 3H**, $p<0.0001$). To evaluate the long-term effects of blocking FA uptake in MNA NBs, we used a patient-derived orthotopic xenograft (PDX) model. We asked whether blocking FA uptake through long-term CB5 treatment could prevent MNA tumor development and prolong animal survival. CB5 treatment was applied 2 weeks after implantation when tumors had not initiated. Mice received CTRL (vehicle) or CB5 (25 mg/kg, b.i.d., i.p.) for 6 weeks and tumor growth was monitored by MRI. CB5 did not prevent tumor initiation. However, chronic CB5 treatment significantly prolonged animal survival ($p=0.004$, **Fig. 3I**) without notable toxicity, suggesting that **blocking FA uptake can suppress primary MNA tumor growth and extend survival**.



Group	Tumor		Tumor incidence
	No	Yes	
CTRL	1	8	89%
CB5	1	9	90%

Fig. 3: NB cell line-derived orthotopic xenograft model. **(a)** LAN5 or SK-N-AS cells were orthotopically implanted in NCr nude mice. Two weeks later mice were treated with vehicle or CB5 (25 mg/kg, b.i.d.) for 2 weeks. **(b)** LAN5 tumor sizes and weights after treatment. Mean±SEM (CTRL=6, CB5=6). **(c)** SK-N-AS tumor volumes (MRI) and weights after treatment. Mean±SEM (CTRL=8, CB5=8). (d–g) TH-MYCN+/+-derived orthotopic allograft model. **(d)** Cells from one TH-MYCN+/+ tumor were orthotopically implanted in NCr nude mice. Two weeks later mice were treated with vehicle or CB5 (25 mg/kg, b.i.d.) for 2 weeks. **(e)** Tumor volumes (MRI) on treatment days 1 and 14 (CTRL=10, CB5=9). (f) Tumor weights at treatment day 14. Mean±SEM (CTRL=10, CB5=9). **(g)** Oil Red O staining of intratumoral lipids. Mean±SEM (CTRL=6, CB5=5 responsive tumors); two-sided unpaired Mann-Whitney test. **(h)** Cells from one TH-MYCN+/+ tumor were orthotopically implanted in syngeneic 129x1/svj wt mice. Two weeks later mice were treated with vehicle or CB5 (30 mg/kg, b.i.d.) for 2 weeks. Mean±SEM (CTRL=14, CB5=13). **(i)** Cells from one MNA patient tumor (P6) were orthotopically implanted in NCr nude mice, and 2 weeks later mice were treated with vehicle or CB5 (25 mg/kg, b.i.d.) for 6 weeks. Tumor incidence analyzed by Fisher's exact test (CTRL=8, CB5=9).

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

We have not tested the anti-tumor activity of combined FASN and FATP2 inhibition due to the fact that this combination was too toxic in our NB *in vitro* models. The combination with conventional chemotherapy had a better profile and was more effective (see below). We are currently testing the anti-tumor activity of TVB-2640 in our pre-clinical models of NB.

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

This sub-aim was completed and results are summarized in our recent publication:
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**. *Nature Communications* 2022, https://doi.org/10.1038/s41467-022-31331-2.

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

Ling Tao Ph.D., post-doctoral associate (100% effort) learned new lab skills necessary for this project, including stable isotope tracing, FA profiling, and additional *in vivo* models. These skills have expanded 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 future research efforts. Throughout the project, she has also developed critical skills that will help foster her academic career. To improve her writing and data analysis skills, and expand her knowledge on translational research she attended the BCM courses on Scientific Writing, Explorative Data Analysis, and Translational Cancer Biology. Recently, she trained one SMART program undergraduate student and one visiting postdoctoral fellow. She has also taught the BCM Molecular Refresher Course on different topics (i.e. cell cycle and chromosome stability, and transcription regulation and RNA sequencing). She has presented her work at multiple avenues including 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, where she presented the results of this project as oral communication (ANR 2021). She was offered an Assistant Professor position at Fudan University, China in 2022.

How were the results disseminated to communities of interest?

TCH Annual Symposium 2023. Targeting lipid metabolism in neuroblastoma. Lingzhi Li – *Oral Presentation*.

Advances in Neuroblastoma Research (ANR) 2021; virtual. Targeting Fatty Acid Transport in MYCN-amplified Neuroblastoma. Ling Tao, Myrthala Moreno-Smith, Giorgio Milazzo, Mahmoud A. Mohammad, Nagireddy Putluri, Giovanni Perini, Cristian Coarfa, Eveline Barbieri – *Oral Presentation*.

New York Academy Cancer Metabolism Symposium 2020; virtual. Targeting Fatty Acid Transport in MYCN-amplified Neuroblastoma. Ling Tao, Myrthala Moreno-Smith, Giorgio Milazzo, Mahmoud A. Mohammad, Nagireddy Putluri, Giovanni Perini, Cristian Coarfa, Eveline Barbieri – *Poster Presentation*.

What do you plan to do during the NCE to accomplish the goals?

N/A

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

N/A

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**

  Journal publications.
  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**. *Nature Communications* 2022, https://doi.org/10.1038/s41467-022-31331-2.

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

  Nothing to Report.

  Other publications, conference papers and presentations.

  - Virtual 2021 ANR. Advances in Neuroblastoma Research (ANR) webinar January 25-26- 27, 2021; https://www.anr2021.org. Oral communication.

- **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:

Not applicable.

QUAD CHARTS:

Not applicable.

9. APPENDICES:

Not applicable.