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14. ABSTRACT
This "Idea award" project evaluates a novel technique of microperfusion to sample the live extracellular glioma microenvironment. IDH-mutant gliomas are particularly vulnerable to NAMPT inhibitors, which help deplete tumor cells of NAD, a key metabolite for cellular function. We test three aims: 1) Can extracellular NAD provide a reflection of intracellular NAD, thereby discerning the intended effect of NAMPTi?; 2) what are the extracellular metabolic alterations that reflect relative sensitivity/resistance to NAMPTi?; 3) What are the candidate biomarkers of cytotoxicity? In the prior year, we established the bilateral murine microperfusion system in patient-derived glioma xenografts (PDXs), in addition to the analysis methods to assay the candidate metabolic biomarkers for each aim. This year, we evaluated the global metabolomic impacts of NAMPT inhibition on NAMPT sensitive or resistant IDH mutant or wild-type glioma xenografts via microperfusion. Findings to date suggest that the tumor-derived metabolic signature, with or without NAMPT inhibition, is strongly enriched for plasma-derived metabolites that resolves over time. Toward next year's goals, we also performed pilot experiments to identify biomarkers of cytotoxicity using diphtheria toxin. The next year will be spent further evaluating the pharmacodynamic impact of NAMPT inhibition and cytotoxicity in these animal models, leveraging updated methods such as isotope tracing and microdialysis to deconvolute the tumor-specific metabolic signature from red blood cell-derived analytes.

15. SUBJECT TERMS
Microperfusion, NAMPT, NAD, glioma, IDH

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1. **INTRODUCTION:**

Gliomas are the most common primary brain tumors in adults. Despite concerted research endeavors, no new drug has surpassed the efficacy of temozolomide since 2005, although these tumors still invariably recur fatally. We do not understand why and how our therapies are failing. As such, we posit that the lack of therapeutic advancements against glioma is due in part to limited feedback obtained from each patient's tumor in response to novel therapies. We posit that this stagnation arises partly from the lack of detailed insights into how a patient's glioma responds at the molecular level to potential treatments. Such invaluable insights could enable early detection of treatment failure or response in order to optimize and tailor effective therapies for each patient. To that end, a subset of gliomas harbor an isocitrate dehydrogenase (IDH) mutation, rendering these tumors more dependent on NAMPT to generate a key cofactor, NAD+. NAMPT inhibition presents a novel opportunity to evaluate the potential to discern pharmacodynamic and cytotoxic extracellular impacts of therapies in glioma. As such, in this study, we explore microperfusion as a novel technique to study the glioma's extracellular microenvironment during the pharmacological reduction of NAD+ via NAMPT inhibition. In this Idea Award, we have proposed to 1) determine the impact of NAMPT inhibition on intracellular/extracellular NAD+ as a pharmacodynamic biomarker; 2) determine the impact of NAMPT inhibition on the glioma extracellular metabolome; and 3) identify potential extracellular markers that might signify therapeutic success as compared to effective cytotoxicity.

2. **KEYWORDS:** NAD+, glioma, metabolism, microperfusion, NAMPT, drug discovery, ATP, extracellular, cytotoxicity

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Major task / Aim 1: Determine the dynamic relationship between intracellular and extracellular NAD+ upon NAMPT inhibition within IDH-mutant glioma **(year 1)**. Milestone(s): Conclude whether or not NAD+ represents a valid candidate pharmacodynamic biomarker of NAMPT inhibition. *~70% complete.*

Major task / Aim 2: Identify which extracellular metabolites convey a pharmacodynamic response to NAMPT inhibition, to generate candidate biomarkers of therapeutic efficacy **(year 2).** Milestone(s): Conclude whether or not the extracellular metabolic signature can be leveraged as a pharmacodynamic biomarker of NAMPT therapy. *~50% complete*

Major Task / Aim 3: Evaluate the quantitative performance of extracellular D-2-hydroxyglutarate (D-2-HG), methylthioadenosine (MTA), and dimethylarginine as biomarkers of therapeutic efficacy against IDH-mutant glioma **(year 3)**. Milestone(s): Conclude whether or not D-2-HG, MTA, or DMA represent valid candidate extracellular biomarkers of therapeutic efficacy and local tumor burden. *~20% complete.*

1) **<u>Major Activities:</u>**

A) Performing bilateral microperfusion of tumor and brain in patient-derived IDH-mutant or wild-type glioma xenografts that are or are not sensitive to NAMPT inhibition.
B) Performing highly sensitive reverse cyclase NAD+ assays to evaluate the impact of NAMPT inhibition on glioma PDXs.
C) Determining the global metabolomic impact of NAMPT inhibition on NAMPT sensitive and resistant glioma PDX, using A132, as well as concurrent treatment with temozolomide (TMZ) for synergy or nicotinic acid (NA) for rescue.
D) Evaluating preliminary signatures of cytotoxicity using diphtheria toxin in glioma PDXs.
E) Understanding how catheter placement impacts the global extracellular metabolome of IDH-mutant gliomas, and the impact of temozolomide on mitigating this signature.

2) **Specific Objectives:**

Major Task/Aim 1: Determine whether or not NAD+ represents a valid candidate pharmacodynamic biomarker of NAMPT inhibition.

Subtask 1: Establish the time-course of intracellular and extracellular NAD+ in intracranial IDH-mutant glioma versus brain upon pharmacologic and genetic NAMPT inhibition, *in vivo*. We previously reported on the development of a mass spectrometry assay for A132 through the Mayo Clinic Pharmacology Core facility. Initial data suggested that A132 was CNS penetrant and that the intratumoral drug concentration at 24 hours were similar to the drug's *in vitro* IC50. A132 could also be detected within microperfusate, we reported that peak free drug levels occurred between 90 minutes and 3 hours after dosing; microperfusate levels were approximately 50% of tissue levels, suggesting potential for pharmacokinetic monitoring. Additionally, to detect very low levels of NAD+ in brain and interstitial fluid, we developed a custom NAD cycling assay with sensitivity down to 50 femtomoles, based on using resorufin fluorescence as an indicator of total NAD+/NADH. When A132 was evaluated in a NAMPT inhibition-sensitive glioma PDX, NAD+ levels in tumor decreased to nearly undetectable levels by 48 hours prior to rebounding above baseline by 72 hours, the latter of which did not occur in normal brain. These results suggested either metabolic compensation via alternative pathways or release of intracellular NAD+ upon effective cell lysis. Although we remain cautiously optimistic about the utility of this assay, we are systematically working to attenuate multiple sources of background NAD+ signal that appears to hamper ability to discern modest changes in extracellular NAD+. These are discussed in greater detail in section 5. Briefly, however, we have needed to revise our protocol to dialyze out low levels of contaminating NAD+ from purchased reagents. Blood contamination of microperfusate samples has become a significant challenge, prompting us to perform renovations of our lab space for prolonged housing of mice. This prolonged experimentation will mitigate the microtrauma of daily catheter insertion/removal. The addition of microdialysis experiments will also be used to exclude blood cells from the collection sample.

Subtask 2: Determine the relative impact of NAMPT inhibition on intracellular versus extracellular NAD+.

Our original proposal leveraged a previously described methodology for centrifugation of tumor tissue to extract extracellular fluid from the bulk tumor for comparison to a paired extracellular fluid-depleted tissue specimen. Our team and other have attempted to replicate this previously published protocol. Unfortunately, it appears that the lipid-rich nature of the infiltrative glioma within the brain microenvironment is prohibitive to meaningful separation of extracellular and intracellular fluid compartments. Higher centrifugation speeds lead to cell damage and cross-contamination of extracellular samples with intracellular contents. Efforts to address this issue *in vitro* are hampered in part by the relatively large extracellular fluid volumes of *in vitro* cultures, reducing sensitivity small changes in a very low abundance metabolite. If further optimization is unsuccessful, bulk tissue samples which are stored from each animal will be leveraged for comparative analyses leveraging optimized methods to mitigate extracellular sample contamination with sources of intracellular NAD+.

Subtask 3: Compare the therapeutic impact on tissue and microperfusate NAD+ to relative tumor size by bioluminescence

We previously reported that we did not observe any change in bioluminescence in a luciferase-positive IDH mutant glioma PDX with A132 administration. However, we then realized that these cells had stochastically lost the IDH mutation that would render them sensitive to NAMPT inhibition. As an alternative approach to evaluate the impact of A132 on NAMPT sensitive glioma PDX burden based on tumor bioluminescence (BLI), and the correlation thereof to NAD+, we administered A132 to GBM6, which is a NAMPT-sensitive glioma PDX line. This required developing a GBM6 line that stably expressed luciferase which was available in early 2023. We confirmed the ability to detect intracranial GBM6 via tumor BLI in an experiment with diphtheria toxin and are now evaluating the impact of A132 on tumor BLI.

Major task / Aim 2: Identify which extracellular metabolites convey a pharmacodynamic response to NAMPT inhibition, to generate candidate biomarkers of therapeutic efficacy.

Subtask 1: Evaluate the extracellular metabolome of NAMPT inhibition within NAMPT-sensitive vs NAMPT-resistant intracranial gliomas, including both IDH-mutant and IDH-WT gliomas.

Summary of key findings from year 1 progress report: Prior to determining the extracellular impact of NAMPT inhibition on NAMPT sensitive and NAMPT resistant gliomas, we needed to establish the baseline metabolome. To that end, our year 1 progress report (Figure 6) reported our initial findings on the most differentially abundant metabolites in IDH-mutant glioma (GBM164) when compared to an internal control (contralateral brain). As expected, 2-hydroxyglutarate (2-HG) was the most differentially abundant metabolite as a well-established oncometabolite of IDH-mutant tumors.

This past year, having identified this baseline signature, we had then asked how TMZ impacts tumor and brain metabolism over 72 hours toward subtask 2 of this aim. To do so,

we also utilized vehicle-treated animals as a control for the impact of catheter placement on the tumor and brain metabolome. We had hypothesized that placement of the catheters would likely minimally impact these metabolomes as compared to the impact of drug, especially since we utilize the accepted best practice protocol of allowing a day for re-annealing of the blood brain barrier after cannula placement prior to starting experiments. Importantly, we have now determined that there is a strongly conserved metabolic signature of catheter placement on both the tumor and brain metabolomes at the 72-hour time-point when compared to the first "0 h" timepoint (Fig. 1). This finding, which is visually apparent from heatmaps of metabolite ranks, was confirmed statistically by use of enrichment analysis, which demonstrated overlapping metabolomes for the impact of catheter on brain over time across all animals at FDRs<0.05.

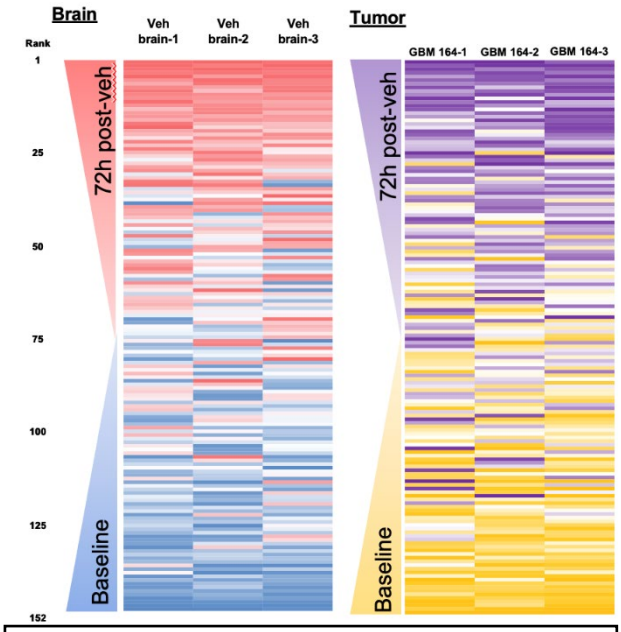

Fig. 1. Catheter insertion has a metabolic impact on glioma patient-derived xenografts and brain.

This impact was particularly profound in the brain, wherein catheter placement resulted in the significant upregulation of over thirty metabolites ($p \leq 0.05$; FC>2; Fig. 2). The impact of vehicle on tumor resulted in upregulation of only 7 metabolites (Fig. 2), likely due to the other injury-associated metabolites already being upregulated in tumor at baseline. Observed consistency in the ranked heatmap, even with inherently heterogeneous tumor samples, suggests that more samples may be needed to detect the potentially smaller fold change over and above that of the baseline tumor signature. In summary, these findings demonstrate that catheter insertion has a significant impact on the metabolome in both tumor and brain. To our knowledge, this metabolic impact of catheter insertion has not been clearly described in the past, and may be of analytical importance to accurately discerning the

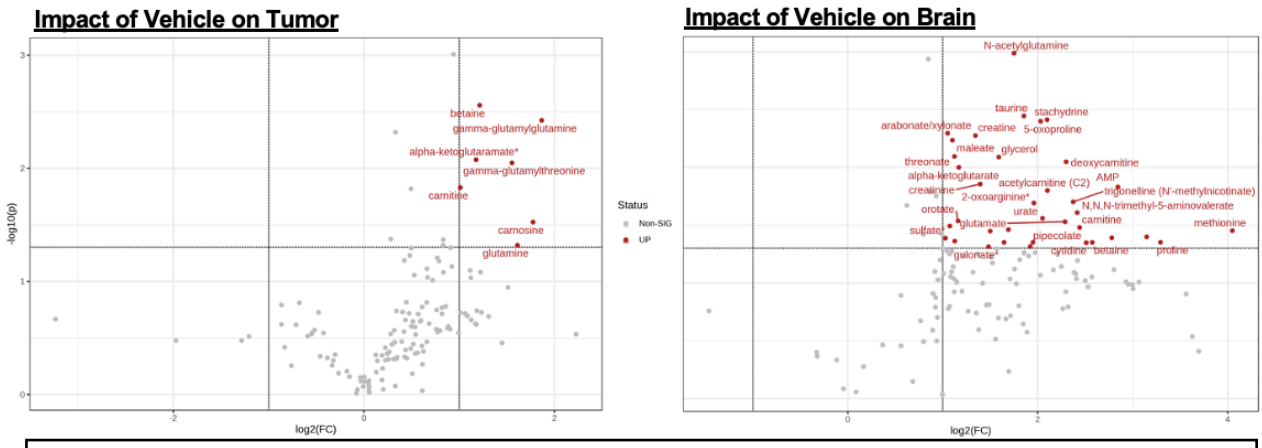

Fig. 2. Catheter insertion results in upregulation of a subset of metabolites in tumor (left) and brain (right) at 72 hours after insertion as compared to baseline.

metabolic pharmacodynamic impacts of candidate therapies in microdialysis/microperfusion experiments.

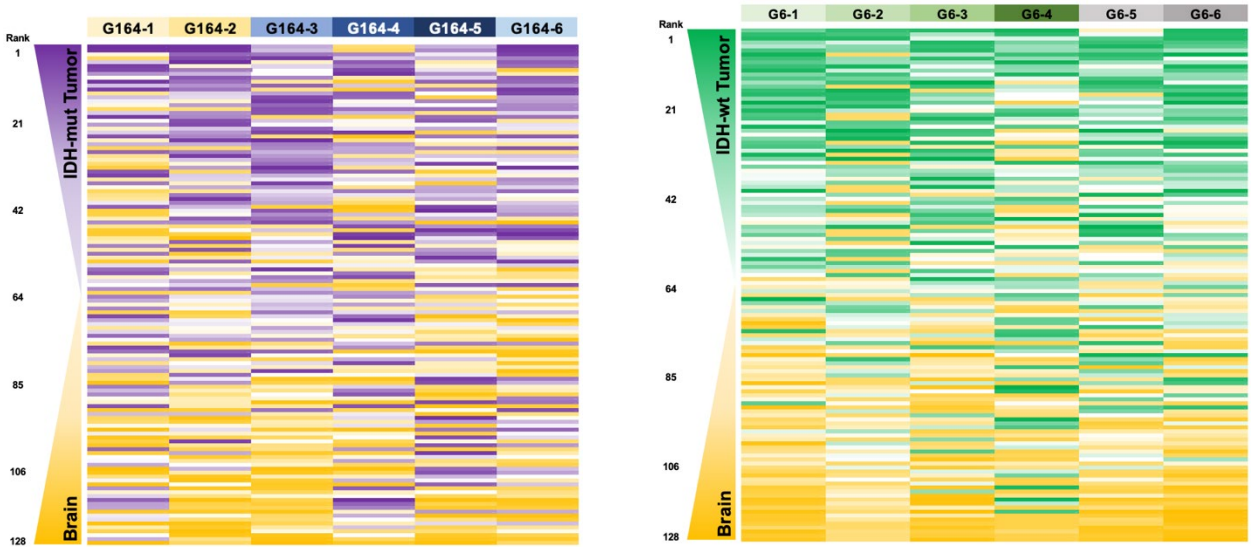

Fig. 3. Tumor metabolic signatures of IDH-mutant (left) and IDH-wild type (right) glioma PDXs as compared to brain based on bilateral microperfusion.

Having now determined that catheter insertion impacts the extracellular metabolome, we then began to evaluate the metabolic signatures of IDH-mutant and wild-type glioma PDXs obtained via microperfusion, both with and without A132. To do so, we calculated the fold-changes for each metabolite between the tumor and brain, and ranked them for comparison across animals. In comparison to the microdialysate data, data obtained via bilateral microperfusion of GBM164 and brain revealed greater heterogeneity across the animals when each tumor was compared to contralateral brain (Figure 3, left). Additionally, evaluation of 2-hydroxyglutarate revealed low or non-existent levels of 2-hydroxyglutarate in a small subset of tumors, suggesting heterogeneity for the IDH-R132H mutation in the replacement GBM164 cell culture despite confirmation of D-2-HG in the new cell culture. Variable tumor growth rates may also have contributed to relatively modest tumor size in some animals. Despite this heterogeneity, some of the more consistently upregulated metabolites in tumor included various phosphatidylcholines, which are found in cell membranes, citraconate/glutaconate (organic acids), butyrate (short chain fatty acid), 3-hydroxybutyrate (ketone body), and itaconate (an intermediate of the Krebs cycle that is central to immunometabolism and anti-inflammation). Similar analyses were performed for IDH-wild type GBM6, which revealed a relatively more consistently enriched signature across animals, although certain animals were outliers, such as G6-4. Evaluation of the fold-tumor-to-brain changes in this animal revealed that only 3 metabolites had fold-changes > 1.5 in this animal, suggesting that there was insufficient tumor present for microperfusion; imaging corroborated this finding. Metabolites that were generally elevated in IDH-wild type as compared to brain included many of the metabolites previously found via microdialysis in GBM164 (Fig. 6 from prior progress report), such as uridine, carnitine, and proline, and agreed with recently published data from our ongoing human microdialysis experiments (not funded by DOD, see PMID37340056). However, data obtained via microperfusion appeared

less consistent than the metabolic signature found via microdialysis. Of note, heme was variably elevated across samples, indicating that blood components may be contributing to heterogeneity across samples. We recently reported that microdialysate of human high-grade glioma is relatively enriched for plasma-associated metabolites when compared to brain, perhaps due in part to relative disruption of the blood-barrier in high-grade tumors (PMID37340056). However, microdialysis excludes red blood cells, whereas whole cells may be recovered by microperfusion—the intracellular contents of which are released when each aliquot is immediately frozen and may therefore impact results.

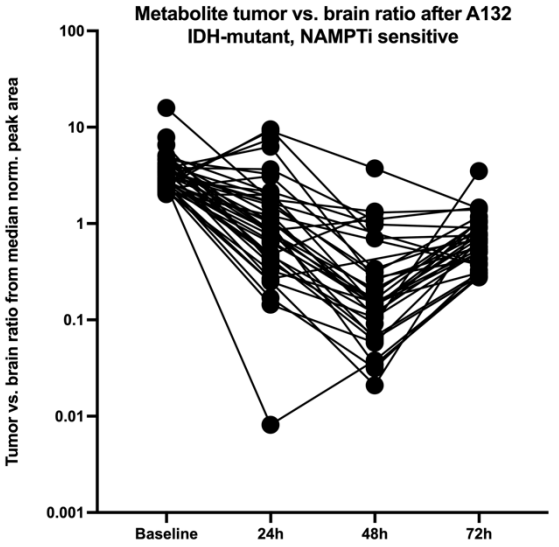

Fig. 4. Impact of A132 (NAMPT inhibitor) on the tumor versus brain ratio of metabolites in IDH-mutant, NAMPTi sensitive glioma. The drug was administered after obtaining baseline ratios.

To minimize batch variance, we had collected samples over the course of several months and sent them all for analysis at the same time utilizing our standardized protocol. As such, the findings of heterogeneity across animals implanted with a single cell line required modified analyses. To help minimize variables, each animal and catheter was leveraged as its own control over time, comparing the post-drug microperfusates to baseline, to help minimize the potential impact of variable tumor burden relative to catheter location. Evaluation of IDH-mutant, NAMPT-inhibitor sensitive glioma revealed a decrease in numerous tumor-associated metabolites based on the tumor versus brain ratio over 48 hours, including carnitines, uridine, ornithine, spermidine, and amino acids including glycine and proline (Fig. 4). Declining carnitine abundance aligned with the hypothesis originally proposed in our Idea Award proposal regarding NAD depletion resulting in accumulation of metabolites upstream of NAD+-dependent enzymes with overflow of cell-permeable metabolites. Alternatively, and more simply, the tumor signature may be mitigated by impaired cellular metabolism. Of note, however, a subset of these metabolites had previously been found in the catheter-associated signature in tumor and brain, suggesting that a component of the A132-related metabolic signature could be attributable to catheter placement, the metabolic signature of which may resolve over time. The metabolic impact of catheter insertion was further supported by presence of highly elevated levels of itaconate, the immunometabolism metabolite associated with anti-inflammation across the GBM PDXs over time (e.g., Fig. 4). However, it is also possible that these metabolites could be attributable to decreases in tumor-associated biology, as carnitines, spermidine, and amino acids each have key roles in glioma metabolism. Mirroring our NAD data from last year's progress report, a subset of metabolites increased at 72 hours despite steadily decreasing throughout 24 and 48 hours. This finding may reflect either lysis of intracellular contents, metabolic adaptation to enable therapeutic resistance, or a combination of both, highlighting the importance of the upcoming Aim 3 studies to help deconvolute the emerging data.

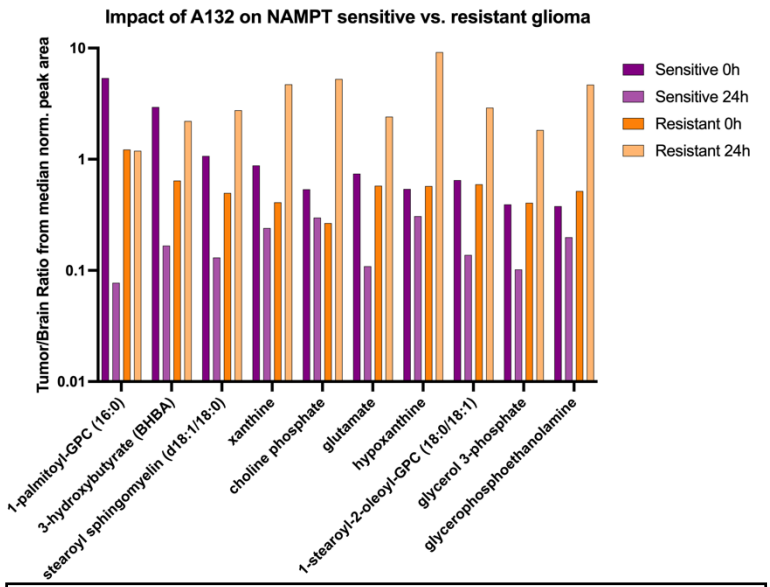

Fig. 5. Differential metabolic impact of A132 on NAMPT sensitive versus resistant glioma.

It is now recognized that chronic NAMPT inhibition facilitates metabolic adaptation in surviving cells within several days. As such, cells lacking NAMPT can provide a robust model of induced resistance to NAMPT inhibition. We therefore evaluated the difference in the extracellular metabolomic response to A132 in NAMPT sensitive versus NAMPT resistant (KO) glioma. Utilizing each mouse's baseline as its control, we observed that phosphatidylcholines/ choline, glycerols, xanthine/hypoxanthine, and glutamate decreased in NAMPT sensitive glioma, but increased in NAMPT resistant glioma—perhaps reflective of the impact of catheter placement itself within the tumor (Fig. 5). These findings suggest that NAMPT inhibition may mitigate a tumor-associated signature that we have now determined exists at baseline within the tumor and may be augmented simply via catheter placement within the tissue. However, given variability at baseline of animals, as well as some of the variability resulting from microperfusion across different days (i.e, variable bloodiness) additional studies will be required to further discriminate between the multiple potential contributors to observed metabolic changes, including via use of microdialysis and with isotope tracing to confirm pharmacologic impacts on metabolic flux through the relevant pathway (further detailed in section 5).

Subtask 2: Evaluate whether or not a NAMPT inhibition-specific specific signature can convey NAMPT inhibition-mediated metabolic changes via use of temozolomide (TMZ), nicotinic acid (NA), and nicotinamide riboside (NR) with bilateral microperfusion.

Summary of key findings from year 1 progress report: We previously performed an initial pilot experiment in mice bearing GBM164 that were treated with TMZ or vehicle. Based on evaluating the top most differentially abundant metabolites between TMZ-versus-vehicle treated tumors, we reported that multiple metabolites increased over time (72h vs baseline) in TMZ-treated tumors, that were not increased with vehicle-treatment, including hypoxanthine/xanthine and the purine nucleoside, inosine, in addition to gluconate. In contrast, the relative abundance at 72h versus baseline for some metabolites was lower in the tumor microenvironment after TMZ as compared to vehicle, including bioenergetic metabolites such as AMP and carnosine.

TMZ GBM 164-1	TMZ GBM 164-2	TMZ GBM 164-3	
6.11	2.22	-1.67	TMZ GBM 164-1: Top 35
1.75	6.19	1.62	TMZ GBM 164-2: Top 35
-1.43	1.7	6.24	TMZ GBM 164-3: Top 35
-6.28	-2.68	2	TMZ GBM 164-1: Bottom 35
-2.95	-6.37	-1.06	TMZ GBM 164-2: Bottom 35
1.48	-0.99	-6.31	TMZ GBM 164-3: Bottom 35

Fig. 6. Enrichment analysis of the impact of TMZ treatment on glioma as compared to baseline.

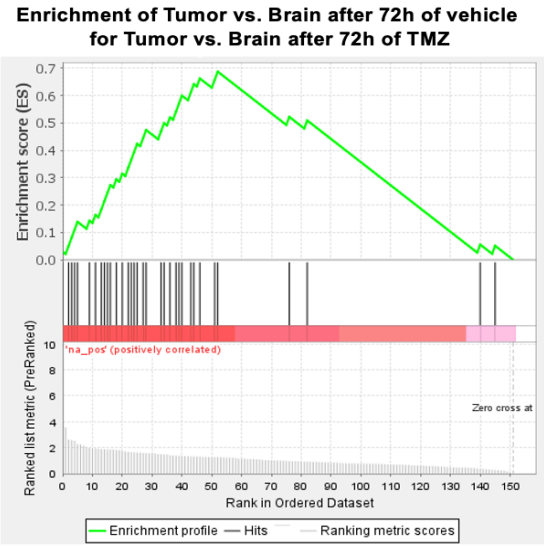

Fig. 7. Enrichment plot of the post-vehicle tumor vs. brain metabolic signature for the post-TMZ tumor vs. brain signature.

Temozolomide is the standard-of-care treatment for patients with high-grade gliomas. As such, novel drugs, including A132, should be tested in combination with TMZ, as any translation into the clinic for primary MGMT- methylated glioma would most likely require co-administration with TMZ. In an initial experiment, we tested the potential synergistic impact of A132 with TMZ. In last year's report, we had asked what metabolites were more upregulated in TMZ-treated tumor than vehicle. However, upon further analysis this year, we here more broadly sought to evaluate the hypothesis that TMZ would have an extracellularly detectable metabolic signature. Enrichment analysis revealed a relatively weaker signature supported by relatively less robust enrichment across animals, when evaluating the impact of TMZ at 72 hours vs baseline, than when comparing 72 hours to baseline in vehicle-treated animals. Based on our finding of a strong metabolic signature induced by catheter insertion in GBM164 (Aim 2, subtask 1), we then asked whether the tumor-associated signature is maintained in the presence of the catheter with and without TMZ treatment. We observed that the tumor-associated signature (when compared to contralateral brain) of TMZ-treated animals remained highly enriched for that of vehicle-treated animals (Fig. 6). We further identified that there was strong enrichment for the

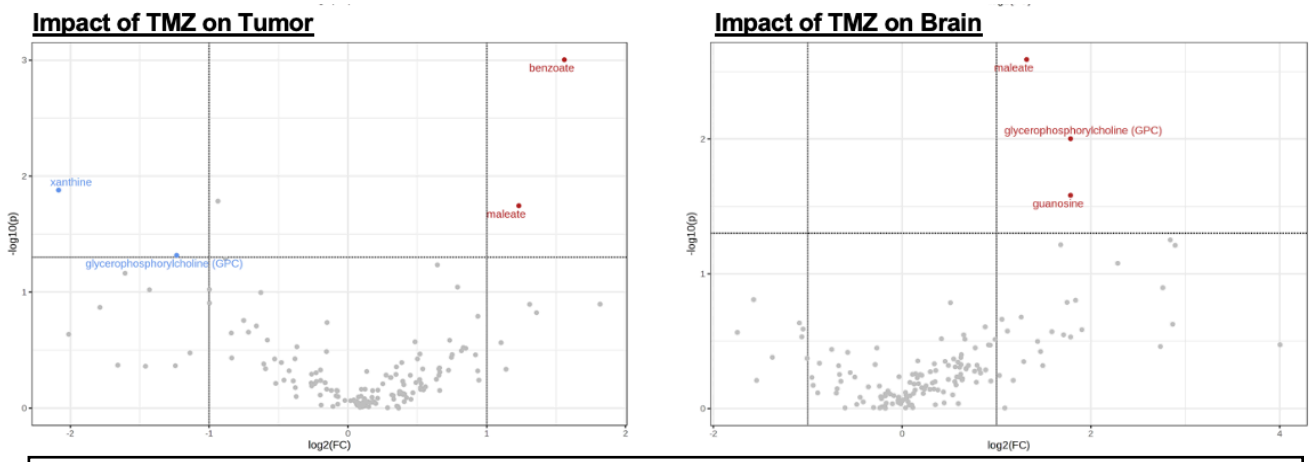

Fig. 8. TMZ results in up/downregulation of 2-3 metabolites in tumor (left) and brain (right) at 48 hours after administration as compared to baseline.

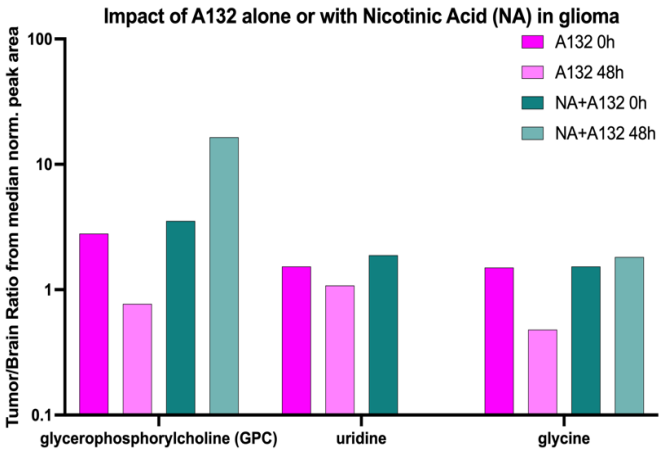

Fig. 9. Metabolic impact of treatment with A132 alone or in combination with nicotinic acid (NA) at 48 hours as compared to baseline.

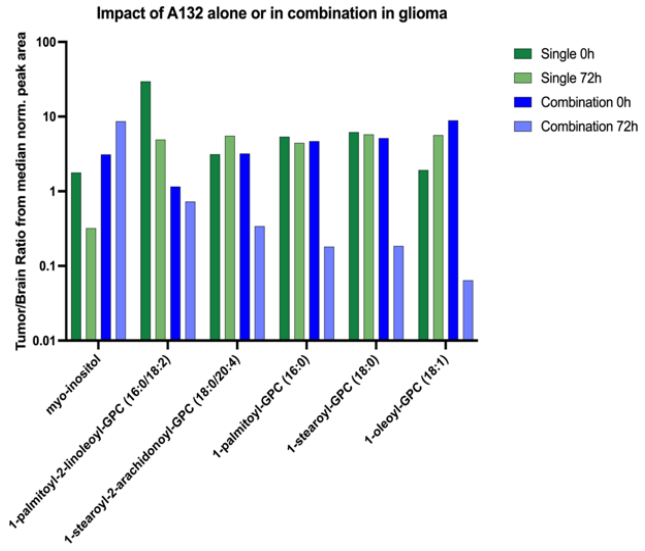

Fig. 10. Metabolic impact of treatment with A132 alone or in combination with nicotinic acid (NA) and temozolomide (TMZ) (combination) at 72 hours as compared to baseline.

tumor versus brain signature of G164 both 72 hours after treatment (TMZ or vehicle) (FDR = 0) (Fig. 7). Unexpectedly, in comparison to vehicle-treated animals, TMZ-treated animals demonstrated a relatively modest number of upregulated metabolites at 72 hours as compared to baseline in both brain and tumor (Fig. 8). This suggested that TMZ may ameliorate injury-induced metabolic upregulation.

We then turned our attention to the impact of NAMPT inhibition with A132 with or without addition of NA, which should help rescue non-sensitive cells in the host to help widen the therapeutic window. For metabolites that were consistently upregulated in tumor, global metabolomic analyses after treatment with either A132 alone or A132 with NA revealed minimal differences (Fig. 9)—perhaps suggesting that NA had relatively little impact within the tumor, as intended. The only consistently tumor-associated metabolites that were different between A132 and A132+NA, as evaluated via brain/tumor ratio, were glycerophosphorylcholine (GPC), uridine, and glycine (Fig. 9). TMZ causes increased cellular depletion of NAD+ increasing potential toxicity, which may be mitigated with NA. Early studies deploying combined administration of A132 and NA with TMZ as compared to A132 alone preliminarily appear to impact a larger cohort of metabolites, including phosphatidylcholines and myo-inositol (a cell-transduction associated carbocyclic sugar) (Fig. 10). Such findings may suggest toxic impacts on the phospholipid bilayer in response to combined use of TMZ+A132, though replication studies are needed.

Major Task / Aim 3: Evaluate the quantitative performance of extracellular D2HG, MTA, and dimethylarginine as biomarkers of therapeutic efficacy against IDH-mutant glioma.

Subtask 1: Relative changes in extracellular concentrations of glioma-associated biomarkers will be evaluated longitudinally upon NAMPT-inhibition and correlated with both BLI and survival. To be completed in year 3.

Subtask 2: Relative biomarker performance will be further assessed via independent cohorts, including combination therapies and unrelated mechanisms of tumor cell ablation.

Although experiments evaluating glioma-associated biomarkers in correlation with tumor bioluminescence (BLI) and survival are part of the year 3 (next year) goals, we have begun pilot studies on this aim. GBM6 is an IDH-wild type glioma xenograft that harbors a CDKN2A/B homozygous deletion. Because of this deletion, 5-methylthioadenosine (MTA) is produced in elevated amounts. GBM164 and 196, the lines proposed for this aim, also harbor this deletion. As such, to preliminarily evaluate the impact of cell death on MTA levels, diphtheria toxin was utilized as proposed in the aim. Preliminarily, a decrease in MTA can be seen by 96 hours after treatment, suggestive that cell death may have occurred by then, hence the decrease in 5-MTA (Fig. 11). Work is ongoing to evaluate 2-HG in IDH-mutant glioma, as well as MTA and dimethylarginine (DMA) in response to NAMPTi (Subtask 1) and other combination therapies/cell ablation (subtask 2).

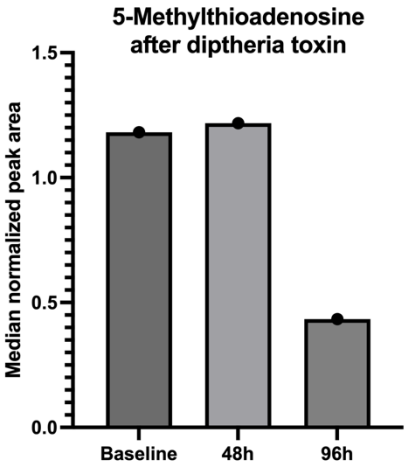

Fig. 11. Metabolic impact of treatment with A132 alone or in combination with nicotinic acid (NA) and temozolomide (TMZ) (combination) at 72 hours as compared to baseline.

3) Significant results and outcomes

Studies and analyses this past year have led to the important finding of catheter insertion-associated metabolic impacts in tumor and brain. Although our initial analytic plan did not adequately anticipate this finding, this observation has implications for analysis of the pharmacodynamic impacts of novel metabolically-targeted therapies via microdialysis/microperfusion. Specifically, our observations suggest that it may be difficult to disentangle the metabolic impacts of catheter insertion from that of the tumor's metabolic signature, particularly as these may share a substantially overlapping metabolome. This finding suggests the need to deploy additional mechanistic tools to elucidate metabolomic impacts of therapy, potentially such as isotope tracing. Isotope tracing is a well-established method that can be utilized to quantitatively evaluate metabolic flux through the tumor, in order to understand if and how certain metabolites are being utilized by the tumor. The clinical relevance of this approach is highlighted by the widespread use of stable isotopes in clinical research, which are not regulated by the FDA, since they are extremely safe and naturally occurring in the environment—albeit in typically very low abundance. We also reproduced our prior observation of a conserved global metabolomic signature of IDH-mutant tumor obtained via microdialysis, characterized by increased 2-hydroxyglutarate, guanosine, BHBA, among other metabolites.

In this Idea Award, we had proposed to leverage microperfusion to increase the abundance and variety of analytes collected from the interstitial fluid. In the past year, we performed global metabolomics analyses on these microperfusate samples. While we had identified a conserved global metabolomic signature of IDH-mutant glioma via microdialysis, this

signature proved more challenging to discern via microperfusion, potentially due in part to variable contamination by RBCs, and their metabolic cargo many metabolites of which appear to overlap the extracellular glioma metabolome, decreasing the signal-to-noise for signature identification. Microperfusion studies moving forward will thus utilize centrifuged microaliquots to help mitigate this variable.

Nevertheless, we adapted our analytical methods to pursue the questions of how 1) NAMPT sensitive vs resistant gliomas respond to NAMPT inhibition, and 2) how addition of NA and TMZ impact the metabolic impact of NAMPT inhibition. Analyses were based upon metabolites that had consistently elevated tumor versus brain fold-changes. Toward the first question, we identified several metabolites that decreased with A132 treatment in NAMPT sensitive cells, including carnitines as hypothesized--many of which rebounded by 72 hours, suggestive of either metabolic adaptation or release of intracellular contents from cell lysis, or a combination thereof. We also identified a subset of metabolites that decreased with A132 inhibition in NAMPT-sensitive glioma, but increased with inhibition in NAMPT resistant glioma, namely phosphatidylcholines/cholines and hypoxanthine/xanthine. Toward the second question, we used microdialysis to determine that TMZ attenuated the metabolic impact of catheter-insertion, which we hypothesize may be due to impaired CNS cell proliferation impeding the glioma-like metabolic signature of catheter-induced gliosis. NA minimally impacted the global metabolome when utilized in combination with A132, but addition of TMZ to NA+A132 appeared to further decrease the abundance of certain phosphatidylcholines. Overall, despite analytical challenges from baseline variability, we have identified candidate pharmacodynamic signatures of NAMPTi in sensitive vs. resistant lines, and begun to discern the relative metabolic impacts of therapeutic combinations. These findings are consistent with the idea that the metabolic impacts of CNS injury, glioma, and metabolically active therapies including NAMPT inhibition can be discerned from the extracellular microenvironment. These observations will be further developed in year 3 via a combination of microperfusion using centrifuged aliquots of microderfusate, microdialysis and isotope tracing experiments, as detailed below.

4) <u>Other achievements</u>

What opportunities for training and professional development has the project provided?
This project has continued to provide training on microperfusion, cell culture work, animal maintenance, imaging, and new techniques for NAD detection to members involved in this project. These techniques have then been taught to other members of the team learning microperfusion and cell culture work for their own independent studies. The members involved in this project have also gained experience in data analysis and figure and manuscript preparation regarding the results presented in this progress report.

How were the results disseminated to communities of interest?
The microperfusion technique and its initial results were presented by Dr. Ali Gharibi Loron at the Gattle Neurosurgery Symposium in October 2022 and the Research Fellows' Association meeting in early 2023. Initial findings from these funded studies have also been presented as a component of multiple invited talks by Dr. Burns. Given the expanded

diversity of analytes inherent to microperfusion over microdialysis, preliminary results are being discussed with engineering colleagues with whom we are collaborating to develop catheters for first-in-human applications of microperfusion. Funding toward this goal based in part on results of this DOD-funded project has been awarded by the state of Minnesota and the Mayo Clinic Center for Clinical and Translational Science.

What do you plan to do during the next reporting period to accomplish the goals?
Follow-up experiments regarding the pharmacodynamic impact of NAMPT inhibition will be performed using centrifuged aliquots of microperfusate, microdialysis and isotope tracing with nicotinamide to improve the signal-to-noise and help extend the mechanistic basis of the alterations observed. The major goal of the next reporting period will be to evaluate the quantitative performance of extracellular D-2-HG, MTA, and dimethylarginine as biomarkers of therapeutic efficacy in IDH-mutant glioma. This will be based on response to NAMPT inhibition, combination therapies, and tumor cell ablation. Interstitial fluid will be collected from GBM164 (sensitive) and196 (resistant), and 164 NAMPT KO (isogenic induced resistant) luciferase positive GBM and assayed for D-2-HG, MTA, and DMA to compare quantitative changes in these candidate biomarkers to changes seen via tumor BLI and survival (in independent non-microdialyzed cohorts). By the end of the next reporting period, we will conclude whether these three candidate extracellular biomarkers could be utilized to evaluate therapeutic efficacy and local tumor burden.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
A key goal of this project was to determine whether tools such as microperfusion or microdialysis could be utilized to discern the metabolic impacts of therapies. Based on our findings of TMZ-induced attenuation of the metabolic signature of catheter-associated injury, and differentially up- or down-regulated metabolites in response to NAMPT inhibition (alone or in combination) in resistant/sensitive lines, our findings support the potential of leveraging the extracellular fluid for pharmacodynamic evaluation. Coincidentally, we have also begun to enroll patients into a separately funded clinical trial evaluating changes in the local tumor and brain adjacent to tumor microenvironment in response to a novel drug combination targeting a different metabolic pathways involved in maintaining intracellular polyamine levels through recently obtained R37 funding via the NIH. Our findings, particularly those illuminating the impact of catheter insertion, suggest the importance of considering how the local microenvironment responds to monitoring interventions, independent of the drug administered. Additionally, as detailed in the technology section below, we are pursuing device development to enable microperfusion in patients with state funding. Because of the heterogeneity identified with microperfusion, but not microdialysis-based studies, likely due to cellular contamination of the extracellular sample, we will leverage centrifuged microperfusate aliquots for future analyses.

What was the impact on other disciplines?
Microdialysis and microperfusion are utilized in many central nervous system (CNS) disease preclinical studies to gather local interstitial fluid at baseline or in response to therapies. Our findings suggest that the insertion of a catheter has metabolic impacts on the CNS microenvironment, particularly in brain. These findings may be of relevance to investigators

evaluating the metabolism of non-glioma CNS diseases via microdialysis/microperfusion. A manuscript is nearing submission to disseminate these findings.

What was the impact on technology transfer?
We previously described our plans for first-in-human studies of microperfusion, in collaboration with Joanneum Research who are the developers of the murine microperfusion system. We submitted an investigational device exemption to the FDA for the human cerebral microperfusion catheters and the clinical grade push-pull pump to perform the studies. Feedback from the FDA helped highlighted additional testing and protocol modifications needed for FDA approval of our first-in-human microperfusion studies. Testing is ongoing to confirm that cytotoxicity is within acceptable limits; once these results are obtained, our revised application will be submitted per the FDA's recommendations. We anticipate deploying the microperfusion catheters within the operating room by mid-2024 with funding for device development provided by the state of Minnesota. Findings from these Department of Defense-funded studies provide valuable insights that will help ensure the optimal design and success of those pending first-in-human studies.

What was the impact on society beyond science and technology?
Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
There were no significant changes in the approach in the past year. The only change was that although we had originally planned on using GBM196 as a NAMPT inhibitor resistant IDH-mutant cell line, we instead utilized GBM164- NAMPT knocked out lines from Aim 1. This was due to the very slow growth rate of the GBM196s and limited availability of the cell line. As our aim was to evaluate the impact of NAMPT inhibition in sensitive versus resistant cell lines, and because NAMPT-KO lines were readily available, we instead use GBM164-KO (resistant) and GBM164 (sensitive) to evaluate the IDH-mutant response to A132. However, as detailed in the next section, we are proposing changes to the approach that will enable us to answer key questions generated from this year's findings.

Actual or anticipated problems or delays and actions or plans to resolve them

1) Microperfusion is a new technique for brain tumors with very little prior work evaluating the metabolic impacts of candidate therapies. We had not anticipated the variability observed in the baseline tumor metabolome given previously highly consistent results with microdialysis. Of relevance, microperfusion does not utilize a membrane, and so can collect red blood cells and other cellular debris that would otherwise be precluded by the membrane. Indeed, some microperfusion samples have been noted to have visible red coloration, which can vary from animal to animal, and even within day to day or aliquot to aliquot. Aliquots were not spun down to remove RBCs, since our original protocol did not utilize this step and our goal was to be consistent. Although we were hopeful that any blood observed could be corrected for analytically this has proven challenging and will be averted through (i) a modified

workflow to maintain catheter in place throughout the experiment minimizing microtrauma of daily re-insertion, and (ii) centrifugation of all aliquots prior to freezing supernatant. This protocol has been successfully utilized by our group for banking human CSF samples.

2) GBM PDXs do not always grow in the same anatomical directions after intracranial implantation. For example, some tumors grow more anteriorly, while others grow evenly in both the anterior and posterior directions. The catheter is inserted into the prior implantation trajectory from the Hamilton needle injection. As such, catheters from animal to animal may not be evenly surrounded by tumor, especially while small. Indeed, review of MRIs revealed heterogeneity of tumor burden across animals; specifically, some animals may have had insufficient tumor burden to robustly differentiate between the metabolic signature of the contralateral brain and tumor. As such, consistent use of more fully developed tumors will improve reproducibility.
3) While informative, global metabolomic analyses does not allow for in-depth evaluation of metabolite usage in specific pathways. To that end, in the following year, we are adjusting our methods to solve these problems: i) Microdialysis will be investigated as a direct comparison to microperfusion, albeit with a modified methodology that includes centrifugation of microperfusate to remove RBC contamination. We have been able to reliably identify tumor-associated signatures using microdialysis. D-2-HG, MTA, and DMA, the focus of year 3 studies, are all dialyzable and reliably quantifiable in our microdialysates, empowering Aim 3 studies to be successfully completed even if microperfusion is not utilized. Key experiments from Aim 2 will be repeated with microdialysis to confirm and expand our findings in year 2. ii) Tumor burden will be quantified in each animal to ensure that they are being implanted after the tumor is adequately robust to ensure optimal catheter targeting for unambiguous comparative analysis of the tumor metabolic signature. iii) As needed, we will utilize isotope tracing with nicotinamide-$^{13}C_6$ when reproducing our findings from Aim 2 to determine how NAMPT inhibition impacts flux of NAD through the NAMPT enzyme. Relative quantification of downstream ^{13}C-labeled metabolites which we expect to observe as a result of flux through TCA cycle and fatty acid oxidation pathways will provide complementary insights regarding the metabolic impacts of NAMPT inhibition, as well as potential emerging mechanisms of cellular resistance.
4) We had previously reported that GBM164, our IDH-mutant human glioma cell line, had stochastically lost its IDH mutation. Utilizing new GBM164, we performed IDH IHC to confirm the presence of the mutant protein and confirmed elevated D-2-HG production in the supernatant. Despite these steps, a subset of the GBM164 animals utilized had low 2-HG levels, suggesting that a partial loss of the enzyme may have compounded our inclusion of animals with smaller tumors. As needed, we will return to earlier passage cells and quantify the percent positivity prior to each tumor implantation. Other cell line models are also available for IDH-mutant glioblastoma; testing their sensitivity to NAMPT inhibitor would be relatively rapid if needed.

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
No human subjects are involved in this study.

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**
  Nothing to report.

- **Journal publications.**
  2 manuscripts are currently pending: 1 describing methods for highly sensitive detection of NAD at fM levels, and another detailing the time-dependent metabolic impacts of catheter placement within the CNS, and the potential of anti-proliferative therapy to attenuate these changes.

- **Books or other non-periodical, one-time publications.**
  Nothing to report.

- **Other publications, conference papers and presentations.**
  Though not the specific title of a national/international presentation, Dr. Burns has discussed this DOD-funded work at multiple invited talks, and acknowledged DOD funding in each of these presentations. A full listing can be provided upon request.

- **Website(s) or other Internet site(s)**
  Nothing to report.

- **Technologies or techniques**
  Improved methodology for highly sensitive detection of NAD. Publication of this methodology currently pending.

- **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?

Name: Terence Burns
Project Role: Primary Investigator
Researcher Identifier (ORCID ID): 0000-0001-7633-3045
Nearest person month worked: 0.1
Contribution to Project: Dr. Burns provides the overall vision for the research program, coordinates and oversees design and conduct the experiments and their analysis.
Funding Support: Mayo Clinic, NIH, Current grant

Name: Ali Loron
Project Role: Research Fellow
Researcher Identifier (ORCID ID): n/a
Nearest person month worked: 8.5
Contribution to Project: Dr. Loron performs the murine microperfusion studies.
Funding Support: Mayo Clinic, current grant

Name: Renee Hirte
Project Role: Research Coordinator
Researcher Identifier (ORCID ID): n/a
Nearest person month worked: 8.7
Contribution to Project: Dr. Hirte developed and performed the NAD assays and analyses.
Funding Support: NIH, current grant

Name: Art Warrington
Project Role: Lab manager
Researcher Identifier (ORCID ID): 0000-0002-3989-975X
Nearest person month worked: 1.8
Contribution to Project: Dr. Warrington oversees regulatory aspects of all studies, including writing and maintaining animal protocols, ensuring compliance with protocols, hazardous waste and controlled substances. He also assists with animal surgeries and training of laboratory personnel and data analysis.
Funding Support: Mayo Clinic, NIH

Name: Shiv Gupta

Project Role: *Co-investigator*
Researcher Identifier (ORCID ID): *0000-0002-9107-9935*
Nearest person month worked: *0.36*
Contribution to Project: *Dr. Gupta an expert in DNA damage responses, as well as PARP and NAD biology. Dr. Gupta will provide practical day-to-day consultation regarding all aspects of the project.*
Funding Support: *Mayo Clinic, NIH*

Name: *Masum Rahman*
Project Role: *Research Fellow*
Researcher Identifier (ORCID ID): *0000-0003-1777-948X*
Nearest person month worked: *3 (through May 2023)*
Contribution to Project: *Dr. Rahman maintains the human glioblastoma xenografts utilized in the study performs tumor cell implantation imaging studies and assists with biochemical analyses.*
Funding Support: *Mayo Clinic, NIH*

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

Ended since prior report:
None

New since prior report (pending at time of prior report):
- Minnesota Partnership for Biotechnology – A first-in-human microperfusion system for in situ CNS discoveries/ Burns/ P008848013 (awarded)
- Glioma intelligence from behind enemy lines/ Burns/ R37CA 276851-1 (awarded)
- A dose escalation and expansion study of sonodynamic therapy with SONALA-001 in combination with Exablate 4000 Type-2 MR-guided focused ultrasound in patients with recurrent or progressive glioblastoma multiforme/ Burns/ SONALA-001

New since prior report (not pending at time of prior report)
- Intra-operative evaluation of focused ultrasound-induced blood-brain barrier disruption/ Burns/ Focused Ultrasound Surgery Foundation

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
Nothing to report

QUAD CHARTS:

Nothing to report

9. APPENDICES:

Nothing to report