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Metformin Pharmacology in Human Cancers: A Proof of Principle Study.

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1.0 INTRODUCTION

1.1. Canonical use and mechanism of action of metformin

Metformin is an oral anti-hyperglycemic drug widely prescribed for the treatment and maintenance of diabetes mellitus type 2 (1). This biguanide drug is a derivative of a compound first isolated from the *Galega officinalis* (French lilac), which has been used to treat type 2 diabetes since the early eighteenth century (2). Metformin's ability to lower fasting blood glucose concentrations is attributed to its ability to suppress hepatic gluconeogenesis and increase glucose uptake and utilization in muscle and visceral tissues; however, its precise molecular mechanism of action is unclear (3, 4). It is generally accepted that one of metformin's main actions is activation of 5' AMP-activated protein kinase (AMPK), to modulate metabolic and lipid signaling. In hepatocytes, metformin treatment leads to an increase in phosphorylated (active) AMPK, and subsequent phosphorylation of Acetyl-CoA carboxylase at Ser79 (P-ACC-Ser79), a direct biomarker of AMPK activity (5). Whether or not this effect is completely responsible for the underlying cause of metformin's anti-hyperglycemic properties remains controversial (3). Other AMPK-independent activities of metformin that may play a considerable role in its pharmacologic effects include weak and transient inhibition of mitochondrial respiratory chain complex I, and tissue sensitization to insulin action (6, 7).

Unlike other anti-diabetic agents, metformin reduces hyperglycemic blood glucose concentrations without stimulating insulin secretion, hypoglycemia, or promoting weight gain. Additionally, through its role as an AMPK agonist, metformin has been shown to decrease free fatty acid concentrations in plasma, and regulate lipid homeostasis in patients (8, 9). This added benefit is especially important as type 2 diabetics are often at higher risk for cardiovascular disease (10). These numerous effects, combined with its relatively benign toxicity profile and low financial cost are what make metformin the recommended first-line therapy for treatment of type 2 diabetes mellitus, despite the controversy and limited understanding of its molecular mechanism of action.

1.2. Metformin Pharmacokinetics

While metformin is generally well-tolerated by patients, the anti-hyperglycemic response between patients is quite variable. With a twice-daily (BID) oral dose of 750 mg, metformin has a plasma elimination half-life of approximately 6 h. Absorption of the tablet occurs predominately in the small intestine, and is mediated through plasma membrane monoamine transporters (PMATs). However, this absorption is often incomplete, resulting in substantial inter-patient variation in drug bioavailability. Mean drug bioavailability is 55% +/- 16%. Because of this, there is large inter-individual variability in metformin pharmacokinetics (11). Steady-state metformin plasma concentrations can range from 54 to 4133 ng/mL (12). While no accumulation in plasma has been seen in patients after multiple doses, metformin elimination is bi-phasic. While initial elimination of metformin from plasma is relatively rapid, there is a second more prolonged elimination phase possibly due to slow uptake and release from a secondary compartment in tissues (11, 13). Metformin exists as a hydrophilic cationic species at physiologic pH, and as such, has severely limited passive diffusion through tissues. Despite this, metformin is still widely distributed throughout the body and is transported to the liver and other peripheral tissues mainly through organic cation transporters 1 and 3 (OCT1/3) (14). Metformin is not metabolized, is poorly plasma protein-bound and is eliminated from the body by renal excretion into the urine (15).

1.3. Metformin as an anti-cancer agent

Within the last decade, a multitude of studies have suggested that metformin has potential use as an anti-cancer therapeutic. Patients with Type 2 diabetes mellitus have a significantly increased risk of developing colon, prostate, endometrial, breast, ovarian, kidney, and pancreatic cancers (16). Epidemiologic studies revealed that diabetic patients receiving metformin had both lower incidence and later onset of cancers compared to diabetics who were not taking metformin (17-23). Diabetics on metformin also had lower incidence of cancers compared to non-diabetics (24). Preclinical studies have shown that metformin elicits anti-proliferative effects in a variety of cancer cell lines both *in vitro* and *in vivo (3, 19, 25)*. However, as with its mechanism of anti-diabetic activity, the mechanism of metformin's anti-cancer activity is unclear and controversial.

The AMPK-dependent inhibition of mTORC1 in cancer cells is one of the potential key mechanisms that facilitates the anti-cancer activity of metformin. AMPK activation leads to mTORC1 inhibition at multiple concentrations via direct phosphorylation of raptor protein, and indirectly through activation of tuberous sclerosis 2 protein (TSC2) (26-28). Metformin treatment of MCF-7 breast cancer cells results in cell growth inhibition via an AMPK-dependent decrease in phospho-S6 (26). As mTORC1 inhibiting agents are already used clinically to treat a number of cancers, metformin may be an already-FDA-approved, less toxic, and relatively cheap mTORC1-inhibiting agent. In fact, in comparing *in vivo* metformin treatment to the mTORC1 inhibitor rapamycin, metformin also decreases activation of AKT upstream of mTORC1 (28). As AKT signaling has been associated with resistance to mTORC1 inhibition, metformin may be rendering a more efficient anti-cancer response than conventional mTORC1 inhibitors.

While the above is a widely accepted mechanism of metformin's anti-cancer activity established *in vitro*, there is another school of thought suggesting that metformin's preventative anti-cancer effects (seen epidemiologically) stem from its ability to systemically regulate insulin signaling independent of metformin's direct action on AMPK. Increased cancer risk associated with Type 2 diabetes mellitus is thought to be a result of constitutively elevated plasma insulin concentrations. Insulin promotes carcinogenesis through insulin receptor signaling, as well as indirectly through increasing concentrations of insulin-like growth factors, sex hormones, and inflammatory processes (29, 30). Metformin can lower circulating insulin concentrations, thus reducing the potential mitogenic effects of insulin on tumor cells (6).

1.4. Translatability of *in vitro* findings to *in vivo*

Despite the disagreement surrounding its molecular mechanism of action, a multitude of clinical trials attempting to provide proof of concept for the direct anti-tumor activity of metformin have been launched and met with conflicting results (25, 31). In investigating why there is so much variability between trials, one problem is that a majority of these trials failed to assess what concentrations of metformin were actually reaching cancerous tissues. As discussed previously, both the pharmacokinetic and therapeutic properties of metformin can vary greatly between patients. Also, a majority of the preclinical evidence claiming metformin as an effective anti-cancer agent were done with supra-pharmacologic concentrations of metformin (5-50 mM), such concentrations are not achievable in humans. We and others have performed preclinical pharmacokinetic studies in animals to obtain a better understanding of achievable tissue concentrations of metformin in animal models of cancer. Dowling *et al*. recently showed that standard mouse models in which metformin elicits anti-cancer activity (at a dose of 0.5 mg/kg/d in drinking water) are likely an approximation of high-dose metformin administration in humans, achieving much higher than typical plasma concentrations than we see with normal human dosing (plasma concentrations ranging from 297 to 16,274 ng/mL, which equal 0.45- 24.3 uM)) (32). We have found that a lower dosing regimen (0.1 mg/kg/d in drinking water) in mice can

still produce a robust anti-cancer effect in MCF-7 xenografts and is sufficient dosing to activate AMPK signaling in tumor tissues. Using this treatment regimen, mouse plasma steady-state concentrations ranged from 389 to 829 ng/mL (0.58-1.24 uM), more reflective of human steady-state plasma concentrations (54 to 4133 ng/mL, which equal 0.08- 6.21 uM). Additionally, tumor tissues reached metformin concentrations in the 100-nM range (unpublished data). In parallel, our studies in cultured MCF-7 cells revealed that treatment with ~1 mM metformin-the lowest dose of metformin required to elicit AMPK activation in culture-achieves intracellular concentrations of approximately 10 uM (unpublished data). As this concentration is substantially above what we see accumulate in tumor tissue, and yet, we still see AMPK activation in said tumor tissue with only nM concentrations, there must be vastly different dynamics of drug response to metformin between *in vivo* and tissue culture (32).

To better understand metformin's anti-cancer effects in humans, it is first crucial to assess what concentration of metformin is achievable in both normal and cancerous tissues, and the degree of inter-patient variability. If the insulin-independent effects of metformin are predominately responsible for its' anti-cancer activity (as preclinical studies widely claim), then assessing the concentrations of metformin in tumor tissues, and whether those concentrations are sufficient to activate AMPK and inhibit mTORC1, are the first steps to beginning to better understand the variability in clinical trial data.

1.5. Study rationale

We propose to conduct a presurgical (proof of principle, window of opportunity) study in patients with surgically resectable thoracic tumors to determine steady-state tissue and plasma concentrations of metformin. To understand the variability in clinical results testing metformin as an anti-cancer agent, it is important to determine the concentrations of metformin that are achievable in tissue. Clinical effects of metformin develop gradually over several days of treatment. Steady-state plasma metformin concentrations are correlated with anti-hyperglycemic response. Thus, achieving steady-state concentrations in this study will allow accurate determination of the most representative concentrations of metformin in normal and cancerous tissues, as well as determine AMPK signaling differences in these tissues. As our Primary Objective is to determine the concentration of metformin in tumors, we will treat patients with metformin extended release **(**ER) (Glucophage® XR), starting at 750 mg PO QD for 4 days, then escalating to 750 mg PO BID for 3-6 days prior to surgery. FDA prescribing information indicates that metformin reaches steady-state plasma concentrations within 24-48 hours after the start of dosing in humans; thus, the 7-to-10-day time frame of our study will allow sufficient time for metformin to reach steady-state plasma concentrations, in addition to time allotted for potential accumulation in tissues. Metformin concentrations will be measured using a validated LC-MS/MS assay.

2.0 OBJECTIVES

2.1 Primary Objective

To determine the intra-tumor concentrations of metformin, with a standard deviation ≤25% of the mean, in patients with solid tumors of thoracic origin administered metformin extended release.

2.2 Secondary Objective

To determine the concentration of metformin in adipose tissue, tumor-adjacent normal tissue, plasma, and whole blood.

2.3 Exploratory Objective

To determine whether metformin alters AMPK activity in tumor cells.

3.0 ELIGIBILITY CRITERIA AND PARTICIPANT REGISTRATION

Inclusion Criteria:

3.1 Patients with confirmed or suspected malignant solid tumor of thoracic origin (*e.g.*, lung, esophageal, thymus, mesothelioma, chest wall, mediastinum, trachea, pleura) with the intent to treat or biopsy by surgery as standard of care. Tumor must be ≥1.5 cm.

3.2 Patients with multicentric disease are eligible. Samples from all available tumors are requested for research purposes.

3.3 Patients with T2DM being treated with metformin (any dose) for a clinical indication at the time of study enrollment are eligible, and will continue metformin treatment as clinically indicated during the presurgical study period. Their dose of metformin will NOT be changed.

Patients not on metformin at the time of study entry must be willing to take metformin extended release (Glucophage® XR, 750 mg QD for 4 days, then 750 mg BID for 3-6 days) for a total of 7-10 days prior to surgery.
Patients do not require a diagnosis of diabetes to be enrolled in the study.

All patients must be willing to keep a drug diary indicating the dates and times of metformin administration.

One patient who has not been treated with metformin will be consented and enrolled. This patient will be asked to provide tumor, adipose, normal lung tissue, and blood samples, and all samples will be collected as described in section 4.2.2. These samples will be taken to serve as a control and are needed in order to optimize the metformin quantification.

3.4 Patients must meet the following clinical laboratory criteria:

- Absolute neutrophil count (ANC) ≥ 1,500/mm^3 and platelet count ≥ 75,000/mm^3.
- Total bilirubin ≤ 1.5 × the upper limit of the normal range (ULN).
- Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) ≤ 3 × ULN.
- eGFR > 60 mL/min/1.73m2 or eCrCL > 60 mL/min
- LDL Question For lung surgery do we need an INR < 1.6

3.5 Ability to give informed consent.

3.6 Patients must be willing to provide 20 mL of blood for research use.

3.7 Patient must be willing to provide consent for use of archived tissue for research.

Exclusion Criteria:

3.8 History of diabetes that is currently being treated without metformin.

3.9 Patients with a documented history of alcoholism, or binge drinking disorder.

3.10 Patients who, at the time of study entry, are not taking metformin for a clinical indication, **and** who will need a radiographic analysis with an iodinated contrast agent during the metformin study treatment period.

This criterion does not apply to patients taking clinically indicated metformin at the time of study entry.

3.11 History of reactive hypoglycemia.

3.12 History of liver disease as defined with LFTs above those in the inclusion

3.13 Known hypersensitivity to metformin.

3.14 Active or history of lactic acidosis, metabolic acidosis, or diabetic ketoacidosis.

3.15 Patients who are pregnant or breastfeeding.

Participant Registration:

3.16 Following completion of pre-treatment evaluations and informed consent, participants will be registered by a Clinical Research Coordinator via the Norris Cotton Cancer Center's clinical trials management system (Velos or equivalent) . At the time of registration, the following information will be recorded:

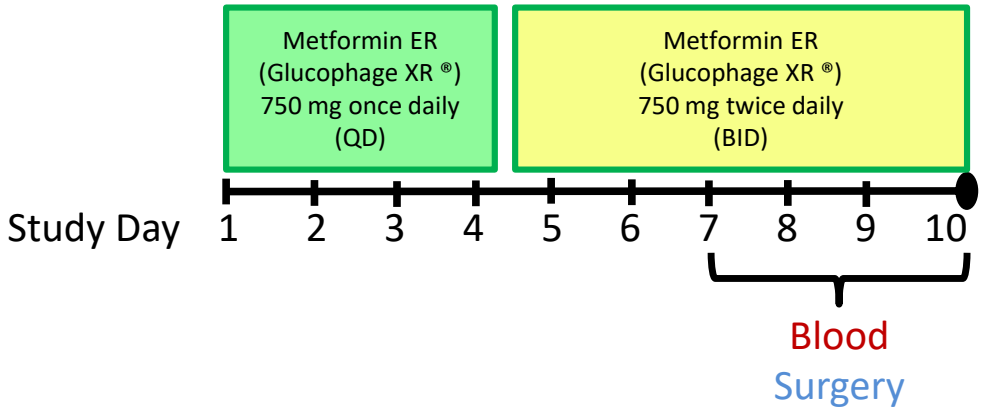
- Patient's name and subject identification number.
- Patient's date of birth.
- Date of treatment start.

3.13 Eligibility criteria will be verified at the time of registration. A case number will be assigned following registration.

4.0 TREATMENT PLAN

4.1 Screening Period

Subjects will provide written informed consent, be evaluated for eligibility criteria, and undergo standard baseline clinical and tumor assessments and staging. Subjects will undergo our standard pre-treatment surgical evaluation.



4.2 Treatment Period

Patients who are not taking metformin at the time of study enrollment will receive oral metformin extended release (Glucophage® XR, 750 mg tablets). Metformin is to be taken with a meal as one 750-mg pill per day for the first 4 days, followed by two 750-mg pills per day (one pill in morning, one pill in evening), for a total pre-surgical metformin regimen of 7-10 days (as depicted in schematic above). The last dose of metformin extended release should be taken at 7:00 pm (+/- 1 hour) the night preceding the surgery.

Patients already taking metformin for a clinical indication at the time of study enrollment will continue their metformin regimen while on study. The last dose of metformin should be taken the night preceding the surgery. A record will be kept regarding which patients are not taking the 750 mg BID dosing of metformin and this will be noted in the analysis.

All patients will record the dates and times of metformin ingestion on the evening before surgery in a drug diary.

A single patient who has not been treated with metformin will be consented and enrolled. This patient will be asked to provide tumor, adipose, normal lung tissue, and blood samples, and all samples will be collected as described in section 4.2.2. These samples will be taken to serve as a control and are needed in order to optimize the metformin quantification.

4.2.1. Presurgical metformin

Patients enrolled will be treated with metformin (administered orally; 750 mg QD for 4 days, then 750 mg BID for 3-6 days; or clinically indicated metformin) for a total of 7-10 days prior to surgery, up until the night before surgery.

4.2.2. Surgery and biological specimens

Specimens will be acquired and processed as described in Appendix A.

Plasma and Whole Blood

On the morning of surgery, 20 mL of blood will be collected for research purposes in the pre-operative area. This blood may be collected at the same time as a routine clinically indicated blood draw or during placement of an intravenous line (before saline is administered), and should occur as close to the time of surgery as possible. Blood will be drawn into (2) 10-mL purple-top (EDTA-treated) tubes, picked up by a member of the Miller Lab within 1 hour of being drawn from the patient, and processed by the Miller Lab as in Appendix A.

In the Miller Lab, a 10-mL blood sample will be centrifuged at 1,000 x *g* for 30 minutes at 4°C to separate plasma, which will be aliquoted into cryovials (1 mL/vial). The other 10-mL blood sample will be aliquoted into cryovials (1 mL/vial) as whole blood. All plasma and whole blood sample aliquots will be stored at -80°C until analysis.

Tumor tissue, adipose tissue, and normal adjacent tissue

Requested specimens: Tumor tissue fragment ≥8 mm in diameter*

Adipose tissue fragment ≥8 mm in diameter*

Normal adjacent tissue fragment ≥8 mm in diameter*

* small tissue fragments may be pooled to yield ≥8 mm of tissue

The patient will undergo surgical resection or biopsy of the tumor(s). During the procedure, the surgeon will attempt to obtain a sample (≥8 mm in diameter) of adipose tissue (likely subcutaneous) and normal adjacent tissue (such as lung, when available) (≥8 mm in diameter) when feasible.

All normal and tumor tissues will be sent to Pathology for dissection. The Pathologist or her/his designate will promptly dissect the surgical specimen(s). ASCO-CAP guidelines require initiation of dissection of a surgical specimen within 1 h of removal from the patient; for this study, minimal lag time ("cold ischemia time") is preferred.

The Pathologist will assess whether the tumor specimen is large enough to procure:

A. tissue for routine diagnostics.
B. ≥6 tumor fragments that are ≥4 mm^3 in all dimensions for research use.
   A 1.5-cm tumor is expected to provide sufficient material for all needs.
   If the tumor is too small to provide adequate tissue for research, the tumor will be considered non-evaluable and the patient will need to be replaced.

Tissue specimens procured for research purposes will be cut into 4-mm^3 fragments, weighed, and individually frozen in cryovials by a member of the Miller lab. Tissue specimens with overt blood content will be pressed between absorbent towels to remove blood prior to weighing. Please see SOP in Appendix A for tissue handling/processing and storage.

All research tissue samples should be frozen within 1.5 h of tissue removal from the patient; a shorter lag time is strongly preferred to preserve phospho-proteins.

4.2.3. Metformin drug information

Metformin (1, 1-Dimethylbiguanide hydrochloride) is a synthetic biguanide derivative. Administered orally, this drug is predominately used to help lower blood glucose concentrations in diabetes mellitus type 2 patients. Metformin effectively lowers blood glucose concentrations in patients by decreasing hepatic glucose output, and increase peripheral glucose uptake and utilization (3, 4). Metformin also decreases circulating insulin and fatty acid concentrations in patients (8).

Preclinical and pharmacodynamic data in humans

Oral metformin is absorbed in the small intestinal tract and taken up through plasma membrane monoamine transporters (PMATs). Metformin is predominantly transported into cells via organic cation transporters (OCT1/3). Metformin has a plasma half-life of 5-6 h (11). Due to inter-patient variability in drug absorption and transport, steady-state metformin plasma concentrations can range from 0.054 to 4.133 ug/mL (0.08- 6.21 uM) (15). Estimated therapeutic concentrations of metformin range from 0.129 to 90 ug/mL (0.19 uM to 132.9 uM) (33).

Selectivity of metformin

Metformin is canonically an agonist of AMPK; however it has also been reported to weakly inhibit mitochondrial respiratory complex 1 *in vitro (5, 7).* In diabetics, metformin systemically lowers blood glucose concentrations by inhibiting hepatic

glucose output and increasing glucose utilization, without causing hyperinsulinemia, hypoglycemia, or promoting weight gain (3). Metformin also systemically increases insulin sensitivity and decreases concentrations of circulating fatty acids (6).

Toxicities and risks of metformin

In worldwide placebo-controlled clinical trials, 781 patients with type 2 diabetes were administered metformin extended release (Glucophage® XR; 500 or 750 mg), and 195 patients received placebo. Adverse reactions reported in >5% of the metformin extended release-treated patients, and that were more common in metformin extended release-treated than placebo-treated patients, are noted in the table below [extracted from Glucophage® product insert (34)].

Adverse Reaction	GLUCOPHAGE XR (n=781)	Placebo (n=195)
	% of Patients	
Diarrhea	9.6	2.6
Nausea/Vomiting	6.5	1.5

.

Diarrhea led to discontinuation of study medication in 0.6% of patients treated with

GLUCOPHAGE XR (metformin extended release). Additionally, the following adverse reactions were reported in ≥1.0% to ≤5.0% of GLUCOPHAGE XR patients and were more commonly reported with GLUCOPHAGE XR than placebo: abdominal pain, constipation, distention abdomen, dyspepsia/heartburn, flatulence, dizziness, headache, upper respiratory infection, taste disturbance. Cholestatic, hepatocellular, and mixed hepatocellular liver injury have been reported with post-marketing use of metformin. In rare cases, metformin treatment can result in lactic acidosis; the main risk factors for this serious adverse event are renal impairment, or overdose of drug. While this lactic acidosis toxicity is uncommon, it is recommended that patients not be prescribed metformin if their plasma creatine values are above 1.5 mg/dL (men) or 1.4 mg/dL (women) (35).

Metformin extended release will be stored, dispensed, and accounted for by the DHMC Investigational Pharmacy.

4.3 Study exit

Following surgery, the patient's participation in the study will end. The patient will then be treated as per standard-of-care by her/his treating physician.

4.4 Evaluable patients

Subjects who proceed to a definitive surgical procedure with A) tumor tissue sufficient to provide specimens for research purposes, and B) histologically-confirmed tumor tissue in the surgical specimen will be considered evaluable. Subjects whose surgical specimens do not contain adequate tumor tissue to assess the Primary Endpoint will not be considered evaluable, and will need to be replaced.

4.5 Analyses

1. *Pharmacokinetic analysis of metformin concentrations in plasma, whole blood, and tissues.*

The concentrations of metformin in all samples of blood, plasma, and tissue (tumor, normal, adipose) will be measured by a liquid chromatography (LC) with tandem mass

spectrometry (MS/MS) assay developed in the NCCC Clinical Pharmacology Shared Resource. The average value from the technical triplicates for each biological specimen will be used for calculations. This methodology is established in the NCCC Clinical Pharmacology Shared Resource for measurement of metformin concentrations in mouse tissues/tumors and cultured cells; this methodology will be re-optimized for analysis of human tissues using specimens collected from the single control subject enrolled in this study, in which specimens will be spiked with metformin to generate standard curves

In brief, the current assay consists of plasma protein precipitation using acetonitrile, and filtration over Phenomenex iPhree cartridges The eluate is dried under nitrogen, resuspended in water, and injected into the LC-MS/MS. In mouse plasma (without full validation), assay accuracy was 91-98%, precision was <15% CV, and recovery was 89%.

2. *Immunohistochemical analysis of AMPK activity of pre- vs. post-metformin treated tumors.*

The goal of this exploratory endpoint is to determine whether metformin treatment alters phosphoprotein markers of AMPK activity in tissues.

In eligible patients, baseline (diagnostic biopsy specimen) and post-metformin (surgical specimen) tumor/normal tissue samples will be sectioned and processed for IHC in the NCCC Pathology Shared Resource. Tissue sections will be immunostained using antibodies against P-ACC(S79), and P-S6(S240/244). P-ACC and P-S6 will be scored using a Histoscore scale (36) that incorporates staining intensity and frequency. We expect metformin treatment to increase P-ACC and decrease P-S6 Histoscores, as metformin treatment should increase AMPK activity.

5.0 DATA COLLECTION, HANDLING AND RECORD KEEPING

5.1 Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Participants will sign an authorization that includes the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization.

Loss of patient confidentiality is a risk of participation. Study participant identities will be kept confidential except as required by law. Subjects' samples will be identified by code only (i.e. linked, but de-identified). Patient samples will be de-identified at the time and site of collection. Electronic case report forms, participant, and study information will be kept in the Velos eResearch password protected database (or equivalent). Additionally, documents containing

participant identifiers, such as those from the medical record to confirm eligibility, will be filed in binders and kept in a locked, secure location at the Norris Cotton Cancer Center.

5.2 Data retention

Following closure of the study, the investigator will maintain all site study records in a safe and secure location. The records are maintained to allow easy and timely retrieval, when needed (e.g., audit or inspection) and, whenever feasible, to allow any subsequent review of data in conjunction with assessment of the facility, supporting systems, and staff. Upon completion of study analysis, research information is stored in Dartmouth College Records Management off-site storage located at 6218 Etna Road, Hanover, NH 03755. Documents are shredded on site after 50 years of storage.

Electronic case report forms, participant, and study information will be kept in the Velos eResearch password protected database (or equivalent) indefinitely.

6.0 STUDY MONITORING, AUDITING, AND INSPECTING

6.1 Safety and Data Monitoring

This study will be monitored by the Data Safety Monitoring and Accrual Committee (DSMAC) of the Norris Cotton Cancer Center. The Committee meets quarterly to review accrual rates and information of all studies that have accrued participants. The DSMAC has the authority to suspend or terminate all research activities that fall within its jurisdiction. In the event that a study is suspended or terminated by the DSMAC, that information will be forwarded to the CPHS office.

6.2 On-Site Monitoring

Clinical research monitoring will be conducted by appropriately trained staff of Dartmouth-Hitchcock Medical Center Clinical Trials Office. This monitoring will include periodic assessment of the regulatory compliance, data quality, and study integrity. Study records will be reviewed and directly compared to source documents and the conduct of the study will be discussed with the investigator. Monitors may request access to all regulatory documents, source documents, CRFs, and other study documentation for on-site inspection. Direct access to these documents is guaranteed by the investigator, who must provide support at all times for these activities.

6.3 Auditing and Inspecting

Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable Dartmouth compliance and quality assurance offices. The investigator will permit study-related audits and inspections by the Dartmouth CPHS, government regulatory bodies, and Dartmouth compliance and quality assurance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data, etc.) The investigator will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.)

7.0 ADVERSE EVENT REPORTING

Patients will be instructed to report the occurrence of any adverse event. An adverse event is any undesirable event occurring with the use of this procedure. Adverse events will be graded according to the NCI Common Toxicity Criteria Version 4.0. A copy of the CTC version 4.0 can

be downloaded from the CTEP home page (http://ctep.info.nih.gov). All appropriate treatment areas should have access to a copy of the CTC version 4.0.

This trial will be independently monitored by the Norris Cotton Cancer Center Data Safety and Accrual Monitoring Committee.

The following definitions will be used to assess causality:

No: The clinical adverse event is definitely unrelated to the study medications (e.g., does not follow a reasonable temporal sequence from study medication administration, present prior to receiving study medication, etc.)

Unlikely: The study medications do not have any reasonable association with the observed experience; however, relationship cannot be definitely excluded.

Possibly: The connection with study medication administration appears feasible, but cannot be excluded with certainty (e.g., follows a reasonable temporal sequence from medication administration, but may also be related to other known factors).

Probably: The clinical experience appears related to the study medications with a high degree of certainty (e.g., follows a reasonable temporal sequence from medication administration and abates upon discontinuation of the medication, cannot be reasonably explained by known characteristics of the patient's clinical state or other modes of therapy administered to the patient, etc.)

8.0 STATISTICAL CONSIDERATIONS

8.1 PRIMARY ENDPOINT – Concentrations of metformin in tumor tissue.

8.2 SECONDARY ENDPOINT – Concentrations of metformin in adipose tissue, tumor-adjacent normal tissue, plasma, and whole blood.

8.3 EXPLORATORY ENDPOINT – Changes in AMPK activity induced by metformin, as determined by changes in P-ACC and P-S6 histoscores between pre- and post-metformin tissue specimens.

8.4 PRIMARY ANALYSIS PLAN – The primary analysis will measure the concentrations of metformin in biological triplicate samples of plasma, whole blood, and tissue specimens from each patient. Metformin concentrations will then be statistically analyzed to determine A) variability between biological triplicates, B) inter-individual variability between patients (within each tissue type; calculate descriptive statistics), and C) relationships between tissue types within each patient (compared by paired *t*-test or two-way ANOVA).

8.5 EXPLORATORY ANALYSIS PLAN – An exploratory analysis will determine whether metformin treatment significantly alters AMPK signaling in tumors compared to baseline. Histoscores from paired (pre- and post-metformin) tissue specimens will be compared by Wilcoxon rank sum test, and the correlation with metformin concentration will be established (the log transformation may be used to comply with the normal distribution assumption). Furthermore, an exploration of the metformin concentrations in plasma blood and tumor tissue and changes in tumor Histoscores will be undertaken using the direct PK-PD model (e.g., sigmoid Emax).

8.6 STATISTICAL TESTING - All statistical tests will be two-sided, and the overall type I error will be 0.05.

8.7 SAMPLE SIZE – We propose to evaluate 20 patients for the Primary Endpoint to account for the wide range (>75-fold) of steady-state plasma concentrations of metformin previously shown in

humans (12). There are no data on intra-tumor concentrations of metformin. As a surrogate, we will use plasma concentrations of metformin under the assumption that plasm and tumors will exhibit similar ranges of variability between patients. We will enroll one additional subject to acquire control tissues, bringing the total study accrual goal to 21 subjects.

The log chi-square test is used to find the minimum detectable coefficient of variation [CV= relative standard deviation (SD)] for metformin in tumor tissues with n=20 sample size (number of tumors and patients). To compute the power of the test, we use the fact that the CV is approximately equal to SD on the log scale. Our one-sided power analysis implies that with n=20, under the null hypothesis that CV=SD/mean=25%, the minimum detectable CV=15% with 90% power and the Type I error alpha=5%.

Based on the high number of patients undergoing surgical resection of lung tumors at DHMC, we project that this study will complete accrual within 24 months.

9.0 HUMAN SUBJECTS

9.1 Subject patient population

Patients with a malignant solid tumor of thoracic origin that will be surgically resected or surgically biopsied as standard of care.

9.2 Consent Procedures

Patients must give a statement of informed consent. The informed consent must meet the requirements of the FDA (21 CFR 50.25 Elements of Informed Consent) and the Committee for the Protection of Human Subjects at Dartmouth College, which is the delegated Dartmouth-Hitchcock Medical Center IRB. Before initiating a trial, the investigator will have written and dated approval from the CPHS of Dartmouth-Hitchcock Medical Center for the trial protocol, amendment(s), written informed consent form, consent form updates, subject recruitment procedures (e.g., advertisements) and written information to be provided to subjects. The investigators, or a person designated by the investigators, will explain the benefits and risks of participation in the study to each subject and to the subject's legally acceptable representative or impartial witness and obtain written informed consent prior to the subject entering the study (before initiation of non-routine tests and administration of study drug). The final form must be agreed to by the CPHS of Dartmouth-Hitchcock Medical Center and must contain all elements in the sample form, in language readily understood by the subject. Each subject's original consent form, signed and dated by the subject or by the subject's legally acceptable representative, and by the person who conducted the informed consent discussion, will be retained by the investigator. In addition, a copy of the informed consent will be given to the subject.

9.3 Potential Risks and Benefits

The physical risk of study participation is low. Subjects taking metformin at the time of study entry will not be subject to physical risks from continuing metformin during the study. Subjects who receive study treatment with metformin extended release for 7-10 days prior to surgery incur physical risks associated with metformin, including diarrhea, nausea/vomiting, abdominal pain, minor hypoglycemia, elevated aminotransferase concentrations, peripheral edema, constipation, dyspepsia/heartburn, flatulence, dizziness, headache, upper respiratory infection, taste disturbance, and liver injury (detailed in Section 4.2.3 and refs. (34, 35)]. Metformin has been reported to rarely cause lactic acidosis that may cause renal impairment, but this was not

reported in 176 comparative trials and cohort studies totaling 35,619 patient-years (37, 38). The results of this investigation may provide novel information on the achievable concentrations of metformin in normal and cancerous tissues, which may shape the development of future treatment regimens to benefit future patients with cancer. Loss of patient confidentiality is a risk of participation. Participation in this study is of low overall risk and has no potential for direct clinical benefit.

9.4 Confidentiality

The pathology specimens from the patient biopsies will be labelled only with the patient case number. The key matching patient identification with the patient case number will be kept in a locked drawer in the PI's office. Only the PI and his designated representatives will have access to this key. No one will have access to the study records but for the Investigators and CRA's, the Dartmouth-Hitchcock CPHS, the FDA, and OPRR.

10.0 PUBLICATION PLAN

At the conclusion of the study, results will be reported in a manuscript in a peer-reviewed journal.

11.0 BIBLIOGRAPHY

1. Nathan, D, Buse, JB, Davidson, MB, Ferrannini, E, Holman, RR, Sherwin, R and Zinman, B. Medical management of hyperglycaemia in type 2 diabetes mellitus: a consensus algorithm for the initiation and adjustment of therapy A consensus statement from the American Diabetes Association and the European Association for the Study of Diabetes. Diabetologia. 52: 17-30 (2009).
2. Witters, LA. The blooming of the French lilac. Journal of Clinical Investigation. 108: 1105-1107 (2001).
3. Viollet, B, Guigas, B, Sanz Garcia, N, Leclerc, J, Foretz, M and Andreelli, F. Cellular and molecular mechanisms of metformin: an overview. Clin Sci (Lond). 122: 253-70 (2012).
4. Rena, G, Pearson, ER and Sakamoto, K. Molecular mechanism of action of metformin: old or new insights? Diabetologia. 56: 1898-906 (2013).
5. Zhou, G, Myers, R, Li, Y, Chen, Y, Shen, X, Fenyk-Melody, J, Wu, M, Ventre, J, Doebber, T, Fujii, N, Musi, N, Hirshman, MF, Goodyear, LJ and Moller, DE. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 108: 1167-74 (2001).
6. Gunton, JE, Delhanty, PJD, Takahashi, SI and Baxter, RC. Metformin rapidly increases insulin receptor activation in human liver and signals preferentially through insulin-receptor substrate-2. Journal of Clinical Endocrinology & Metabolism. 88: 1323-1332 (2003).
7. El-Mir, MY, Nogueira, V, Fontaine, E, Averet, N, Rigoulet, M and Leverve, X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. Journal of Biological Chemistry. 275: 223-228 (2000).
8. DeFronzo, RA and Goodman, AM. Efficacy of metformin in patients with non-insulin-dependent diabetes mellitus. The Multicenter Metformin Study Group. N Engl J Med. 333: 541-9 (1995).
9. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. Lancet. 352: 854-65 (1998).
10. Hurst, RT and Lee, RW. Increased incidence of coronary atherosclerosis in type 2 diabetes mellitus: mechanisms and management. Ann Intern Med. 139: 824-34 (2003).
11. Graham, GG, Punt, J, Arora, M, Day, RO, Doogue, MP, Duong, JK, Furlong, TJ, Greenfield, JR, Greenup, LC, Kirkpatrick, CM, Ray, JE, Timmins, P and Williams, KM. Clinical Pharmacokinetics of Metformin. Clinical Pharmacokinetics. 50: 81-98 (2011).
12. Christensen, MM, Brasch-Andersen, C, Green, H, Nielsen, F, Damkier, P, Beck-Nielsen, H and Brosen, K. The pharmacogenetics of metformin and its impact on plasma metformin steady-state concentrations and glycosylated hemoglobin A1c. Pharmacogenet Genomics. 21: 837-50 (2011).
13. Timmins, P, Donahue, S, Meeker, J and Marathe, P. Steady-state pharmacokinetics of a novel extended-release metformin formulation. Clinical Pharmacokinetics. 44: 721-9 (2005).

14. Shu, Y, Sheardown, SA, Brown, C, Owen, RP, Zhang, S, Castro, RA, Ianculescu, AG, Yue, L, Lo, JC, Burchard, EG, Brett, CM and Giacomini, KM. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. J Clin Invest. 117: 1422-31 (2007).
15. Tucker, GT, Casey, C, Phillips, PJ, Connor, H, Ward, JD and Woods, HF. Metformin kinetics in healthy subjects and in patients with diabetes mellitus. Br J Clin Pharmacol. 12: 235-46 (1981).
16. Giovannucci, E, Harlan, DM, Archer, MC, Bergenstal, RM, Gapstur, SM, Habel, LA, Pollak, M, Regensteiner, JG and Yee, D. Diabetes and cancer: a consensus report. Diabetes Care. 33: 1674-85 (2010).
17. Bodmer, M, Meier, C, Krahenbuhl, S, Jick, SS and Meier, CR. Long-term metformin use is associated with decreased risk of breast cancer. Diabetes Care. 33: 1304-8 (2010).
18. Decensi, A, Puntoni, M, Goodwin, P, Cazzaniga, M, Gennari, A, Bonanni, B and Gandini, S. Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. Cancer Prev Res (Phila). 3: 1451-61 (2010).
19. Evans, JM, Donnelly, LA, Emslie-Smith, AM, Alessi, DR and Morris, AD. Metformin and reduced risk of cancer in diabetic patients. BMJ. 330: 1304-5 (2005).
20. Jiralerspong, S, Palla, SL, Giordano, SH, Meric-Bernstam, F, Liedtke, C, Barnett, CM, Hsu, L, Hung, MC, Hortobagyi, GN and Gonzalez-Angulo, AM. Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer. J Clin Oncol. 27: 3297-302 (2009).
21. Li, D, Yeung, SC, Hassan, MM, Konopleva, M and Abbruzzese, JL. Antidiabetic therapies affect risk of pancreatic cancer. Gastroenterology. 137: 482-8 (2009).
22. Libby, G, Donnelly, LA, Donnan, PT, Alessi, DR, Morris, AD and Evans, JM. New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. Diabetes Care. 32: 1620-5 (2009).
23. Wright, JL and Stanford, JL. Metformin use and prostate cancer in Caucasian men: results from a population-based case-control study. Cancer Causes Control. 20: 1617-22 (2009).
24. Xu, H, Aldrich, MC, Chen, Q, Liu, H, Peterson, NB, Dai, Q, Levy, M, Shah, A, Han, X, Ruan, X, Jiang, M, Li, Y, Julien, JS, Warner, J, Friedman, C, Roden, DM and Denny, JC. Validating drug repurposing signals using electronic health records: a case study of metformin associated with reduced cancer mortality. J Am Med Inform Assoc. 22: 179-91 (2015).
25. Chae, YK, Arya, A, Malecek, MK, Shin, DS, Carneiro, B, Chandra, S, Kaplan, J, Kalyan, A, Altman, JK, Platanias, L and Giles, F. Repurposing metformin for cancer treatment: current clinical studies. Oncotarget. 7: 40767-40780 (2016).
26. Dowling, RJ, Zakikhani, M, Fantus, IG, Pollak, M and Sonenberg, N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. Cancer Research. 67: 10804-12 (2007).
27. Mohammed, A, Janakiram, NB, Brewer, M, Ritchie, RL, Marya, A, Lightfoot, S, Steele, VE and Rao, CV. Antidiabetic Drug Metformin Prevents Progression of Pancreatic Cancer by Targeting in Part Cancer Stem Cells and mTOR Signaling. Transl Oncol. 6: 649-59 (2013).
28. Zakikhani, M, Blouin, MJ, Piura, E and Pollak, MN. Metformin and rapamycin have distinct effects on the AKT pathway and proliferation in breast cancer cells. Breast Cancer Res Treat. 123: 271-9 (2010).
29. Jalving, M, Gietema, JA, Lefrandt, JD, de Jong, S, Reyners, AK, Gans, RO and de Vries, EG. Metformin: taking away the candy for cancer? Eur J Cancer. 46: 2369-80 (2010).
30. Poloz, Y and Stambolic, V. Obesity and cancer, a case for insulin signaling. Cell Death Dis. 6: e2037 (2015).
31. Camacho, L, Dasgupta, A and Jiralerspong, S. Metformin in breast cancer - an evolving mystery. Breast Cancer Res. 17: 88 (2015).
32. Dowling, RJO, Lam, S, Bassi, C, Mouaaz, S, Aman, A, Kiyota, T, Al-Awar, R, Goodwin, PJ and Stambolic, V. Metformin Pharmacokinetics in Mouse Tumors: Implications for Human Therapy. Cell Metabolism. 23: 567-568 (2016).
33. Kajbaf, F, De Broe, ME and Lalau, JD. Therapeutic Concentrations of Metformin: A Systematic Review. Clinical Pharmacokinetics. 55: 439-59 (2016).
34. Glucophage XR product insert. https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0ahUKEwjcrMLO8cPXAhWGZiYKHVe1DoMQFggrMAA&url=https%3A%2F%2Fpackageinserts.bms.com%2Fpi%2Fpi_glucophage.pdf&usg=AOvVaw2zAbyMgwlF7wxaXv60vtv3.
35. Lipska, KJ, Bailey, CJ and Inzucchi, SE. Use of metformin in the setting of mild-to-moderate renal insufficiency. Diabetes Care. 34: 1431-7 (2011).
36. Wadamori, N, Shinohara, R and Ishihara, Y. Photoacoustic depth profiling of a skin model for non-invasive glucose measurement. 2008 30th Annual International Conference of the Ieee Engineering in Medicine and Biology Society, Vols 1-8. Doi 10.1109/Iembs.2008.46504945644-5647 (2008).
37. Bray, GA, Edelstein, SL, Crandall, JP, Aroda, VR, Franks, PW, Fujimoto, W, Horton, E, Jeffries, S, Montez, M, Mudaliar, S, Pi-Sunyer, FX, White, NH, Knowler, WC and Grp, DPPR. Long-Term Safety, Tolerability, and Weight Loss Associated With Metformin in the Diabetes Prevention Program Outcomes Study. Diabetes Care. 35: 731-737 (2012).
38. Bolen, S, Feldman, L, Vassy, J, Wilson, L, Yeh, HC, Marinopoulos, S, Wiley, C, Selvin, E, Wilson, R, Bass, EB and Brancati, FL. Systematic review: comparative effectiveness and safety of oral medications for type 2 diabetes mellitus. Ann Intern Med. 147: 386-99 (2007).

Appendix A

Standard Operating Procedure for Fresh Tissue Acquisition by Surgery and a Blood Sample (D17188- presurgical metformin study)

1) **Consent**
The patient will be asked to provide informed consent by a treating surgeon, oncologist, or designated personnel at a presurgical consultation visit.
The personnel obtaining consent will notify the CRC [Kayla Fay (Kayla.A.Fay@hitchcock.org)] that a patient has been consented, and the CRC will pick up Consent Form.
The CRC will register the patient into the research database (Velos), and give the patient a unique research identification number (ID#):
"D17188_###" starting with D17188-001 as patient 001.

2) **Blood specimen** (day of surgery; prior to surgery)
20 mL of venous blood (ideally, in two 10-mL tubes) will be obtained prior to surgery (as close to the time of surgery as possible) in the pre-operative area, which may be done during a routine pre-surgical blood draw or placement of an i.v. line.
Blood should be collected in **purple-top (EDTA) tubes** labeled with the patient's name and time of draw.
The person drawing the blood will immediately notify the Miller Lab that the blood specimen is ready for pick-up. A Miller Lab member will pick up the specimen within 1 hour from the Operating Room (OR) Control Desk (located on the 4th floor near the ICU).

Miller Lab e-mail: Todd.W.Miller@Dartmouth.edu
(**preferred**) Miller Mobile phone: 603-359-3463
Miller Lab phone: 603-653-6043
Miller Office phone: 603-653-9284

In the Miller Lab, one 10-mL sample of blood will be aliquoted into cryovials (1 mL/vial, approx. 10 vials) and frozen at -80°C as **whole blood**.
One 10-mL sample of blood will be centrifuged at 1,000 x *g* for 30 min at 4°C (metformin is stable), and **plasma** (supernatant) will be aliquoted into cryovials (1 mL/vial, approx. 5 vials) and frozen at -80°C.

3) **Tumor, adipose, and normal tissue specimens** (by surgery)
The CRC will notify Todd.W.Miller@Dartmouth.edu **with maximal advance notification** of the date of surgery.
The CRC will notify the Pathology Department individuals and Miller Lab individuals listed below 24 hours prior to the time of surgery with notice that a surgical specimen will be arriving and that tissue is to be acquired for this study to **minimize time between surgery and freezing**.
The surgical specimen may provide a diagnostic specimen; excess tissue will be used for research purposes.

Pathology Dept. e-mails: Laura.J.Tafe@hitchcock.org
LABPathPA@hitchcock.org
Miller Lab: Todd.W.Miller@Dartmouth.edu
(**preferred**) Miller Mobile phone: 603-359-3463
Miller Lab phone: 603-653-6043
Miller Office phone: 603-653-9284

Requested specimens: Tumor tissue fragment ≥8 mm in diameter*
Adipose tissue fragment ≥8 mm in diameter*
Normal adjacent tissue fragment ≥8 mm in diameter*

*** small tissue fragments may be pooled to yield ≥8 mm of tissue**

All normal and tumor tissue specimens will be sent to Pathology for dissection.

The Pathology Department individual who receives the surgical specimens will immediately notify the Miller Lab that specimens have arrived, and a Miller Lab member will come to Pathology to acquire tissue.

The Miller Lab member will pre-weigh cryovials, and write the vial weight on tube.

To Pathology, the Miller Lab member will bring cryovials, which need to be labeled with the patient study ID (D17188_###), tissue type, date, time of surgery, and time of freezing.

The Pathologist (Laura Tafe) or her delegate will assess whether the tumor specimen is large enough to procure:

A. tumor tissue for routine diagnostics.
B. ≥6 tumor fragments that are ≥4 mm in all dimensions for research use.

A 1.5-cm tumor is expected to provide sufficient material for both needs.
All research samples should be acquired within 1.5 h of tissue removal from the patient; a shorter lag time is strongly preferred to preserve phospho-proteins.

In Pathology, research tissue specimens with overt blood content will be gently pressed between absorbent towels to **remove blood**. Tissues will be cut into fragments ≥4 mm in all dimensions, and individually placed into cryovials (1 fragment per vial) on ice.

The Miller Lab member will then promptly re-weigh the vials, write the new weight on the vial, freeze vials in liquid nitrogen, and store vials at -80°C.

Time from patient to freezing should be minimized.

4) The following data will be recorded in the specimen log book (template in Appendix B) in the Miller Lab:
   Research ID#
   Date and time of blood draw and surgery
   Time that blood, plasma, and tissue fragments are frozen, and numbers of aliquots/specimens
   Tissue locations and tumor subtypes

Appendix B
Specimen log template

D17188 Research Blood and Tissue Collection **Subject Number:_____**

	Sampling Date and Time	Freeze time	Numbers of aliquots/specimens	Notes
Blood				
Plasma				
Tumor tissue				Tumor subtype and location:
Normal tissue				Tissue location:
Adipose tissue				Tissue location: