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THE METABOLOMIC EFFECTS OF METFORMIN ON COLON CANCER

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THE METABOLOMERIC EFFECTS OF METFORMIN ON COLON CANCER

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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Lexington, Kentucky

Co-Directors: Dr. Tianyan Gao, PhD, Professor of Cellular and Molecular Biology and Dr. B. Mark Evers, MD, Director of Markey Cancer Center

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THE METABOLOMIC EFFECTS OF METFORMIN ON COLON CANCER

Metformin is an oral biguanide that is prescribed to over 120 million people worldwide for the treatment of conditions including type II diabetes mellitus, polycystic ovarian syndrome, and gestational diabetes. This hypoglycemic agent is rapidly emerging as a potential cost-effective anti-oncogenic agent. Over the past decade multiple epidemiologic studies have consistently associated metformin with decreased cancer incidence and cancer-related mortality. More recently numerous preclinical and clinical studies have demonstrated anti-cancer effects of metformin, leading to the proposal of numerous clinical trials to better understand this drug and its mechanisms of action.

Previously experts believed metformin primarily targeted AMP-activated protein kinase (AMPK), a crucial cellular energy sensor, but more recent data suggest the impact of metformin has a multi-faceted impact on various metabolic pathways. Current understanding of the potential anti-cancer effects of metformin raises the intriguing possibility of a duality of action, suggesting that metformin has the ability to act directly on a tumor while also indirectly lowering insulin levels in the host. This complexity creates challenges in determining the true impact of this drug in the clinical and translational setting.

Despite an increase in investment, only one in every 10 new molecular therapeutic agents that enters clinical development receives approval from the Food and Drug Administration. This warrants a demand for better designed clinical trials with more elegant and robust analyses of relevant primary endpoints to determine which targeted therapies are cost-effective, and more importantly which agents will provide the best care for our patients. Stable isotope resolved metabolomics (SIRM) is a powerful tool capable of robust analyses that can address these questions. Using these capabilities we have determined that metformin does significantly impact cellular metabolism by shifting colon cancer cells into glycolytic overdrive, ultimately leading to decreased proliferation and protein synthesis in cancer cells. This study contributes to the literature and implores that we continue to elucidate the full potential of this drug, especially in the setting of personalized medicine where select patients may receive maximal benefit from this agent.

KEYWORDS: Metformin, colon cancer, metabolomics, glycolysis
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1.1 Colon Cancer

Colon and rectal cancers are the second leading cause of cancer related death in the United States with an estimated 150,000 new diagnoses to occur in 2015. Colon cancer is an insidious, ruthless process that takes advantage of our own genetic and cellular pathways and wreaks havoc to create an environment conducive to tumor growth and development. Colonic mucosa is deemed to be at risk for the development of malignancy in the setting of genetic instability which sets the stage for a precursor lesion (1). Cell proliferation increases due to altered metabolism, decreased apoptosis, and unlimited replication which leads to a cancer in situ and more developed adenomas. Later activated protease systems, immune modulation, and angiogenesis or blood vessel development to supply these tumors, leads to all the requirements for the development of an invasive disease and ultimately a metastatic cancer (2). Risk factors for the development of colon cancer include genetic predisposition, dietary factors such as a diet high in red meat and animal fat, low-fiber diets, and low overall intake of fruits and vegetables. Obesity and lifestyle choices such as cigarette smoking, alcohol consumption, and sedentary habits have also been associated with increased risk for colorectal cancer (3-5).

1.1.2 Development and Diagnosis of Colon Cancer

Initial symptoms of colon cancer frequently include abdominal and or pelvic pain, blood in the stools, unintentional weight loss, and eventually sepsis if obstruction and/or perforation of the intestine occur (6). Diagnosis of is often made prior to the development of severe symptoms based on screening and surveillance, especially in high risk populations. It is estimated that full implementation of screening guidelines could cut mortality rate from colorectal cancer in the United States by an estimated additional 50% (7). Other studies such as fecal occult blood testing, carcinogenic embryogenic antigen (CEA), and tissue biopsy aid in the initial diagnosis of colon cancer. Patients may also be evaluated for the presence of metastatic disease, or spread of cancer to distant
organ sites, using computer tomography (CT) imaging of the chest, abdomen, and pelvis, and positron emission tomography (PET) scanning to assess for distant hypermetabolic disease processes.

1.1.3 Staging and Current Treatments of Colon Cancer

Five year survival rates for colon cancer are largely dependent on the stage of disease at the time of diagnosis and treatment. Stages of colon cancer are classified as Stage I-IV. These stages are developed based on the disease burden present in the primary tumor (T), lymph nodes (N), and metastasis (M), which is referred to as TNM staging (8). Stage I disease is defined as T1 (tumor invades submucosa) or T2 (tumor invades muscularis propria), without evidence of nodal or metastatic disease. Stage II is defined as tumors described as T3, (Stage IIA - tumor extends through the muscularis propria), T4a (Stage IIB - tumor penetrates to the surface of the visceral peritoneum), or T4b (Stage IIC - tumor directly invades or is adherent to local organs or structures), but still does not have disease burden in the lymph nodes or at distant organ sites. Stage III is defined as cancer that has spread to the lymph nodes and Stage IV describes metastatic disease that has spread to a single distant organ (Stage IVA, M1a), or is present in multiple different organs and/or the peritoneum (Stage IVB, M1b) (9).

Patients diagnosed with Stage I disease have an average survival of over 90%, whereas patients diagnosed with Stage IV disease have a more dismal prognosis with five year survival rates of around 5% (10). When considering the prognosis for all-comers with this disease, which includes patients with Stages I, II, III, and IV colon cancer, the five year survival rate is 65.9% with an estimated 50,000 deaths to occur this year (11). When broken down by stage at presentation and year survival rates, the prognosis actually sounds much more dismal. 39% of patients present with localized disease which has that higher 90% survival rate previously mentioned, while 36% of present with regionally progressive disease with a 5 year survival rate of 70.8%. With consideration to patients with distal disease, which compromises 20% of this population, survival
drops to 13.1% at five years (12).

Current recommendations for the treatment of colon cancer include surgery and chemotherapy. Depending on the location and extent of the primary tumor, surgical options include right colectomy, extended right colectomy, left colectomy, sigmoid colectomy, and total abdominal colectomy with ileorectal anastomosis (13). These procedures may be performed with a minimally invasive approach (i.e. laparoscopic or robotic) in select patients (14, 15). Patients with stage IV disease limited to the liver and/or lung may benefit from resection of these distant metastatic tumors (16, 17). Patients with residual disease or advanced stage colon cancers are treated with chemotherapy adjuvantly (postoperative) which commonly include 5-fluorouracil (5-FU) with leucovorin or capecitabine, either alone or in combination with oxaliplatin (18, 19). Advances in targeted therapies and personalized medicine has prompted treatment with biologic agents such as Bevacizumab (Avastin), Cetuximab (Erbitux) (20-22). These agents are effective at reducing tumor burden and increasing both disease free and overall survival, but frequently have significant side effects such as severe nausea, vomiting, weight loss, and risk of infectious complications due to immunosuppression.

Recent studies have suggested using off label agents in combination with traditional chemotherapy and biologic agents (23, 24). Metformin, which is an oral hypoglycemic agent traditionally used to treated Type II diabetes mellitus, offers an alternative that will allow decrease doses, and therefore toxicity, of aggressive traditional chemotherapy. We also speculate using this drug during the pre-surgical and immediate post-surgical interval when no other therapeutic intervention such as chemotherapy can safely be offered could potentially lead to decreased micro-metastatic disease which results in later recurrences. Furthermore, patients who cannot tolerate aggressive chemotherapy due to age or other medical conditions could significantly benefit from this safe and less toxic alternative.
1.2 The History and Development of Metformin

Metformin was first described in the scientific literature in 1919, by Emil Werner and James Bell, as a product in the synthesis of \(N,N\)-dimethylguanidine, however it had been in use since medieval times in Europe (1). In the 17th century a English botanist determined the extracts of *Galega officinalis*, also known as French lilac or goat’s rue, could be used to treat polyurea (i.e. increased frequency of urination) associated with the development of diabetes mellitus (2). The active ingredient in the French lilac that produced the lowering of blood glucose was shown to be galegine or isoamylene guanidine, but guanidines alone are too toxic for routine use at high doses (3). The development of biguanides (two linked guanidine rings) were effective and safer than traditional botanicals. Metformin and other oral biguanides such as phenformin and buformin gained popularity in the early 1920’s as effect oral hypoglycemics used to treat diabetes mellitus. Within a decade the use of these agents was discouraged due to ready availability of subcutaneous insulin which was fast-acting and easier to titrate, as well as the increased risk of renal and/or liver failure with increasing doses of biguanides (4-7). Renewed interest in oral biguanides emerged in the 1950’s when scientists began studying the pharmacokinetic effects of the drug and determined previous studies were flawed based on a misunderstanding of the mechanisms and dosing of biguanides (1).

Unfortunately as a number of lactic acidosis and cardiac related deaths increased, phenformin was removed from the market in the United States (US) in 1977. Despite concerns in the US, endocrinologists in France and Scotland felt comfortable continuing to use it cautiously. In 1995 the benefits of metformin were rediscovered in Europe during the United Kingdom (UK) Prospective Diabetes Study which was a randomized, multicenter clinical trial that followed 3867 patients over 10 years (8). Independently of blood glucose control, metformin reduced the risks of myocardial infarction and mortality of all causes and is considered to be safer than other biguanides because it is less lipophilic due to its structure containing two methyl substitutes as opposed to a large phenylethyl side chain, which is seen in phenformin (3, 9) (Figure 1.1).
Figure 1.1. Metformin is less lipophilic because it has two methyl side groups.

Metformin is less lipophilic than because of the two methyl substituents on metformin compared to the larger phenylethyl side chain in phenformin.
Metformin was approved by the Food and Drug Administration (FDA) in 1994 and Glucophage (i.e. “glucose eater”) was the first branded formulation marketed by Bristol-Meyers Squibb (10). It is now prescribed to over at least 120 million people worldwide and is the most commonly used drug for type II diabetes (11).

Since then metformin has also been approved to treat polycystic ovarian disease, non-alcoholic fatty liver disease, and gestational diabetes (12-15). In 1924, the Nobel laureate Otto Heinrich Warburg first hypothesized the existence of a connection between cellular metabolism and malignancy (16). In 1971, Dilman et al. suggested that oral biguanides hold promising potential as potent anti-aging and anti-oncogenic agents (17). Over the past three decades substantial data has surfaced suggesting that metformin may exert cancer and chemopreventive effects by suppressing the transformative and hyperproliferative processes that initiate carcinogenesis (18). Metformin has been shown to have anti-proliferative and anti-metabolic effects against cancer in many different types of cancer including breast, prostate, lung, colon, liver, and ovarian, but there is scarce data describing the specific mechanism metformin utilizes to target these oncogenic cells, notably colon cancer cells (19-24). Furthermore, although metformin has been studied as a molecular inhibitor, there currently is scant literature describing specifically which patients may benefit most from this therapy (25, 26). These issues highlight the need for further of the precise mechanism of this drug and which patients may receive the most benefit from, specifically those with colon cancers.

1.3 Metformin: Mechanisms of Action

Metformin is considered to be a hypoglycemic agent because it lowers blood glucose concentrations in patients with type II diabetes without causing physiologically overwhelming hypoglycemia. Metformin is also frequently described as an insulin sensitizer leading to reduction in insulin resistance and significant reduction of plasma fasting insulin level (27). Metformin is also responsible for inhibiting hepatic gluconeogenesis, however multiple
mechanisms including enzymatic variations in glucose-6-phosphatase (G6P), pyruvate carboxylase-phosphoenolpyruvate carboxykinase (PC-PEPCK), and inhibition of mitochondrial glycerophosphate dehydrogenase (28-31). Metformin also increases anaerobic glucose metabolism in the intestine and decreases gluconeogenesis, glycogenolysis, and fatty acid oxidation in the liver. It is responsible for insulin-mediated glucose uptake, decreases glycogenesis, and decreases fatty acid oxidation in skeletal muscle (11) (Figure 1.2). Central to the understanding of the molecular mechanisms of metformin is the traditionally accepted target 5′ adenosine monophosphate-activated protein kinase (AMPK) (Figure 1.3).

AMPK is a crucial cellular energy sensor because it promotes adenosine triphosphate production (ATP) by increasing the expression of catabolic proteins while conserving ATP by switching off biosynthetic pathways (32). AMPK is activated by increases in AMP:ATP or ADP:ATP ratios, which occur when cellular energy status has been compromised. This frequently occurs in response to metabolic stresses, such as oncogenesis, that either interfere with ATP production or that accelerate ATP consumption. AMPK and its orthologues exist universally as heterotrimeric complexes comprising a catalytic α-subunit and regulatory β- and γ-subunit (33). The function of the catalytic subunits of AMPK is very important to its relationship with tumor biology. For many decades AMPK has been thought to be the main target of Metformin in both its direct and indirect metabolic functions. It consists of three protein subunits that together make a functional enzyme, conserved from yeast to humans. Triggering the activation of AMPK can be carried out when the γ subunit of AMPK undergoes a conformational change so as to expose the active site (Thr-172) on the α subunit. AMPK is activated by increases in AMP and ADP as the cellular concentrations of ATP, ADP and AMP change. In the basal state, sites 1 and 3 in the γ-subunit are occupied by ATP. Replacement of ATP by ADP or AMP at site 3 during moderate stress promotes phosphorylation of Thr172. Replacement of ATP by AMP at site 1 during more severe stress causes a further tenfold allostERIC
Metformin impacts metabolism in the intestine, liver, and skeletal muscle.

Metformin increases anaerobic glucose metabolism in the intestine and decreases gluconeogenesis, glycogenolysis, and fatty acid oxidation in the liver. It also increases insulin-mediated glucose uptake, decreases glycogenesis, and decreases fatty acid oxidation in skeletal muscle. All of this results in systemic hypoglycemia (Adapted from Bailey, Diab Vasc Dis Res, 2009).
Figure 1.3. 5' adenosine monophosphate-activated protein kinase (AMPK) is an enzyme that plays a role in cellular energy homeostasis.

Triggering the activation of AMPK can be carried out when the γ subunit of AMPK undergoes a conformational change so as to expose the active site (Thr-172) on the α subunit. AMPK is activated by increases in AMP and ADP as the cellular concentrations of ATP, ADP and AMP change (Hardie et al. Nat Rev Mol Cell Biol 2012;13:251-262; used with permission).
activation. As cellular energy status returns to normal, AMP at site 1 and ADP or AMP at site 3 are progressively replaced by ATP. This promotes the dephosphorylation of Thr172 and a return to the basal state (33). The major upstream kinases of AMPK are the LKB1–STRAD–MO25 complex and the Ca2+/calmodulin-activated protein kinase kinases (34, 35). The LKB1–STRAD–MO25 complex provides a high basal level of phosphorylation at Thr172 that is modulated by the binding of AMP to the AMPK γ-subunit, which promotes phosphorylation and inhibits dephosphorylation. In tumor cells that have lost the tumor suppressor LKB1 owing to somatic mutations, treatments that increase AMP and ADP levels do not normally activate AMPK because basal activity is too low for the effects of nucleotide binding on phosphorylation status to become evident. This emphasizes that the effects of AMP and/or ADP on Thr172 phosphorylation status are a result of their binding to AMPK, and such effects are independent of the upstream kinases and phosphatases that phosphorylate or dephosphorylate Thr172 (32).

Although metformin activates AMPK, this may not explain all of the therapeutic effects of the drug. Metformin, at least in part, does exert its therapeutic effects by activating the AMPK. However, the site of its action, as well as the mechanism to activate AMPK, remains elusive. Other mechanisms of action have been proposed for metformin such as inhibition of mitochondrial complex I, inhibition of glucagon-induced elevation of cyclic adenosine monophosphate (cAMP), and consequent activation of protein kinase A (PKA), inhibition of mitochondrial glycerophosphate dehydrogenase (31, 36, 37). The exact mechanism of metformin has not been fully elucidated and warrants further review.

1.3.1 Indirect Mechanisms of Action

The indirect insulin-dependent effects of metformin are mediated by its ability to activate 5’ adenosine monophosphate-activated protein kinase AMPK and inhibit gluconeogenesis in the liver and stimulate glucose uptake in muscle. The resulting reduction in circulating insulin alleviates activation of
PI3K/AKT/mTOR pathway signaling in cancer cells. The indirect effects of metformin are due to decreased blood glucose primarily by suppressing glucose production by the liver, or hepatic gluconeogenesis (Figure 1.4). The direct effects of metformin include increased insulin sensitivity, enhances peripheral glucose uptake (by inducing the phosphorylation of GLUT4 enhancer factor), decreases insulin-induced suppression of fatty acid oxidation, and decreases absorption of glucose from the gastrointestinal tract (38, 39). Increased peripheral use of glucose may be due to improved insulin binding to insulin receptors, but this theory also applies to its role as an anti-cancer agent (40, 41). Metformin diminishes hepatic glucose output leading to lower systemic glucose and insulin levels, which could impair malignant growth indirectly without requiring accumulation of metformin in the tumor. High insulin levels associated with obesity, insulin resistance and type 2 diabetes may promote tumorigenesis. Insulin-like growth factors, insulin, IGF-1 and IGF-2, secreted by visceral or mammary adipose tissue have significant paracrine and endocrine effects. These effects can be exacerbated by increased steroid hormone production (41). The insulin receptor lies upstream of a number of growth-promoting pathways including the PI3K/AKT/mTOR signaling network which are implicated in oncogenesis (42, 43).

1.3.2 Direct Mechanisms of Action

The direct insulin-independent effects of metformin are mediated by activation of AMPK and a reduction in mammalian target of rapamycin (mTOR) signaling and protein synthesis in cancer cells (44). The tumor suppressors liver kinase B1 (LKB1) and TSC2 are important in mediating the effects of metformin on AMPK and mTOR respectively; however, metformin may also inhibit mTOR independently of LKB1, AMPK and TSC2 (45). Metformin can act directly on cancer cells by inhibiting cell progression by suppressing mTOR signaling, mitochondrial glucose oxidation, and/or reducing stability of hypoxia inducing factor (HIF) under hypoxic conditions.
Figure 1.4. Metformin has direct and indirect effects on cellular and molecular signaling.

The indirect effects of metformin are due to decreased blood glucose primarily by suppressing glucose production by the liver, or hepatic gluconeogenesis. The direct effects of metformin include increased insulin sensitivity, enhances peripheral glucose uptake (by inducing the phosphorylation of GLUT4 enhancer factor), decreases insulin-induced suppression of fatty acid oxidation, and decreases absorption of glucose from the gastrointestinal tract (Adapted from Luengo et al. BMC Biol 2014;12:82).
1.4 Metformin’s Impact as an Anti-Cancer Agent

Insufficient activity of AMPK allows uncontrolled cell growth, despite the conditions of cellular stress such as those occurring during tumorigenesis, making AMPK an attractive target for anti-cancer therapy. Although numerous AMPK-activating drugs have been described, only metformin is widely used clinically and available in cost-effective generic formulations. Other AMPK activators are currently less suitable for routine clinical use due to the higher rates of lactic acidosis (phenformin) or low specificity and potency (5-aminoimidaz-ole-4-carboxamide ribonucleoside). Novel, more specific AMPK activators are still in preclinical development (46). A rapidly increasing body of preclinical and clinical evidence argues for the use of metformin as an anti-oncogenic agent.

1.4.1 Molecular and Cellular Signaling Targets of Metformin

Protein synthesis is one of the most energy-consuming processes in the cell and, as such, is a major target of AMPK signalling under conditions of cellular energy stress. Upon activation by metformin, AMPK phosphorylates TSC2, stimulating its GTPase-activating protein (GAP) activity towards the small GTPase RHEB, which causes a reduction in mTOR signaling. mTOR controls protein synthesis by regulating the phosphorylation of key proteins involved in mRNA translation, such as the 4E-BPs and S6Ks (Figure 1.5). Inhibition of mTOR signaling leads to a reduction in phosphorylation of downstream effectors like 4E-BPs and S6Ks, therefore resulting in decreased protein synthesis and subsequent cancer cell proliferation (47). As indicated earlier, LKB1 is the immediate AMPK activator in response to cellular energy stress and phosphorylates a key AMPK residue (Thr172) obligate for catalytic activity (22, 33, 48). Cells lacking LKB1 exhibit decreased AMPK activation and elevated mTOR signaling even under conditions of cellular energy stress or treatment with AMPK agonists (49). LKB1 is also required for metformin-mediated AMPK activation. A variety of cells lacking LKB1, are resistant to the growth inhibitory
Figure 1.5. Multiple molecular mechanisms have been proposed to explain how metformin decreases cell metabolism and proliferation.

Although AMPK is thought to be the main mediator of the potential anti-carcinogenic effects of metformin, the molecular mechanism of metformin is incompletely understood: metabolic inhibition of the mitochondrial respiratory chain (complex I) results in increased glycolysis and acetyl-CoA carboxylase (ACC) activation results in decreased lipid synthesis and increased oxidation. Activation of AMPK is also thought to result in decreased proliferation by cell cycle arrest and protein synthesis inhibition via decrease of CD1/CDK and inhibition of mTOR complex I, respectively (Habibollahi et al. J Nucl Med 2013;54:252-258).
effects of metformin in vitro LKB1 and TSC2 represent key factors in determining tumor sensitivity to the effects of metformin (22).

AMPK phosphorylates p53, a tumor suppressor responsible for regulating cell cycle arrest (50, 51). AMPK activation induces phosphorylation of p53 on serine 15, and this phosphorylation is required to initiate AMPK-dependent cell-cycle arrest. AMPK-induced p53 activation promotes cellular survival in response to glucose deprivation, and cells that have undergone a p53-dependent metabolic arrest can rapidly reenter the cell cycle upon glucose restoration. However, persistent activation of AMPK leads to accelerated p53-dependent cellular senescence. Thus, AMPK is a cell-intrinsic regulator of the cell cycle that coordinates cellular proliferation with carbon source availability (52).

Metformin also exerts its downstream therapeutic effects such as regulating lipid synthesis by inhibiting Acetyl-CoA carboxylase (ACC) and regulating mTOR signaling by activating TSC2 (38, 53). Uptake of fatty acids into mitochondria, which seems to be the rate-limiting step in β-oxidation, is promoted by AMPK via phosphorylation and in activation of the ACC2 isoform of ACC (33). This results in a drop in concentration of the ACC product, malonyl CoA, an inhibitor of fatty acid entry into mitochondria mediated by the carnitine O-palmitoyltransferase 1 (CPT1) system (54, 55). Malonyl-CoA is a critical bifunctional molecule, i.e., a substrate of fatty acid synthase (FASN) for acyl chain elongation (fatty acid synthesis) (56). Since ACC is a rate-limiting enzymes in de novo fatty acid synthesis as it catalyzes ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. Loubiere et al. determined metformin does not alter the concentration of malonyl-CoA and that the inhibitory effect of metformin on lipogenesis in prostate cancer cell lines is primarily due to a cellular energy deficit (57). Furthermore, they determined metformin decreases ATP in a dose-dependent manner, and this diminution is significantly correlated with the inhibition of lipogenesis.

1.4.2 Metabolic and Bioenergetic Changes Initiated by Metformin

Altered cellular metabolism is a hallmark of cancer cells (16, 58). Tumor
cells adapt their metabolism to provide the necessary substrates to continue to immortally proliferate (59, 60). Sensitivity to the anti-tumoral effects of metformin may also be governed by expression levels of the organic cation transporters (OCT1). Metformin enters the cell by OCT1, where it then accumulates in the mitochondria (Figure 1.6). There, metformin inhibits complex I of the electron transport chain and mGDP, resulting in decreased NADH oxidation. One of the best described mechanisms of metformin is by inhibition of complex I, the first component of the mitochondrial electron transport chain (58). Complex I inhibition by metformin interrupts mitochondrial respiration and decreases proton-driven synthesis of ATP, causing cellular energetic stress and elevation of the AMP:ATP ratio. Decreased electron chain activity suppresses tricarboxylic acid (TCA) cycle flux and decreases mitochondrial ATP synthesis, and these actions result in increased AMPK signaling, decreased cAMP/PKA signaling, decreased gluconeogenesis and increased glycolysis (28).

Madiraju et al. determined metformin non-competitively inhibits the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase, resulting in an altered hepatocellular redox state, reduced conversion of lactate and glycerol to glucose, and decreased hepatic gluconeogenesis by using whole-body mitochondrial glycerophosphate dehydrogenase knockout mice (31). Acute and chronic low-dose metformin treatment effectively reduced endogenous glucose production, while increasing cytosolic redox and decreasing mitochondrial redox states. mGPD transports cytosolic reducing equivalents from NADH into the mitochondria via the glycerol-phosphate shuttle. Interestingly, inhibition of mGPD and complex I both compromise the ability of mitochondria to oxidize cytosolic NADH and decrease the entry of these reducing equivalents into the electron.

Mitochondria are highly sensitive to stress and are susceptible to oxidative damage which accompanies inflammation and subsequently an ideal environment for tumor establishment and proliferation (61). The sensitivity of measuring mitochondrial stress may offer novel insight into translational questions regarding mitochondria as an energy sensor and targeted molecular therapies for cancer like metformin (26) (Figure 1.7).
Figure 1.6. Metformin inhibits mitochondrial metabolism.

Metformin enters the cell by organic cation transporter 1 (OCT1), where it then accumulates in the mitochondria. There, metformin inhibits complex I of the electron transport chain and mGDP, resulting in decreased NADH oxidation. Decreased electron chain activity suppresses tricarboxylic acid (TCA) cycle flux and decreases mitochondrial ATP synthesis. These actions result in increased AMPK signaling, decreased cAMP/PKA signaling, decreased gluconeogenesis and increased glycolysis (Luengo et al. BMC Biol 2014;12:82).
Figure 1.7 XF Extracellular Flux Analysis can be used to determine the bioenergetic health index.

The specific metabolic downstream effects Metformin causes by mitochondrial inhibition can be determined by performing a mitochondrial stress test using measurements of cellular oxygen consumption rate (OCR) (Chacko et al., Clin Sci (Lond) 2014;127:367-373).
1.4.3 Epidemiologic Evidence of Metformin as an Anti-Cancer Agent in Colon Cancer

Evans et al. were the first to report a potential association of metformin use with reduced cancer incidence. In diabetics receiving metformin (as opposed to other therapies), overall cancer incidence was lowered (odds ratio (OR) 0.86 and 95% confidence interval (95% CI) (0.73–1.02) and there was evidence of a dose response in relation to the total duration of use or number of prescriptions dispensed (62). Landman et al. showed that metformin use was associated with lower cancer mortality compared to no metformin use (hazard ratio 0.23–0.80) and that the effect was dose dependent (63). Specifically to colon cancer, Cho et al. recently reported that in patients taking metformin independently associated with decreased adenoma incidence (OR=0.738, 95% CI=0.554–0.983, P=0.03) (64). Table 1.1 reviews epidemiologic studies specific to colon cancers.

Although epidemiological studies have fairly consistently reported reduced cancer incidence and/or mortality in diabetic patients who receive metformin in standard clinical doses (1500–2250 mg/day in adults), these studies have important methodological limitations. Most were conducted retrospectively and many sampled their cases from hospital or clinical registries rather than population-based registries, thereby limiting external validity and introducing potential selection biases (62, 64). Inclusion criteria varied (65). Some studies did not exclude individuals with prior diagnosis of cancer, thus introducing a possible reverse causation bias (65). Many studies included patients exposed to a variety of treatments for diabetes complicating the analysis of metformin associations (62, 65-67). However, despite the flaws in the epidemiologic data there is valid evidence to support that this drug potentially has anti-oncogenic capabilities.
Table 1.1. Recent epidemiologic studies evaluating metformin colon cancer.

<table>
<thead>
<tr>
<th>Study type</th>
<th>Outcome</th>
<th>Sample size</th>
<th>Comparison</th>
<th>Results (HR/OR/RR, 95% CI)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrospective cohort</td>
<td>Incidence</td>
<td>62,809</td>
<td>Sulfonurea</td>
<td>HR-1.8 (1.29-2.53)</td>
<td>Currie et al. (2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Metformin + sulfonurea</td>
<td>HR-1.43 (1.05-1.94)</td>
<td></td>
</tr>
<tr>
<td>Prospective cohort</td>
<td>Incidence</td>
<td>480,984</td>
<td>Any vs. no metformin</td>
<td>HR-0.36 (0.13-0.98)</td>
<td>Lee et al. (2011)</td>
</tr>
<tr>
<td>Retrospective cohort</td>
<td>Mortality</td>
<td>595</td>
<td>Any vs. no metformin</td>
<td>HR-1.45 (1.08-1.93)</td>
<td>Lee et al. (2012)</td>
</tr>
<tr>
<td>Retrospective cohort</td>
<td>Incidence</td>
<td>995,843</td>
<td>Any vs. no metformin</td>
<td>RR-0.643 (0.490-0.845)</td>
<td>Tseng (2012)</td>
</tr>
<tr>
<td>Retrospective cohort</td>
<td>Adenoma incidence</td>
<td>12,186</td>
<td>Any vs. no metformin</td>
<td>OR-0.738 (0.554-0.983)</td>
<td>Cho (2014)</td>
</tr>
</tbody>
</table>

Abbreviations: Hazard ratio (HR), Odds ratio (OR), Confidence Interval (CI).
1.4.4 Pre-clinical Evidence of Metformin as an Anti-Cancer Agent in Colon Cancer

Despite extensive funding in research and development to cure colon cancer, few agents pass preclinical trials to demonstrate efficacy in humans. These advancements most often rely on murine models to screen for efficacy before progressing to clinical trials. Previously there has been little focus on the validation of these models, even in the era of targeted therapy where understanding the genetic signatures of tumors under study is critical. Table 1.2 represents recent animal models used to evaluate metformin in colon cancer.

Each of these models provides valuable information regarding metformin. Li et al. determined with either vitamin D3 or metformin alone, combined use of vitamin D3 and metformin showed more pronounced effect in reducing the numbers of aberrant crypt foci (ACF) and tumor in the colon and was associated with downregulation of S6P expression, via the AMPK (IGF1)/mTOR pathway (68).

Algire et al. determined administration of metformin led to the activation of AMPK, the inhibitory phosphorylation of ACC and increased apoptosis as estimated by poly (ADP-ribose) polymerase (PARP) cleavage in mice fed a high-energy diet on growth of an in vivo colon cancer model in mice injected with MC38 colon carcinoma cells (23). Zafaar et al. evaluated the effects of induced diabetes with metformin treatment in combination with or without oxaliplatin and determined that metformin did have anti-proliferative effects with this murine model (69). Buzzai et al. determined that metformin is selectively toxic to p53 deficient cells. They used paired isogenic colon cancer cell lines HCT116 p53(+/+) and HCT116 p53(-/-) treatment with metformin selectively suppressed the tumor growth of HCT116 p53(-/-) xenografts (50). Hosono et al. used an azoxymethane (AOM) model with a 6 week intraperitoneal injection of metformin (250mg/kg/d) and determined that metformin suppresses colonic epithelial proliferation via the inhibition of the mTOR pathway through the activation of AMPK (70).
Table 1.2. Recent murine models using metformin to study colon cancer.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Animal</th>
<th>Model</th>
<th>Dose</th>
<th>Route</th>
<th>Effect on pathways</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Wister rat</td>
<td>DSS</td>
<td>120 mg/kg/d</td>
<td>i.p.</td>
<td>decrease PI3K/mTOR</td>
<td>Li, 2015</td>
</tr>
<tr>
<td>Male</td>
<td>C57BL/6</td>
<td>5×105 MC38</td>
<td>50 mg/kg</td>
<td>d.w.</td>
<td>decrease FASN, Akt</td>
<td>Algire, 2010</td>
</tr>
<tr>
<td>Male</td>
<td>p53 WT vs KO</td>
<td>HCT116 s.c.</td>
<td>250 mg/kg</td>
<td>i.p.</td>
<td>p53(-/-) less autophagy</td>
<td>Buzzai, 2007</td>
</tr>
<tr>
<td>Male</td>
<td>Swiss albino</td>
<td>DMH</td>
<td>100 or 200 mg/kg</td>
<td>d.w.</td>
<td>decrease angiogenesis</td>
<td>Zafaar, 2014</td>
</tr>
<tr>
<td>Male</td>
<td>BALB/c</td>
<td>AOM</td>
<td>(250 mg/kg/d)</td>
<td>i.p.</td>
<td>aberrant crypt foci</td>
<td>Hosono, 2010</td>
</tr>
</tbody>
</table>

Abbreviations: DMH-dextran sodium sulfate (DSS), azoxymethane (AOM), intraperitoneal (i.p.), drinking water (d.w.), subcutaneous (s.c.), mTOR mammalian target of rapamycin, fatty acid synthase (FASN), 1,2-dimethylhydrazine (DMH).
Although each of these recent models contributes to the literature, collectively there is such a profound variety in available models with regard to cancer induction model, delivery of treatment, doses of treatment, and varieties and combinations of treatments it is difficult to draw substantial conclusions which would warrant proceeding with performing a clinical trial. The need for translational data prompts researchers to seek more clinically applicable models by reviewing human data obtained through clinical and translational programs.

1.4.5 Clinical Evidence of Metformin as an Anti-Cancer Agent in Colon Cancer

Gaining access to human and clinical specimens is a priority to better understand the true mechanisms of metformin and other molecular inhibitors that act in a multi-faceted manner. Baba et al. used immunohistochemistry data to determine that phosphorylated AMPK expression in colorectal cancer is associated with superior prognosis among p-MAPK3/1-positive cases, suggesting a possible interaction between the AMPK and MAPK pathways influencing tumor behavior (71). Julien et al. obtained and implanted 54 human colorectal tumors in immunodeficient mice and rats, representing 35 primary tumors, 5 peritoneal cancers and 14 metastases (72). Furthermore, they determined through extensive in vivo analysis they were able to compare the molecular profile with the drug sensitivity of each tumor mode and found that the key role of KRAS mutation in cetuximab resistance. Using patient derived xenografts (PDX) Nunes et al. IGF2 upregulation (16%), which was mutually exclusive with IRS2, PIK3CA, PTEN, and INPP4B alterations, supporting IGF2 as a potential drug target (73). Development of targeted therapeutics required translationally relevant preclinical models with well-characterized cancer genome alterations. Colorectal PDXs are appropriate tools to identify both new targets and predictive biomarkers of response/resistance to targeted therapies.
1.5 The Challenges of Moving Metformin from the Bench to the Bedside for Cancer Treatment

1.5.1 Review of Previous Clinical Trials Using Metformin as an Anti-Cancer Agent in Colon Cancer

Prospective randomized clinical trials designed to use metformin for the treatment of breast and prostate cancers are just now reaching accrual and/or offering relevant data regarding the efficacy of using this treatment as a molecular inhibitor to target cancer (74-76). Most of the breast cancer trials that have met accrual and finished data collection or have evaluated initial data determined there was no difference in mortality or proliferative biomarkers when comparing their treatment groups (77-80). A couple of recently performed trials did show significant decreases in proliferative markers such as Ki67 (81, 82). Interestingly in their recent randomized placebo controlled trial Bonanni et al. determined that the metformin effect on Ki-67 change relative to placebo was not statistically significant, with a mean proportional increase of 4.0% (95% CI, -5.6% to 14.4%) 4 weeks apart, but did show significantly different effects according to insulin resistance, particularly in luminal B tumors (19). Kalinsky et al. determined that although there was no reduction in ln(ki-67) after metformin (p=0.98) or compared to controls (p = 0.47) that there was a significant reduction in BMI, cholesterol, and leptin (83).

Thus far there is little data regarding completed clinical trials observing the effect of metformin on colon cancer. Table 1.3 offers a brief overview of the current clinical trials on available on the National Cancer Institute (NCI) site (www.clinicaltrials.gov). Multiple trials are currently ongoing while others had difficulty reaching accrual. Hosono et al. performed a prospectively randomized trial on 26 nondiabetic patients with aberrant crypt foci to treatment with metformin (250 mg/d, n = 12) or no treatment (control, n = 14) (84). They examined proliferative activity in colonic epithelium (via proliferating cell nuclear antigen labeling index) and apoptotic activity (via terminal deoxynucleotidyl
Table 1.3 Recent and ongoing clinical trials using metformin for colon cancer.

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Institute</th>
<th>Design</th>
<th>Trial Arms</th>
<th>Inclusion Criteria</th>
<th>Primary End Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT01926769*</td>
<td>Gachon University</td>
<td>Non-randomized</td>
<td>1. FOLFOX6 + Metformin</td>
<td>Advanced CRC</td>
<td>DFS, OS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. FOFIRI + Metformin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01930864+</td>
<td>Barretos Cancer Hospital, Gil Medical Center</td>
<td>Non-randomized</td>
<td>1. Irinotecan 350mg/m² q21d + metformin up to 2500mg/d</td>
<td>Refractory to oxali-platin, 5-FU, irinotecan</td>
<td>Non-progression at 12 weeks</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Irinotecan alone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01632020#</td>
<td>University of Arkansas (Slow accrual)</td>
<td>Randomized, double-blinded placebo</td>
<td>1. Metformin 850mg PO BID 14-21 days</td>
<td>Stage I-IV colon or rectal cancer</td>
<td>Proliferation status of CRC tumor and adjacent normal tissue following Metformin therapy</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Study ID</th>
<th>Institution</th>
<th>Design</th>
<th>Treatment</th>
<th>Phase</th>
<th>Comparator</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT01816659#</td>
<td>M.D. Anderson Cancer Center (Slow accrual)</td>
<td>Randomized, double-blinded placebo</td>
<td>1. Metformin 850mg PO BID 14-21 days 2. Control</td>
<td>Stage I-IV colon or rectal cancer</td>
<td>Compare Ki67 biopsy vs. surgery</td>
</tr>
<tr>
<td>NCT01725490*</td>
<td>Yonsei University</td>
<td>Randomized, double-blinded placebo</td>
<td>1. Metformin 500mg PO Qday 2. Metformin 1500mg PO Qday 3. Control</td>
<td>FAP</td>
<td>Specimen number and size of polyps</td>
</tr>
<tr>
<td>NCT02201381!</td>
<td>Health Clinics Limited (Pending discussions with regulatory ethical bodies)</td>
<td>Prospective (QOL measure / form)</td>
<td>1. Atorvastatin 80mg Qday 2. Metformin up to 1000mg once a day 3. Doxycycline 100mg day for 1 Qday month 4. Mebendazole 100mg Qday</td>
<td>Stage IV</td>
<td>PFS</td>
</tr>
<tr>
<td>NCT01312467©</td>
<td>National Cancer Institute (NCI)</td>
<td>Single arm</td>
<td>1. Metformin 500mg PO Qday</td>
<td>Colon cancer, Colon adenoma, Obesity</td>
<td>Change in activated S6serine 235 over 12 weeks</td>
</tr>
</tbody>
</table>

Abbreviations: Disease free survival (DFS), Overall survival (OS), Colorectal
cancer (CRC), per oral (PO), twice daily (BID), Familial Adenomatous Polyposis (FAP), every day, (Qday), Quality of life (QOL).

*Recruiting +Not recruiting #Terminated !Suspended ©Completed.
transferase dUTP nick-end labeling). At 1 month, the metformin group had a significant decrease in the mean number of ACF per patient (8.78 +/- 6.45 before treatment versus 5.11 +/- 4.99 at 1 month, P = 0.007), whereas the mean ACF number did not change significantly in the control group (7.23 +/- 6.65 versus 7.56 +/- 6.75, P = 0.609). To date this is the first reported trial of metformin for inhibiting colorectal carcinogenesis in humans provides preliminary evidence that metformin suppresses colonic epithelial proliferation and rectal aberrant crypt foci formation in humans, suggesting its promise for the chemoprevention of colorectal cancer.

1.5.2 Elucidation of the Metabolomic Impact of Metformin in Colon Cancer

Most of the clinical trials that have been completed thus far must combine different tumor biology, patients with variable health backgrounds, and off-target primary outcomes in order to meet accrual and continue to secure funding (85-87). Evaluating molecular end points can be hindered by difficulties in procuring tumor tissue before and after drug administration and by heterogeneity in previous exposure to cancer therapies.

To determine the true effects of metformin on colon cancer cells will require differentiation of the relative importance of indirect (insulin-mediated) and direct (insulin-independent) effects. The currently accepted mechanisms of action give rise to a broad range of therapeutic targets, leading to a large number of potential clinical markers of metformin benefit, so clinical and translational scientists must choose wisely and aim well when choosing which drugs to use and which molecular pathways to target (88). Recently developed high-throughput experimental technologies, summarized by the terms genomics, transcriptomics, proteomics and metabolomics provide for the first time ever the means to comprehensively monitor the molecular level of disease processes (89). These advances can characterize a drug target's physiology and improving the overall efficiency of pharmaceutical research processes. Fendt et al. used treated prostate cancer cell lines with metformin and performed labeled $^{13}$C-glucose and $^{13}$C-glutamine to measure the incorporation of labeled carbon from
either nutrient into the tricarboxylic acid cycle metabolite α-ketoglutarate by gas chromatography - mass spectrometry (90). They determined that metformin inhibition of glutamine anaplerosis in the presence of metformin further attenuated proliferation while increasing glutamine metabolism rescued the proliferative defect induced by metformin (Figure 1.8).

The Warburg effect describes the biochemical anomaly that occurs when a shift in glucose metabolism occurs from mitochondrial oxidative phosphorylation to glycolysis, therefore the tumor is phenotypically exhibiting the effects of anaerobic metabolism (i.e. increased lactate production) (91). These metabolic hallmarks can be readily assessed either in vitro or in vivo by metabolic technologies to monitor responsiveness and resistance to novel targeted drugs.

1.5.2a Stable Isotope-Resolved Metabolomics (SIRM)

Metabolomics can be defined as a “systemic interrogation of the metabolome in terms of metabolite concentration, structure, and transformation of pathways” (92). One of the most reliable methods of measuring the complexity of oncogenic metabolomic abnormalities, namely of glucose metabolism and phospholipid turnover, is by SIRM (93). Isotopes are different forms of a respective element. The atoms have the same number of protons, but different numbers of neutrons. For example, when considering the isotopes of carbon, the “most common” carbon is $^{12}$C, as it is stable and quite abundant in the environment (98.89%). It is important to point out that $^{12}$C is balanced in the sense that it has 6 protons and 6 neutrons, therefore the term “isotope” has no impact on the nuclear stability. Similarly $^{13}$C, which has 6 protons and 7 neutrons, is a staple isotope, is not radioactive, but is much less abundant (1.11%) than $^{12}$C. $^{13}$C, if it has 6 protons and 8 neutrons, is a radioisotope and is not considered to be a stable isotope; however, this isotope is extremely rare.

Although stable isotopes do have the potential to become unbalanced, and therefore unstable, this generally does not happen as the difference
Figure 1.8 Metformin specifically blocks glutamine from contributing to glycolytic metabolism in prostate cancer cells.

Previous authors determined that metformin attenuates proliferation in prostate cancer cells by inhibiting complex I of the respiratory chain and that metformin increases the dependency of the prostate cancer cell lines on reductive glutamine anaplerosis of the tricarboxylic acid (TCA) or Kreb’s cycle. Therefore, they suggest a combination therapy between metformin and inhibitors of the glutamine pathway might have a therapeutic benefit in prostate cancer (Fendt et al. Cancer Res. 2013; 73:4429-4438; used with permission).
between $^{12}$C and $^{13}$C is very small and it does not have the ability to participate in the bond-breaking step in the metabolic sequence (94). Finally, isotopomers should not be confused with isotopes. Isotopomers are compounds of identical chemical structure but are different in isotopic composition at individual atoms (92). This important concept is how different structures can be identified once they are labeled (Figure 1.9).

Sample Processing

From a clinical and translational, as well as ethical, standpoint it is imperative to note that $^{13}$C is naturally occurring in the environment and does not pose a health risk to animals or humans (95). Animal or human tissues may be labeled by various methodologies. In vivo labeling generally requires access to either a intravascular compartment (intravenous) or a body compartment (intraperitoneal, subcutaneous) so the $^{13}$C may be ultimately absorbed and circulated throughout the body in order to effectively label the tissues. Once the tissues are adequately labeled the clinical and translational scientist must take extreme caution to produce excellent precision and accuracy during sample collection and processing to ensure high quality, structured data (96). The ultimate goals of excellent sample processing are as follows: 1) to maintain biochemical integrity during sampling; 2) to efficiently and reproducibly recover metabolites from biospecimens with high throughput; 3) to increase the coverage of the metabolome on limited quantities of biospecimens; 4) to determine trace level and/or labile metabolites in the presence of stable and abundant species, with high throughput; 5) to enable large-scale metabolite identification and automation of metabolite assignment; 6) to identify de novo or obtain crucial structural information of unknowns, with or without the use of metabolite; databases; 7) to facilitate metabolite quantification without the need for authentic standards (93).

Briefly, sample processing involves sample homogenization and sample extraction. During homogenization samples must be broken into very fine particles (i.e. <10µm) without compromising their biochemical integrity (96).
Figure 1.9 $^{13}$C-6 Glucose labeling identifies specific components of the citric acid cycle.

Stable Isotope-Resolved Metabolomics (SIRM) refers to the labeling of stable isotopes which are different forms of a respective element (i.e. $^{13}$C versus $^{12}$C). The atoms have the same number of protons, but different numbers of neutrons. An isotopomer is a compound of identical chemical structure but differs in isotopic composition at individual atoms which allows for the analysis and editing of different metabolites after SIRM (Image courtesy of Teresa Fan, PhD).
Typical homogenization techniques may include manual grinding with a pestal (liquid \( N_2 \) compatible) or mechanical liquid \( N_2 \) homogenizers. For very small samples (i.e. biopsy samples, small \textit{ex vivo} labeled tissues), in order to maximize the amount of sample retrieved, it may be necessary to homogenize these samples with glass beads using a micro ball mill directly in solvents that denature and precipitate the proteins without affecting their biochemical integrity.

Sample extraction includes separation of the polar fraction metabolites from the lipid fraction and protein fractions using acids or solvents. Lyophilization should be employed after polar extraction in order to remove water, trichloroacetic acid (TCA), and ammonium bicarbonate. Sixty percent acetonitrile, acetone, or chloroform/methanol/water are ideal solvents for extraction because they are compatible with acid-labile metabolites (i.e. fructose-2,6-bisphosphate, UDP-\( N \)-acetylglucosamine) and the solvent extraction method can be integrated with the solvent quenching steps (96). Of note, TCA extraction is an excellent is superior with regard to sample throughput because the removal of the volatile TCA can be done with lyophilization, but it can lead do degradation of some metabolites like glutamine.

One of the benefits of metabolomic analysis is that a robust amount of data can be gained from careful attention to ensure quality labeling and precision sample preparation, however if this standard is not respected then \( ^{13}C \) labeling will not occur on all of the appropriate positions and the sample will be compromised. This error will likely require re-analysis or re-extraction, and will not yield the robust statistical analysis that is standard when samples are prepared with care and precision.

\textbf{1.5.2b Nuclear Magnetic Resonance (NMR)}

NMR is a powerful tool that can be used to identify and quantify metabolites in an unfractionated mixture like serum or media samples or crude cell/tissue extracts (97). When SIRM is used, NMR can determine the positional isotopomer distributions of metabolites derived from a precursor enriched in stable isotopes such as \( ^{13}C \) and \( ^{15}N \) via metabolic transformations. NMR readily
determines each positional isotopomer as a distinct species without the need for fragmentation, however one disadvantage of NMR is its relatively low sensitivity (97). With regard to 2D experiments, as $^{13}$C is a magnetic nucleus, whereas $^{12}$C is not, a $^{13}$C NMR experiment recorded at natural abundance with proton decoupling will consist of a series of singlets (i.e. one for each carbon atom), but if a molecule is enriched with $^{13}$C, adjacent carbons will couple and also “split” like what is seen in the $^1$H spectrum observed in homonuclear scalar experiments. For screening purposes 1D HSQC (heteronuclear single quantum coherence) can help select metabolites that are $^{13}$C enriched, but sometimes this is an overestimation of labeling since also takes into account additional $^{13}$C atoms (98). When combined with mass spectrometry, NMR provides a very accurate measurement of complementary information.

### 1.6 Purposes and Aims of Studies

Although the exact mechanisms of metformin are not entirely understood, there is robust literature that defines the hallmarks of cellular and molecular signaling in colon cancer cell lines with regards to protein synthesis, fatty acid oxidation, and cell cycle arrest (23, 51, 99). Approaching this project, the primary research questions is what exactly are the metabonomic effects of metformin on colon cancer cells, especially since this has already been determined in other cancers such as breast and prostate (90, 100)? Furthermore, additional questions stem from the tremendous phenotypic and genotypic variability seen when comparing individuals within our patient population. How can we expect one drug to work exactly the same way as a molecular inhibitor for every patient? The world of metabolomics opens amazing opportunities to analyze each patient’s cancer and potentially find personalized targeted therapies for responders and potentially elucidate more beneficial therapies through pathway discovery for non-responders (74, 92, 101) (**Figure 1.10**).
Figure 1.10 Specific impact of metformin on the citric acid cycle in colon cancer cells.

Previous data suggests that metformin does decrease colon cancer cell proliferation and metabolism, especially when used in combination with chemotherapeutic agents. However, the current literature lacks information regarding exactly how and where metformin impacts colon cancer metabolism in vitro patient derived cell lines, patient derived xenografts in murine models, and in ex vivo human tissues studies. The aim of our multi-faceted study is to determine the specific glycolytic mechanisms of how metformin impacts human colon cancers and to determine if certain molecular inhibitors may be more beneficial than other for select patients.
The hypothesis is that each colon cancer cell line and collected human tissue will have its own unique metabolic profile. I also hypothesize that a newly created colon cancer cell line from a PDX that was originated from a locally invasive cancer will be less responsive to metformin treatment and provide a more realistic concept of human patient tumor biology. I anticipate there will be notable changes through the citric acid cycle, and it is very likely that metformin will increase lactate production since it forces most cells into a more glycolytic form of metabolism. Furthermore, we anticipate that a newly created PDX will provide an excellent and reproducible background on which we can treat samples with in vivo murine models for future drug discovery models.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Cell lines

Colon cancer cell lines SW480, KM20, HT29, and HCT116 were authenticated November 2011 (Genetica DNA Laboratories, Cincinnati, OH). Cancer cell line PDX was created from human tissues collected (Appendix A) at the University of Kentucky after three generations of tumor implantation into (severe combined immunodeficiency SCID) mice (Appendix B). Table 2.1 contains specific information for cell lines.

2.2 Animal studies

All animals were housed in a specific pathogen-free animal facility (060-immunodeficient animal room) at the University of Kentucky BioPharmacy Complex with 12 hour light-dark cycle. Animals received water and chow ad lib. All animal procedures were reviewed and approved by the University of Kentucky IACUC (Appendix B).

2.2.1 Implantation of PDX

SCID mice underwent implantation of human derived tissues obtained from consenting patients with colon cancer undergoing surgical resection at the University of Kentucky. Interaction with these patients and the collection of these tissues was approved by the University of Kentucky IRB (Appendix A).

The general procedure for the subcutaneous implantation of involves preparation of the tumor by washing it with 2% Penicillin/Streptozosin/Fungizone in phosphate buffered saline (PBS) at least three times for all colon samples. Original patient derived tissue is flash frozen in liquid nitrogen (LN₂) for protein analysis and additional tissue is placed in 10% formalin for 24 hours, the 70% ethanol to later be paraffin blocked and prepared for immunohistochemistry (IHC). Next the tumor is cut into very small pieces (i.e. tumor slurry) and a portion is placed in 100µL of Matrigel (Corning, Bedford, MA) and remains on ice while transported to the animal facility for implantation.
<table>
<thead>
<tr>
<th></th>
<th>SW480</th>
<th>KM20</th>
<th>HT29</th>
<th>HCT116</th>
<th>PDX</th>
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Abbreviations: (DMEM), (FBS), carcinoembryonic antigen (CEA), transforming growth factor (TGF), cancer (CA).
Additives: sodium pyruvate, non-essential amino acids, multivitamins
The animal facility is reserved and sterile equipment is prepared and covered to ensure sterility. The animal is anesthetized with isofluorane and oxygen at 2L/min. The skin is prepped with betadine and alcohol and covered with sterile towels. The skin is incised with an 11” scalpel and a subcutaneous flank pocket is developed using an 18” gauge blunt needle. The tumor slurry is pipetted into the pocket, ensuring there is no spillage of contents. The skin is gently grasped while a buried subcuticular stitch using 5-0 absorbable suture (Ethicon, Somerville, NJ) to approximate the skin edges. The skin is cleaned with alcohol and the animal is allowed to gently wake up. Further details regarding this procedure can be found in Appendix B.

2.2.2 Randomized trial of intraperitoneal metformin injection

Ten 8-week old SCID mice, all weighing over 25.0 grams were implanted with a single third generation PDX as described above. All implanted tumor volumes were from an equally divided second generation PDX and mixed with 1.5mL of Matrigel (Corning, Bedford, MA). Each mouse received 100µL of the 3rd generation tumor/Matrigel slurry delivered subcutaneously to the left flank by strict sterile technique. After 22 days all ten mice developed subcutaneous tumors. Mice were randomized into two groups. They were weighed and tumors were measured on days 1, 7, and 14. The control group received a daily intraperitoneal injection of 30µL of sterile normal saline for 14-16 days of treatment. The metformin group received a daily injection 30µL of Metformin (200mg/kg/day) for 14-16 days. The mice received water and regular chow ad lib.

2.2.3 13C-glucose tissue and plasma labeling

Prior to sacrifice, in order to achieve 13C-glucose tissue and plasma labeling a 20% solution of 13C-Glc in PBS (1.075 M) was sterile-filtered and 100 µL of this solution (i.e. 20 mg of 13C-Glc) were injected into the lateral tail vein while the mouse was under intermittent anesthesia. Mice were injected with three sequential injections of 100 µl of 20 % glucose 15 minutes part (i.e. at 0, 15, 30, with sampling at 45 minutes post injection). Approximately 50 µL of blood samples were taken intraorbitally at timed intervals, chilled on ice 5 minutes after
standing at room temperature and separated into plasma and blood cells by centrifugation at 4°C at 3,500 g for 15 minutes. Plasma was immediately flash-frozen in liquid N2 for storage prior to metabolite extraction.

2.2.4 Tissue harvest

Mice were sacrificed humanely by cervical dislocation at different times post 13C-Glc injection, and the following xenograft was resected. Dissected tissue was blotted, washed with water, blotted and flash-frozen in liquid N2 within 3 minutes of euthanasia. Additional tissues were flash frozen and placed in 10% formalin for 24 hours followed by 70% ethanol for Western blotting and paraffin blocking prior to sectioning for immunohistochemical staining.

Mice were sacrificed over the course of three days. The mice were “evenly” sacrificed (sacrifice day 1-1 control, 1 metformin; sacrifice day 2-2 control, 2 metformin; sacrifice day 3-1 control, 1 metformin).

2.3 Collection of human tissues

The clinical and translational scientist is responsible for identifying and contacting potential patients for the gastrointestinal tissue protocol which received IRB approval in November 2013 (Appendix A). If the patient agrees to participate in the study the clinical and translational scientist obtains consent which is secured in locked cabinet and all electronic patient identifiers are maintained in a password and firewall protected secure file. The biospecimen core and/or operating room circulator is requested to call or page when the specimen is available. The clinical and translational scientist retrieves tissues with ice and distributes and stores the tissues accordingly to other lab personnel.

2.4 Ex vivo tissue labeling/incubation

Tissues intended for labeling were collected and glucose-free DMEM + 0.45% 13C 6-Glucose + 10% dialyzed FBS +antibiotics was prepared. Tissues were washed with antibiotic/antifungal PBS x 3 and then resuspended tissue pieces were placed in 5-10 mL of freshly made and pre-warmed tracer medium in a T25 flask. Next, 0.2 mL of aliquot of tracer medium was removed from the flask
and stored in a 1.5 mL microfuge tube and flash-frozen and store at -80°C. The T25 flask was placed on shaker x 24 hours in 37°C, 5% CO2 incubator. At the end of the tracer treatment, it was removed from shaker and record the weight of the flask. Next the medium was transferred into a 15 mL centrifuge tube and centrifuge at 3500xg or higher speed for 10 minutes at 4°C. Immediately after medium removal, transfer the tissue pieces to a microcentrifuge tube. Wash the tissue pieces on ice with ice-cold PBS 3 times. Remove supernatant and place tube in LN2.

2.5 Cellular and molecular signaling

2.5.1 Proliferation analysis

All cell lines (Table 2.1) were plated in a 6 well dish at 1.0x10⁶ cell number and allowed to seed for 12 hours. Conditions were treatment control, 1mM metformin, and 5 mM metformin at time points of 24 hours, 48 hours, and 72 hours. At each time point the cells were washed with PBS and 500µL of the crystal violet staining solution (0.5% crystal violet in 20% methanol) to the cells in the 6-well dish. These cells were fixed and stained for 20 minutes at room temperature. Next plate was generously rinsed and allowed to dry overnight. The next day the dried and stained cells were stained with 500 µL of 1% SDS and placed on a shaker for 20 minutes to dissolve the stain. The 200 µL of SDS/cell solution was added into a 96 well plate and measured at an absorbance of 570nm.

2.5.2 Western blotting analysis

All cell lines (Table 2.1) were plated in a 6 well dish at 5.0x10⁶ and allowed to seed for 12 hours. Then the cells were treated with varying doses of metformin (control, 1mM, 5mM) for 24 hours. The cells were then collected and lysed with TNN buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM NP40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 25 µg/ml each of aprotinin, leupeptin, and pepstatin A] at 4°C for 30 min. Lysates were clarified by centrifugation (10,000 x g for 30 min at 4°C), and protein concentrations were determined using the
Bradford method (102). Briefly, total protein (100 µg) was resolved on a 10% polyacrylamide gel and transferred to Immobilon-P nylon membranes. Filters were incubated overnight at 4°C in a blocking solution (Tris-buffered saline containing 5% nonfat dried milk and 0.1% Tween 20), followed by a 1-hour incubation with primary antibodies. Primary antibodies analyzed were p-ACC (Cell Signaling #11818) ACC (Cell Signaling #3676), p-AMPK (Cell Signaling #2535), AMPK (Cell Signaling #5831, p-S6 (Cell Signaling #2217) and β-actin. Filters were washed three times in a blocking solution and incubated with horseradish peroxidase-conjugated second antibodies for 1 h. After three additional washes, the immune complexes were visualized by the enhanced chemiluminescence detection system.

2.5.3 Immunohistochemistry

All 10 animals from the randomized trial of intraperitoneal metformin injection and all collected human tissues (immediate post resection and ex vivo incubated) were analyzed for expression of proliferation marker Ki67 (Cell Signaling #9027) and apoptotic marker cleaved caspase-3 (Cell Signaling #9661). Aperioscope digital imaging was used to determine a quantitative score for nuclear staining (Ki67) and positive pixel count (cleaved caspase-3). The extent of expression score was assessed on a scale of 0 to 3 the intensity score was also measured on a scale of 0 to 3 (negative = 0, weak = 1, moderate = 2, strong = 3). Multiplication of the values for intensity and extent of expression provided a score for immunoreactivity.

2.6 Metabolic studies

2.6.1 Seahorse analysis

Cellular OCR was used to measure basal respiration, non-mitochondrial respiration, ATP-linked respiration, and proton leak. ECAR was used to monitor glycolysis. Each cell line was plated in specialized XF96 plates (Seahorse Bioscience, North Billerica, MA). Each cell line was plated at a concentration of 3.0x10^4 cells/well. Twelve hours after seeding cells were treated with control (ddH2O) or 1mM of metformin (Sigma-Aldrich, St. Louis, MO). Plated cells were
maintained for 24 hours in a 37°C/5% CO₂ incubator. One hour prior to assay, cells were equilibrated with bicarbonate-free low buffered DMEM medium without any supplement, or supplemented with glucose or glutamine as indicated, in a 37°C non-CO₂ incubator. Reagents required for the experimental protocol were prepared in assay medium, loaded into reservoirs and automatically injected into plates as required for each assay.

2.6.1a Oxygen consumption rate (OCR)

Experimental design followed the protocol provided by Seahorse Bioscience. Briefly, cells were metabolically perturbed by the addition of three different compounds in succession and the OCR was measured prior to and after injection of each compound. First, cells were injected with oligomycin (1 µM working concentration), which inhibits ATP synthesis and identifies the percentage of OCR devoted to ATP synthesis. The second compound, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (0.6 µM working concentration), an uncoupling agent, collapses the mitochondrial membrane potential; a rapid consumption of energy and oxygen without ATP generation follows. FCCP was used to calculate the maximum and spare respiratory capacity of cells. Finally, cells were exposed to a combination of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor (1 µM working concentration). This combination inhibits mitochondrial respiration and allows calculation of the mitochondrial and non-mitochondrial fractions contributing to respiration.

2.6.1b Extracellular acidification rate (ECAR)

For the first ECAR assessment, non-glycolytic acidification was obtained in the absence of extracellular glucose and pyruvate. Next, cells were exposed to three compounds, with measurements after each injection. The first injection, a saturating concentration of glucose (10 mM), causes an increase in ECAR and defines the rate of glycolysis under basal conditions. The second injection, oligomycin (1 µM working concentration), inhibits mitochondrial respiration and shifts energy production toward glycolysis, revealing the maximum glycolytic
capacity of the cells. The final injection, 2-deoxy-D-glucose (2-DG; 100 mM), a glucose analog, inhibits glycolysis. The difference between glycolytic capacity (Measurement 2) and glycolysis (Measurement 1) defines the glycolytic reserve.

2.6.2 Metabolomic analysis

2.6.2a Cell and media experiments

SW480 and PDX cells were plated at 60-70% confluency in 10 cm dishes. The conditions for both cell lines were as follows: unlabeled control, unlabeled treated with 1mM metformin, $^{13}$C-glucose labeled control, $^{13}$C-glucose labeled treated with 1mM metformin. Prior to placing labeled medium on the cells all cell lines were was twice with PBS. Each plate was weighed before and after extracting 100µL of media (0 hour time point) which was immediately flash frozen. Cells were allowed to incubate in 37°C/C0₂ for 24 hours. Each plate was weighed before and after extraction of 100 µL of media (24 hour time point) and this was also flash frozen. The remaining media was transferred into a 15 ml conical tube and centrifuged at 3,500xg for 20 minutes at 4°C. Supernatant was removed and 100 µl was aliquoted in a 2 ml screw top tube (USA Scientific 1420-8700) for metabolite extraction. 5 mL of cold non-sterile PBS buffer was added onto the plate, and gently rotated on the plate to wash all surface of the plate prior to being suctioned off. Remove PBS by vacuum-suction and repeat.

2.6.2b Cell quenching and collection

1 mL of cold acetonitrile was added to cover the whole plate and allowed to stand for 5 minutes. Next 0.55 mL of nanopure water + 0.2 ml 0.2 mM Tris-HCl pH 8 was added as an internal standard for NMR to the plate. Then the cells were scraped and placed into a 15mL tube for a final acetonitrile to water ratio of 2:1.5 (v/v).

2.6.2c Cell and media experiments using acetone extraction

One lyophilized NMR aliquot of the polar fraction resulting from CH₃CN-H₂O-CHCl₃ extraction of tissue sample on ice and the sample is dissolved in 100 µL of nanopure water and mixed. Then 400 µL of ice cold 100% acetone was
added to perform protein precipitation and vortexes prior to being placed in -80°C for 30 minutes to after a second centrifugation for 20 minutes @ 14,000 rpm in 4°C the supernatant may be transferred to a 2mL screw top tube and the protein pellet is further extracted with 100 µL of 60% acetonitrile and repeated to preserve as much sample as possible. The samples are flash frozen and then transferred to the lyophilizer. Then the dry samples are dissolved in 55 µL of 50% D2O with 50 nmoles DSS (With EDTA) per 0.1 mL and vortex to mix the buffer with the extract and centrifuge for 20 minutes @ 14,000 rpm, 4°C before loading into 1.7 mm NMR tubes to attain a minimum column height of 36 mm.

2.6.2d Tissue extraction for animal and human samples

Frozen tissues were ground in liquid N² to < 10 µm particles in a 6750 Freezer/Mill (Retsch, Inc., Newtown, PA) and extracted simultaneously for soluble and lipidic metabolites as follows. Up to 20 mg of frozen tissue powder in 15 ml polypropylene conical centrifuge tube (Sarstedt, Newton, NC) containing three 3 mm diameter glass beads was vigorously shaken in 2 mL of cold acetonitrile (mass spectrometry grade, stored at -20°C) to denature proteins, followed by addition of 1.5 mL nanopure water, and 1 mL HPLC-grade chloroform (Fisher Scientific). The mixture was shaken vigorously until achieving a milky consistency followed by centrifugation at 3,000 g for 20 minutes at 4°C to separate the polar (top), lipidic (bottom), and tissue debris layers (interface). The polar and lipidic layers were recovered sequentially and the remaining tissue debris (mainly denatured proteins) was extracted again with 0.5 mL chloroform:methanol:butylated hydroxytoluene (BHT) (2:1:1 mM) and centrifuged at 4°C, 22,000xg for 20 min to separate the three phases again. The residual polar and lipid fractions were pooled with respective main fractions. All three fractions were vacuum-dried in a speedvac device (Vacufuge, Eppendorf, New York, NY) and/or by lyophylization. The dry weight of tissue debris was obtained for normalization of metabolite content. The polar extracts were redissolved in 100 % D2O containing 30 nmol perdeuterated DSS (2,2'-dimethyl-2-silapentane-5-sulfonate, Cambridge Isotope Laboratories, Andover, MA) as internal chemical
shift and concentration reference for NMR measurement.

2.6.2e NMR Spectroscopy

NMR spectra were recorded at 14.1 T on a Varian Inova spectrometer equipped with a 5 mm inverse triple resonance cold probe, at 20 °C. 1-D $^1$H NMR spectra were recorded with an acquisition time of 2 s and a recycle time of 5 s to minimize peak saturation. 1-D $^1$H Spectra were typically processed with zero filling to 131 k points, and apodized with an unshifted Gaussian and a 0.5 Hz line broadening exponential. Concentrations of metabolites and $^{13}$C incorporation were determined by peak integration of the $^1$H NMR spectra referenced to the intensity of DSS methyl groups, with correction for differential relaxation, as previously described (103-105).

$^{13}$C profiling was achieved using 1-D $^1$H-(106) HSQC experiments recorded with a recycle time of 1.5 s and $^{13}$C GARP decoupling during the proton acquisition time of 0.15 s. 1-D HSQC spectra were processed with zero-filling to 16 k points and apodized using an unshifted Gaussian function and 6 Hz line broadening.

TOCSY and HSQC-TOCSY spectra were recorded with an isotropic mixing time of 50 ms, a B1 field strength of 8 kHz, and acquisition times of 0.341 s in t2 and 0.05 s in t1. The free induction decays were zero-filled once in t2, and linear predicted and zero filled to 4096 points in t1. The data were apodized using an unshifted Gaussian and a 1 Hz line broadening exponential in both dimensions. Positional $^{13}$C incorporation into labeled metabolites was quantified as previously described (104, 107).

2.7 Statistical analysis

Proliferation data was normally distributed and was therefore compared with a paired two-tailed t-test comparing the control condition to each of the treatment conditions within the respective time point. Statistical analyses were performed with SAS 9.3 (Cary, NC) and a p-value of <0.05 was the standard for statistical significance. Seahorse data was analyzed using a Wilcoxon signed
rank test because the data was not normally distributed and required statistical analysis with a non-parametric test to ensure statistical significance was valid when present. Paired tests using the Wilcoxon signed rank test were employed to assess differential expression of ordinal biomarker immunoreactivity scores between matched normal versus tumor and normal versus metastatic tissues as this data did not appear to be normally distributed based on histogram and required analysis with a non-parametric test. The correlations between biomarkers were measured using Spearman’s correlation coefficient non-normally distributed data. All results are expressed as mean ± SE.
CHAPTER THREE: RESULTS

Introduction

Reprogrammed energy metabolism is a hallmark of cancer cells and is rapidly emerging as a potential target for therapeutic intervention (108). To overcome metabolic stress, cancer cells activate several pro-survival pathways. Activation of AMPK, an established metabolic stress sensor, occurs with even modest decreases in ATP production. This activation promotes enhanced activity of catabolic pathways that generate more ATP and inhibits anabolic pathways (32). AMPK is certainly implicated in the decreased proliferation, lipid synthesis, and protein synthesis observed in human colon cancers, but alone it cannot explain why colon cancer cells exhibit such a significant increase in glycolysis when under metabolic stress.

Madiraju et al. demonstrated that non-competitively inhibits the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase, resulting in an altered hepatocellular redox state, reduced conversion of lactate and glycerol to glucose, and decreased hepatic gluconeogenesis. Acute and chronic low-dose metformin treatment effectively reduced endogenous glucose production, while increasing cytosolic redox and decreasing mitochondrial redox states (31). While this novel mechanism certainly contributes to the effect of metformin on whole body energy metabolism, it is likely one of many mechanisms that contributes to metformin’s actions as an anti-cancer agent.

Fendt et al. determined that metformin decreased glucose oxidation and increased dependency on reductive glutamine metabolism in both cancer cell lines and in a mouse model of prostate cancer. Inhibition of glutamine anaplerosis in the presence of metformin further attenuated proliferation, whereas increasing glutamine metabolism rescued the proliferative defect induced by metformin. These data suggest that interfering with glutamine may synergize with metformin to improve outcomes in patients with prostate cancer (90). Corominas-Faja et al. showed that certain breast cancer cells treated with metformin had an accumulation of 5-formimino-tetrahydrofolate, one of the one-
carbon units responsible for de novo synthesis of purines and pyrimidines (100). We hypothesized that since metformin has similar anti-cancer effects on colon cancer cells that it has in breast and prostate cancer cells that there are novel mechanisms relevant to colon cancer that can be determined with metabolomic analysis. This study focuses on the effects cellular and molecular, as well as metabolic changes seen with the use of metformin on four well-establish colon cancer cell lines and a new, yet aggressive colon cancer cell line. Furthermore, we suggest that metabolomic analysis of colon cancer cells treated with metformin can determine if the drug has effects that can be seen with SIRM and if not can identify where and how certain cancer cell lines and/or human tissues are resistant.

Results
3.1 Cellular and molecular signaling

Metformin decreases colon cancer cell proliferation and increases p-AMPK and p-ACC while decreasing p-S6

3.2 In vitro cellular and molecular signaling

Treatment with metformin results in decreased proliferation and in all cell lines at 5mM for 24 hours, but only in cell lines HT29 and HCT116 was there an effect prior to 72 hours or at a lower dose than 5 mM. Both of these cell lines are less aggressive than S480, KM20, or PDX and the both of these cell lines had virtually no expression of p-S6 with the 5mM dose. SW480 cell line did have a notable decrease in expression of p-S6 and a moderate increase of p-AMPK and p-ACC on Western blot (Figure 3.1A). Only at the 5mM dose of metformin after 72 hours of treatment did metformin significantly affect the proliferation of SW480 cells (Figure 3.1B).

The pattern of response seen in KM20 cells is very to the behavior that is exhibited by SW480 cells. Both have a notable increase in p-S6 with the 5mM
Figure 3.1A Western blot analysis identifies that metformin increases p-ACC and p-AMPK, and decreases p-S6 in SW480 cells.

Increasing doses of Metformin (1mM and 5mM) for a period of 24 hours increases the phosphorylation of p-ACC and p-AMPK and decreases the amount of p-S6.

Figure 3.1B Metformin decreases cell proliferation in SW480 cells.

Metformin only significantly decreases proliferation at doses of 5mM after 72 hours of treatment in SW480 cells (p=0.002).
dose (Figure 3.2A). However, both of these metastatic cell lined do not experience anti-proliferative effects with metformin treatment unless they have been treated with 5mM for at least 72 hours (Figure 3.2B). The HT29 cell line originated from a woman with grade I well-differentiated colon adenocarcinoma in the 1970s. It does not have any very notable genetic mutations and only expresses carcinoembryonic antigen (CEA). The notable difference over 24 hours between the control cell, 1mM, and 5mM is marked (Figure 3.3A). It is also notable that metformin has an anti-proliferative effect on these cells a day earlier than other three more aggressive colon cancer cell lines (5mM 48 hours p=0.005, 5mM 72 hours p=0.002) (Figure 3.4B).

Interestingly, although metformin has a much more dramatic impact on proliferation in HCT116 cells, the increase in p-AMPK and p-ACC and the decrease in p-S6 in HT29 cells compared to the expression seen by Western blotting with the HCT116 cell line suggests that metformin has more of an impact on the pathways that regulate glycolysis, fatty acid synthesis metabolism, and a decrease in protein synthesis (Figure 3.4A). The HCT116 cell line probably has the most impressive response to the anti-proliferative properties of metformin because it has a marked dose dependent response at 48 hours (5mM p=0.003), but at 72 hours even the 1mM doses significantly decreases cancer cell proliferation in HCT 116 cells (72 hours 1 mM p=0.007, 5mM p=0.001) (Figure 3.4B). Not surprisingly, the PDX cell line had virtually no response to treatment with metformin at 24 hours (Figure 3.5A) and displayed decreased cell proliferation only after 72 hours of in vitro treatment (Figure 3.5B).

PDX in from patients with colon cancer maintains histologic integrity despite many generations. This is a very aggressive cell line that originated this year after our lab performed implanted this aggressive locally advanced stage IV adenocarcinoma into a SCID mouse (Figure 3.6).
Figure 3.2A Western blot analysis identifies that metformin increases p-ACC and p-AMPK, and decreases p-S6 in KM20 cells.

Increasing doses of Metformin (1mM and 5mM) for a period of 24 hours increases the phosphorylation of p-ACC and p-AMPK and decreases the amount of p-S6. There is a significant decrease in the cells treated with the 5mM dose (p=0.010).

Figure 3.2B Metformin decreases cell proliferation in KM20 cells.

Metformin only significantly decreases proliferation at doses of 5mM after 72 hours of treatment in KM20 cells.
Figure 3.3A Western blot analysis identifies that metformin increases p-ACC and p-AMPK, and decreases p-S6 in HT29 cells.

Increasing doses of Metformin (1mM and 5mM) for a period of 24 hours markedly increases the phosphorylation of p-ACC and p-AMPK and decreases the amount of p-S6 suggesting that in this cell line is an increase in glycolysis, decrease in fatty acid synthesis, and a decrease in protein synthesis and cell proliferation.

Figure 3.3B Metformin decreases cell proliferation in HT29 cells.

Metformin significantly decreases proliferation at the 5mM dose after 48 hours (p=0.005) and even more so at 72 hours (p=0.002) in HT29 cells. This data suggests that colon cancers with tumor characteristics similar to the HT29 cell line may be more responsive to metformin as an anti-cancer agent.
Figure 3.4A Western blot analysis identifies that metformin increases p-ACC and p-AMPK, and decreases p-S6 in HCT116 cells.

Increasing doses of Metformin (1mM and 5mM) for a period of 24 hours increases the phosphorylation of p-ACC and p-AMPK and decreases the amount of p-S6. This cell line is more responsive than the metastatic cell lines in the study, but it is not as responsive as the HT29 cells.

Figure 3.4B Metformin decreases cell proliferation in HCT116 cells.

Metformin significantly decreases proliferation at the 5mM dose after 48 hours (p=0.003) and even more so at 72 hours (p<0.001) in HCT 116 cells. Most importantly, this cell line also demonstrates a significant decrease in cell proliferation when treated for 72 hours with 1mM of the study drug (p=0.007).
Figure 3.5A Western blot analysis identifies that metformin has less impact on p-ACC and p-AMPK, and p-S6 when PDX is compared to other commonly used colon cancer cell lines.

Increasing doses of Metformin (1mM and 5mM) for a period of 24 hours show little impact on the phosphorylation of p-ACC and p-AMPK, nor does it seem to decrease the amount of p-S6.

Figure 3.5B High doses of metformin decreases cell proliferation in PDX cells after 72 hours.

Although little change is seen at 24 hours of treatment and there actually seems to be an initial increase in metabolism at 24 hours in cells treated with metformin, the drug does decrease proliferation at 48 hours (although not significantly, p=0.444).
Figure 3.6. Example of patient derived tissue implantation (A-D) and resulting patient derived xenograft (PDX) (E-H).

A small pocket is created with an 11” blade scalpel (A). Next the subcutaneous pocket is developed with a blunt 18”gauge needle and the matrigel/tumor slurry is deposited into the subcutaneous pocket (B,C). The wound is closed with 5-0 absorbable suture. After one month of allowing the tumor to develop, a subcutaneous PDX will form (E). The skin is incised and gently dissected away from the tumor (F). The tumor is removed, cleansed, and sectioned (G). The tumor is then implanted into another mouse (steps A-D) to create the next generation of the PDX (H).
Patients who undergo gastrointestinal cancer surgery at the University of Kentucky are offered the option to participate in clinical research. Table 3.1 is a deidentified record of patients whose tissues have evolved into xenografts researchers can use to explore novel cancer therapies and molecular mechanisms. For the most part these tumors maintain their histological integrity after multiple generations through SCID mice, therefore these tissues are clinical relevant and represent a translational science advance (Figure 3.7).

3.3 In vivo cellular and molecular signaling

Ten SCID mice were implanted with third generation tumor from patient 93 as was described in the previous methods section. Approximately three weeks after their initial implantation all ten mice began to uniformly develop tumors. There was no significant difference (p=0.39) when comparing the two randomized groups (Figure 3.8) (Table 3.2).

The two groups started the trial at roughly the same weight, however the treatment group was notably leaner than the control group after the first week (Figure 3.9) (Table 3.3). The mice did gain weight over the course of the next week. Although their tumor size was no different when compared to their counterparts, it is hard to know if this weight gain was due to increased tumor size or metabolic adaptation to metformin. Weight loss is a side effect of metformin so the initial weight loss was not shocking. While these mice were smaller than the control group on their respective days of sacrifice, this difference was not statistically significant (p=0.051).

Metformin has little impact on pS6, p-AMPK, or p-ACC in SCID mice carrying the aggressive PDX from the initial tumor of patient 93 (Figure 3.10). However, observing the IHC staining reveals a decrease in Ki67 therefore suggesting that metformin does indeed impact tumor proliferation in vivo (Figure 3.11). There is no significant difference between the metformin group and the control group when evaluating the apoptotic marker cleaved caspase-3. These findings suggest that an aggressive human tumor may not respond to short
Table 3.1. Deidentified record of tumors progressing to successful patient derived xenograft (PDX).

<table>
<thead>
<tr>
<th>Patient</th>
<th>93</th>
<th>109</th>
<th>130</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/ Gender</td>
<td>63/Male</td>
<td>79/Male</td>
<td>63/Male</td>
</tr>
<tr>
<td>Surgery</td>
<td>Resection of metastasis</td>
<td>Sigmoid colectomy/en bloc bladder</td>
<td>extended right colectomy/ileocolic anas.</td>
</tr>
<tr>
<td>Tumor size</td>
<td>.5cm (one abdominal wall deposit)</td>
<td>2.9x2.8x2.5cm</td>
<td>2.5x1.8x1.8cm</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>Poorly differentiated</td>
<td>Invasive poorly differentiated</td>
<td>invasive moderately differentiated</td>
</tr>
<tr>
<td>Tumor type</td>
<td>AdenoCa-medullary subtype</td>
<td>adenocarcinoma</td>
<td>adenocarcinoma</td>
</tr>
<tr>
<td>Stage</td>
<td>T3Nb1</td>
<td>T4N2</td>
<td>T3N*M1a(liver)</td>
</tr>
<tr>
<td>Number +LN</td>
<td>2 out of 23</td>
<td>4 out of 20</td>
<td>0 out of 14</td>
</tr>
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<td>Extramural invasion</td>
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<td>No</td>
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<tr>
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<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lymphvascular invasion</td>
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<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Tumor inf. Lymphs</td>
<td>&gt;3 HPF</td>
<td>&lt;3</td>
<td>moderate</td>
</tr>
<tr>
<td>Mutations</td>
<td>increased MSI, KRAS negative</td>
<td>loss of nuclear expression of MLH1 and PMS2</td>
<td>loss of MLH1 and PMS2, +BRAFV600E</td>
</tr>
<tr>
<td>Outcome to date</td>
<td>No disease to date (April 2015)</td>
<td>&lt;1 year PFS</td>
<td>Died 6 days post op; sepsis/cardiac failure</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------------------</td>
<td>------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>Notes:</td>
<td>Recurrent right colon CA; 1st surgery 2012</td>
<td>Palliative with Xeloda and Bevacizumab</td>
<td>Sigmoid colectomy-CA 1976; Left colon polyp 1991; Met to liver- treated with Folfox; Avastin neoadjuvantly</td>
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</tbody>
</table>

Abbreviations: lymph node (LN), CA (cancer), high power field (HPF), microsatellite instability (MSI), progression free survival (PFS), anastomosis (anas).
Figure 3.7. Hemotoxylin and Eosin staining of patient derived xenografts (PDX) through subsequent generations.

Three patients (patient 93, 109, and 130) provided tissues that have maintained their histological integrity. This resource offers a tremendous opportunity to pursue translational cancer questions with human derived tissues that provide a more realistic structure in which to study tumor microenvironment.
Figure 3.8. Severe combined immunodeficiency (SCID) tumor volume over the study period.

There was no significant difference with respect to tumor size when comparing the control group to the metformin group (p=0.39).
Table 3.2. Severe combined immunodeficiency (SCID) mice metformin trial tumor size.

This table corresponds to the raw data used to generate Figure 3.8.

<table>
<thead>
<tr>
<th>Ear tag no.</th>
<th>Treatment Group</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14-16</th>
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<td></td>
<td></td>
<td>L</td>
<td>W</td>
<td>H</td>
</tr>
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<td>0.5</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>613</td>
<td>Control</td>
<td>0.4</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>616</td>
<td>Control</td>
<td>0.3</td>
<td>0.4</td>
<td>0.1</td>
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<td>0.3</td>
<td>0.2</td>
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<td>Control</td>
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<td>0.3</td>
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</tr>
<tr>
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<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
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<td>Met</td>
<td>0.3</td>
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</tr>
<tr>
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<td>Met</td>
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<tr>
<td>626</td>
<td>Met</td>
<td>0.3</td>
<td>0.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Abbreviations: L, length (cm); W, width (cm), H, height (cm), Met, Metformin
Figure 3.9. Severe combined immunodeficiency (SCID) weight over the study period.

The treatment group initially lost weight which is likely due common side effects from metformin, however this weight loss did not prove to be a significant factor by the end of the murine trial (p=0.051).
Table 3.3. Severe combined immunodeficiency (SCID) mice metformin trial weights.

This table corresponds to the raw data used to generate Figure 3.9.

<table>
<thead>
<tr>
<th>Ear tag number</th>
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<th>Day 1 (gm)</th>
<th>Day 7 (gm)</th>
<th>Day 14-16 (gm)</th>
</tr>
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<tbody>
<tr>
<td>610</td>
<td>Control</td>
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<tr>
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<td>Control</td>
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<td>33.2</td>
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<td>616</td>
<td>Control</td>
<td>31.9</td>
<td>32</td>
<td>32.3</td>
</tr>
<tr>
<td>620</td>
<td>Control</td>
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<td>29.6</td>
</tr>
<tr>
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<td>Control</td>
<td>30.5</td>
<td>30.9</td>
<td>31.2</td>
</tr>
<tr>
<td>611</td>
<td>Metformin</td>
<td>30.4</td>
<td>30.1</td>
<td>31</td>
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<td>32</td>
<td>30.5</td>
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<tr>
<td>618</td>
<td>Metformin</td>
<td>31</td>
<td>31</td>
<td>31.2</td>
</tr>
<tr>
<td>626</td>
<td>Metformin</td>
<td>27.8</td>
<td>27.7</td>
<td>28.3</td>
</tr>
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Figure 3.10. Western blotting analysis of PDX tumors from control and metformin treated mice show minimal differences in p-ACC, p-AMPK, and p-S6.

Ten severe combined immunodeficiency (SCID) mice received left subcutaneous flank implanted with third generation tumor from a third generation PDX. Based on cell signaling patterns there is a slight increase in p-ACC and p-AMPK expression and a decrease in p-S6 expression in the treatment group suggesting that metformin does cause some downstream changes in glycolysis, lipid metabolism, and protein metabolism in mice harboring PDX.
Figure 3.11. Immunohistochemistry (IHC) staining of patient derived xenograft (PDX) tumors from control and metformin treated mice show a decrease in Ki67 in the treatment group, but no significant difference in cleaved caspase-3.

Ten severe combined immunodeficiency (SCID) mice received left subcutaneous flank implanted with third generation tumor from PDX. Tumors were allowed to grow to 0.5x0.5 cm and then were randomized into two treatment groups. The control group (n=5) received a 30 uL IP injection of saline each day for 14 days and the treatment group (n=5) received a 30uL (200mg/kg) intraperitoneal injection Metformin for 14-16 days.
course (i.e. 1 to 3 days) of *in vitro* treatment with a molecular inhibitor, but over a course of time exposed to both the direct and indirect effects of the drug *in vivo*, researchers may be able to elucidate more information regarding an individualized tumor in a relatively controlled environment.

### 3.4 Ex vivo tissue labeling

*Ex vivo* tissue labeling offers a relatively simple technique to elegantly observe human tissues and cancers in an environment that is at least applicable to the human tumor microenvironment that previously accommodated the respective neoplasm. During the same time we performed $^{13}$C6 labeling of freshly resected tissues from patients we also incubated tissue for IHC analysis to evaluate for sample quality. We determined that specimens maintain their tissue structure and integrity despite 24 hours of being gently shaken in labeling media (Figures 3.12). We also determined that larger tumors may be sectioned and a PDX can be performed utilized as well. We were able to compare one patient’s tissues both as a fresh primary sample and then later after one generation through a SCID mouse (Figure 3.13). In the era of personalized medicine, this strategy offers a safe, relatively easy, and cost-efficient way to “trial” multiple different therapies on one patient without any worries for side effects or subsequent therapy related morbidity or mortality to the patient.

### 3.5 In vitro metabolic data

After determining that different colon cancer cell lines had varying metabolic responses to metformin based on their baseline phenotype using various molecular markers such p-AMPK, p-ACC, p-S6, Ki67, and cleaved caspase-3 and direct proliferation studies of cells, we then sought to probe further into the metabolic profiles of all five cell lines to determine if the varied responses to a single molecular inhibitor, metformin, could be elucidated by mitochondrial stress tests and measurements of glycolytic energy responses. Collectively the metabolic impact of metformin significantly decreases mitochondrial respiration while increasing glycolysis. But this did not fully explain
Figure 3.12. (Patient 109) *Ex vivo* labeled tissues are evaluated using immunohistochemistry (IHC) staining to observe for markers of cell proliferation (Ki67) and apoptosis (cleaved caspase 3).

*Ex vivo* labeled tissues are being used to determine the direct effects of tumor microenvironment in a controlled environment. There was no difference in the markers evaluated within these respective samples.
Figure 3.13 (Patient 130) Another example of ex vivo labeled tissues are evaluated using immunohistochemistry (IHC) staining to observe for markers of cell proliferation (Ki67) and apoptosis (cleaved caspase 3).

Ex vivo labeled tissues are being used to determine the direct effects of tumor microenvironment in a controlled environment. There was no difference in the markers evaluated within these respective samples.
why certain cell lines, namely SW480 and PDX, could also increase glycolysis and decrease mitochondrial respiration in an effort to more efficiently metabolism energy substrates, but have such little change to their overall metabolic phenotype. This prompted us to use SIRM to evaluate SW480 and PDX cells and their medial, in addition to murine in vivo labeled tissues and human ex vivo labeled tissues to determine the specific impact metformin has on glycolysis occurs by increasing $^{13}$C-6 Lactate and decreasing $^{13}$C-6 Alanine. However we also found that in these two particular cell lines there appears to be a “recycled” pathway that allows for continued metabolism despite a stressed environment which normally forces cells into a more conservative and energy saving phenotype.

3.6 Seahorse analysis of OCR and ECAR

Cellular OCR was used to measure basal respiration, non-mitochondrial respiration, ATP-linked respiration, and proton leak and ECAR was used to monitor glycolysis, glycolytic reserve, and glycolytic capacity.

Upon evaluating OCR in our five cell lines of interest we determined certain patterns applied to each cell line. For example, we determined earlier that the two cell lines that were not as phenotypically aggressive (i.e. HT29 and HCT116) had more phosphorylation changes of factors associated with energy balance (p-AMPK), fatty acid oxidation (p-ACC), and protein synthesis (p-S6), as well as decreased proliferation measured by cell count. Also, we determined that the three more aggressive and/or metastatic cell lines (SW480,KM20, and PDX) were less responsive to metformin when evaluate with markers of cellular and molecular metabolism.

However, upon evaluating the mitochondrial respiration, with the exception of HCT116 and HT29 and non-mitochondrial respiration, each cell line has a significant decreases in maximal respiration, non-mitochondrial respiration, ATP-linked respiration, and proton leak (Figures 3.14-3.18). Briefly, a decrease in non-mitochondrial respiration suggests mitochondrial respiration is dysfunctional.
Figure 3.14. SW480 Oxygen consumption rate (OCR).

A. 24 hours of 1mM treatment with Metformin results in a significant decrease in OCR. B. The overall phenotype of the cell changes from an energetic to a more glycolytic state form of metabolism when observing both OCR and extracellular acidification rate (ECAR). C. Both basal respiration and non-mitochondrial respiration are significantly decreased with metformin treatment, suggesting mitochondrial respiration has peaked and other energy pathways are being utilized. D. ATP-linked respiration is significantly decreased with metformin treatment, suggesting a decrease in the respiration used to drive mitochondrial ATP synthesis. E. Maximal respiration is significantly decreased with metformin treatment, suggesting substrate oxidation and respiratory chain activity increase in an attempt to maintain mitochondrial membrane potential. F. Proton leak is significantly decreased suggesting oxidation phosphorylation is incompletely coupled.
Figure 3.15. KM20 Oxygen consumption rate (OCR).

A. 24 hours of 1mM treatment with Metformin results in a significant decrease in OCR. B. The overall phenotype of the cell changes to a more glycolytic strategy of metabolism. The phenotypic change in this cell line is more marked compared to SW480, HT29, HCT116, and UKPDX cells. C. Both basal respiration and non-mitochondrial respiration are significantly decreased with metformin treatment, suggesting mitochondrial respiration has peaked and other energy pathways are being utilized. D. ATP-linked respiration is significantly decreased with metformin treatment, suggesting a decrease in the respiration used to drive mitochondrial ATP synthesis. E. Maximal respiration is significantly decreased with metformin treatment, suggesting substrate oxidation and respiratory chain activity increase in an attempt to maintain mitochondrial membrane potential. F. Proton leak is significantly decreased suggesting oxidation phosphorylation is incompletely coupled.
Figure 3.16. HT29 Oxygen consumption rate (OCR).

A. 24 hours of 1mM treatment with Metformin results in a significant decrease in OCR. B. The overall phenotype of the cell changes to a more glycolytic form of cellular metabolism, but this is not as marked as seen in other cell lines. C. Basal respiration is significantly decreased with metformin treatment, but non-mitochondrial respiration is not. Overall this cell line responds more to metformin treatment and this is because mitochondrial inhibition of complex I is increased in this particular cell line, thus further sensitizing colon cancers with this phenotype to metformin treatment. D. ATP-linked respiration is significantly decreased with metformin treatment, suggesting a decrease in the respiration used to drive mitochondrial ATP synthesis. E. Maximal respiration is significantly decreased with metformin treatment, suggesting substrate oxidation and respiratory chain activity increase in an attempt to maintain mitochondrial membrane potential. F. Proton leak is significantly decreased suggesting oxidation phosphorylation is incompletely coupled and mitochondrial respiration is significantly decreased, likely through inhibition of complex I.
Figure 3.17. HCT116 Oxygen consumption rate (OCR).

A. 24 hours of 1mM treatment with Metformin results in a significant decrease in OCR. B. The overall phenotype of the cell changes to a more glycolytic form of metabolism. C. Basal respiration is significantly decreased with metformin treatment, but non- mitochondrial respiration is not. Overall this cell line responds more to metformin treatment and this is because mitochondrial inhibition of complex I is increased in this particular cell line, thus further sensitizing colon cancers with this phenotype to metformin treatment. This pattern is similar to HT29 cells, which also had a significant response to 24 hours of treatment with 1mM Metformin. D. ATP-linked respiration is significantly decreased with metformin treatment, suggesting a decrease in the respiration used to drive mitochondrial ATP synthesis. E. Maximal respiration is significantly decreased with metformin treatment, suggesting substrate oxidation and respiratory chain activity increase in an attempt to maintain mitochondrial membrane potential. F. Proton leak is significantly decreased suggesting oxidation phosphorylation is incompletely coupled and mitochondrial respiration is significantly decreased, likely through inhibition of complex I.
Figure 3.18. PDX Oxygen consumption rate (OCR).

A. 24 hours of 1mM treatment with Metformin results in a significant decrease in OCR. B. The overall phenotype of the cell changes to a more glycolytic form of metabolism. C. Basal respiration is significantly decreased with metformin treatment, but non-mitochondrial respiration is not. Overall this cell line responds more to metformin treatment and this is because mitochondrial inhibition of complex I is increased in this particular cell line, thus further sensitizing colon cancers with this phenotype to metformin treatment. D. ATP-linked respiration is significantly decreased with metformin treatment, suggesting a decrease in the respiration used to drive mitochondrial ATP synthesis. E. Maximal respiration is significantly decreased with metformin treatment, suggesting substrate oxidation and respiratory chain activity increase in an attempt to maintain mitochondrial membrane potential. F. Proton leak is significantly decreased suggesting oxidation phosphorylation is incompletely coupled and mitochondrial respiration is significantly decreased.
because of an overload or inhibition of complex I and complex III (109). Therefore, HT29 and HCT116 (Figures 3.16 and 3.17), both of which are cell lines that are quite responsive to metformin based on the fact that cellular and molecular signaling is “maxed out” with regard to metformin’s inhibition of respiratory complex I, a well-known mediator of metformin efficacy (58). The three more aggressive cell lines have respective OCRs that are profoundly affected by metformin, therefore they must be getting energy substrates from another source. By observing the phenotypic changes in KM20 (Figure 3.15), it seems that this cell line, which is metastatic and extremely chemo-resistant, nearly exhausts its glycolytic pathways (Figure 3.20) by significantly increasing glycolysis (p=0.040) and eventually limiting glycolytic reserve (p<0.001) in order to deal with metformin's metabolic assault. Unfortunately SW480 and PDX do not follow this pattern, and in fact, although PDX does increase its glycolytic function, it is the only cell line that does not have an significant increase in glycolysis (p=0.304) (Figures 3.19-23). SW480 significantly increases its glycolysis levels, but not as markedly as KM20. Furthermore, by taking a closer look at glycolytic reserve in these three aggressive cell lines (Figures 3.20, 3.21, 3.23) a pattern emerges that KM20 makes glycolysis its energy back-up plan and has a significantly reduced glycolytic reserve as a trade-off. In contrast, SW480, which also significantly increases its glycolytic levels, has a significant, yet more modest decrease in glycolytic reserve. Surprisingly, PDX has a significant decrease in glycolytic reserve without the significant increase in glycolysis (Figure 3.23). Therefore, a more sensitive metabolic test is required to determine what the back-up system these two aggressive cell lines (i.e. SW480 and PDX) use to evade the inhibitory effects of metformin as an anti-cancer agent.

3.7 $^{13}$C-NMR and $^1$H analysis of SW480 and PDX cells and media

Given that we hypothesized that certain more aggressive cell lines had an alternate source of energy substrates to allow its evasion from metformin as a targeted therapy we sought a more elegant way to answer this question. If there are significant changes then SIRM labeling would allow us to see if and where in
Figure 3.19. SW480 Extracellular acidification rate (ECAR).

A. Overall there are significant differences in ECAR in SW480 cells treated with metformin versus those that are not for 24 hours, although these changes in base levels and glycolytic capacity are not significantly different (B, D). There are significant changes in both glycolysis (C) and glycolytic reserve (E), suggesting that metformin does force the cells into a more glycolytic form of metabolism in order to continue proliferation and metabolism of energy substrates while decreasing reserve and later potentially exhausting this form of metabolism.
Figure 3.20. KM20 Extracellular acidification rate (ECAR).

A. Overall there are significant differences in ECAR in KM20 cells treated with metformin versus those that are not for 24 hours although the decreases in basal levels and glycolytic capacity are not significantly different (B, D). There are significant changes in both glycolysis (C) and glycolytic reserve (E) suggesting that metformin induces an increase in glycolysis and forces cells into a more glycolytic strategy of energy metabolism while also decreasing amount of glycolytic reserve remaining to continue this source of metabolism.
Figure 3.21. HT 29 Extracellular acidification rate (ECAR).

A. Overall there are significant differences in ECAR in HT29 cells treated with metformin versus those that are not for 24 hours. Similar to other cell lines there is little if no changes in both base levels and glycolytic capacity. There are significant changes in both glycolysis (C) and glycolytic reserve (E) suggesting that metformin does glycolysis and forces cells into a more glycolytic strategy of energy metabolism while also decreasing amount of glycolytic reserve remaining to continue this source of metabolism. Most notably, these changes are more profound in this cell line which mirrors the cell signaling and proliferation data, again suggesting that patients with tumor phenotypes similar to this cancer cell line may exhibit a more positive response to metformin as an anti-cancer agent.
Figure 3.22. HCT116 Extracellular acidification rate (ECAR).

A. Overall there are significant differences in ECAR in HCT116 cells treated with metformin versus those that are not for 24 hours. Similar to other cell lines there is little if no changes in both base levels and glycolytic capacity. There are significant changes in both glycolysis (C) and glycolytic reserve (E) suggesting that metformin does glycolysis and forces cells into a more glycolytic strategy of energy metabolism while also decreasing amount of glycolytic reserve remaining to continue this source of metabolism. Most notably, these changes are more profound in this cell line which mirrors the cell signaling and proliferation data, again suggesting that patients with tumor phenotypes similar to this cancer cell line may exhibit a more positive response to metformin as an anti-cancer agent.
Figure 3.23. PDX Extracellular acidification rate (ECAR).

A. Overall there are significant differences in ECAR in UKPDX cells treated with metformin versus those that are not for 24 hours. Similar to other cell lines there is little if no changes in both base levels and glycolytic capacity. Surprisingly, glycolysis is increased in this aggressive and novel cell line, but there is not a statistically significant difference between the treatment and control groups (p=0.304). Glycolytic reserve is significantly decreased (p<0.001) (E) suggesting in more aggressive colon adenocarcinomas metformin may cause cells to start using an alternate energy source prior to completely exhausting their glycolytic reserve.
glycolysis these aggressive cell lines were able to evade molecular inhibitors that
normally induce a metabolic stress state by increasing glycolysis. The
interrogation of the metabolome to answer complex molecular questions focuses
on human disease by metabolite profiling for biomarker discovery, as well as
pharmacology and therapeutics. Recent advances in stable isotope tracer-based
metabolomic approaches enable unambiguous tracking of individual atoms
through compartmentalized metabolic networks directly in human subjects, which
made this resource an ideal tool to elucidate the complexities of the direct and
indirect actions of metformin, especially in its relationship with aggressive
molecularly evasive cancers.

The metabolomic analysis of the SW480 cells and PDX cells as well as
their respective media samples gave tremendous insight into our question and
hypothesis and confirmed that there is an alternate energy source that is being
utilized in addition to glycolysis during cellular metabolism when cells are treated
with metformin. Given the labeled stable isotopes identified during the $^{13}$C6-glucose HSQC and $^{13}$C6-glucose, $^1$H analysis were namely $^{13}$C-lactate and small
peaks in $^{13}$C-alanine. Also, notably there are decreases in $^{13}$C-4-glutamine
suggesting that metformin may preferentially increase mitochondrial oxidation
therefore shunting glutamine into the system and forcing the cell to use this high
energy substrate as its best resource in a high stress system. The qualitative
data available through analysis of the media also suggested that metformin
causes cells to preferentially use alanine and secrete lactate as a waste product,
and that perhaps the alanine is being "recycled" through another pathway to
ensure there is always a pyruvate pool available with ample energy substrates.

In Figure 3.24 NMR analysis with $^{13}$C6-Glc HSQC determined that SW480
cells treated with metformin versus those that were not treated with metformin
exhibit peaks in $^{13}$C-lactate and decreases in the peaks expressing evidence of
$^{13}$C-alanine metabolism. Also, notably there are decreases in $^{13}$C-4-glutamine,
$^{13}$C-4-glucose-total glutathione, and $^{13}$C-3-aspartate, as well as a slight increase
in $^{13}$C-G1-uridine diphosphate glucose. These findings indicate a decrease in
nucleotide synthesis in cells treated with Metformin. Figure 3.25 shows similar
Figure 3.24 SW480 colon cancer cells $^{13}$C6-Glc HSQC.

NMR analysis with $^{13}$C6-Glc HSQC determined that SW480 cells treated with metformin versus those that are not exhibit peaks in $^{13}$C-lactate and small peaks in $^{13}$C- alanine. Also, notably there are decreases in $^{13}$C-4-Gln, $^{13}$C-4-Glu-GSH+GSSG, and $^{13}$C-3-Asp, as well as a slight increase in $^{13}$C-G1-UDPG.
Figure 3.25. SW480 colon cancer cells $^{13}$C6-Glc, $^1$H.

NMR analysis with $^{13}$C6-Glc, $^1$H determined that SW480 cells treated with metformin versus those that are not exhibit peaks in $^{13}$C-lactate and decrease in $^{13}$C-alanine. Also, notably there are decreases in $^{13}$C-4-Gln, $^{13}$C-4-Glu-GSH+GSSG, and $^{13}$C-3-Asp.
peaks with a different $^1$H chemical shift (0.81-2.89 ppm) which also determined that SW480 cells treated with metformin versus those that are not exhibit peaks in $^{13}$C-lactate and small peaks in $^{13}$C-alanine, as well as the corresponding changes in nucleotide synthesis. Figure 3.26 represents $^1$H shift between 5.14-6.56 ppm for SW480 colon cancer cells that are not labeled with $^{13}$C-glucose. Analysis of unlabeled SW480 cells also revealed that the control groups had peaks in the $^{12}$C-G1-Glycogen, $^{12}$C-G1-uridine diphosphate N-acetylglucosamine, $^{12}$C-G1- uridine diphosphate N-acetylglucosamine, $^{12}$C-1'-UXP (uracil nucleotide) suggesting they may use fructose 6-phosphate and glutamine as alternate energy sources. Figure 3.27 represents the $^1$H shift analysis (0.81-2.89 ppm) of SW480 colon cancer cell medium associated with cell treatment with $^{13}$C6-Glucose. Interestingly the media also had notable $^{13}$C-3-Lactate peaks suggesting that the cells preferentially use glutamine and alanine as their “primary” back-up energy sources and create a more acidic surrounding environment by releasing more lactate into the surrounding media.

We observed similar results when we analyzed the metabolic changes seen in PDX 93 cells treated with Metformin versus those treated with the control media. Figure 3.28 represents the $^{13}$C6-Glucose HSQC analysis of PDX colon cancer cells treated with metformin versus those that were not treated with metformin. Here, were also noted that our PDX colon cancer cells also exhibit primarily increased peaks in $^{13}$C-lactate and small peaks in $^{13}$C-alanine, again suggesting that these cells get there additional energy source from additional pyruvate pools. Figure 3.29 demonstrates that the $^1$H shift (0.81-2.89 ppm) for PDX colon cancer cells also shows an increase in $^{13}$C-lactate. Figure 3.30 shows that results of $^1$H shift (0.81-2.89 ppm) in unlabeled PDX colon cancer cells. These results mirror those seen in the preexisting SW480 cell line and suggest the control groups had peaks in the $^{12}$C-G1-Glycogen, $^{12}$C-G1-uridine diphosphate N-acetylglucosamine, $^{12}$C-G1-uridine diphosphate N-acetylglucosamine, $^{12}$C-1'-UXP (uracil nucleotide) suggesting they may use fructose 6-phosphate and glutamine as alternate energy sources. Figure 3.31 demonstrates that effects of metformin on the medium of PDX colon cancer
cells with and without metformin. The $^{13}$C6-glucose analysis of the $^1$H shift (0.81-2.89 ppm), although not as pronounced due to the decreased amounts of protein, that the PDX cells also demonstrate increases in $^{13}$C-lactate suggesting that metformin causes these cells to use alternate energy pathways, although they are not purely glycolytic.
Figure 3.26. SW480 colon cancer cells unlabeled, $^1$H.

Unlabeled SW480 cells revealed the control groups had peaks in the $^{12}$C -G1- Glycogen, $^{12}$C -G1-UDPGlcNAc, $^{12}$C -G1-UDPGalNAc, $^{12}$C -1'-UXP (uracil nucleotide) suggesting they may use fructose 6-phosphate and glutamine as an alternate energy sources.
Figure 3.27. SW480 colon cancer cell medium $^{13}$C₆-Glc, $^1$H.

Interestingly the media also had notable $^{13}$C-3-Lac peaks suggesting that the cells preferentially use glutamine and alanine as their "primary" back-up energy sources and create a more acidic surrounding environment by releasing more lactate into the surrounding media.
Figure 3.28. PDX colon cancer cells $^{13}$C6-Glc HSQC.

PDX colon cancer cells also exhibit primarily increased peaks in $^{13}$C-lactate and small peaks in $^{13}$C- alanine, again suggesting that these cells get there additional energy source from additional pyruvate pools.
Figure 3.29. PDX colon cancer cells $^{13}$C$_6$-Glc, $^1$H.

This sample also shows an increase in $^{13}$C-lactate and small peaks in $^{13}$C-alanine, but the peaks are somewhat less clear with this example because there is likely a low level of protein and possible issues with the sample causing the data to be less precise.
Similarly to SW480 cells the unlabeled PDX cells showed increases in unlabeled SW480 cells revealed increased uptake at $^{12}$C-G1-Glycogen, $^{12}$C-G1-UDPGlcNAc, $^{12}$C-G1-UDPGalNAc, $^{12}$C-1'-UXP suggesting they may use fructose 6-phosphate and glutamine as an alternate energy source and may be a precursor to downstream lipid metabolism.
Figure 3.31. PDX colon cancer cell medium $^{13}$C6-Glc, $^1$H.

Although not as pronounced due to the decreased amounts of protein, the PDX cells also demonstrate increases in $^{13}$C-lactate suggesting that metformin causes these cells to use alternate energy pathways, although they are not purely glycolytic.
CHAPTER FOUR: CLINICAL TRIAL DESIGN

4.1 Objectives

Primary Objectives

To estimate the percent increase of AMPK phosphorylation, by immunohistochemistry, in patients receiving a minimum of 14-days of Metformin given preoperatively, in stage I-IV colon cancer patients.

Secondary Objectives

1. To examine the difference in proliferative expression of Ki-67 using immunohistochemistry comparing the two treatment populations.

2. To determine the expression of liver kinase B1 (LKB1), p-mTOR Phospho-S6K, and acetyl CoA using Western blotting in this population of subjects.

3. Post-hoc genetic analysis of surgical specimens to determine presence of K-ras, hereditary nonpolyposis colon cancer (HNPCC), phosphoinositide- 3-kinase gene (PI3KCA), and mismatch repair (MMR).

4. To delineate the effect of Metformin on glycolysis and downstream tumor metabolism in human colon cancer tissues through the use of nuclear magnetic resonance imaging (NMR) and mass spectrometry (MS) metabolomics analysis.

4.2 Background

Study Disease(s): Colon Cancer

Colon cancer is the third leading cause of cancer mortality worldwide, has become increasingly prevalent in Western populations, and is associated with both obesity and Type II diabetes mellitus (DMII) (110, 111). Colon cancer is increasingly prevalent in Appalachia, and our cancer center serves a large number of colon cancer victims from this area (112). Metformin is an oral antidiabetic drug in the biguanide class and is FDA approved as the first line drug of choice for the treatment of DMII, but has also been shown to have potential anti-cancer effects (11, 113). Our central hypothesis is that the predominant role
of Metformin in colon cancer cells is to activate AMPK which has downstream impacts on decreasing protein metabolism through its effect Akt/mTOR pathway, and decreasing fatty acid metabolism through decreasing ACC and its targeted impact on fatty acid metabolism. Moreover, we speculate that Metformin’s impact on these molecular growth factors will inhibit colon cancer cell growth, proliferation, and tumor metabolism during the time interval after diagnosis and prior to surgery, and may even offer a therapeutic option for patients whom cannot tolerate aggressive traditional chemotherapy agents.

**Study Drug: Metformin**

Metformin is an oral antidiabetic drug in the biguanide class and is FDA approved as the first line drug of choice for the treatment of DMII, but has also been shown to have potential anti-cancer effects (11, 112). The primary anti-tumor molecular target of Metformin is AMP-activated protein kinase (AMPK), which is a serine/threonine protein kinase (39). AMPK plays a crucial role in monitoring systemic and cellular energy status and which protects crucial cellular functions under energy-restricted conditions such as metabolic stress, i.e. tumor development (45). Specific downstream metabolic mechanisms proposed to be affected by Metformin’s action on AMPK in colon cancers are i) protein metabolism and translation by the mammalian target of rapamycin (mTOR) pathway and ii) acetyl co-enzyme A (ACC) pathway in lipid metabolism (18, 114, 115).

In addition to its function as a gluconeogenesis suppressor, Metformin has recently been shown to possess strong anti-cancer properties. It has been shown to reduce proliferation, induce apoptosis, cause cell cycle arrest, and improve response to breast tumor xenografts in pre-clinical studies (18, 51, 99, 116-118). Pre-clinical studies of the impact of Metformin on lung, prostate, colon, and pancreatic cancer have been explored (20, 22, 24, 69). Metformin is a safe therapeutic agent that has been previously used in many cancer clinical trials studying breast cancer (19, 81, 82). For pre-clinical treatment studies similar to the study we propose, safe doses range from 500mg two times per day up to
1,700 mg per day (19, 82, 119). Recent pharmacokinetic data shows that effective metformin induced inhibition of hepatic gluconeogenesis can be achieved with bi-daily dosing of metformin to achieve 74-100 µM peak average values of serum concentrations (31).

Metformin’s most common side effects are gastrointestinal, and these symptoms are often resolve spontaneously and can often be avoided by gradual escalation of dosage. We do not plan to escalate the dose of metformin in this study, but similarly to previously performed studies in breast cancer and other ongoing trials exploring colon cancer, we intend to give two to three week trials of clinically accepted levels of this agent. Metformin treatment has not been associated with hypoglycemia unless used in conjunction with other glucose-lowering medicines (sulfonylureas or insulin). In U.S. clinical trials, about 4% of participants were unable to continue metformin due to adverse effects. Serious adverse events are infrequent and generally limited to lactic acidosis, which occurs only in persons with renal or hepatic insufficiency or other contraindications (120).

Contraindications to this drug include CHF, metabolic acidosis with or without coma, DKA, renal disease (serum creatinine >1.5 mg/dL in males or >1.4mg/dL in females), abnormal creatinine clearance resulting from shock, septicemia, or myocardial infarction, radiologic contrast study for 48 hr after, and current lactation (9). Metformin has a bioavailability of 50-60% and the peak plasma time of the regular release form is 2-3 hours. It is eliminated by the kidneys (90% by tubular secretion) at 450-540 ml/min with a t\(^{1/2}\) of 4-9 hours (121).

**Rationale**

Colon cancer is a devastating disease that is increasing in incidence regionally, nationally, and worldwide. Colon cancer is strongly associated with obesity, now considered an epidemic in the United States. Our surrounding Appalachian population has the highest rates of both obesity and colon cancer in the country, and could benefit significantly from our proposed pilot study. Our
proposed project using Metformin as a safe, low cost, targeted therapy offers an agent which can affect the correlated conditions of colon cancer and obesity. Current recommendations for the treatment of colon cancer include surgery and chemotherapy. Metformin offers an alternative that will allow decrease doses, and therefore toxicity, of aggressive traditional chemotherapy. We also speculate using this drug during the pre-surgical and immediate post-surgical interval when no other therapeutic intervention such as chemotherapy can safely be offered could potentially lead to decreased micro-metastatic disease which results in later recurrences. Furthermore, patients who cannot tolerate aggressive chemotherapy due to age or other medical conditions could significantly benefit from this safe and less toxic alternative.

Epidemiologic data in human colon cancers suggests patients taking Metformin for the treatment of diabetes have better outcomes than their non-diabetic counterparts, which seems counterintuitive as these patients often have other comorbidities such as obesity which contribute to less optimal outcomes (67, 121). Both in vitro and in vivo animal data in colon cancer also suggest Metformin does have an anti-tumor effect, but the direct metabolic implications of the drug have yet to be determined (122, 123). Previous studies have shown the anti-tumor effects of Metformin in breast, prostate, gastric, and pancreatic cancers (83, 124-126). However, a randomized trial studying the specific metabolic effects of Metformin in human colon cancer has yet to be performed.

We therefore propose to explore a novel, innovative approach studying the metabolic effects of Metformin in human colon cancer tissues using a prospective, double-blinded, randomized control pilot study at the University of Kentucky Markey Cancer Center. We intend to determine the metabolic impact of Metformin treatment in patients with colon cancer by examining the role of Metformin on protein metabolism and tumor cell proliferation in colon cancer tissues, as well as delineating the effect of Metformin on fatty acid metabolism in human colon cancer tissues.
Correlative Studies Background

AMPK analysis by IHC staining

We plan to analyze both the pre and post treatment specimens to determine phosphorylation changes of AMPK in the Metformin treated specimens. IHC staining is an established and well characterized technique to identify and analyze AMPK in both pre-clinical and clinical studies (71, 127, 128). AMPK is an enzyme that plays a role in cellular energy homeostasis. It is also acts as an effector of metformin action on glucose uptake in cellular processes (129). Since Metformin targets AMPK we intend to use it as a primary biomarker in our study. We anticipate that Metformin treated tissues will show increased phosphorylation changes in AMPK when compared to specimens not treated with Metformin. We plan to prepare and stain these tissues in our laboratory at the Markey Cancer Center and have the resources and equipment to carry out all procedures. We plan to quantify and compare differences in AMPK phosphorylation with Aperioscope imaging and quantification.

Ki67 analysis by IHC staining

We plan to analyze both the pre and post treatment specimens to determine the proliferative changes present by comparing and quantifying the positive staining of Ki67 in the tissues. Ki67 is a nuclear protein that is associated with and may be necessary for cellular proliferation, and it is also associated with ribosomal RNA protein transcription (130, 131). There are multiple pre-clinical and clinical studies supporting the evaluation of Ki67 as a proliferative marker in tumor biology studies and that it can be accurately quantified and used as a reliable marker (131). We anticipate that tumor specimens which have not been exposed to the study drug will have an increased observance of the staining of Ki67, thus indicating an increase of proliferation in tissues not exposed to Metformin. We plan to stain all tissues for this biomarker by the same protocol to maintain consistency and we plan to quantify and compare differences in Ki67 staining with Aperioscope imaging and quantification.
**LKB1 analysis by Western blotting**

LKB1 is a primary upstream kinase of AMPK and exerts its growth suppressing effects by activating a group of ~14 other kinases, comprising AMPK and AMPK-related kinases (48). Activation of AMPK by LKB1 suppresses growth and proliferation when energy and nutrient levels are scarce. Activation of AMPK-related kinases by LKB1 plays vital roles maintaining cell polarity thereby inhibiting inappropriate expansion of tumor cells (34). LKB1 leads to disorganization of cell polarity and facilitates tumor growth under energetically unfavorable conditions (132). We plan to analyze both the pre and post treatment specimens to determine the presence of LKB1. We anticipate an increase in LKB1 in the tissues not exposed to Metformin as this kinase which is upstream of the anticipated target biomarker as it is allows tumor growth in settings of cellular stress. Western blotting analysis will allow us to study qualitative data of protein expression related to LKB1.

**p-mTOR and phospho-S6K analysis by Western blotting**

mTOR and phospho-S6K are both effectors of cell growth and proliferation via the regulation of protein synthesis in tumor cell growth and metabolism we intend to analyze with Western blotting. mTOR is a phylogenetically conserved serine/threonine kinase which is phosphorylated and activated by Akt, but can also auto phosphorylate when experiencing metabolic stress (133). mTOR regulates protein synthesis through the phosphorylation and inactivation of the repressor of mRNA translation, eukaryotic initiation factor 4E-binding protein (4E-BP1), and through the phosphorylation and activation of S6 kinase (S6K1) (53).

Pre-clinical studies have shown that metformin potentiates the effects of paclitaxel in endometrial cancer cells through inhibition of cell proliferation and modulation of the mTOR pathway and clinical studies in human tumor xenografts show that Metformin treatment results in a downregulation of mTOR and downstream effectors such as pS6K (42, 134). We intend to use Western blotting analysis to study qualitative data of protein expression related to the mTOR and pS6K inhibition we expect to observe in Metformin treated tissue specimens.
Acetyl-CoA analysis by Western blotting

Acetyl-CoA is produced during the second step of aerobic cellular respiration, pyruvate decarboxylation, which occurs in the matrix of the mitochondria and its main function is to convey the carbon atoms within the acetyl group to the citric acid cycle to be oxidized for energy production. This metabolic molecule is extremely important in the understanding of cellular metabolism and for this reason we would like to include it in our qualitative Western blotting analysis studies. Preclinical data shows that fatty acid oxidation is increased in the presence of Metformin and downstream effectors involved in fatty acid metabolism such as acetyl CoA and fatty acid synthase (FASN) are increased in models of cellular stress (23, 106, 135). We anticipate that the Metformin treated tissues will show an increase in the protein expression of acetyl CoA using Western blotting analysis.

Genetic studies of surgical specimens

Genetic analysis of all tumor specimens is important as we need to understand the implications of any genetic differences we find in comparing the pre and post treatment specimens. We intend to observe mutations for K-ras, PI3KCA, HNPCC, and MMR, all of which are important genetic mutations in colon cancer known to effect tumor biology (136-139). It is currently unknown if or how Metformin treatment changes expression of any of these genetic profiles. Unfortunately adequate tumor biopsy tissue will not be available in quantity or in a timely manner in order to stratify patient to a respective treatment arm based on genetic information, however a post-hoc analysis to determine if there is a relationship between Metformin and any of these mutations will present novel and interesting data.

Metabolomic analysis of specimens with NMR and MS

Metabolomic analysis is a new and exciting high throughput analysis which will allow for rapid progress that will significantly advance the fields of colon cancer physiology, endocrinology, and metabolism. Ultimately, our findings will provide insight into the specific mechanisms by which Metformin acts on
human colon cancer tissues could provide other insights into other novel avenues of research for drug development in the treatment and control of this devastating disease. This technology has been utilized by other groups to study the pharmacokinetics of Metformin and suggest its effect in in vitro studies, but has yet to be used to study Metformin and/or colon cancer in human tissue studies (100, 106, 140). Fan et al. has shown that human tissue studies in lung cancers can be performed using both NMR and MS (92, 94, 97). We anticipate that tissues treated with Metformin will show decrease levels of glycolysis and downstream effects of tumor metabolism, but these secondary outcomes will involve drug and pathway discovery in Metformin and its relationship to colon cancer.

4.3 Patient Selection

Eligibility Criteria

- Patients must have histologically or cytologically confirmed colon cancer stage I-IV.
- Patients must have surgically resectable disease
- Age $\geq 18$ years and $< 90$ years of age. Because no dosing or adverse event data are currently available on the use of metformin in patients $<18$ years of age, children are excluded from this study, but will be eligible for future pediatric trials.
- ECOG performance status $\leq 2$ (Karnofsky $\geq 60\%$, see Appendix A).
- Life expectancy of greater than 3 months.
- Patients must have normal hepatic and renal function as defined below:
  - AST(SGOT)/ALT(SGPT) $\leq 2.5 \times$ institutional upper limit of normal
  - creatinine $< 1.5$ mg/dL
- The effects of metformin on the developing human fetus are unknown. For this reason and because biguanide agents are known to be teratogenic (Category B), women of child-bearing potential and men must agree to use
adequate contraception (hormonal or barrier method of birth control; abstinence) prior to study entry and for the duration of study participation. Should a woman become pregnant or suspect she is pregnant while she or her partner is participating in this study, she should inform her treating physician immediately. Men treated or enrolled on this protocol must also agree to use adequate contraception prior to the study, for the duration of study participation, and 4 months after completion of metformin administration.

- Ability to understand and the willingness to sign a written informed consent document.

**Exclusion Criteria**

- Patients who are not able to undergo surgical resection of their colorectal cancer
- Stage 0 (carcinoma in situ) and suspected but not biopsy proven colon cancer
- Surgical intervention at another institution for treatment of colorectal cancer prior to being treated at University of Kentucky
- Subjects with a history of previous malignancy treated with chemotherapy or radiation within the last 6 months.
- Current pregnancy or breast feeding. If patient becomes pregnant during their time in the study they will be removed from the study.
- Pre-operative testing that includes any of the following:
  - Creatinine level (renal function test)- > 1.5 mg/dL
  - AST/ALT > 2.5 ULN
  - Glucose testing that reveals persistent (> 3 laboratory measurements) hypoglycemia without patient being in fasting state.
- Patients with known brain metastases should be excluded from this clinical trial because of their poor prognosis and because they often develop
progressive neurologic dysfunction that would confound the evaluation of neurologic and other adverse events.

History of allergic reactions attributed to compounds of similar chemical or biologic composition to metformin.

Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements.

Pregnant women are excluded from this study because metformin is an agent with the potential for teratogenic or abortifacient effects (Category B). Because there is an unknown but potential risk for adverse events in nursing infants secondary to treatment of the mother with metformin, breastfeeding should be discontinued if the mother is treated with metformin.

HIV-positive patients on combination antiretroviral therapy are ineligible because of the potential for pharmacokinetic interactions with metformin.

Inclusion of Women and Minorities

- Both men and women of all races and ethnic groups are eligible for this trial.
- We shall enroll 50% women in this study as previous census and patient population data show that this is an appropriate representation of the state, region, and disease demographic.
- At least 10% of the sample we include we will recruit will comprise minority participants. We also plan to enroll at least 40% of our population from native Appalachians.
4.4 Registration Procedures

Protocol Review and Monitoring Committee and Institutional Review Board Review

Before implementing this study, the protocol, the proposed informed consent form and other information to subjects, must be reviewed by the Markey Cancer Center’s Protocol Review and Monitoring Committee and the University of Kentucky Institutional Review Board (IRB). A signed and dated statement that the protocol and informed consent have been approved by the IRB must be maintained in the Markey Cancer Center Clinical Research Office (MCC CRO) regulatory binder. Any amendments to the protocol, other than administrative ones, must be approved by the study sponsor and the UK IRB.

Enrollment Guidelines

Eligible patients will be identified by the principal investigator and co-investigators of this study. Potentially eligible patients will be screened in the University of Kentucky Markey Cancer Center clinics by the investigators, study personnel, and the Principal Investigator (PI). Upon obtaining proper consent, patients will be enrolled into the study.

Informed Consent

The goal of the informed consent process is to provide people with sufficient information so they can make informed choices about whether to begin or continue participation in clinical research. The process involves a dynamic and continuing exchange of information between the research team and the participant throughout the research experience. It includes discussion of the study’s purpose, research procedures, risks and potential benefits, and the voluntary nature of participation.

The informed consent document provides a summary of the clinical study and the individual’s rights as a research participant. The document acts as a starting point for the necessary exchange of information between the investigator and potential research participant. Also, research participants and their families
may use the consent document as an information resource and reference throughout participation in the trial. The informed consent document is often considered the foundation of the informed consent process; it does not, however, represent the entirety of the process. Nor is the informed consent document a risk-management tool for the investigator and/or institution.

The investigator must explain to each subject (or legally authorized representative) the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it may entail. Each subject must be informed that participation in the study is voluntary and that he/she may withdraw from the study at any time and that withdrawal of consent will not affect his/her subsequent medical treatment or relationship with the treating physician.

This informed consent should be given by means of a standard written statement, written in non-technical language. The subject should read and consider the statement before signing and dating it, and should be given a copy of the signed document. If the subject cannot read or sign the documents, oral presentation may be made or signature given by the subject’s legally appointed representative, if witnessed by a person not involved in the study, mentioning that the patient could not read or sign the documents. No patient can enter the study before his/her informed consent has been obtained. The informed consent form is considered to be part of the protocol, and must be submitted by the investigator with the protocol at the time of IRB review.

Compliance with Laws and Regulations

The study will be conducted in accordance with U.S. Food and Drug Administration (FDA) and International Conference on Harmonization (ICH) Guidelines for Good Clinical Practice (GCP), the Declaration of Helsinki, any applicable local health authority, and IRB requirements. The PI or designee will be responsible for obtaining continuing and not less than annual IRB re-approval throughout the duration of the study. Copies of the Investigator's annual report to the IRB and copies of the IRB continuance of approval must maintained by the
MCC CRO. The PI or designee is also responsible for notifying the Data and Safety Monitoring Committee of the MCC and the UK IRB of any significant adverse events that are serious and/or unexpected, as per SOP’s of those entities.

4.5 Treatment Plan

Enrollment and Screening Process

Prior to any study-required tests, subjects must first provide written informed consent to participate in this study. All lab tests and radiographic studies should be completed within 1 week prior to registration/initiation of treatment. These studies will include, but are not limited to the following: Complete history, physical examination, and evaluation of Zubrod Performance Status. Pathological biopsy or cytologically proven colon cancer. CT of the abdomen and pelvis with contrast or PET/CT Bone scan, only if the patient has bone pain and/or elevated alkaline phosphatase. CBC; serum chemistry tests to include alkaline phosphatase, glucose, creatinine, electrolytes, AST (SGOT), and total bilirubin. All radiographic studies should be completed within 1 week prior to registration/initiation of treatment.

Agent Administration

Treatment will be administered on an outpatient basis. Reported adverse events and potential risks are described in Section 7. The patient will discontinue the study if they experience adverse events during the 14-21 days of treatment with the research drug. No investigational or commercial agents or therapies other than those described below may be administered with the intent to treat the patient’s malignancy. The patients will receive a reminder of their choice (i.e. text messaging, email, phone call) from the research coordinator. We will also request that the patient keep either an electronic or hard copy medication diary to be given to research staff on the day of their scheduled operation. Plasma obtained in the pre-operative holding area prior to surgery will be analyzed for presence of Metformin in the patient’s circulatory system. Any patient having a plasma sample without evidence of the study drug will be excluded from the
study.

**Metformin**

Metformin will be supplied by the IDS pharmacy and will be given at a dose of 850 mg BID for a minimum of 14 days and a maximum of 21 days prior to surgery. Patients will not receive any prophylactic or supportive care regimens during the use of this study drug. We will ensure all patients have not had imaging with contrast studies within one week of starting study drug as this could significantly impair renal function.

**General Concomitant Medication and Supportive Care Guidelines**

Metformin is metabolized via hepatic and intestinal CYP3A1/2 and is renally excreted. Therefore, we anticipate there will be little interaction with medications that are metabolized by the cytochrome P450 system, but we will take care to protect patients from drugs with increased renal elimination, most notably patients undergoing radiographic imaging with renally cleared contrast dyes. Because there is a potential for interaction of metformin with other concomitantly administered drugs eliminated through renal secretion, the case report form must capture the concurrent use of all other drugs, over-the-counter medications, or alternative therapies. The Principal Investigator should be alerted if the patient is taking any agent known to affect or with the potential to affect renal function.

**Duration of Therapy**

In the absence of treatment delays due to adverse event(s), treatment will consist of a single 14-21 day period of oral metformin ingestion, followed by surgical resection of the subject’s colon cancer per institutional standards, or until one of the following criteria applies:

- Disease progression
- Intercurrent illness that prevents further administration of treatment,
- Unacceptable adverse event(s),
• Patient decides to withdraw from the study, or

• General or specific changes in the patient’s condition render the patient unacceptable for further treatment in the judgment of the investigator.

**Duration of Follow Up**

Patients will be followed for 4 weeks after removal from study or until death, whichever occurs first. Patients removed from study for unacceptable adverse event(s) will be followed until resolution or stabilization of the adverse event.

**Criteria for Removal from Study**

Patients will be removed from study when any of the criteria listed in Section 5.5 applies. The reason for study removal and the date the patient was removed must be documented in the Case Report Form.

**4.6 Dosing Delays/Dose Modifications**

Below are dose modification tables for the following adverse events: nausea, vomiting, diarrhea, hypoglycemia, renal dysfunction, and hepatic dysfunction.

<table>
<thead>
<tr>
<th>Nausea or Vomiting</th>
<th>Management/Next Dose for Metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ Grade 1</td>
<td>No change in dose</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Hold until ≤ Grade 1. Resume at same dose level.</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Off protocol therapy</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Off protocol therapy</td>
</tr>
</tbody>
</table>

*Patients requiring a delay of >2 weeks should go off protocol therapy.**Patients requiring > two dose reductions should go off protocol therapy.

Recommended management: antiemetics.
<table>
<thead>
<tr>
<th>Diarrhea</th>
<th>Management/Next Dose for Metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ Grade 1</td>
<td>No change in dose</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Hold until ≤ Grade 1. Resume at same dose level.</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Off protocol therapy</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Off protocol therapy</td>
</tr>
<tr>
<td></td>
<td>*Patients requiring a delay of &gt;2 weeks should go off protocol therapy.</td>
</tr>
<tr>
<td></td>
<td>**Patients requiring &gt; two dose reductions should go off protocol therapy.</td>
</tr>
</tbody>
</table>

Recommended management: Loperamide antidiarrheal therapy  
Dosage schedule: 4 mg at first onset, followed by 2 mg with each loose motion until diarrhea-free for 12 hours (maximum dosage: 16 mg/24 hours)

<table>
<thead>
<tr>
<th>Diarrhea</th>
<th>Management/Next Dose for Metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjunct anti-diarrheal therapy is permitted and should be recorded when used.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Renal dysfunction</th>
<th>Management/Next Dose for Metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ Grade 1</td>
<td>No change in dose</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Hold until ≤ Grade 1. Resume at same dose level.</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Off protocol therapy</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Off protocol therapy</td>
</tr>
<tr>
<td></td>
<td>*Patients requiring a delay of &gt;2 weeks should go off protocol therapy.</td>
</tr>
<tr>
<td></td>
<td>**Patients requiring &gt; two dose reductions should go off protocol therapy.</td>
</tr>
</tbody>
</table>

Recommended management: Per NCCN guidelines
<table>
<thead>
<tr>
<th>Hepatic dysfunction</th>
<th>Management/Next Dose for Metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ Grade 1</td>
<td>No change in dose</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Hold until ≤ Grade 1. Resume at same dose level.</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Hold* until &lt; Grade 2. Resume at one dose level lower, if indicated.**</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Off protocol therapy</td>
</tr>
</tbody>
</table>

*Patients requiring a delay of >2 weeks should go off protocol therapy.

**Patients requiring > two dose reductions should go off protocol therapy.

Insert any recommended management guidelines, if appropriate.

<table>
<thead>
<tr>
<th>Hypoglycemia</th>
<th>Management/Next Dose for Metformin</th>
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<tbody>
<tr>
<td>≤ Grade 1</td>
<td>No change in dose</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Hold until ≤ Grade 1. Resume at same dose level.</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Off protocol therapy</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Off protocol therapy</td>
</tr>
</tbody>
</table>

*Patients requiring a delay of >2 weeks should go off protocol therapy.

**Patients requiring > two dose reductions should go off protocol therapy.

Recommended management: antiemetics.

Insert any recommended management guidelines, if appropriate.
4.7 Adverse Events: List and Reporting Requirements

Adverse event (AE) monitoring and reporting is a routine part of every clinical trial.

**Expected Toxicities**

**Adverse Events List**

Adverse Event List(s) for Metformin

**More common**
- Abdominal or stomach discomfort
- cough or hoarseness
- decreased appetite
- diarrhea
- fast or shallow breathing
- fever or chills
- general feeling of discomfort
- lower back or side pain
- muscle pain or cramping
- painful or difficult urination
- sleepiness

**Less common**
- Anxiety
- blurred vision
- chest discomfort
- cold sweats
- coma
- confusion
- cool, pale skin
- depression
- difficult or labored breathing
- dizziness
• fast, irregular, pounding, or racing heartbeat or pulse
• feeling of warmth
• headache
• increased hunger
• increased sweating
• nausea
• nervousness
• nightmares
• redness of the face, neck, arms, and occasionally, upper chest
• seizures
• shakiness
• shortness of breath
• slurred speech
• tightness in the chest
• unusual tiredness or weakness
• wheezing

Rare
• Behavior change similar to being drunk
• difficulty with concentrating
• drowsiness
• lack or loss of strength
• restless sleep
• unusual sleepiness

Serious side effects include the following:
• Renal failure most common in patients with impaired CrCl
• Lactic acidosis most common in patients with diabetic ketoacidosis or pre-existing metabolic derangement

Adverse Event List(s) for Other Agent(s) Oral Glucose Tablet?
• Hyperglycemia
• Excitability
Adverse Event Characteristics

• **CTCAE term (AE description) and grade:** The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 4.0. A copy of the CTCAE version 4.0 can be downloaded from the CTEP web site: http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.ht.

• **For expedited reporting purposes only:**
  o aEs for the agent(s) that are listed above should be reported only if the adverse event varies in nature, intensity or frequency from the expected toxicity information which is provided.
  o Other AEs for the protocol that do not require expedited reporting are outlined in the next section (Expedited Adverse Event Reporting) under the sub-heading of Protocol-Specific Expedited Adverse Event Reporting Exclusions.

  ▪ **Attribution of the AE:**
    - Definite – The AE is *clearly related* to the study treatment.
    - Probable – The AE is *likely related* to the study treatment.
    - Possible – The AE may be related to the study treatment.
    - Unlikely – The AE is *doubtfully related* to the study treatment.
    - Unrelated – The AE is *clearly NOT related* to the study treatment.

• **Expedited Adverse Event Reporting**

  For MCC Investigator-Initiated Trials (IITs), investigators **must** report to the Overall PI any serious adverse event (SAE) that occurs after the initial dose of study treatment, during treatment, or within 30 days of the last dose of treatment on the local institutional SAE form. This applies to the following categories:
• **Grade 3 (severe) Medical Events** – Only events that are Unexpected and Possibly, Probably or Definitely Related / Associated with the Intervention.

• **ALL Grade 4 (life threatening or disabling) Medical Events** – Unless expected AND specifically listed in protocol as not requiring reporting.

• **ALL Grade 5 (fatal) Events** regardless of study phase or attribution

  **Note:** If subject is in Long Term Follow Up, death is reported at continuing review.

  **Note:** Abnormal laboratory values are not considered medical events, unless determined to be causative of SAE by the investigator or grade 5.

  The following table (Table 4.1) outlines the required forms and reporting structure for clinical trials. Table 4.2 outlines MCC reportable complications.
<table>
<thead>
<tr>
<th>Study type</th>
<th>Expedited reporting to MCC</th>
<th>Expedited reporting to External Agency</th>
<th>Non-expedited AE</th>
<th>Form</th>
<th>IRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIT where MCC Investigator holds the IDE or IND</td>
<td>Grade 3 – Unexpected AE PLUS Possibly, Probably or Definitely Related ALL Grade 4 Unless expected AND listed in protocol as not requiring reporting. ALL Grade 5 (fatal) Events</td>
<td>FDA: Suspected AE that is serious and Unanticipated (not listed in IDB or consent)</td>
<td>OnCore and DSMC reporting only</td>
<td>Mandatory Medwatch 3500a for Serious and unanticipated OnCore for all AEs, including SAEs</td>
<td>Yes if it meets the IRB reporting requirements: Unanticipated Problem and/or Serious AE (use IRB AE reporting form for all correspondence with IRB)</td>
</tr>
<tr>
<td>IIT by MCC Investigator of commercially available agent</td>
<td>Grade 3 – Unexpected AE PLUS Possibly, Probably or Definitely Related ALL Grade 4</td>
<td>FDA: Suspected AE that is serious and Unanticipated (not listed in IDB or consent)</td>
<td>OnCore and DSMC reporting only</td>
<td>Voluntary Medwatch 3500 for Serious and unanticipated OnCore</td>
<td></td>
</tr>
<tr>
<td>(non-IND and non-IDE)</td>
<td>Unless expected AND listed in protocol as not</td>
<td>for all Aes, including SAEs</td>
<td></td>
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</tbody>
</table>


Table 4.2. MCC reportable complications.

<table>
<thead>
<tr>
<th>Attribution</th>
<th>Gr. 2 &amp; 3 AE Expected</th>
<th>Gr. 2 &amp; 3 AE Unexpected</th>
<th>Gr. 4 AE Expected</th>
<th>Gr. 4 AE Unexpected</th>
<th>Gr. 5 AE Expected or Unexpected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unrelated</td>
<td>Not required</td>
<td>Not required</td>
<td>5 calendar days#</td>
<td>5 calendar days</td>
<td>24 hours*</td>
</tr>
<tr>
<td>Unlikely</td>
<td>Not required</td>
<td>Not required</td>
<td>5 calendar days</td>
<td>5 calendar days</td>
<td>24 hours*</td>
</tr>
<tr>
<td>Possible</td>
<td>Not required</td>
<td>5 calendar days</td>
<td>5 calendar days#</td>
<td>24 hours*</td>
<td></td>
</tr>
<tr>
<td>Probable</td>
<td>5 calendar days</td>
<td>5 calendar days</td>
<td>24 hours*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Definite</td>
<td>5 calendar days</td>
<td>5 calendar days</td>
<td>24 hours*</td>
<td></td>
<td></td>
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</tbody>
</table>

# If listed in protocol as expected and not requiring expedited reporting, event does not need to be reported.

* For participants enrolled and actively participating in the study or for AEs occurring

Protocol-Specific Expedited Adverse Event Reporting Exclusions for this protocol only, the AEs/grades listed below do not require expedited reporting to the Overall PI or the MCC DSMC, however, they still must be reported through the routine reporting mechanism (i.e. case report form). IRB reporting is as outlined in IRB SOP: [http://www.research.uky.edu/ori/SOPs_Policies/C2-0350-](http://www.research.uky.edu/ori/SOPs_Policies/C2-0350-).

<table>
<thead>
<tr>
<th>CTCAE SOC</th>
<th>Adverse Event</th>
<th>Grade</th>
<th>Hospitalization / Prolongation of Hospitalization</th>
<th>Attribution</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
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</table>
Expedited Reporting to External Agencies

The Overall PI will comply with the policies of all external funding agencies and the UK IRB regarding expedited reporting, as per the UK IRB’s SOP: 
http://www.research.uky.edu/ori/SOPs_Policies/C4-0150-. 

Expedited Reporting to the FDA

The Overall PI, as study sponsor, will be responsible for all communications with the FDA. The Overall PI will report to the FDA, regardless of the site of occurrence, any serious adverse event that meets the FDA’s criteria for expedited reporting following the reporting requirements and timelines set by the FDA.

Expedited Reporting to Hospital Risk Management

Participating investigators will report to the UK Office of Risk Management any participant safety reports or sentinel events that require reporting according to institutional policy.

Routine Adverse Event Reporting

All Adverse Events must be reported in routine study data submissions to the Overall PI on the OnCore case report forms. AEs reported through expedited processes (e.g., reported to the IRB, FDA, etc.) must also be reported in routine study data submissions.

4.8 Pharmaceutical Information

Metformin Product description:

Metformin will be dosed in single 850 mg tablets and dispersed to the patients in a blinded fashion by the clinical research technician. The study drug will be purchased through the UK-Chandler Pharmacy in Lexington, KY and will all be pharmaceutical grade medication acceptable for human use.

Solution preparation

The study medication will be prepared in a standard fashion by the UK Chandler Pharmacy. Please see package insert for details.
**Route of administration:**

Patients will take Metformin 850 mg PO two times per day, Q 12 hours. We suggest taking medication in an upright position to prevent aspiration and staying well hydrated while taking the study drug. Patients taking Glucose 10 mg PO two times per day, Q 12 hours will also be encouraged to maintain an upright position to prevent aspiration and staying well hydrated while taking the study drug.

Metformin should be taken with meals to reduce gastrointestinal side effects. Glucophage can cause lactic acidosis leading to hypoxic states and impairment of renal function. Metformin should not be administered in pregnant women and breast feeding mothers unless clearly indicated. Metformin tablet should be taken as whole without chewing or crushing. Glucophage is not recommended in patients with active hepatic disease. It is advised to avoid alcohol intake while on Metformin treatment as alcohol potentiates lactic acidosis.

**Agent Ordering:**

We will obtain all study drug materials through the UK-Chandler Pharmacy. All study drugs will be de-identified and labeled in such a fashion that only the clinical research nurse and the pharmacist will be aware of which study drug the patient is given. Patient will be given amount of study drug necessary for treatment up until the night before their surgery. Their medication container will contain their name, study number, and contact information for the clinical research nurse.

**4.9 Biomarker, Correlative, and Special Studies**

Pre-treatment samples will be collected at the colonoscopy suite and surgical specimens will be retrieved with the pathologist in the pathology suite. The patient samples will be retrieved immediately and cryopreserved with liquid nitrogen after diagnostic biopsy during the colonoscopy procedure and the surgical procedures, respectively. Another portion of the tissues will be collected in cold 10% formalin and prepared for IHC staining. Our lab as well as our
collaborator’s lab (Fan) have an extensive background in tissue collection, cryopreservation, processing, and storage

Biomarker Studies

**AMPK analysis by IHC staining**

We plan to analyze both the pre and post treatment specimens to determine phosphorylation changes of AMPK in the Metformin treated specimens. IHC staining is an established and well characterized technique to identify and analyze AMPK in both pre-clinical and clinical studies (71, 127, 128). AMPK is an enzyme that plays a role in cellular energy homeostasis. It is also acts as an effector of metformin action on glucose uptake in cellular processes (129). Since Metformin targets AMPK we intend to use it as a primary biomarker in our study. We anticipate that Metformin treated tissues will show increased phosphorylation changes in AMPK when compared to specimens not treated with Metformin. We plan to prepare and stain these tissues in our laboratory at the Markey Cancer Center and have the resources and equipment to carry out all procedures (Harris, Gao). We plan to quantify and compare differences in AMPK phosphorylation with Aperioscope imaging and quantification.

**Ki67 analysis by IHC staining**

We plan to analyze both the pre and post treatment specimens to determine the proliferative changes present by comparing and quantifying the positive staining of Ki67 in the tissues. Ki67 is a nuclear protein that is associated with and may be necessary for cellular proliferation, and it is also associated with ribosomal RNA protein transcription (130, 131). There are multiple pre-clinical and clinical studies supporting the evaluation of Ki67 as a proliferative marker in tumor biology studies and that it can be accurately quantified and used as a reliable marker (131, 141). We anticipate that tumor specimens which have not been exposed to the study drug will have an increased observance of the staining of Ki67, thus indicating an increase of proliferation in tissues not exposed to Metformin. We plan to stain all tissues for this biomarker by the same protocol to maintain consistency and we plan to
quantify and compare differences in Ki67 staining with Aperioscope imaging and quantification.

**LKB1 analysis by Western blotting**

LKB1 is a primary upstream kinase of AMPK and exerts its growth suppressing effects by activating a group of ~14 other kinases, comprising AMPK and AMPK-related kinases (48). Activation of AMPK by LKB1 suppresses growth and proliferation when energy and nutrient levels are scarce. Activation of AMPK-related kinases by LKB1 plays vital roles maintaining cell polarity thereby inhibiting inappropriate expansion of tumor cells (34). LKB1 leads to disorganization of cell polarity and facilitates tumor growth under energetically unfavorable conditions (132). We plan to analyze both the pre and post treatment specimens to determine the presence of LKB1. We anticipate an increase in LKB1 in the tissues not exposed to Metformin as this kinase which is upstream of the anticipated target biomarker as it is allows tumor growth in settings of cellular stress. Western blotting analysis will allow us to study qualitative data of protein expression related to LKB1.

**p-mTOR and phospho-S6K analysis by Western blotting**

mTOR and phospho-S6K are both effectors of cell growth and proliferation via the regulation of protein synthesis in tumor cell growth and metabolism we intend to analyze with Western blotting. mTOR is a phylogenetically conserved serine/threonine kinase which is phosphorylated and activated by Akt, but can also auto phosphorylate when experiencing metabolic stress (133).

Pre-clinical studies have shown that metformin potentiates the effects of paclitaxel in endometrial cancer cells through inhibition of cell proliferation and modulation of the mTOR pathway and clinical studies in human tumor xenografts show that Metformin treatment results in a downregulation of mTOR and downstream effectors such as pS6K (42, 134). We intend to use Western blotting analysis to study qualitative data of protein expression related to the mTOR and pS6K inhibition we expect to observe in Metformin treated tissue specimens.
ACC analysis by Western blotting

is produced during the second step of aerobic cellular respiration, pyruvate decarboxylation, which occurs in the matrix of the mitochondria and its main function is to convey the carbon atoms within the acetyl group to the citric acid cycle to be oxidized for energy production. This metabolic molecule is extremely important in the understanding of cellular metabolism and for this reason we would like to include it in our qualitative Western blotting analysis studies. Preclinical data shows that fatty acid oxidation is increased in the presence of Metformin and downstream effectors involved in fatty acid metabolism such as ACC and fatty acid synthase (FASN) are increased in models of cellular stress (23, 106, 135). We anticipate that the Metformin treated tissues will show an increase in the protein expression of acetyl CoA using Western blotting analysis.

Genetic studies of surgical specimens

Genetic analysis of all tumor specimens is important as we need to understand the implications of any genetic differences we find in comparing the pre and post treatment specimens. We intend to observe mutations for K-ras, PI3KCA, HNPCC, and MMR, all of which are important genetic mutations in colon cancer known to effect tumor biology (136-139). It is currently unknown if or how Metformin treatment changes expression of any of these genetic profiles. Unfortunately adequate tumor biopsy tissue will not be available in quantity or in a timely manner in order to stratify patient to a respective treatment arm based on genetic information, however a post-hoc analysis to determine if there is a relationship between Metformin and any of these mutations will present novel and interesting data.

Metabolomic analysis of specimens with NMR and MS

Metabolomic analysis is a new and exciting high throughput analysis which will allow for rapid progress that will significantly advance the fields of colon cancer physiology, endocrinology, and metabolism. Ultimately, our findings will provide insight into the specific mechanisms by which Metformin acts on...
human colon cancer tissues could provide other insights into other novel avenues of research for drug development in the treatment and control of this devastating disease. This technology has been utilized by other groups to study the pharmacokinetics of Metformin and suggest its effect in in vitro studies, but has yet to be used to study Metformin and/or colon cancer in human tissue studies (100, 106, 140). Fan et al. has shown that human tissue studies in lung cancers can be performed using both NMR and MS (92, 94, 97). We anticipate that tissues treated with Metformin will show decrease levels of glycolysis and downstream effects of tumor metabolism, but these secondary outcomes will involve drug and pathway discovery in Metformin and its relationship to colon cancer.

4.10 Study Calendar

Schedules shown in the Study Calendar (Table 4.3) below are provided as an example and should be modified as appropriate.

Baseline evaluations are to be conducted within 1 week prior to start of protocol therapy. Scans and x-rays must be done ≤4 weeks prior to the start of therapy. In the event that the patient’s condition is deteriorating, laboratory evaluations should be repeated within 48 hours prior to initiation of the next cycle of therapy.
Table 4.3. Study calendar.

<table>
<thead>
<tr>
<th></th>
<th>Pre-Study</th>
<th>Wk. 1</th>
<th>Wk. 2</th>
<th>Wk. 3</th>
<th>Off Study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metformin vs Placebo</strong></td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>(if indicated)</td>
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<tr>
<td>Informed consent</td>
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<tr>
<td>Demographics</td>
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<tr>
<td>Medical history</td>
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<tr>
<td>Concurrent meds</td>
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<tr>
<td>Physical exam</td>
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<tr>
<td>Vital signs</td>
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<tr>
<td>Performance status</td>
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<td>CBC w/diff, plts</td>
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<tr>
<td>Serum chemistry&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>EKG (as indicated)</td>
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<tr>
<td>Adverse event evaluation</td>
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<tr>
<td>Tumor measurements</td>
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<td>X</td>
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<tr>
<td>Radiologic evaluation</td>
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<td>X</td>
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<tr>
<td>B-HCG</td>
<td>X&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Other tests, as appropriate</td>
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<tr>
<td>Other correlative studies</td>
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</tbody>
</table>
4.11 Measurement of Effect

This is a pilot study of metformin in the preoperative setting and as such, is not intended to have a significant cancer therapy effect.

4.12 Data Reporting / Regulatory Requirements

Adverse event lists, guidelines, and instructions for AE reporting can be found in Section 7.0 (Adverse Events: List and Reporting Requirements).

Data Reporting

This study will require data submission and reporting via the OnCore Database, which is the official database of the Markey Cancer Center Clinical Research and Data Management Shared Resource Facility (CRO). Instructions for submitting data is listed in Study-Specific Data Management Plans created by CRO staff.

Responsibility for Data Submission

Study staff are responsible for submitting study data and/or data forms to OnCore as per the Markey Cancer Center CRO SOP’s. This trial will be monitored by the MCC Data and Safety Monitoring Committee (DSMC) on a schedule determined by the Protocol Review and Monitoring Committee at the initial PRMC review. THE CRO staff is responsible for compiling and submitting data for all participants and for providing the data to the Principal Investigator for review.

4.13 Statistical Considerations

Study Design/Endpoints

Draft Sample Size Calculation:

**Study Design and Sample Size.** A (two armed) one-arm trial will be employed wherein upregulation of AMPK will be compared from pre to post-treatment tissues within the same patient. AMPK will be measured using standard technique of immunohistochemistry (IHC) tissue staining which is routinely performed in our lab (Harris, Gao). Changes in quantitatively measured
AMPK IHC score from pre to post-treatment samples will be evaluated. Using published data on histoscore of AMPK alpha 2 levels, we will assume that the mean increase from pre to post-treatment sample will be equal to 75 (SD of the difference = 125), resulting in a moderate effect size equal to 0.60 (128). A sample of 26 patients will provide 80% power to detect this effect size based on a two-sided, nonparametric Wilcoxon signed ranks test with 5% significance level. In order to account for at most a 10% possibility of unpaired pre and post sample in a patient, we will enroll a total of 30 patients into the trial.

A secondary, exploratory goal of the study is to compare high-throughput metabolomics parameters between pre and post treated samples. We assume at least 3% of metabolites are truly differentially expressed with a 2-fold difference between pre versus post treatment samples. The standard deviation of metabolite expression is assumed to be 25% of the mean value based on our experience. Based on Monte Carlo simulations, a sample of 10 patients (with matching pre and post specimens) will provide over 80% power to detect metabolite changes at 5% FDR based on a paired t-test.

**Statistical Analysis.** Data analysis for quantitative levels of AMPK, Ki-67 and other biomarkers will involve descriptive summaries and graphical presentations of pre, post-treatment and changes from pre to post evaluations. Comparisons of changes in biomarker levels will be performed using nonparametric Wilcoxon signed ranks test (or paired t-test, if appropriate). Correlations between biomarkers will be assessed using measures of correlation coefficient such as Spearman’s correlation.

Bioinformatics methods including computational processing and statistical analysis for differential analysis will be employed for the metabolomics studies. Specifically, before downstream statistical data analysis can begin system biochemistry and bioinformatics methods will be employed in collaboration with Dr. Fan and her team. Statistical analysis for the processed metabolite endpoints will involve pairwise comparisons for each endpoint based on paired t-tests as well as general linear and linear mixed models to evaluate kinetics over
stratification factors.

Interim Analyses

Interim analysis will be performed after 15 patients have been accrued and relevant data is reviewed. We will ensure there is a difference in our primary endpoint, phosphorylation, and also determine that all tissues specimens are being processed and stored appropriately for NMR and MS analysis.

Data Management. The study statistician and staff from the Biostatistics and Bioinformatics Shared Resource Facility (B2SRF) of the Markey Cancer Center will work closely with the study PI and the CRO at Markey in the development of eCRFs for the study. A draft data collection form has been devised as part of the protocol submission. In addition to the standard eCRF forms available in Oncore, this draft data collection form will serve as a guide for development of additional protocol-specific eCRFs in Oncore. Specifically, the statisticians will attend several meetings including the Protocol Initiation Meeting (PIM) to address all statistical considerations for this protocol including data collection forms and database for quantitative assessments of AMPK, Ki-67 and other biomarkers. The OnCore clinical trial management system, managed by Markey’s CRO, will be the primary database repository of clinical data from all patients enrolled into this trial. Data will be accessed by the study statistician on a regularly-scheduled basis to perform statistical programming for conduct of data quality control, data management, generation of interim reports and statistical analysis. The B2SRF has developed an automated mechanism for generating the interim reports of accrual and safety data. In collaboration with the study team, procedures will be developed for timelines for data quality control, resolution of data queries, and final data analysis. Finally, access of the high-throughput metabolomics data and linkage with the clinical data will be arranged between the study PI, statistician and Dr. Fan’s study team.
CHAPTER FIVE: DISCUSSION

5.1 Overview of determined mechanisms of action

The oral anti-diabetic drug metformin is beneficial for at least a subset of cancer patients, but there continues to be a staunch debate about whether metformin can directly affect tumor metabolism or if it can actually reduce the risk of cancer at all (142). The majority of existing data regarding the effect of metformin on tumorigenesis has focused on breast and prostate cancer. As the literature is currently lacking data specific to the metabolomic effects of metformin on combined in vitro, in vivo, and ex vivo data in colon cancers this prompted us to systematically determine this agent’s specific effects on tumor cells and tissues in these settings. We carefully designed our experiments to first evaluate in vitro cellular and molecular mechanisms in multiple and varied colon cancer cell lines to determine which phenotypes best respond to metformin. We determined that responsive colon cancer cell lines involve the canonical and known mechanisms of action involving metformin such as increased phosphorylation of AMPK and ACC and downregulation of pS6 indirectly through the PI3K/mTOR pathway. However, we determined that “resistant” cancer cell lines don’t follow this pattern and participate in adaptive metabolic behaviors to evade effects of metformin. Our next step was to follow these experiments with studies to determine the metabolic alterations metformin triggers in certain cancer cell lines through mitochondrial complex I inhibition. This gave us further insight into how more aggressive cancer cell lines such as SW480, KM20, and our newly synthesized PDX evade targeted molecular therapies. Using SIRM metabolomics and NMR analysis we determined that in cancer cell lines that evade metformin as a targeted agent tend to preferentially utilize alanine as an seemingly renewable energy substrate. Determining how and where alanine is “renewed” is a potential next step with our current hypotheses including that it may be recycled through additional pyruvate pools or that a high rate of recycling via the pentose cycle suggests that a significant fraction of cellular NADPH is generated by the pentose cycle as opposed to generation by the malate-pyruvate shuttle and this is the actually “additional” energy substrate (143).
5.2 The potential impact of metabolomics on preclinical and clinical data

Our next step in determining the specific mechanisms of metformin in colon cancer was to collect human colon cancer tissues in order to develop truly translational *in vivo* and *ex vivo* models to specifically evaluate aggressive tumors and use metabolomic analyses to determine how we can potentially target them. These models maintain their structural and oncogenic integrity through multiple generations and are reproducible. This provides a realistic canvas to test new molecular inhibitors and anti-oncogenics on human tissues without any risk to the patient. Using human tissues in conjunction with SIRM offers the best opportunity to create novel research models of human colon cancer that are clinically applicable (92, 144).

Improving our current work with *in vitro* and *in vivo* models is paramount to producing research that is clinically valid and relevant. Many current animal models used to study colon cancer use quite a variety of species, as well as methods of oncogenic vector introduction, and even variable doses of drugs with and without combinations of other therapies that may or may not be effective (145). As translational researchers we must aim to make tedious and expensive animal work as clinically relevant as possible (73, 146). However, xenotransplantation of human tissue into animals requires ethical training and consideration and clinical and translational scientists must agree to take on the tremendous responsibility of doing no harm on the bench or at the bedside (147, 148). Long-standing cancer cell lines are still common sources of scientific data when evaluating colon cancer, and although high throughput metabolomics can be used for these types of studies, the great power of metabolomic analysis is that real, human cancer tissues can literally be collected at the bedside, the surgeon’s bedside, and processed and stable isotope labeled within minutes. Hours to days later scientists, physicians, and surgeons can begin to gather valuable data that can potentially lead to better tailored therapies for patients in the form of molecular inhibitors and anti-oncogenic immunomodulators. For example Jahnke et al. developed a label-free monitoring system to directly analyze the chemosensitivity of undissociated tumor tissue. Using a preparation
of tumor micro-fragments (TMF) established from melanoma biopsies, we characterized the tissue organization and biomarker expression by immunocytochemistry. Robust generation of TMF was established successfully and demonstrated on a broad range of primary melanoma tumors and tumor metastases. Using isolated TMF, sensitivity to six clinically relevant chemotherapeutic drugs (dacarbazine, doxorubicin, paclitaxel, cisplatin, gemcitabine, and treosulfan) was determined by impedance spectroscopy in combination with a unique microcavity array technology we developed. In parallel, comparative analyses were performed on monolayer tumor cell cultures. Lastly, we determined the efficacy of chemotherapeutic agents on TMF by impedance spectroscopy to obtain individual chemosensitivity patterns (149).

5.3 Clinical and Translational Scientists, Cash, and Clinical Trials

Some of the major impediments to these valuable studies are funding, lack of collaboration between scientists and direct health care providers, and difficulty design valid clinical trials that will successfully meet accrual while also assessing relevant primary endpoints (150, 151). Unfortunately, The US is experiencing a severe shortage of underrepresented biomedical researchers (152). Even more discouraging, Hu et al. determined Inflation-adjusted NIH funding for surgical research decreased 19% from $270M in 2003 to $219M in 2013, with a shift from R-awards to U-awards. Proportional funding to outcomes research almost tripled, while translational research diminished. Nonsurgical departments have increased NIH application volume over the last 10 years; however, surgery's application volume has been stagnant. To preserve surgery's role in innovative research, new efforts are needed to incentivize an increase in application volume (153). Programs like the Center for Clinical and Translational Studies and the current T32 grant at the University of Kentucky offer an excellent opportunity for residents who intend to pursue a career in academia and need the training and guidance to be part of developing projects and trials that will be beneficial to patients and cost-effective for funding organizations.
5.4 Conclusions and Further Directions

In conclusion, we embarked on a project to determine the true mechanism of action of metformin on colon cancer cells. We determined that metformin decreases cell proliferation and protein synthesis through the mTOR pathway, and increases metabolic stress points by the activation of p-AMPK and p-ACC in certain less aggressive cell lines such as HT29 and HCT116. However, determining the specific mechanisms of more aggressive and elusive cell lines like SW480, KM20, and PDX required more investigative work through metabolic analysis with measurements of OCR and ECAR to determine if and where they may be mitochondrial complex stress points that could be targeted. We determined that very aggressive cell lines will seek other energy sources to continue to grow and live. We used SIRM and $^{13}$C-NMR to determine that certain aggressive colon cancer cell lines evade the glycolytic inducing stress of metformin by seek renewable energy sources available via the Kreb’s cycle and potential the pentose phosphate pathway. Also, we determined that metformin does anti-proliferative effects on human tissues carried in a PDX treated with and without metformin for a two week period. Finally we have performed initial ex vivo $^{13}$C-glucose labeling of human tissues and with and without metformin and intend to perform the necessary analysis to determine if the effect in human tissues mirrors our findings in two aggressive colon cancer cell lines, one that was developed during my training and offers a solid clinical and translational model of developing in vitro, in vivo, and ex vivo studies with a single human tissue.

Finally, I hope that additional future directions lead to a continued career as an academic surgeon with active basic science and clinical and translational projects throughout my career.
APPENDIX A. Institutional Review Board Approval for Gastrointestinal Tissue Collection Protocol

Background

Gastrointestinal malignancies in the United States are increasing in incidence, and Kentucky leads the nation in number of colorectal cancer and pancreatic cancer deaths in both men and women in data from 2004-2008 according to the American Cancer Society. The Center for Disease Control evaluated cancer rates in Appalachia and determined that the incidence of gastrointestinal malignancies for this population is even higher than other parts of the United States. In order to better understand tumor growth and metastasis of these diseases, we must evaluate tumor metabolism and microenvironments responsible for the aggressive nature of these processes. Biospecimens and data collected from cancer patients and people without cancer are paramount to understanding the metabolic function of these tumors and developing modalities that can impede or halt their growth. We will use de-identified biospecimens collected from leftover resected patient tissues for non-human research purposes to study molecular pathways and signaling related to cancer growth. We also intend to obtain serum and plasma from these patients prior to the initiation of their surgical procedure. This protocol describes the methods used to protect human subjects for Markey Cancer Center (MCC) sponsored/sanctioned research studies that use biospecimens and data collected from such people. It details the standard operating procedure we intend to use to consent patients for biospecimen collection and collection of these specimens by our honest brokers.

Fresh tissue including normal and primary tumor is prospectively collected in patients already undergoing surgical resection for gastrointestinal malignancy and inflammatory bowel diseases. Frozen previously banked specimens are also available. We also intend to collect one total milliliter of serum and plasma in the pre-operative holding area from the patient prior to surgery. A technician in the Markey Cancer Center Biospecimen and Tissue Procurement Shared Resource Facility (MCC BSTP SRF) will act as an honest broker and will provide
specimens completely de-identified of PHI to Dr. Evers’ lab personnel for non-human research purposes. The tissues, serum, and plasma samples collected will be analyzed by Markey Cancer Center laboratory staff by methods to analyze tumor metabolism and cellular and molecular signaling. While this research will not affect the care of the patients providing the samples, their tissues will be used to better understand mechanisms that contribute to tumor signaling, growth, and metastasis. We intend to develop methods to slow, or potentially inhibit these pathways. Better understanding of these pathways and strategies to diminish them can result in development of treatment options for future patients suffering with malignancies of the gastrointestinal tract.

Objectives

To obtain bio specimens from an honest broker to perform non-human research using tissues from surgical specimen while protecting the rights of the individuals from whom the specimens and information was obtained. Non-human research experiments will include protein and biomarker analysis using Western blotting, cell culture, molecular signaling pathway analysis and metabolic analyses using ELISA (enzyme-linked immunosorbent assay), study of genetic polymorphisms using PCR (polymerase chain reaction), and genetic and epigenetic testing using DNA microarrays. Only the biospecimens mentioned in this protocol will be used for non-human research, and their retrieval will have no impact on patient treatment or plan of care.

Biospecimens

Tissue including primary tumor and/or nonneoplastic tissue that automatically comes with the surgical specimen (e.g. mesenteric fat) is prospectively collected in patients already undergoing resection for gastrointestinal disease. After the specimen is acquired it will be evaluated by the pathology department to determine what tissue is necessary and unnecessary for clinical workup and pathologic staging of the tumor. Fresh (unprocessed) tissue designated as unnecessary for clinical care will therefore be available for research, as it represents material in excess of what is needed for clinical care.
and, thus, would otherwise be disposed. Tissues will be stored in solution of phosphate buffered solution and 20mg/ml fetal bovine serum and deidentified by Brent Hallahan, both of whom are honest brokers in the MCC BSTP SRF. This sort of approach to fresh tissue processing by the BSTP has already been approved by the IRB (protocol 04-0454). The specimens will then be collected by Evers lab associates.

Regarding the use of already-existing archival formalin-fixed paraffin embedded material (FFPE) housed in the department of pathology, FFPE tissue blocks will be obtained from archival clinical material stored in the pathology department. These are cases that have already been processed, evaluated, and signed out by an attending pathologist. In most instances, after tissue sections are cut from such blocks, the remaining FFPE tissue is no longer needed for patient care. Judging when a given FFPE tissue block is no longer needed will be the responsibility of Dr. Horbinski, the MCC BSTP SRF Director and a board-certified anatomic pathologist. Dana Napier, a histotechnician in the MCC BSTP SRF, will act as an honest broker and will provide FFPE specimens completely de-identified of PHI to Markey Cancer Center lab personnel for non-human research purposes.

Patients who also consent to providing serum and plasma biospecimens under the IRB protocol # 04-0454 will have a blood sample drawn from a previously placed intravenous catheter at the same time as specimen retrieval specified in IRB protocol #04-0454. These specimens will not be obtained by separate venipuncture, fingerstick, heelstick, or earstick. One milliliter of blood will be aliquoted from the sample drawn for the IRB protocol # 04-0454 for our purposes and poses minimal risk and discomfort to the patient. After retrieval the sample will be placed on ice and the honest broker will de-identify the sample prior to contacting Markey Cancer Center laboratory personnel for biospecimen retrieval. The de-identified specimen will be given a study code or number and the laboratory personnel will have no access to patient health information.

Regarding patients who have already consented to tissue collection using
IRB protocol # 04-0454 we are requesting the ability to retrospectively obtain plasma and serum samples that are archived in the MCC BSTP SRF. Dana Napier a histotechnician in the MCC BSTP SRF, will act as an honest broker and will provide these specimens completely de-identified of PHI to Markey Cancer Center lab personnel for non-human research purposes.

Data

Honest brokers will be responsible for entering, tracking, and maintaining the identity of the samples through electronic database tables. For research purposes, the honest brokers will assign a unique de-identified study code to each sample. The following data will be collected by the MCC BSTP SRF: Patient demographics, specimen characteristics, specimen tracking information, cancer pathology where applicable, cancer registry information (diagnosis, staging, treatment, survival, historical cancer diagnoses, etc.), patient survey information, biospecimen analytic results, environmental specimen analytic results, etc. The information will be stored in database tables that reside on access-restricted servers managed by the MCC Informatics Shared Resource Facility (ISRF). Some, but not all database tables may contain PHI. Database tables will utilize one or more unique identifiers (such as medical record number) with the associated unique de-identified study code to associate records across database tables. Database tables may be managed by one or more software applications such as caTISSUE and custom applications developed by the ISRF. All computer systems and applications will require authentication through unique logins and passwords. Computer systems, network, and applications will be maintained with due diligence to modern security standards. Servers will reside behind appropriately configured firewalls managed by UK Communications and monitored by ISRF personnel. Access to patient information will be restricted to the honest brokers and clinical research personnel who come into contact with patients prior to their operation for consenting purposes. Only de-identified, coded or aggregate information will be given to investigators/study personnel by the honest brokers, and honest brokers will provide laboratory staff with de-
identified tissue, plasma, or serum specimen. As stated above, the honest brokers will maintain the identity of the samples through unique identifiers such as medical record numbers and each sample will be assigned a unique de-identified study code. Study personnel will only obtain samples with the specific, de-identified study code.

**Study population**

Biospecimens and data from adult patients of UK Healthcare and MCC affiliates will be collected. Patients undergoing surgical resection for gastrointestinal malignancy or inflammatory bowel disease in which the treatment procedure includes resection of all or portions of visceral fat or mesentery will meet inclusion criteria for the study. Any archival FFPE tissue from a cancer patient obtained before 2008 is automatically included, as it is likely that the individual from whom the tissue was removed is no longer available to give specific consent. As this represents a repository for future research, its size is not limited.

**Subject recruitment methods and privacy**

BSTP employees identify potential donors by monitoring the UK Healthcare data base for patients undergoing surgery for gastrointestinal diseases, and who are/will be treated at UK Healthcare or an affiliate health care provider. They will be asked to participate and will be asked to sign the general banking protocol consent form (already IRB approved, IRB# 04-0454) For the use of specimens for existing studies, the possible uses are covered in the general banking consent form. This consent includes a discussion of the possible uses and risks for their specimens that adequately inform the subject for the limited types of existing and future studies of banked specimens that are eligible to receive specimens. Privacy will be maintained by not providing the specimen to investigators with any unique patient identifiers.

**Informed consent process**

Patients will be consented by BSTP-SRF and clinical research personnel
using the general banking combined consent and authorization form from the Markey Cancer Center General Specimen Banking Protocol (IRB# 04-0454). A waiver of informed consent has been requested for the existing FFPE block data base, serum, and plasma specimens and is requested in this application for the use of clinical/epidemiologic data.

**Research Procedures**

This protocol involves obtaining biospecimens that will be used for non-human research only. Non-human research experiments will include protein and biomarker analysis using Western blotting, cell culture, molecular signaling pathway analysis and metabolic analyses using ELISA (enzyme-linked immunosorbent assay), study of genetic polymorphisms using PCR (polymerase chain reaction), and genetic and epigenetic testing using DNA microarrays.

Molecular pathway inhibitors and modulators of genetic and epigenetic expression will be developed in efforts to learn how to slow down and/or inhibit the processes that contribute to tumor metabolism and growth. Only leftover tissues will be used for non-human research, and will have no impact on patient treatment or plan of care.

**Resources**

This protocol will utilize physical resources and established operations within the department of pathology and the BSTP. The department of pathology’s surgical pathology laboratory will be used to obtain fresh (unprocessed) specimens from clinical specimens that are not needed for clinical care. Plasma and serum samples will be collected at the same time as plasma and serum samples are collected for patients consenting to biospecimen donation using IRB protocol # 04-0454. Archival FFPE material as well as archived serum and plasma samples will be obtained from material stored in the pathology department.

Storage of the biospecimens will follow established procedures. The material obtained from fresh specimens is available for immediate use or can be
frozen and stored in the MCC BSTP laboratory for future use. The archival clinically derived FFPE material is stored in the department of pathology.

Once de-identified tissues are received from the honest broker, researchers associated with the Evers lab will perform experiments as mentioned above. Data will be stored within the MCC ISRF. Database tables may be managed by one or more software applications such as caTISSUE and custom applications developed by the ISRF. Access to patient information will be restricted to authorized study, clinical research personnel, MCC biospecimen core personnel, and ISRF personnel. All computer systems and applications will require authentication through unique logins and passwords. Computer systems, network, and applications will be maintained with due diligence to modern security standards. Servers will reside behind appropriately configured firewalls managed by UK Communications and monitored by ISRF personnel. Only coded or aggregate information will be given to investigators. Information will not be provided using personal identifiers.

**Personnel**

All key personnel and the individuals acting as honest brokers will have training in human subject protection. Basic science research personnel associated with Dr. Evers’ lab will only have contact to the honest broker in order to obtain the de-identified tissue.

**Potential risks**

Privacy and confidentiality see data section and request for waiver of informed consent.

**Safety precautions**

Use of a unique identifier so that the donor of the specimen/data remains anonymous to the user of the specimen. Use of hospital or General consent to confirm intent. Tissue will be retrieved from resected specimens who are involved in the anticipated surgical specimen with regards to appropriate oncological margins.
Benefit vs risk

No direct benefit. Indirect benefit to everyone including subject if information that leads to more effective ways to prevent, treat or cure cancer are found. No physical risks as specimen/data are collected as part of routine patient care. There is a minimal risk of breach or privacy/confidentiality as noted above.

Available alternatives

None.

Research materials

Existing and to be collected material in conjunction with routine patient care.

Privacy and confidentiality

As noted above all material stored and distributed to investigators using unique (non-patient) identifiers and an “honest broker” system that isolates the identity of the source of the material being used for research from the researcher. All data stored in limited access and password protected manner.

Payment

None

Cost to subject

None

Data and Safety monitoring

By Markey scientific advisory committee. The ultimate responsibility for oversight of data and specimen handling is the Director of Markey Cancer Center who is listed as the PI of this application.

Subject Complaints

Subjects may address questions or complaints to Craig Horbinski, MD,
PhD, The Markey Cancer Center director of research, UK Healthcare customer service, the UK Hospital ethics committee or the IRB.

Research involving non-English speaking subjects

HIV/AIDS

Not available.

PI-sponsored FDA regulated research

Not available.
APPENDIX B: Institutional Animal Care and Use Committee Approval for Patient Derived Xenografts for the Implantation of Human Tissues

USDA Pain Category: D

Preoperative Evaluation: Animals and occupied animal cages should be placed on a preheated circulating water heating pads set to 90-100F to prevent hypothermia which can result in slowed metabolism, prolonged induction, and extended postsurgical recovery times.

Preoperative xenografts preparation: All patient tissues will be handled with sterile, autoclaved instruments and be processed under a sterile hood. Patient tissues will be cleansed with Phosphate buffered solution (Sigma) and 2% penicillin/streptomycin (Sigma) and fungizone (LifeSciences). Once cleansed the tissues will be cut into 5mm pieces and mixed with 100 uL of sterile Matrigel (BDBiosciences). This tissue/Matrigel mixture will be placed in sterile 1.5mL microcentrifuge tubes and placed in an opaque container with ice for transport to the animal facility.

Type of surgery to be performed: Surgical Implantation: Patient derived xenografts, colon, pancreatic

Is this a Recovery or Non-recovery Type of Surgery: Recovery

Duration of anesthesia/surgery: 15-30 min

Describe pre-op health assessment: Animals will be visually examined to ensure they are healthy and normally active. Individual animal body weights will be determined and noted on the pink DLAR Surgery Cards.

Describe use of pre-anesthetic medications: Buprenorphine 0.05-0.1 mg/kg SC (only when using Isoflurane anesthesia).

Operative Anesthesia: Isoflurane (1-4% in O2, inhaled-nosecone)

How will anesthetic depth be measured? Adequate depth of anesthesia will be assessed by presence of regular steady respirations and absence of a withdrawal reflex (toe or tail pinch) or corneal reflexes.
Surgical table, incision site and surgeon preparation: Surgical table, incision site and surgeon preparation. Standard procedures for rodent survival surgery will be followed as described in the IACUC Policies, Procedures and Guidelines on Rodent Surgery. Procedures will be performed in a designated area of the laboratory specifically prepared in advance for that purpose.

Following induction of anesthesia an artificial tear or ophthalmic ointment is applied to both eyes. The skin will then be shaved and scrubbed thoroughly with Povidone iodine or Chlorhexidine solution. The animal's body temperature will be maintained with heating pads or lamps, as necessary. The surgeon will wear a mask and sterile gloves. Instruments will be steam autoclaved prior to use and sterilized with a glass bead sterilizer between animals. Assurance is made that the temperature of instruments from the hot bead sterilizer are normalized before use. The pump will be prepared aseptically.

Support equipment: Isoflurane vaporizer with gas scavenging (if using isoflurane), recirculating water heating pad(s), and glass bead sterilizer.

Describe surgical procedure Prior to surgery mice will be assessed for normal activity, inquisitiveness, condition of hair coat, eating, drinking, defecation, urination, appearance of eyes, breathing rate, gait and bodyweight. If any abnormal condition is observed, the animal will not be used and the PI or one of the co-investigators will consult with a DLAR veterinarian.

Adequate anesthesia will be assessed by checking eye blinking, response to toe pinch, palpebral reflex muscle relaxation, lack of response to incision, respiratory rate and depth.

If animals respond to any of these, they are not adequately anesthetized and anesthesia will continue until these signs are absent. Additional isoflurane will be administered through inhalation for isoflurane (via cone placed over the animal’s nose and connected via a hose to isoflurane vaporizer) as necessary.

At all times, sterile techniques will be followed. Instruments will be initially steam sterilized. Between animals, instruments will be rinsed with sterile saline and heat sterilized (glass bead sterilizer). Two sets of instruments will be used
alternately in the event that surgery is being performed on >4 animals per day.

Persons performing the surgery will scrub their hands with bactericidal scrub and wear a clean lab coat, sterile surgical gloves, and a surgical mask. The surgical table will be disinfected and covered with sterile drapes.

The surgical site(s) (abdominal fossa located caudal to the margin of the ribcage and ventral to the transverse spinous processes of the lumbar vertebra) will be shaved in case of SCID mice. The surgical sites will be cleaned with a Betadine swab stick from the center of the incision to the periphery. This process will be repeated with 70% alcohol prep pads. Scrubbing will be alternated between the Betadine and alcohol and repeated at least three times, ending with the Betadine. The surgical sites will be draped with sterile drapes.

Once the surgical sites have been prepared, disinfected and covered with a sterile drape, small flank incision just inferior to the scapula will be made using an 11” blade scalpel. The subcutaneous tissues will be bluntly dissected using a hemostat. The PDX/matrigel mixture prepared under sterile conditions in the lab with by introduced into the subcutaneous pocket using sterile large opening micropipette tips to deposit the PDX. Once the PDX has been deposited the incision will be closed with a subcuticular 5-0 Vicryl (Ethicon) suture, or wound clips. The wound will be cleaned with alcohol wipe. Drapes will be removed and the animal will be allowed to recover from anesthesia.

Reference article for the caecal injection of CRC cells: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2557075/

**Post-surgery/anesthetic recovery monitoring plan:** Postoperatively, animals will be placed on warm heating pads and monitored for signs of recovery, including signs of pain or discomfort (lethargy, hunched position). The animals’ progress will be monitored daily by members of the Mark Evers lab. Post-operative animals, will be given buprenorphine (opioid) analgesia 0.1mg/kg by IP every 12 hours for 48 hours.

As part of our standard post-operative care, mice will be monitored for
normal grooming behavior, clean coat, clear eyes and normal locomotion and inquisitiveness. The incisions will be checked for swelling and redness. If the animals do not appear normal or if problems occur with the incisions, a DLAR veterinarian will be asked to examine the animal and recommend treatment (or euthanasia if needed).

**Wound Closure**: Surgical wounds will be closed with 5.0 Vicryl (Ethicon) absorbable sutures, or wound clips.

**Will post-operative analgesics be administered?** Yes

**Indicate Post-Operative Medications and Describe Postop Care:**

1. Post-operative animals, will be given buprenorphine (opioid) analgesia 0.1mg/kg by IP every 12 hours for 48 hours.

2. A pink DLAR Surgery Card with the date the procedure was performed will be used to mark cages after surgery. Animals that have had surgery will be observed daily (5-7 days) by the PI, or a member of the research team, and the results of observations recorded (e.g. back of pink card). The animals will be observed with regard to normal activity, inquisitiveness, condition of hair coat, eating, drinking, defecation, urination, appearance of eyes, breathing rate, gait, and body condition. The animals will also be evaluated for vocalization, dehydration, and appearance of the surgical wound (e.g. red edges, swelling, or exudates), and suture/wound clip displacement. The cards will be removed after suture/staple removal.

3. Mice will be observed twice weekly for tumor growth and size. Endpoints requiring sacrifice include decreased range of motion, poor oral intake, tumors > 2 cm, lethargy, or signs of animal discomfort. Tumors greater than 10% animal body weight will require humane euthanasia.

4. If abnormal conditions are observed, the PI or a co-investigator will consult with a DLAR veterinarian.

**When will sutures/staples be removed?** Absorbable sutures. Not applicable. 10-14 days for wound.
APPENDIX C: Center for Clinical and Translational Sciences Seed Grant
The metabolomic effects of Metformin on colon cancer in human colon tissues

Applicant:
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Immediate Supervisor:
B. Mark Evers, MD

Primary Mentor:
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Core Support, Markey Cancer Center
University of Kentucky, Department of Molecular and Cellular Biochemistry
(859) 323-3454
Tianyan.gao@uky.edu

Amount requested:
$5,000
Budget and Budget Justification

<table>
<thead>
<tr>
<th>Metformin Metabolomic Study Budget</th>
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<tbody>
<tr>
<td>Incubation and Laboratory Supplies</td>
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<tr>
<td>----------------------------------</td>
</tr>
<tr>
<td>Laboratory computer for research nurse</td>
</tr>
<tr>
<td>$^{13}$C labeled media and supplies</td>
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<tr>
<td>Tissue analysis fees</td>
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<tr>
<td>NMR/MS analysis</td>
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<tr>
<td>Computational fees</td>
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<tr>
<td>Medication costs</td>
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<td>Metformin Hydrochloride</td>
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**Overview:**

Funding for this project is requested for 1) equipment to assist in the record keeping for the clinical research nurse and supplies for tissue preparation, 2) partial cost of nuclear magnetic resonance imaging (NMR) and mass spectrometry (MS) for tissue analysis and 3) study drug purchase. This project will continue enrollment until a maximum of 33 subjects complete one experiment.

**Incubation and Laboratory Supplies:**

We are requesting a laptop PC ($700) for the clinical research nurse to keep all data with patient health information in a secure, password protected location. We anticipate one year lab costs for basic supplies including $^{13}$C labeled media and tissue preparation supplies to cost ($500). This will result in $1,200 in cost.

These supplies will be necessary to process tissues collected from the subjects in our study. The PC will be a one-time purchase and the cost of the supplies from our current providers has remained static for the past three years.
**Tissue analysis fees:**

NMR and MS sample analysis costs forty dollars per hour of sample analysis. The pair based approach of tissue incubation will result in sixty six total samples from thirty three patients will result in a cost of $2,640 dollars. Computational data analysis will cost an additional $600, resulting in a total cost of $3,240.

**Medication costs:** The Markey Cancer Center laboratory will purchase all study medications from a United States retailer, and study medication will be of pharmaceutical grade and quality as defined by the FDA. Cost of Metformin for tissue incubation is $76 for 100 gram. We anticipate medication costs for patient sample incubation to be approximately $560 over the course of the study for thirty three patients.
Abstract:

Colon cancer is the third leading cause of cancer mortality worldwide and is associated with obesity and Type II diabetes (110) (111). Metformin is an FDA approved oral antidiabetic drug which has also been shown to have anti-cancer effects (11, 18). The primary anti-tumor molecular target of Metformin is AMP-activated protein kinase (AMPK), a serine/threonine protein kinase (113). AMPK plays a crucial role in monitoring systemic and cellular energy status and crucial cellular functions under energy-restricted conditions such as metabolic stress, i.e. tumor development. Specific downstream mechanisms proposed to be affected by Metformin’s action on AMPK in colon cancers are i) protein metabolism by the mammalian target of rapamycin (mTOR) pathway and ii) acetyl co-enzyme A (ACC) pathway in lipid metabolism (45, 114). Previous studies have shown the anti-tumor effects of Metformin in breast, prostate, gastric, and pancreatic cancers (83, 124-126). However, a trial studying the specific metabolic effects of Metformin in colon cancer has yet to be performed. We propose a novel, innovative approach studying the metabolic effects of Metformin in colon cancer tissues using a prospective patient matched tissue pilot study. Our proposed project is fundamentally different from previous studies using Metformin since we will use human tissues as opposed to in vitro cell lines or animal tissues to examine this drug’s anti-oncogenic effects. Furthermore, we will determine the specific metabolic effects of the drug which contribute to its ability to target colon cancer cells. This innovative study will provide insight into further understanding of colon cancer metabolism and novel strategies for targeted therapies.

Study Personnel:

Jennifer W. Harris, M.D., Principal Investigator, is currently a post-doctoral fellow recipient of a T32 training grant and general surgery resident at the University of Kentucky. Dr. Harris has considerable experience in human tissue collection and tissue processing and analysis, as well as treatment of clinical diseases such as metabolic syndrome and colon and rectal cancers. The
proposed project is an extension of metabolic studies performed at the University of Kentucky Markey Cancer Center exploring tumor microenvironment and signaling in human colon cancers. Dr. Harris’ responsibilities will be to coordinate clinical study and perform metabolic studies in collaboration with Dr. Gao and Dr. Fan.

**Mark Evers, M.D., Consultant**, is currently the director of the Markey Cancer Center and surgeon at the University of Kentucky. Dr. Evers has an extensive background in conducting clinical trials and is an expert in cancers of the gastrointestinal tract. He will serve as a mentor and provide guidance to assist in coordinating the progression of the study and collaboration of the group as a whole.

**Tianyan Gao, PhD, Co-Investigator**, is currently an Associate Professor in the Department of Cellular and Molecular Biology at the University of Kentucky. Dr. Gao has an extensive background in conducting protein and ribonucleic acid analysis which will be necessary for the analysis of patient samples and cellular data collection. Dr. Gao will assist with specimen analysis and data interpretation.

**Teresa Fan, PhD, Co-Investigator**, has a longstanding history in quality human tissue collection and analysis, and is considering an expert in the field of isotope labeling of human tissues for metabolic study using nuclear magnetic resonance imaging and mass spectrometry. Dr. Fan will share her expertise in our proposed study by assisting us with the use of this state of the art technology and interpretation of data and results.

**Clinical Research Nurse, R.N, B.S.N.** required, will be responsible for ethical registering and consenting patients for the study, obtaining and labeling study medications for the patients, being on call for patient questions, blinding the treatment arms to the care providers and patients, and collecting clinical data on study patients. We plan to interview several clinical research nurses all of whom have a strong background in human trials at the University of Kentucky for this position which will offer a stipend to their additional pay.
**Heidi Weiss, PhD, Consultant**, is a Professor of Biostatistics at the University of Kentucky. Dr. Weiss will conduct the data analysis with her statistical expertise both during and at the conclusion of our proposed study.

**Resources and Environment:**

Utilizing a multidisciplinary approach with significant surgical and clinical experience from the Colorectal Surgery Division of General Surgery at University of Kentucky (Beck, Hourigan, Patel), metabolism and obesity (Fan, Lane, Higashi), colon cancer treatment/study design (Gao, Evers), and biostatistics/computational biology (Weiss/Moseley) we plan to develop a patient matched tissue human pilot study using Metformin as a metabolic chemotherapeutic agent against colon cancer. Comparing patient matched samples incubated with and without Metformin we will determine the short-term effects of Metformin treatment by comparing protein expression of effectors involved in protein synthesis, tumor cell migration, and tumor metabolism. We will also use isotope labeling to determine protein and fatty acid metabolism differences between the pre-treatment and post-treatment samples.

The Markey Cancer Center at the University of Kentucky Medical Center is a NCI (National Cancer Institute) designated center that provides cutting edge surgical and medical cancer care to Kentucky and surrounding states. The endoscopy and surgical services provided at this center have the expertise and surgical volume to support this trial. A clinical research nurse will be designated to enroll patients and organize and record of data to ensure the success of this trial. We have worked closely with the Markey Cancer Center Biospecimen Core to collect gastrointestinal tissues from surgical patients. Over the past six months we have enrolled over seventy five percent of patients from whom we requested consent for tissue collection. We do plan to financially compensate participants as we are requesting that patients in this study reliably take a study drug for two weeks. Plasma samples to confirm Metformin use will be drawn in the preoperative area to ensure usage of drug and study cooperation.

Our laboratory is located in the Biomedical/Biological Sciences Research
Building and has all of the necessary equipment and supplies we need for tissue storage, culture, and preparation. We have spent the last year refining our tissue collection and preparation techniques with assistance from the Markey Cancer Center Biospecimen Core and lab personnel from the Fan lab to ensure the appropriate protocol is followed and those samples are ready for metabolomics analysis. We will work with the Fan lab using state of the art metabolomics analysis to perform 1D nuclear magnetic resonance (NMR) and gas chromatography mass spectrometry (GC-MS) to assess the difference between patient matched samples collected from our study population.

Finally, we plan to work closely with our biostatistician colleagues Heidi Weiss, PhD and Hunter Moseley, PhD to analyze and interpret the data we collect for our outlined experiments. Dr. Weiss has an extensive background in development and data analysis of clinical trials, and Dr. Moseley is an expert in the analysis and interpretation of metabolomics data.

**Specific Aims:** Metformin is a safe, low cost, FDA approved drug for the treatment of type II diabetes mellitus, but has also been shown to have potential anti-cancer effects (11, 18). Epidemiologic data in human colon cancers suggests patients taking Metformin for the treatment of diabetes have better outcomes than their non-diabetic counterparts, which seems counterintuitive as these patients often have other comorbidities such as obesity which contribute to less optimal outcomes (67, 121). Both in vitro and in vivo animal data in colon cancer also suggest Metformin does have an anti-tumor effect, but the direct metabolic implications of the drug have yet to be determined (122, 123). Thus far the only human clinical trials have been performed on patients with benign colon diseases and not colon cancer (154). We are proposing a novel human tissue pilot study to determine the underlying metabolic effects of Metformin in colon cancer.

Our **central hypothesis** is that the predominant role of Metformin in colon cancer cells is to activate AMPK which has downstream impacts on decreasing protein metabolism through its effect on Akt/mTOR pathway, and decreasing
fatty acid metabolism through decreasing ACC and its targeted impact on fatty acid metabolism. Moreover, we speculate that Metformin’s impact on these molecular growth factors will inhibit colon cancer cell growth and proliferation, and may even offer a therapeutic option for patients whom cannot tolerate aggressive traditional chemotherapy agents. To examine our hypothesis and achieve our long term goal of better defining the metabolic impact of Metformin in human colon cancers, we have planned experiments with the following.

**Aim 1: To determine the role of Metformin on protein metabolism and tumor cell proliferation in colon cancer tissues.**

**Hypothesis:** Patient colon cancer tissues treated with Metformin will have decreased expression of markers indicative of protein metabolism and tumor cell proliferation compared to samples treated with the placebo condition.

**Approach:** Surgically resected patient colon cancer tissues will be treated ex vivo with Metformin. We will use the well-established techniques of Western blotting and immunohistochemistry staining to evaluate protein metabolism markers AMPK, Akt, mTOR, and phosphor-S6 ribosomal protein and cell proliferation marker Ki-67 and markers of fatty acid metabolism such as fatty acid synthase (FASN).

**Impact:** This study will establish that tumors in patient tissue samples treated with Metformin have less growth potential due to a decrease in protein metabolism and ability to grow and proliferate secondary to the targeted molecular effects of Metformin.

**Aim 2: To delineate the effect of Metformin on cellular metabolism in human colon cancer tissues.**

**Hypothesis:** Patient tissues treated with Metformin will have altered cellular metabolism and particularly decreased fatty acid synthesis in evaluated colon cancer tissues compared to patients treated with the placebo condition.

**Approach:** We will utilize state of the art metabolomic techniques $^{13}$C-labeled NMR and GC-MS to study specific metabolic pathways in patient derived
colon cancer tissues.

**Impact:** This patient matched human tissue study will establish that tumors in patients treated with Metformin are indeed less metabolically active compared to tissues of patient tissues incubated without Metformin.

To achieve our aims, we have assembled a multidisciplinary team with significant expertise in colon cancer physiology and function (Harris, Evers, Gao), metabolism and obesity (Evers, Fan), and biostatistics/computational biology (Weiss, Moseley). Our highly collaborative team has the requisite expertise, novel model systems and state-of-the art technology to make rapid progress that will significantly advance the fields of colon cancer physiology, endocrinology, and metabolism. Ultimately, our findings will provide insight into the specific mechanisms by which Metformin acts on human colon cancer tissues could provide other insights into other novel avenues of research for drug development in the treatment and control of this devastating disease.

**Research Strategy**

**Significance:** Colon cancer is the third leading cause of cancer mortality worldwide has become increasingly prevalent in Western populations (111). This devastating disease was the second leading cause of cancer mortality in the United States in 2008, and is projected to increase in prevalence given its association a sedentary lifestyle and a high/low fiber diet (155-157). Obesity and its associated comorbid conditions have become an increasingly difficult challenge for healthcare providers worldwide, and in the U.S. adult obesity rates rose from 14% in 1978 to over 30% in 2000 with over 50% of Americans projected to be obese in 2030 (158). Obesity and its associated comorbid conditions, namely Type II diabetes mellitus, are suggested as culprits for increasing the risk of developing colon cancer, as well as increasing both the morbidity and mortality of patients who fall victim to this disease (41, 159, 160).

Metformin is an oral antidiabetic drug in the biguanide class and is FDA approved as the first line drug of choice for the treatment of DMII, but has also
be shown to have an anti-tumor effect on colon cancer cells in in vitro and animal studies (123, 161, 162).

Metformin acts through both insulin dependent and insulin independent pathways and this makes it an attractive option for targeted molecular therapy as it has the potential to inhibit multiple pathways involved in cancer cell metabolism (40). The consensus of the published literature agrees that the main effect of Metformin on tumorigenic cells occurs at the convergence of multiple metabolic pathways, namely at the molecular activator AMPK which is of paramount importance as it plays a significant role in how cells respond under metabolic stress conditions (36, 70, 163, 164). AMPK acts on protein metabolism through the Akt/mTOR pathway and on fatty acid metabolism through mechanisms involving ACC (47, 135). Little is known regarding the effect of Metformin on the nuclear and mitochondrial changes which occur downstream from these molecular pathways.

Our proposal is significant as we intend to discover these downstream mechanisms which profoundly affect tumor growth and cancer cell proliferation.

**Innovation:** Our innovative approach to determine the metabolic impact of Metformin will involve the analysis of patient tissues using state-of-the-art approaches. This system combines the use of stable isotope tracers with mass spectrometry (MS) and nuclear magnetic resonance imaging (NMR) analyses, to enable robust and global mapping of Metformin’s impact on fatty acid metabolism, lipid absorption, metabolism, and storage. All of these factors contribute to a cancer cell’s ability to acquire nutrition and survive under metabolic stress conditions.

Another novel and innovative detail to our proposal is the design of our study. Patients will undergo surgical resection of all or part of their colon. Multiple tissue samples of the tumor will be collected and a portion of the sample will be incubated in growth media with Metformin while another portion of the sample will be incubated in growth media without Metformin. We will be able to create a human tissue pilot study using Metformin in which we compare the
metabolic effects of the drug on patient matched samples. This approach offers an innovative qualitative as well as quantitative analysis of the differences in both patient matched samples as well as samples from patient tissues receiving the two different study conditions.

**Approach:** Our work on the in vitro effects of Metformin on colon cancer cell lines was initially funded by the T32 funding mechanism (Trainee Grant 5T32CA160003-03). We found that there is a considerable difference in the cellular growth and metabolism of colon cancer cell lines HT29, HCT116, SW480, and KM20, all of which are colon cancer cell lines derived from human colon cancers. We also successfully submitted a human gastrointestinal tissue collection protocol to the University of Kentucky IRB which aims to collect colon cancer tissues from human patients (IRB# 13-0753-P2H). We were strongly advised to seek additional funding to explore performing protein analysis and metabolomic studies on tissues acquired from patients who have colon cancer.

**Human tissue pilot study design:** Patients seeking treatment at the Markey Cancer Center who have been recently diagnosed with Stage I- Stage IV colon cancer will discuss study involvement with the clinical research nurse designated to the study. Pre-clinical data will be reviewed to determine if the patient has any of the excluding criteria that would make them ineligible for the study. Exclusion criteria for the study include the following:

- Ages < 21 or > 90 years old
- Stage 0 (carcinoma in situ)
- Metformin treatment within the past 6 months for any other condition
- BMI < 18 or > 40
- Previous malignancies treated with chemotherapy or radiation
- Surgical intervention at another institution for treatment of colorectal cancer prior to being treated at University of Kentucky
- Current pregnancy or breast feeding. If patient becomes pregnant during
their time in the study they will be removed from the study.

The patient will be enrolled in the study if they agree to informed consent to provide tissues for analysis. After surgical resection, the patient samples will be retrieved immediately and either cultured under different conditions ex vivo in medium or cryopreserved directly in liquid nitrogen. Our lab as well as our collaborators lab (Fan), have an extensive background in tissue collection, cryopreservation, processing, and storage (92, 94, 97). According to our power analysis we need to have a total of thirty three patients in order to adequately be able to ascertain statistical significance, if any, between the patient matched samples. The specific analysis of these tissues and the approaches in which we will successfully complete our proposed aims are discussed in further detail below.

**Specific Aim 1: To determine the role of Metformin on protein metabolism and tumor cell proliferation in colon cancer tissues.**

Metformin acts through insulin dependent mechanisms mainly in the liver, and this contributes to increased systemic glucose by means of gluconeogenesis in the liver which releases glucose into the blood stream (Figure A.1). This effect increases the amount of circulating insulin and subsequent binding of insulin the insulin receptors on cells in the body. This cellular stimulation results in increased cellular catabolism and downstream protein synthesis and cell growth. However, the insulin independent mechanism of Metformin acts intracellularly and increases the effects of AMPK. Intracellularly AMPK decreases the cells ability to participate in protein synthesis in two ways: i) it increases the activity of TSC2 which is a tumor suppressor of the mTOR complex, and also directly decreases the activity of the mTOR complex. Increased AMPK activity also decreases the activity of ACC which is the first gateway of the cell to participate in fatty acid metabolism.

Metformin acts on this pathway in an insulin dependent manner and results in decreased fatty acid production and subsequent storage. Our intended experiments after collection of surgical specimen human tissues will use
Figure A.1. Metformin is an oral antihypoglycemic drug that plays an important role in regulating cellular metabolism via AMPK and mTOR.
biochemical and immunological techniques to measure protein expression of protein synthesis and fatty acid metabolism. Freshly collected tissues will be incubated in Metformin containing or control medium and cellular lysis will be performed to isolate protein samples for Western blotting. After cell lysate and protein isolation, Western blotting will be performed and we will probe for markers for cell energy utilization such as AMPK, protein synthesis including mTOR and phospho-S6, as well as markers of fatty acid metabolism such as ACC and fatty acid synthase (FASN) (44, 165). Alternatively, the treated tissues can be fixed and prepared as paraffin-embedded tissue blocks. To measure cell proliferation we will use the standard technique of immunohistochemistry (IHC) tissue staining which is routinely performed in our lab (Harris, Gao). We will only evaluate the surgical specimens with IHC as the biopsy samples will not provide enough tissue to perform the paraffin preparation step required for IHC. We will probe for a well-known marker of cell proliferation Ki-67, as well as FASN (43).

Statistical considerations for the endpoints of protein metabolism will include comparisons of pre- and post-treatment tissues, and also comparison of tissues from patients in the different treatment conditions using both descriptive and graphical presentations. Similar comparisons comparing qualitative data for IHC will include a comparison of patient tissues after different treatment conditions to assess for changes in markers indicating decreased cell proliferation. We will quantify the number of cells stained with the respective markers and perform a two tailed t-test comparing the expression of cell proliferation markers in the two groups (Weiss).

**Anticipated results, potential problems, and alternative solutions:**

There will be a decrease in protein synthesis and fatty acid metabolism markers in the Metformin treated tissues compared to those not treated with Metformin.

Potential problems could include loss of sample due to inappropriate tissue preparation or errors in performing Western blotting. To prevent either of these problems we will only allow experience technicians to process samples
and perform Western blotting. We anticipate IHC staining results will show that post-treatment samples will have a decreased expression of Ki67 and FASN. One potential limitation is that the surgically resected tissues may undergo apoptosis during the ex vivo treatment period. We will monitor the level of apoptosis by analyzing the expression of apoptosis markers such as cleaved caspase 3 to make sure that tissues are mostly viable after the control treatment. We are currently conducting pilot experiments using mouse colon tissues to determine the optimal incubation time needed. Our experiments with human tissues will be adjusted accordingly.

**Specific Aim 2: To delineate the effect of Metformin on cellular metabolism in human colon cancer tissues.**

It is well established in the colon cancer literature that an increase in fatty acid metabolism and fatty acid production promotes the growth of colon cancer cells by increasing the activity of ACC and its downstream effectors (166-168).

Previous authors have also shown Metformin impacts fatty acid metabolism in vitro and in animal models, but the literature lacks evidence of these effects actually being present in humans and there have only been analyses to evaluate protein and nuclear markers (23, 50, 169). We are also interested in detecting any global alteration in cellular metabolism (e.g. Increased TCA cycle activity) upon Metformin treatment. Our intended experiments after collection of the surgical specimen human tissues will use similar techniques to measure protein expression of protein synthesis and fatty acid metabolism. To label freshly collected tissues, mucosal slices will be incubated with $^{13}$C-glucose containing medium for 24 hours and processed as described previously. SIRM analysis will be performed by a combination of NMR, Gas chromatography-mass spectrometry (GC-MS), and Fourier transform mass spectrometry (FT-MS) (92). Our co-investigator has over twenty years of experience with this technique and equipment (Fan). The extent of $^{13}$C-labeling lipid precursors in both treatment groups of patient matched samples, including glycerol-3-phosphate and citrate, will be obtained from GS-MS analysis while intact triglycerides, phospholipids,
and fatty acids will be acquired from FT-MS analysis (Figure A.2). Based on these $^{13}$C-labeled enrichment patterns of lipids in Metformin treated and non-treated human patient matched colon cancer samples and their precursors, the dynamics of colon cancer cells and their de novo fatty acid and lipid synthesis pathways in the two treatment groups will be compared, thus delineating if Metformin treatment does indeed affect de novo fatty acid and lipid synthesis. Labeling the tissues and using this advanced technology will tell us exactly when and where in the Krebs cycle these metabolic changes are taking place.

Statistical analysis will include using two sample t-tests with Hochberg's p-value adjustment due to the simultaneous testing of multiple metabolites. Software simulation will be performed 1000 times to determine the sample size that will provide adequate statistical power to detect the hypothesized difference between treatment groups with control of family-wise error rate. Statistical analysis for the processed endpoints from the fatty acid synthesis and fatty acid oxidation studies will involve pairwise comparisons for each treatment condition, each pair matched sample, and time points of software analysis. Linear and mixed models to evaluate metabolic kinetics will be measured. Multivariate statistical analyses will be used for the discovery of non-targeted metabolic changes.

**Anticipated results, potential problems, and alternative solutions:**

We anticipate that there will be a decrease in fatty acid synthesis in Metformin treated patient tissues compared to their matched non-treated specimens. In addition, we expect to detect various metabolic alterations associated with metformin treatment. We do not anticipate problems with tissue collection or processing, nor do we anticipate problems with profiling the lipid and polar metabolites including their $^{13}$C-labeling patterns using in-house tools established in the metabolomics center at the University of Kentucky through collaboration with Dr. Fan. Dr. Weiss, our biostatistics collaborator will perform multivariate statistical analysis for discerning non-targeted metabolic changes.
Figure A.2. NMR and GC-MS analyses.

(A) NMR analysis. Expected $^{13}$C-positional isotopomer patterns of glycolytic and Krebs cycle metabolites synthesized from [U-$^{13}$C]-Glc by mammalian cells (Fan et al.); (B) GC-MS analysis. Each 1H peak arose from protons directly attached to $^{13}$C and the peak assignment denotes the $^{13}$C-carbon. Thus, the peak intensity reflects $^{13}$C abundance of the attached carbon (Fan et al.).
from the very large metabolomics datasets for any unexpected but meaningful metabolic signature associated with AMPK or ACC manipulation. This is the value of the metabolomics approach for discovery of new functional links for signaling pathways.

**Training Plan:**

1. Training expectations associated with research

   The funds we are requesting through the CCTS Seed grant will be applied to this study by providing materials for data collection and analysis, as well as tissue sample analysis and study medication. These funds will specifically cover the expenses of the most translational aspects of this novel and innovative study. As post-doctoral fellow/surgical resident who is interested in oncology this project literally takes a novel idea from the bench to the bedside, involves patient intervention in the clinical, pre-operative, and surgical setting, and brings the tissues they generously provide for bedside to bench research. This approach embodies the heart of clinical and translational science and the use of NMR and GC-MS metabolomics analysis is an elegant method of elucidating information that will significant advance this topic in the surgical and oncology literature.

2. Plan for meeting these expectations

   We will obtain both IRB approval as well as approval from the Markey Cancer Center Clinical Trial Board to conduct this project. We anticipate starting patient enrollment and tissue collection in October 2014 and concluding enrollment and tissue collection in April 2015. Funds provided by the CCTS Seed grant will be applied to the project as outlined in the proposal by funding supplies for tissue collection and preparation, a computer for the clinical research nurse, and human patient sample analysis. We plan to complete tissue and data analysis in May 2015 and begin work on our findings and a manuscript in June 2015.

3. Objective criteria for meeting these criteria

   The data and safety monitoring board will meet monthly and discuss data
collection, monitoring, and any adverse events. At these bi-monthly meetings the board will confirm the following criteria:

- patient enrollment is meeting its proposed trajectory and is documented correctly
- patient data is satisfactorily being collected and protected by the clinical research nurse
- tissues are being collected in a timely manner and being appropriately prepared for analysis
- data and data analysis is shared with the board as it is collected at each meeting

4. Schedule of meetings with applicant and primary mentor

The applicant already has scheduled weekly meetings with the primary mentor, Dr. Gao and scheduled bi-weekly meetings with the immediate supervisor, Dr. Evers. These meetings can be recorded and all parties can sign a document that can be copied and recorded for the records of the data and safety monitoring board, as well as the CCTS.
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VITA

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EDUCATION:

Residency: University of Kentucky
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July 2010- present

Fellowship: Markey Cancer Center
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Graduate School: University of Kentucky
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July 2013- present

Medical School: M.D., Quillen College of Medicine
at East Tennessee State University
Johnson City, Tennessee
July 2006- May 2010

Undergraduate: B.S. East Tennessee State University
Johnson City, Tennessee
Major: Biology, Minor: Philosophy
August 2002- May 2006

HONORS/AWARDS:

Residency:

2010-2011 Clinical Teaching Award- given to one surgery resident each year by the graduating medical student class for excellence in teaching and clinical instruction
2011-2012  Medical Student Teaching Award- given by the third year medical students for excellence in medical student education

2012-2013  Medical Student Teaching Award- given by the third year medical students for excellence in medical student education

**Medical School:**

2010  Recipient of Bieber Scholarship- given to a graduating student that has a keen interest in research and has faced particular hardship during medical school

2009  2nd place poster presentation, Medical Student division at Appalachian Student Research Forum, Johnson City, Tennessee

**Undergraduate:**

2006  Magna Cum Laude
Biology Honors in Discipline graduate
ETSU Track and Field Academic MVP
ETSU Track and Field Letterman

2005  TIAA-CREF Academic All- Southern Conference Team
ETSU Track and Field Academic MVP
Institute of Quantitative Biology research program at ETSU
Howard Hughes Medical Institute Grant recipient

2004  TIAA-CREF Academic All- Southern Conference Team
Institute of Quantitative Biology research program at ETSU
NIH Institute for Quantitative Biology Grant recipient

2003  ETSU Track and Field Scholarship (2003-2006)
Biology Honors Scholarship (2003-2006)

2002  Ralph Stuart Stowers Scholarship- awarded to Valedictorian of Richlands High School each year and covers one year of all expenses to four year university of student’s choice
CERTIFICATIONS AND LISCENSURES:

- State of Kentucky Medical Licensure, 46460 - current expires 2/28/2016
- Advanced Trauma Life Support Instructor - 2011 - current
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- Advanced Cardiac Life Support provider - 2004-current
- Basic Life Support provider - 2002- current
- Swiftwater Rescue Technician – 2004 - 2010

PUBLISHED MANUSCRIPTS:


2. Joseph D. Valentino, MD, Jing Li, PhD, Kate Zaytseva, PhD, W. Conan Mustain, MD, Victoria A. Elliott, BS, Ji Tae Kim, PhD, **Jennifer W. Harris**, MD, Katherine Campbell, MD, Heidi Weiss, PhD, Chi Wang, PhD, Jun Song, MD, Lowell Anthony, MD, Courtney M. Townsend Jr., MD, B. Mark Evers, MD. Co-Targeting the PI3K and RAS Pathways for the Treatment of Neuroendocrine Tumors. Clinical Cancer Res. 2014 Mar 1;20 (5):1212-22.


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PUBLISHED BOOK CHAPTERS:

