Predictors of Cancer Cell Response to Metformin

by

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A thesis submitted in conformity with the requirements for the degree of Master’s of Science
Institute of Medical Science
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Abstract

There is great interest in transitioning the antidiabetic drug metformin for use in cancer, with evidence suggesting it can improve outcome following radiotherapy (RT). Its benefit may arise from inhibiting cancer cell proliferation, abrogating tumour hypoxia through respiratory inhibition, or directly radiosensitizing cancer cells. With metformin entering clinical trials, it is crucial to identify relevant biomarkers for patient benefit. AMPK and p53 were previously identified to facilitate metformin’s radiosensitization, while OCT and MATE transporters mediate its intracellular concentration: we thus investigated these as contributors to metformin’s radiosensitizing, anti-proliferative, or respiratory-inhibitory effects. We found that loss of AMPK or p53 exacerbated sensitivity to metformin’s inhibition of proliferation, while OCT expression was sufficient to confer response to metformin’s respiratory inhibition. Together, loss of AMPK and p53 signaling, and OCT expression, represent putative biomarkers of response to inhibition of proliferation and respiration, respectively, and may be further explored in a clinical setting.
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List of Abbreviations

4EBP1: 4E-binding protein 1

ABC: ATP-binding cassette

ACC: acetyl-CoA carboxylase

ADA: American Diabetes Association

ADP: adenosine diphosphate

AICAR: 5-aminoimidazole-4-carboxamide riboside

AMP: adenosine monophosphate

AMPK: 5’-AMP-activated protein kinase

ASO: antisense oligonucleotide

ATCC: American Type Tissue Collection

ATM: ataxia telangiectasia mutated

ATP: adenosine triphosphate

AUC: area-under-the-curve

CAMKKβ: Ca^{2+}/calmodulin-dependent kinase kinase β

cAMP: cyclic AMP

CCLE: Cancer Cell Line Encyclopedia

CDK: cyclin dependent kinase

CHK: checkpoint kinase

C_{max}: maximal plasma concentration
CREB: cAMP response element binding

CRT: chemotherapy and radiation

CRT-C2: CREB-regulated transcription co-activator 2

DDR: DNA damage response

DFS: disease-free survival

DHAP: dihydroxyacetone phosphate

DNA: deoxyribonucleic acid

EASD: European Association of the Study of Diabetes

EF5: 2-(2-nitro-1H-imidazole-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide

ER: estrogen receptor

ETC: electron transport chain

F2,6BP: fructose-2,6-bisphosphate

FBP1: fructose-1,6,-bisphosphatase

FBS: fetal bovine serum

G-3-P: glycerol-3-phosphate

G6Pase: glucose-6-phosphatase

GDSC: Genomics of Drug Sensitivity in Cancer

GFR: glomerular filtration rate

GLUT: glucose transporter

GOF: gain of function

GPAT: glycerol phosphate acyl-transferase

GTEx: Genotype-Tissue Expression project
GTP: guanosine triphosphate

Gy: Gray

HbA1c: hemoglobin A1c

HBO: hyperbaric oxygen therapy

HCC: hepatocellular carcinoma

HER2: human epidermal growth factor receptor

HIF-1α: hypoxia-inducible factor 1α

HMG-CoA reductase: 3-hydroxy-3-methylglutaryl CoA reductase

HNC: head and neck cancer

HNF-4α: hepatocyte nuclear factor 4α

HNSCC: head and neck squamous cell carcinoma

HPV: human papilloma virus

IC_{50}: half maximal inhibitory concentration

IGF: insulin-like growth factor

IGFR: insulin-like growth factor receptor

IR: ionizing radiation

IRS: insulin receptor substrate

KO: knockout

LDH: lactate dehydrogenase

LKB1: liver kinase B1

LRR: locoregional recurrence
MATE: multidrug and toxin extrusion

MDM2: mouse double minute 2 homolog

MEF: mouse embryonic fibroblast

MFS: major facilitator superfamily

mGPD: mitochondrial glycerophosphate dehydrogenase

mmHg: millimetre of mercury

MPP+: 1-methyl-4-phenylpyridine

mTOR: mammalian target of rapamycin

NADH: nicotinamide adenine dinucleotide

NIH: National Institute of Health

OAT: organic anion transporter

OCR: oxygen consumption rate

OCT: organic cation transporter

OCTN: Na$^+$-zwitterion/cation transporter

OS: overall survival

p70S6K: p70-S6 kinase

pCR: pathological complete response

PEPCK: phosphoenolpyruvate carboxykinase

PFK: phosphofructokinase

PFK/FBPass: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

PGC-1α: PPAR-γ-co-activator-1α
P_i: inorganic phosphate

PI3K: phosphoinositide-3-kinase

PIP_2: phosphatidylinositol(4,5)-bisphosphate

PIP_3: phosphatidylinositol(3,4,5)-trisphosphate

PK: pharmacokinetics

PKA: protein kinase A

Q: ubiquinone

QH_2: ubiquinol

qPCR: quantitative polymerase chain reaction

Rag: ras-related GTPase

RAPTOR: regulatory associated protein of mTOR

REDD1: regulated in development and DNA damage responses 1

Rheb: rho enriched in brain

RNA: ribonucleic acid

RNAi: RNA interference

RPKM: reads per kilobase transcript per million reads

RT: radiotherapy

shRNA: short hairpin RNA

SHP: small heteromeric partner

siRNA: small interfering RNA

SLC: solute carrier

SMR: small multidrug resistance
SNP: single nucleotide polymorphism

T2D: type 2 diabetes

TEA⁺: tetraethylammonium

TFIA: transcription factor 1A

TMD: transmembrane domain

TSC2: tuberous sclerosis 2

URAT: urate transporter

Vd/F: volume of distribution

VEGF: vascular and endothelial growth factor

WT: wild type
1 Literature Review

There is great interest in repurposing the antidiabetic drug metformin for use in cancer. A multitude of observational and retrospective studies, combined with laboratory and preclinical data, have provided rationale for investigation of its use as a cancer therapeutic. In this chapter, the scientific literature regarding metformin will be reviewed, with emphasis on areas that may contribute to its reported benefit in cancer. Proposed mechanisms of its action in diabetes and cancer, in addition to factors thought to contribute to interpatient variability in metformin response, will be discussed. Consideration of the latter will be important in order to identify candidate biomarkers, which is crucial for effective clinical implementation of metformin as a cancer preventative or therapeutic.

1.1 Metformin as first-line therapy for type 2 diabetes

Metformin (1,1-dimethylbiguanide) is an orally administered drug most widely prescribed to counter hyperglycemia in patients with type 2 diabetes (T2D)\(^1\). It was first introduced in the 1950s and currently is recommended as first-line oral therapy in the treatment of diabetes by the American Diabetes Association (ADA) and the European Association of the Study of Diabetes (EASD)\(^2\). Currently, metformin is often the first drug used in treating newly diagnosed T2D patients, though it may also be used in combination with sulfonylureas.

1.1.1 Discovery & use in diabetes

The benefits of metformin were harnessed as early as in medieval Europe, when *Galega officinalis* (French lilac or goat’s rue) was used as an herbal remedy for polyuria. The use of this plant in treating diabetic symptoms owes to its rich content of guanidine, which has hypoglycemic activity. Though guanidine itself proved too toxic for clinical use, its discovery prompted the development of the biguanides metformin and phenformin in the 1920s, which were then introduced clinically in Europe in the 1950s. Phenformin was withdrawn from the market in most countries in the 1970s due to its high risk of causing lactic acidosis; however as a well tolerated drug with few adverse side effects, metformin went on to attain approval for use in the United States in 1995\(^3,4\).
1.1.2 Pharmacokinetics & distribution

Metformin is administered orally in preparations ranging from 500mg-2500mg, which results in plasma concentrations of 1.0-3.0 mg/L, or approximately 0.006mM-0.02mM\textsuperscript{5}. The maximum daily dose approved for metformin use is 3000mg, recommended for a person weighing 60kg. In humans, metformin has a bioavailability of approximately 50-60\%, with incomplete gastrointestinal absorption following a single dose\textsuperscript{6,7}. Absorption continues for 6-10h following administration, during which time the drug passes through the stomach and small intestine\textsuperscript{8}. Absorption of metformin occurs predominantly in the small intestine, with negligible absorption taking place in the stomach or large intestine\textsuperscript{9}. Within the small intestine, concentrations of metformin are significantly higher than in other tissues or plasma\textsuperscript{10,11}, indicating high concentrations of metformin within enterocytes and possible absorption in a paracellular manner\textsuperscript{12}. Metformin is chemically stable, not metabolized, and subsequently eliminated via excretion into the urine\textsuperscript{6}.

Peak plasma levels of metformin occur within 3h after oral administration of a single dose, and can range from 1.0 to 1.6 mg/L after a 500mg dose, to 3mg/L after a 1500mg dose\textsuperscript{6}. Renal excretion of metformin occurs at 3.5 times glomerular filtration rate (GFR), and has a terminal half-life of approximately 20h, with the bulk of metformin elimination occurring within the first 8h after administration\textsuperscript{6}. Metformin does not bind plasma proteins, and its plasma concentrations drop rapidly, with a half-life of 6h\textsuperscript{13}. The volume of distribution following oral administration of metformin is approximately 600L, though as only 50\% is absorbed, the actual volume of distribution is likely closer to 300L. Nonetheless, there is substantial tissue uptake of metformin, with elevated concentrations identified in the kidneys, adrenal glands, pancreas and liver\textsuperscript{5}.

1.1.3 Safety Profile

Metformin is tolerated well following administration. The most common complaint among metformin users is gastrointestinal upset, which is circumvented by giving the drug in divided doses with meals throughout the day\textsuperscript{14}. Though exact therapeutic ranges of metformin are unclear, concentrations above 5mg/L, or 0.03mM, are regarded as elevated\textsuperscript{15}. High plasma concentrations of metformin can cause lactic acidosis, a life-threatening condition characterized by blood pH below 7.5, and plasma lactate concentrations greater than 5.0mM\textsuperscript{16}. Patients with
lactic acidosis presented with plasma metformin levels ranging from 20-107mg/L. Incidence of lactic acidosis is rare, with approximately 3.3 cases per 100 000 patient years of metformin treatment, and risk factors include old age and renal failure.

1.2 Mechanisms of metformin’s antidiabetic activity

1.2.1 Metformin as a mitochondrial inhibitor

The primary target of metformin is within the mitochondria, whereby it was shown to transiently and specifically inhibit respiratory complex I, NADH dehydrogenase, in the electron transport chain. This resulted in decreased NADH oxidation and proton-driven ATP synthesis, consequently eliciting a cellular energy deficit due to a shift in ATP/ADP/AMP equilibrium to favour increased AMP levels. This was concluded from studies involving intact and permeabilized hepatocytes from mice and rats, as well as in isolated mitochondria. In these studies, inhibition of cellular oxygen uptake by metformin was exacerbated when incubated only in the presence of glutamate and malate, which are Complex I substrates. Incubation with succinate, a Complex II substrate, in combination with rotenone, a Complex I inhibitor, however, rescued the inhibition of oxygen consumption following metformin treatment. Treatment with metformin also decreased ATP:ADP ratios, suggesting a mechanism by which metformin selectively inhibits respiratory complex I and decreases electron flow through the electron transport chain, resulting in reduced ATP production by oxidative phosphorylation (illustrated in Figure 1:1).

The exact mechanism by which metformin enters the mitochondria or associates with complex I is unclear; however one study using a copper-sensitive probe observed that metformin bound mitochondrial copper ions, and this interaction was crucial to carry out its metabolic activity. It has also been proposed that metformin is actively transported into the mitochondria, as this could account for the nontoxicity of its respiratory inhibition via compensatory negative feedback: as metformin inhibits its target, synthesis of ATP is reduced and would limit further uptake of metformin and the extent of its mitochondrial inhibition.
Though much remains uncertain about the direct target and mechanism of metformin’s action, it is clear that it inhibits mitochondrial activity, which affects downstream signaling pathways responsible for exertion of its antidiabetic activity.

**Figure 1:** Primary targets of metformin. Metformin enters mitochondria by an unknown mechanism, whereby it inhibits Complex I of the electron transport chain. Its inhibitory action decreases electron flow through the respiratory chain, which is thought to ultimately reduce ATP production by ATP synthase. Abbreviations: I, complex I; NAD, nicotinamide adenine dinucleotide; Q, ubiquinone; QH$_2$, ubiquinol; mGPD, mitochondrial glycerophosphate dehydrogenase; G-3-P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; III, complex III, Cyt c$_{ox}$, cytochrome c (oxidized); Cyt c$_{red}$, cytochrome c (reduced); IV, complex IV; V, ATP synthase; ADP, adenosine diphosphate; P$_i$, inorganic phosphate; ATP, adenosine triphosphate.
1.2.2 AMPK-dependent suppression of hepatic glucose production

It has been indicated that the action of metformin in the liver serves mainly to oppose the action of the hormone glucagon by inhibiting hepatic gluconeogenesis. It has been proposed to do so by inhibiting gluconeogenic enzymes\(^{22-24}\) as well as reducing hepatic uptake of gluconeogenic substrates\(^{25}\). As discussed previously, metformin exerts an inhibitory effect on the mitochondria, which decreases electron transport through the respiratory chain and ATP production by oxidative phosphorylation. Reduced ATP production yields an increase in cellular AMP:ATP ratios, which is detected by, and results in activation of, 5'-AMP-activated protein kinase (AMPK). Once active, AMPK modulates a variety of downstream processes in order to restore cellular ATP levels following its depletion in response to metformin. Its promotion of macronutrient catabolism and inhibition of biosynthetic pathways is thought to contribute greatly to the ability of metformin to achieve decreased hepatic glucose output.

AMPK is a phylogenetically conserved serine/threonine protein kinase that monitors systemic and cellular energy status, and modulates energetic pathways to protect cellular function under energy-restricted conditions\(^{26}\). It occurs as a heterotrimer, comprising of a catalytic \(\alpha\) subunit and regulatory \(\beta\) and \(\gamma\) subunits; though within humans, each of these subunits occur as functionally redundant isoforms encoded by distinct genes, yielding up to 12 possible heterotrimeric combinations\(^{26,27}\).

The ability of AMPK to detect changes in cellular energy status is conferred by regulatory AMPK-\(\gamma\) subunits, which contain 3 binding sites for adenine nucleotides\(^{28-30}\). Under normal metabolic conditions, equilibrium of ATP:ADP:AMP within the cell is maintained at a ratio of approximately 100:10:1 due to the action of the enzyme adenylate kinase favouring production of ADP (ATP+AMP -> 2ADP)\(^{31}\). AMPK-\(\gamma\) binding sites have similar affinity for AMP, ADP and ATP; hence under unstressed conditions, low levels of cellular AMP result in competition primarily between ADP and Mg-ATP\(^4\) for AMPK-\(\gamma\) binding\(^{28}\). During energetic stresses such as hypoxia, glucose deprivation or exposure to mitochondrial inhibitors, depletion of cellular ATP reverses adenylate kinase activity toward the production of ATP and AMP instead, displacing ATP:ADP ratios and increasing AMP levels, which allows for its competition with ATP and ADP at AMPK-\(\gamma\) binding sites\(^{26,32}\). Once bound to AMPK-\(\gamma\), AMP induces a
conformational change in the subunit in order to amplify activation of AMPK, which occurs by phosphorylation of Thr-172 in the AMPK-α subunit; in addition, the change in conformation also protects against dephosphorylation of Thr-172. ADP is also capable of protecting against dephosphorylation upon being bound to AMPK-γ, however it is unable to induce allosteric activation\textsuperscript{28,32,33}. The major upstream kinase responsible for Thr-172 phosphorylation is the tumour suppressor liver kinase B1 (LKB1)\textsuperscript{34–36}; thus in tumour cells lacking LKB1, increased phosphorylation at Thr-172 in response to greater cellular AMP levels is diminished. Thr-172 can also be phosphorylated by Ca\textsuperscript{2+}/calmodulin-dependent kinase kinase β (CAMKKβ), resulting in AMPK activation in response to increases in intracellular Ca\textsuperscript{2+} concentrations, which can occur independently of changes in AMP levels\textsuperscript{37–39}.

Activation of AMPK following onset of cellular energy crisis stimulates catabolic pathways that generate ATP, such as glycolysis and β-oxidation of fatty acids\textsuperscript{27}. It achieves this by phosphorylating key metabolic enzymes and modulating gene expression through the induction of transcription factors and co-activators\textsuperscript{40}. For example, AMPK inhibits acetyl-CoA carboxylase (ACC), a critical enzyme that catalyzes the rate-limiting reaction in β-oxidation of fatty acids, which ultimately promotes fatty acid oxidation while inhibiting synthesis of free fatty acids, which can impair insulin signaling\textsuperscript{41–43}. Enhanced fatty acid oxidation may mediate metformin’s ability to alter fatty acid metabolism and improve hepatic steatosis, as was seen in a previous study using mice receiving metformin\textsuperscript{44}. AMPK also stimulates glycolysis via phosphorylation of phosphofructokinase-2 (PFK-2)\textsuperscript{45}, and thus can directly reduce glucose output\textsuperscript{46}. It was demonstrated that metformin’s suppression of glucose production and lipogenesis was mitigated following AMPK inhibition both using the nonselective inhibitor Compound C\textsuperscript{42}, and forced expression of dominant negative AMPK. Furthermore, use of 5-aminoimidazole-4-carboxamide riboside (AICAR), an adenosine analogue that upon uptake within cells is converted to ZMP, which mimics AMP and artificially activates AMPK, inhibited gluconeogenesis in hepatoma cells\textsuperscript{47}. AMPK also promotes glucose uptake into cells through induction of GLUT1 located at the plasma membrane, and translocation of GLUT4 from intracellular storage vesicles to the plasma membrane in skeletal muscle, hepatocytes, and adipocytes\textsuperscript{48–50}. Thus, activation of AMPK by metformin is a critical determinant of its inhibition of hepatic glucose production through its stimulation of fatty acid and glucose catabolism, and promotes increased uptake of glucose as well.
In order to restore cellular energy levels, AMPK also suppresses biosynthetic pathways that consume ATP by phosphorylating metabolic enzymes or regulatory proteins. AMPK phosphorylation targets switch off almost all anabolic processes, including: fatty acid synthesis through inactivation of ACC1, triglyceride and phospholipid synthesis through inactivation of glycerol phosphate acyl-transferase (GPAT), cholesterol synthesis through inactivation of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), glycogen synthesis through inactivation of glycogen synthase, protein synthesis through inactivation of mammalian target of rapamycin (mTOR) via activation of tuberous sclerosis 2 (TSC2) and regulatory-associated protein of mTOR (RAPTOR), and ribosomal RNA synthesis through inactivation of transcription factor IA (TF1A). Thus, AMPK activation is crucial in mediating an adaptive response to energetic stress and conserving cellular ATP levels (illustrated in Figure 1).

Previously, AMPK was considered responsible for inhibition of gluconeogenic gene transcription. AMPK was thought to regulate the phosphorylation and subsequent localization of CRTC2 (CREB [cAMP-response-element-binding]-regulated transcription co-activator 2), which, in its non-phosphorylated form, associates with nuclear phosphorylated CREB to drive the expression of PGC-1α (PPAR-γ co-activator-1α) and its gluconeogenic target genes PEPCK (phosphoenolpyruvate carboxykinase) and G6Pase (glucose-6-phosphatase). Phosphorylation of CRTC2 on Ser-171 by AMPK prevents its nuclear localization and subsequently inhibits the transcription of gluconeogenic genes. Requirement of AMPK for the reduction of gluconeogenic gene expression was demonstrated via overexpression of a constitutively active AMPK variant, stimulation of CREB-binding protein in hepatocytes by metformin and AICAR, and disruption of constitutively-active CRTC recruitment to promoters of gluconeogenic genes via metformin or expression of constitutively active AMPK in hepatoma cells. Similarly, deletion of liver kinase B1 (LKB1), the upstream kinase regulating basal activation of AMPK, increased expression of gluconeogenic and lipogenic enzymes, and negated the glucose-lowering effect of metformin in mice fed a high-fat diet. Together, these data contributed to the conclusion that AMPK activation directly regulated gluconeogenesis.

1.2.3 AMPK-independent suppression of hepatic glucose production

In spite of the literature reviewed above, emerging evidence has indicated an AMPK-independent mechanism by which metformin can inhibit hepatic gluconeogenesis. A study by
Foretz et al. demonstrated that metformin decreased hepatic glucose production in transgenic mice lacking both AMPK and its upstream activator kinase, liver kinase B1 (LKB1), indicating the presence of an AMPK-independent mechanism of action for metformin. Metformin was also able to lower glucose output in a murine model overexpressing PGC-1α, in spite of increased protein levels of PEPCK and G6Pase, supporting the notion that metformin impacts gluconeogenesis primarily by modulating enzymatic function rather than depending on AMPK-mediated alteration of their expression.

The increased AMP:ATP ratio following mitochondrial inhibition by metformin also antagonizes cAMP synthesis driven by glucagon. Glucagon signaling ultimately promotes gluconeogenesis, glycogenolysis and ketogenesis, processes that are hyperactive in diabetic patients owing to their elevated glucagon levels, and leads to an increase in hepatic glucose output. Increased AMP levels inhibit the adenylate cyclase enzyme responsible for catalyzing the conversion from ATP to cAMP. Thus, the action of metformin reduces cAMP levels and opposes its stimulation of protein kinase A (PKA) and phosphorylation of its downstream target 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK/FBPase), a bifunctional enzyme responsible for regulating intracellular levels of fructose-2,6-bisphosphate (F2,6BP). F2,6BP is both an allosteric activator of the glycolytic enzyme phosphofructokinase 1 (PFK), and an allosteric inhibitor of the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBP1), and is a key intermediary in balancing rates of glycolysis and gluconeogenesis. PFK/FBPase1 phosphorylation by glucagon-stimulated PKA yields conformational changes in the enzyme favouring FBPase 1 activity and reduction of intracellular F2,6BP, ultimately promoting gluconeogenesis over glycosis. In the absence of phosphorylation by PKA, such as that following reduced intracellular cAMP in the presence of metformin, PFK activity is favoured over FBPase, yielding increased F2,6BP and resultant increase in glycolytic flux and suppression of gluconeogenesis. Thus, metformin is proposed to suppress hepatic gluconeogenesis via a direct AMPK-dependent mechanism, as well as an indirect cAMP-mediated, AMPK-independent mechanism that directly opposes glucagon signaling (Figure 1:3).

A very recent study presented data indicating a different mechanism by which metformin can affect mitochondrial function and elicit decreased systemic plasma glucose levels. In these experiments, Sprague-Dawley rats treated either acutely or chronically with metformin did not
undergo alteration in their hepatocellular energy status, with ATP:ADP ratios remaining constant\textsuperscript{67}. This was consistent with a previous study in which a lack of alteration in ATP:ADP ratios in rat hepatoma cells following metformin treatment was reported\textsuperscript{68}. Interestingly, rats receiving metformin still had lower plasma glucose and insulin levels than their untreated counterparts, suggesting a mechanism of action for metformin’s glucose-lowering effect apart from modulation of cellular energy charge. Consistent elevation of plasma lactate levels, lactate:pyruvate ratios (indicating increased cytosolic redox state), and decreased β-hydroxybutyrate:acetoacetate ratios (reflecting decreased mitochondrial redox state), led the authors to hypothesize that metformin suppressed one of two main redox shuttles. Treatment with biguanides selectively inhibited activity of mitochondrial glycerophosphate dehydrogenase (mGPD), which converts dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G-3-P), and feeds electrons to the mitochondrial electron transport chain (\textit{Figure 1:3}). Furthermore, the authors were able to phenocopy metformin’s glucose-lowering effect systemically by both by using antisense oligonucleotide (ASO) knockdown of hepatic mGPD in rats, and a whole-body knockout mGPD mouse model. Metformin was able to non-competitively inhibit recombinant human mGPD, and direct binding was supported by structure-modeling studies. From these data, the authors concluded that metformin targets the mGPD shuttle, which results in elevation of cytosolic redox state. The subsequent decrease in conversion of lactate to pyruvate catalyzed by lactate dehydrogenase (LDH) ultimately prevents catabolism of pyruvate in inverse glycolysis and in this novel manner is able to decrease hepatic gluconeogenesis\textsuperscript{67}.

\textbf{1.2.4 Non-hepatic activity of metformin}

The activity of metformin is not restricted to the liver, however; its ability to enhance glucose uptake in peripheral tissues is also attributed to its systemic glucose-lowering effect. It has been shown to activate AMPK both in isolated rodent skeletal muscle, as well as in human skeletal muscle whereby increased AMPK activity induces translocation of GLUT-4 glucose transporters to the plasma membrane and facilitates insulin-independent glucose uptake, mimicking conditions of muscle contraction\textsuperscript{69}. Metformin has also been shown to improve tyrosine kinase activity of both the insulin receptor and insulin-receptor substrate 2 (IRS-2)\textsuperscript{70,71}, which contribute to improved insulin sensitivity in skeletal muscle\textsuperscript{72}. 
In all, though much remains to be clarified about metformin’s exact mechanism of action, it is able to successfully reduce systemic glucose and insulin levels, which has rendered it such an effective therapeutic for diabetic patients.

**Figure 1.2** Metabolic effects of AMPK. Increased relative AMP following onset of energetic crisis activates AMPK, which acts to restore cellular energy levels. AMPK promotes glucose uptake through stimulation of GLUTs 1 and 4, and promotes catabolism of glucose and fatty acids through induction of PFK2 and inhibition of ACC1, which limits β-oxidation. AMPK also restricts a variety of biosynthetic processes including ribosomal RNA synthesis, triglyceride and phospholipid synthesis, cholesterol synthesis, fatty acid synthesis, and protein synthesis. Abbreviations: GLUT1/4, glucose transporters 1/4; PFK2, phosphofructokinase 2; TF1A, transcription factor 1A; GPAT, glycerol phosphate acyltransferase; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; ACC1/2, acetyl coA carboxylase 1/2; TSC2, tuberous sclerosis 2; RAPTOR, regulatory-associated protein of mTOR; mTOR, mammalian target of rapamycin; LKB1, liver kinase B1; AMPK, 5’AMP-activated protein kinase.
Figure 1:3 Metformin reduces hepatic glucose output. Metformin’s inhibition of respiratory complex I increases relative cellular AMP levels, which activate AMPK and favour glucose uptake, glycolysis and β-oxidation of fatty acids. Its AMP-dependent inhibition of adenylate cyclase also opposes glucagonic activity, which is elevated in diabetic patients. Inhibition of adenylate cyclase decreases cAMP levels and resultant activation of PKA, which normally acts to promote gluconeogenesis and suppress glycolysis. Metformin has recently been shown to non-competitively inhibit the redox shuttle mGPD, which usually converts G-3-P to DHAP, and passes electrons to the mitochondrial electron transport chain. mGPD inhibition results in an increased cytosolic redox state, ultimately preventing the activity of LDH in converting lactate to pyruvate and thus reduces gluconeogenesis in this manner.

Abbreviations: OCT, organic cation transporter; AMP, adenosine monophosphate; AMPK, 5’-AMP activated protein kinase; MATE, multidrug and toxin extrusion; cAMP, cyclic AMP; PKA, protein kinase A; mGPD, mitochondrial glycerophosphate dehydrogenase; G-3-P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; NADH, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase.
1.3 Metformin transporters facilitate its intracellular and systemic effects

As metformin is positively charged at physiological pH, it requires the presence of membrane transporters in order to cross the plasma membrane and exert its specific intracellular activity. Metformin depends on a family of transporters from the solute carrier (SLC) class of membrane proteins: the organic cation transporters (OCT), which are responsible for its uptake, and multidrug and toxin extrusion (MATE) transporters, which excrete it.

1.3.1 Organic cation transporters (OCTs)

OCTs are a family of polyspecific bidirectional uniporters comprising of 3 homologs (OCT1, OCT2, OCT3), and they are responsible for the uptake of a wide variety of organic cations, including metformin as well as endogenous compounds, xenobiotics and heavy metals\textsuperscript{73,74}. Under normal physiological conditions, they favour electrogenic uniport to transport organic cations into cells, and operate independently of Na\textsuperscript{+} and proton gradients; however they are also capable of mediating electroneutral exchange of organic cations in conditions where near-saturating concentrations of substrate are found on both sides of the membrane\textsuperscript{75,76}. OCTs belong to the superfamily of human solute carriers 22A (SLC22A), which also include Na\textsuperscript{+}-zwitterion/cation cotransporters (OCTNs), organic anionic transporters (OATs) and urate transporters (URAT)\textsuperscript{77}. Members of the SLC22A superfamily have consistent membrane topology comprising of 12 α-helical transmembrane domains (TMDs) and intracellularly-localized N- and C- termini, with an extracellular loop between TMD1 and TMD2 containing glycosylation sites and an intracellular loop between TMD6 and TMD7 containing phosphorylation residues\textsuperscript{78}. Several isoforms of this family are expressed at the sinusoidal membrane of hepatocytes to reflect their role in uptake of hepatic substrates, including the dominant metformin importer, OCT1.

Structurally, OCTs bear similar topology to the remainder of the SLC22A superfamily; however in addition OCTs possess several unique highly conserved motifs between TMD2 and TMD3, as well as between TMD8 and TMD9. An 11-residue sequence located before TMD2 is unique to the OCTs as well, and considered an OCT family signature sequence\textsuperscript{78}. The homology present in N- and C- termini of OCTs, as well the conservation of specific cysteine, glycine and
proline residues reputedly involved in determining substrate selectivity/affinity and pore structure\textsuperscript{79,80}, support the notion that these isoforms originated as a past gene duplication event\textsuperscript{81}.

The OCT1 and 2 homologs were originally discovered in rats, and were shortly followed by the discovery and cloning of its orthologs in humans\textsuperscript{82–84}. OCT1, 2 and 3 are encoded by the genes \textit{SLC22A1}, \textit{SLC22A2} and \textit{SLC22A3} respectively, each of which span 11 exons and 10 introns\textsuperscript{76,81,85–87}, and occur within a cluster located on chromosome 6.6q26. OCTs 1, 2 and 3 are composed of 554, 555, and 556 amino acids, respectively\textsuperscript{88,89}.

OCT homologs differ greatly in their tissue-specific expression, as well as across species\textsuperscript{90}. In humans, OCT1 is primarily expressed in the basolateral membrane of hepatocytes\textsuperscript{83}, although its expression has also been detected at moderate levels in gastric enterocytes and renal proximal and distal tubule cells\textsuperscript{91–93}, and to a lesser extent, in colon, skeletal muscle, lung, mammary epithelial cells, and some neurons\textsuperscript{75,94–98}. OCT2 demonstrates a more restricted expression pattern, being most heavily expressed in the peritubular membrane of renal proximal tubule cells\textsuperscript{99}, although it is expressed to lesser degrees in small intestine, colon, lung, and brain\textsuperscript{75,96–98,100}. OCT3 has a much broader tissue expression pattern in comparison, and has been detected in many organs, including the brain\textsuperscript{101}. Its highest expression is found in skeletal muscle, liver, placenta and heart\textsuperscript{96,99,102,103}. In all, the high expression of OCT1 and OCT3 in hepatocytes, and of OCT2 in renal cells, is consistent with their primary physiological roles as facilitators of drug uptake, disposition and detoxification; however, their distribution among other cell types implies their involvement in a broad array of physiological processes.

1.3.2 Multidrug and toxin extrusion transporters (MATEs)

MATEs are a family of transporters controlling the secretion of drugs and xenobiotics. They act in concert with OCTs to mediate vectorial renal secretion of cationic compounds across tubular epithelial cells. Unlike OCTs, which depend upon transmembrane potential at the basolateral surface to facilitate movement of cationic substrates, MATEs are driven by the transmembrane H\textsuperscript{+} gradient at brush-border membranes and are bidirectional H\textsuperscript{+}/organic cation antiporters\textsuperscript{104,105}. They belong to the class of multidrug transporters, along with other transporter families including the major facilitator superfamily (MFS), small multidrug resistance family (SMR) and ATP-binding cassette family (ABC). MATEs were originally identified by norfloxacin-accumulation experiments in \textit{Vibrio parahaemolyticus} and \textit{Escherichia coli};
homologs of these proteins have been identified in many other organisms thereafter\textsuperscript{106}. Orthologous human MATE1 and MATE2 were first identified through homology screening using the NorM antiporter in \textit{V. parahaemolyticus} as a reference\textsuperscript{107}. A kidney-specific variant of MATE2, specified as MATE2-K, was cloned and functionally characterized shortly thereafter\textsuperscript{108}.

MATE proteins usually range in length of 400-550 amino acid residues, with a predicted membrane topology of 12 helical TMDs\textsuperscript{76}. Highly conserved sequences occur in extracellular loops between TMD1 and TMD2, and TMD7 and TMD8, in intracellular loops between TMD2 and TMD3, and TMD8 and TMD9, as well as in regions between TMD4 and TMD5, and TMD10 and TMD11. Like the members of the OCT family, the high degree of conservation in these sequences suggest that MATE proteins originated from a past duplication event of a common ancestral gene\textsuperscript{109}. In humans, MATE1 and MATE2 are encoded by the genes \textit{SLC47A1} and \textit{SLC47A2} respectively, which are located on chromosome 17p11.2\textsuperscript{107}.

MATE2-K is the only human MATE2 protein for which transport activity has been functionally characterized. Like MATE1, it is a polyspecific bidirectional antiporter capable of translocating a variety of cationic substrates including tetraethylammonium (TEA), 1-methyl-4-phenylpyridine (MPP\textsuperscript{+}) and metformin\textsuperscript{108}. In general, MATE1 and MATE2-K demonstrate similar substrate specificity for most cationic compounds tested, with the exception of the platinum-derived anticancer agent oxaliplatin, which was a better substrate of MATE2-K\textsuperscript{76,110}.

Human MATE1 is strongly expressed in the apical regions of proximal and distal convoluted tubules in the kidney\textsuperscript{107,108}. It has also been detected in abundance in liver and skeletal muscle, and to a lesser degree in heart\textsuperscript{107}. MATE2 is also primarily expressed in kidney, though it has been detected in brain as well\textsuperscript{108}. The variant MATE2-K is expressed primarily in the kidney, specifically in the brush border membranes of proximal tubules\textsuperscript{108}. The predominant expression of MATE1 and MATE2 in the kidney supports the notion that they mediate the final step of excretion of organic cations\textsuperscript{107}. There is great species-dependency in the tissue distribution of MATEs however, with mouse MATE1 and rat MATE1 demonstrating considerably wider distribution patterns than their human homologs\textsuperscript{105,107,111}. In particular, mouse MATE1 protein was detected in brain, heart, stomach, small intestine, bladder, thyroid and adrenal glands, in addition to the kidney and liver, suggesting that MATEs may contribute to other physiological processes in addition to renal excretion\textsuperscript{107,111}.
1.3.3 OCT1 is critical for metformin to act in the liver

Metformin acts preferentially upon the liver due to the high expression of OCT1 in hepatocytes, which facilitates cellular uptake of metformin; this may account for the high concentrations of metformin observed in the liver relative to those found systemically. The importance of OCT1 in metformin distribution to hepatocytes was first established in a study by Wang et al., whereby tissue distribution following intravenous injection of metformin was monitored in mice that were either wild-type (+/+) or knockout (-/-) for Oct1. Metformin concentrations were significantly decreased in the livers, and to a lesser extent in the intestines, of Oct1(-/-) mice. These results were corroborated in a parallel study by Jonker et al., which also reported increased excretion of organic cations in mice lacking Oct1 in addition to their decreased uptake in the liver and intestine. A subsequent study by this group using knockout Oct2(-/-) mice and double-knockout Oct1/2 (-/-) mice indicated that the loss of Oct1 exclusively affected organic cationic disposition in the liver and intestine, while in Oct2(-/-) mice, no significant difference was found in the distribution of organic cations compared to that of wild-type mice. Furthermore, loss of both Oct1 and Oct2 in double-knockout mice yielded impaired renal secretion of organic cations and substantially increased plasma concentrations, suggesting redundancies in both transporters regarding their function in renal clearance. It has been proposed that in conditions of impaired OCT1 function, metformin uptake can be dependent on the OCT3 homolog, which is also expressed in the liver in less abundance, but was characterized with an affinity for metformin similar to that of OCT1 in cell-based transport and inhibition assays.

High hepatic OCT1 expression may account for the observation that the liver is one of few organs capable of accumulating metformin in normal and streptozotocin-diabetic mice. Further data contributing to the importance of OCT1 in mediating metformin response include impaired AMPK activation following metformin treatment seen in primary mouse hepatocytes with OCT1 deletions, and abolished ability of metformin’s glucose-lowering effect in Oct1-deficient mice. Additionally, increased hepatic OCT1 expression in obese mice fed high-fat diets corresponded with increased hepatic accumulation of metformin. However, functional redundancies identified between OCT1 and OCT2 in mediating renal excretion of TEA⁺ suggest that the role of OCT2, along with remaining metformin transporters that have been not been as
closely studied, may be more important in determining metformin disposition and pharmacokinetics than previously suggested.

1.3.4 Genetic variation in OCTs and MATEs

OCTs and MATEs are highly polymorphic in ethnically diverse populations, and may represent factors contributing to variation in patient response\textsuperscript{118-120}. Many variants occurring in these transporters have been identified and functionally characterized \textit{in vitro}. Their role in affecting metformin disposition and efficacy in diabetic patients has comprised the subject of several studies (summarized in Table 1:1). Polymorphisms in OCT1 have been studied in most detail, though data indicates functional variation in OCT2, MATE1 and MATE2 also contribute to patient response. Hence, it is apparent that the contributions of OCTs and MATEs in determining metformin disposition, pharmacokinetics and response in patients, are far more important than were suggested by previous animal studies by Jonker et al., in which knockout of Oct2 was not accompanied by alterations in organic cation disposition in a mouse model\textsuperscript{114}. 
Table 1.1 Studies addressing genetic variation in metformin transporters and response. These include parameters of pharmacokinetics (PK) including area-under-the-plasma-time-curve (AUC), maximal plasma concentration ($C_{\text{max}}$) and volume of distribution ($V_d/F$). Glycosylated hemoglobin A1c (HbA1c) levels are used as markers of average long-term plasma glucose concentrations and monitored routinely in diabetic patients.

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<td>990</td>
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<td>Increased HbA1c reduction</td>
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<td>Reduced transport</td>
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1.4 Metformin and cancer

1.4.1 Metformin and decreased cancer incidence

In recent years, metformin has been associated with a reduced incidence of cancer in diabetic patients, sparking interest in the repositioning of this antidiabetic drug into treatment of neoplastic disease\textsuperscript{138}. The association was striking in light of the fact that diabetes mellitus has been associated with a 1.2-2.0-fold increase in cancer incidence\textsuperscript{139}. In contrast with other common antidiabetic treatments, metformin was independently associated with a reduced risk of cancer incidence by almost 40\%\textsuperscript{139}. A multitude of observational and retrospective studies conducted thereafter investigated the association between metformin use and incidence in several cancer types, including those of the liver\textsuperscript{140–147}, colorectum\textsuperscript{140,144,147–158}, pancreas\textsuperscript{140,144,147,151,153,155–157,159}, stomach\textsuperscript{144,147,148}, breast\textsuperscript{18,147,148,151–153,156,157,160–163}, lung\textsuperscript{147,148,152,157,164,165}, ovary\textsuperscript{148,155,166,167}, prostate\textsuperscript{147,148,151,153,156,157,168–172}, kidney, esophagus, skin, uterus and bladder\textsuperscript{140,148,153,173}.

Though not all reports agree on a universal association between metformin use and reduced incidence in diabetic patients, recent meta-analyses have indicated that use of metformin particularly lowers risk of colorectal\textsuperscript{174}, hepatocellular\textsuperscript{174}, lung\textsuperscript{175}, and pancreatic\textsuperscript{176} cancers; though this may be an artifact of having a greater population of patients to examine due to the relatively large number of studies conducted to investigate these cancer sites. The results of these studies must be interpreted cautiously however, as they are retrospective in nature and thus prone to bias and confounding inherent in their design\textsuperscript{173,177}. In all, current data suggest that the role of metformin as a cancer prophylactic may be limited to specific types of cancer, though several clinical trials are currently underway to clarify these findings.

1.4.2 Metformin and improved outcome to cancer therapy

Metformin use has also been associated with improved outcome in diabetic patients undergoing treatment for cancer. Jiralerspong and colleagues first reported a link between metformin use and increased pathological response in diabetic breast cancer patients receiving neoadjuvant chemotherapy in a retrospective study involving 2529 patients, of which 68 were diabetic patients taking metformin\textsuperscript{178}. Another retrospective study by Skinner et al. examining a smaller cohort of 285 esophageal cancer patients receiving concurrent chemotherapy and radiation (CRT) reported a significantly greater pathologic complete response (pCR) rate in 29
metformin users, with metformin use associated with increased pCR in a dose-dependent manner\textsuperscript{179}. The same group also reported an increase in pCR rate, loco-regional control and disease-free-survival (DFS) after 5 and 10 years in 20 diabetics receiving metformin in a retrospective analysis of 482 rectal cancer patients treated with neoadjuvant CRT followed by surgical resection\textsuperscript{180}.

Therapeutic benefits have also been reported when metformin use was combined with radiotherapy (RT) alone. A retrospective analysis in a relatively small cohort of 30 head-and-neck patients indicated reduced locoregional recurrence (LRR) and increased overall survival (OS) in 10 patients receiving metformin\textsuperscript{181}. Large-scale retrospective studies also reported improved outcome following metformin use in castrate-resistant prostate cancer patients\textsuperscript{172,182}. However, in another retrospective cohort study involving 130 breast cancer patients treated with RT, metformin use in diabetic patients was independently and significantly associated with increased acute skin toxicity, indicating that its potential to sensitize cancer cells to radiation must also be balanced against its toxicity\textsuperscript{183}. Nonetheless, these findings collectively provide strong rationale to further examine a possible therapeutic benefit of metformin when used in combination with cancer treatment.

1.4.3 Benefits of metformin in nondiabetic patients

There is limited data surrounding the effect of metformin in non-diabetic cancer patients. In a short-term clinical trial involving 9 nondiabetic patients, suppression of colorectal aberrant crypt foci following metformin treatment was reported, and is currently undergoing a trial of longer duration\textsuperscript{184}. Another trial identified reduction of Ki67, a marker of cellular proliferation, in biopsy samples from a pilot cohort of 8 non-diabetic breast cancer patients who received metformin pre-operatively\textsuperscript{185}. Furthermore, a pilot window-of-opportunity study in prostate cancer patients receiving metformin pre-operatively identified reduced Ki67 staining in prostatectomy samples 3 weeks following initial biopsy\textsuperscript{186}. This finding is consistent with numerous preclinical and laboratory studies, which have demonstrated the ability of metformin to inhibit cell proliferation, and have spurred numerous clinical studies investigating incorporation of metformin as a neoadjuvant to cancer treatment. Currently, there are 173 clinical trials registered with the NIH to investigate metformin and cancer.
1.5 Mechanisms of metformin’s anticancer activity

1.5.1 Indirect inhibition of cancer cell proliferation

The action of metformin in the liver, by mechanisms discussed previously, promotes reduction of systemic glucose and insulin concentrations, provided that they were elevated at baseline. Reduction of systemic glucose levels can potentially limit its use by cancer cells. Furthermore, increased plasma levels of insulin, or insulin-like growth factors (IGFs), can promote growth of tumour cells expressing the insulin receptor or insulin-like growth factor receptor (IGFR). Increased binding of these receptors stimulates hyperactivation of signaling pathways promoting cell survival and proliferation\textsuperscript{187}. Without being directly taken up by cancer cells, the ability of metformin to lower systemic insulin reduces signaling and activity of the insulin receptor and its downstream induction of cellular growth and proliferation in tumour cells, constituting an indirect mechanism by which it can affect insulin-responsive cancer in hyperinsulinemic patients (\textit{Figure I:4}).

1.5.2 Direct inhibition of cancer cell proliferation

1.5.2.1 AMPK dependent effects

Metformin can also act upon cancer cells directly. As discussed previously, mitochondrial inhibition by metformin leads to activation of AMPK, which is a major downstream effector of its antitumour activity. AMPK stimulates catabolic pathways that generate ATP while suppressing biosynthetic pathways that consume ATP in order to restore cellular energy levels and mitigate energy crisis imposed by metformin\textsuperscript{27}. Accordingly, cancer cells that lacked LKB1 or AMPK activity were more sensitive to cell death following glucose deprivation, which emphasizes the importance of this signaling axis in coordinating adaptation to energetic stress\textsuperscript{36,188–190}.

Activation of AMPK in the presence of metformin is indirect and occurs downstream of increases in cellular AMP and ADP levels following inhibition of mitochondrial complex I\textsuperscript{42}. This was demonstrated in cell lines expressing AMPK-\gamma with an inactivating mutation, which were rendered insensitive to AMP and ADP, and thus unable to undergo activation following treatment with metformin and other known AMPK activators\textsuperscript{191}. Recent data by Madiraju and colleagues have challenged this particular mechanism\textsuperscript{67}; nonetheless AMPK activation by
metformin has been well characterized. As discussed previously, the ability of AMPK to restrict anabolic, energy-consuming pathways is thought to largely contribute to the antiproliferative effect of metformin. In particular, negative regulation of mTOR by AMPK constitutes a link between monitoring of cellular energy status and inhibition of cancer cell growth by metformin. Frequently deregulated in cancer, mTOR is a catalytic subunit composed of two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which together regulate cellular growth and are responsible for the integration of many hormonal signaling and energy-sensing pathways, including input from insulin, AMPK and other growth factors.

mTORC1 regulates protein translation through its inactivation of 4E binding protein 1 (4E-BP1) and activation of ribosomal S6 kinase (p70S6K) via RAPTOR interaction, and controls translation of cell growth regulators such as cyclin D1, vascular endothelial growth factor (VEGF), and hypoxia-inducible factor 1α (HIF-1α), the latter of which promote angiogenesis and potentially radiation tolerance. Hyperactivation of mTOR contributes to the uncontrolled growth and proliferation characteristic of cancer cells. AMPK negatively regulates mTORC1 activity by phosphorylating TSC2 and stimulating GTPase activity within the TSC1:TSC2 complex, which then acts upon Rheb, a G-protein promoting mTORC1 activity while GTP-bound. GTPase activity of TSC1:TSC2 converts Rheb to its GDP-bound state, which inactivates it and results in suppression of mTOR activity. Furthermore, AMPK also directly phosphorylates RAPTOR, which further contributes to quenching of mTOR activity. The ability of AMPK to oppose deregulated mTOR signaling further contributes to its cytostatic, antitumour effect, and contributes to a mechanism by which metformin can directly inhibit cancer cell proliferation.

The ability of metformin to inhibit cancer cell growth was first reported in a panel of cancer cell lines originating from breast, prostate and ovary, whereby metformin decreased cellular proliferation in a dose-dependent manner, increased AMPK activation, and decreased phosphorylation of mTOR and its downstream effector p70S6K. Silencing of AMPK abrogated both growth inhibition and mTOR suppression by metformin, suggesting AMPK-dependence in mediating metformin’s intracellular effects. Similar observations were made in mouse embryonic stem cells, human breast cancer cells irrespective of ER or HER2 status, prostate, colon, endometrial and pancreatic cancer cell lines, whereby metformin increased AMPK phosphorylation and suppressed mTOR signaling resulting in inhibition of cellular proliferation.
Loss of TSC2 in mouse embryonic fibroblasts (MEFs), an AMPK target that negatively regulates mTOR, reversed mTOR suppression and yielded increased protein translation and cellular growth\textsuperscript{202}, indicating the importance of AMPK-mTOR signaling in mediating growth inhibition.

1.5.2.2 Interaction between AMPK and p53

AMPK is also capable of phosphorylating p53 at serine-15 (Ser-15), which couples energy status detection to cell cycle regulation. The transcription factor p53 was one of the first identified tumour suppressors\textsuperscript{207} and is activated by a variety of stresses routinely encountered by cells, such as DNA damage, oxidative stress, hypoxia, and oncogene activation\textsuperscript{208}. Its induction can mediate an adaptive response to cellular stress by promoting processes such as cell cycle arrest, growth inhibition, and autophagy. Alternatively p53 activation can eliminate severely damaged cells through the induction of senescence or apoptotic cell death\textsuperscript{208–210}. Structurally, p53 is composed of an acidic N-terminal transactivation domain, which is required for activating target genes, a proline-rich domain, a core DNA-binding domain, which facilitates sequence specific binding to p53-response elements in DNA, a tetramerization domain, which mediates interaction of p53 monomers to form dimers and tetramers, and a C-terminal regulatory domain, which is the site of regulation by upstream signaling pathways\textsuperscript{211,212}. To activate transcription, p53 forms a homotetramer in cells and binds to its response elements in the DNA sequence\textsuperscript{213–215}. The target genes that are upregulated mediate the downstream effects of p53, which can limit the proliferation and survival of premalignant cells and protect against the development of tumours\textsuperscript{207,208,216}. Thus, p53 mutation or inactivation is essential to the formation of most human tumours\textsuperscript{217}, with the majority occurring as point mutations in the DNA-binding domain and impairing its DNA-binding and transactivation functions\textsuperscript{216}.

In normal unstressed cells, p53 is present at very low concentrations owing to its rapid turnover by E3 ubiquitin ligases. In particular, the MDM2/MDMX protein complex ubiquitinates p53 and targets it for proteasomal degradation, and is thus critical for maintaining low steady-state p53 levels in order to restrict its impact on cellular fate\textsuperscript{218,219}. Under conditions of genotoxic stress, p53 undergoes a variety of posttranslational modifications that disrupt its interaction with MDM2 and contribute to its stabilization, nuclear accumulation, and biochemical activation of its transcription factor activity\textsuperscript{220}. Many upstream kinases can activate p53 in conditions of various cellular stresses: ataxia telangiectasia-mutated (ATM) mediates p53 stabilization following
ionizing radiation (IR), checkpoint kinases 1/2 (CHK1/2) do so following DNA damage, and AMPK facilitates p53 activation primarily under metabolic stress\textsuperscript{221–223}. Upon activation, p53 transactivates its downstream effectors, in particular the multifunctional protein p21, which is a cyclin-dependent kinase (CDK) inhibitor widely regarded as a universal inhibitor of cell cycle progression, and is responsible for restraining cells at G\textsubscript{1}/S and S-phase checkpoints\textsuperscript{224–226}. Upregulation of gene products such as Bax, Bcl-2 and Bcl-XL promote apoptosis upon localization to, and subsequent release of, mitochondrial cytochrome c and subsequent induction of cleaved-caspase-3, -6 and -7\textsuperscript{227}.

Though an interaction between AMPK and p53 has been characterized, it is unclear whether metformin can directly activate p53. Activated AMPK was sufficient to induce a p53-dependent checkpoint and cell cycle arrest at G\textsubscript{1} following glucose starvation\textsuperscript{223}; this was demonstrated in studies using cell lines expressing constitutively-active AMPK\textsuperscript{223} as well as in hepatoma cells treated with AICAR\textsuperscript{228}. Additionally, metformin has been reported to induce cell cycle arrest and cell death in cancer cell lines, with p53 dependency arising under conditions of nutrient starvation and cell death occurring through both apoptosis and necrosis\textsuperscript{229}. In one study, prostate cancer cell lines treated with metformin underwent cell cycle arrest through downregulation of cyclin D1 expression by p53-dependent activation of regulated in development and DNA damage responses 1 (REDD1), a negative regulator of mTOR\textsuperscript{230}. This suggested a requirement for p53 activation, independent of AMPK, in mediating metformin’s anti-proliferative effects. More recently, a mechanism linking AMPK activation to p53 stabilization following metformin treatment was described, involving phosphorylation of p53-regulatory MDMX by AMPK, which enhanced its sequestration by 14-3-3σ and facilitated time-dependent stabilization of p53\textsuperscript{231}. Furthermore, metformin has also been reported to induce cell cycle arrest and apoptosis through downregulation of cell cycle promoters such as cyclin D1 and E2F1, and upregulation of p53 targets p21 and p27\textsuperscript{203–205,232,233}, irrespective of p53 mutaton status\textsuperscript{232}. Conversely, another study using matched colon carcinoma cell lines differing in p53 status found that p53-deficient cells exhibited impaired survival and increased apoptosis following metformin treatment\textsuperscript{234}, implying that loss of p53 prevents cells exposed to metabolic stress from reducing energy consumption, and subjects them to energy crisis and programmed cell death. This latter finding may suggest contextual lethality of metformin in cancer cells having genetic defects in tumour suppressor proteins, such as p53, whereby metformin is
selectively toxic to cells unable to adapt to energy deficiency. This effect, however, is likely to vary owing to the heterogeneity in metabolic and genetic characteristics of tumours. Nonetheless, vastly conflicting data in the literature indicates that the requirement of p53 to cause cell cycle arrest in cancer cells treated with metformin remains controversial.

1.5.2.3 AMPK independent effects

Several studies have also indicated an AMPK-independent mechanism of action for metformin. It was demonstrated in MEFs and prostate cancer cells that silencing of REDD1, and not AMPK, circumvented the ability of metformin to inhibit mTOR and subsequent proliferation; thus p53-dependent activation of REDD1 was identified as a novel AMPK-independent mechanism by which metformin can decrease mTOR activity. Alternatively, metformin was able to inhibit mTOR and cellular proliferation in prostate cancer cells with siRNA knockdown of both AMPK catalytic subunits α1 and α2. In mouse embryonic fibroblasts (MEFs) lacking TSC2 or both AMPK subunits α1 and α2, metformin was still able to inhibit mTOR and p70S6K. Instead, using MEFs expressing constitutively activated Rag B, which is a Ras-related GTPase, p70S6K phosphorylation remained intact in the presence of biguanides. This may describe an alternate mechanism by which metformin is able to inhibit mTOR signaling through inhibition of Rag-GTPases and their recruitment and lysosomal activation of mTOR under conditions of amino acid withdrawal.

Irrespective of its exact mechanism of action (summarized in Figure 1:4), numerous laboratory studies have demonstrated that metformin can suppress cellular growth and elicit apoptosis. There is also evidence indicating that metformin selectively targets cancer stem cells, which falls in line with in vivo studies in which metformin inhibited growth of tumour xenografts in mice, and reduced the incidence and size of mammary tumours in Her2/neu transgenic mice. Thus, the ability of metformin to inhibit proliferation in cancer cells, as has been demonstrated in several laboratory and preclinical studies, represents an exciting benefit that it can confer when potentially combining with cancer therapy in future clinical trials.

1.5.3 Limitations of preclinical studies

Many of these studies, however, have faced criticism for their use of metformin concentrations several fold higher than those achievable clinically, which may have
overestimated the degree of energetic stress and AMPK activation feasibly conferred by metformin\textsuperscript{243}. \textit{In vitro} studies investigating metformin commonly use concentrations ranging from 1.0mM to excess of 20mM, while preclinical models are often given metformin at doses of 250mg/kg: these dosages are many-fold higher than are safely recommended for diabetic patients, and unfeasible for clinical implementation. However, inter-species allometric scaling dictates that larger organisms often require smaller drug doses on a mg/kg basis than their smaller counterparts, due to their different pharmacokinetic processes, metabolic rates, size and protein binding, among other factors. By allometric scaling, doses administered to mice are 12.3 times greater than their equivalent human mg/kg dose, such that mice injected with 250mg/kg metformin receive the same dosage of metformin as a diabetic patient receiving approximately 1000mg of metformin daily, which is a dose well within the clinically recommended range\textsuperscript{244}. Nonetheless, metformin has been reported to slow tumour growth in mouse models when administered at doses as low as 50mg/kg, which is promising for its potential clinical benefits when administered at tolerable doses\textsuperscript{204,242}. Considerations regarding an appropriate dose for metformin use in cancer treatment remain the subject of many preclinical studies and an important question that must be addressed prior to clinical transition.

Another limitation of inhibitory effects of metformin as reported \textit{in vitro} is that they may be misrepresented as a consequence of cell culture models used. Cancer cells are grown in media containing extremely high concentrations of glucose and growth factors, and thus are highly unrepresentative of physiological conditions. Under these conditions, which are optimized for maximal growth and proliferation, higher concentrations of metformin may be required to elicit growth-inhibitory effects than would be necessary under physiological conditions\textsuperscript{243,245}. In support of this, Menendez and colleagues reported increased sensitization of HER2-positive and triple-negative breast cancer cell lines to metformin under glucose-starved conditions\textsuperscript{245}. More recently, it was reported that the ability of metformin to induce AMPK and inhibit proliferation of pancreatic cancer cells was exacerbated when cultured in low-glucose medium\textsuperscript{206}; similar results were reported in a nonresponsive breast cancer cell line, whereby metformin’s growth-inhibitory effects were enhanced in normoglycemic medium\textsuperscript{246}. These results may indicate contextual synthetic lethality between metformin-induced growth inhibition and glucose deprivation, such as found in the tumour microenvironment, and underscore the importance of physiologically representative \textit{in vitro} models. Nevertheless, in spite of the limitations of
preclinical methods used to address metformin’s direct inhibition of cancer cells, it is evident that metformin shows great promise as an anti-proliferative agent that may improve response to treatment modalities by slowing regrowth of resistant tumour cells and decreasing relapse rates.

Figure 1: Proposed mechanisms by which metformin inhibits cancer cell proliferation. Metformin alters cellular energy charge through its direct respiratory inhibition, activating AMPK. Among its promotion of catabolic processes, its negative regulation of mTOR via phosphorylation of TSC2 and RAPTOR inhibits its promotion of cellular growth and proliferation through its downstream effectors 4EBP1 and p70S6K. AMPK can also phosphorylate p53, which regulates cell cycle and can induce metabolic checkpoint at G1 as well as apoptosis. Induction of p53 by metformin is controversial but has been demonstrated in a REDD1-dependent manner. REDD1 can also negatively regulate mTOR, which may constitute an alternative mechanism by which metformin can oppose mTOR signaling. Metformin may also inhibit the RAG GTPases, which activate and recruit mTOR under conditions of amino acid withdrawal, and exert its antiproliferative activity in this mechanism as well. Metformin also inhibits proliferation indirectly by decreasing systemic glucose and insulin levels. This reduces binding of insulin or IGFs to their receptors, and downstream signaling through the PI3K-Akt axis, which ultimately promotes mTOR activity. Abbreviations: OCT, organic cation transporter; MATE, multidrug and toxin extrusion; REDD1, regulated in development and DNA damage responses 1; LKB1, liver kinase B1; AMPK, 5’AMP-activated protein kinase; TSC2, tuberous sclerosis 2; RAPTOR, regulatory-associated protein of mTOR; mTOR, mammalian target of rapamycin. 4EBP1, 4E binding protein 1; p70S6K, p70S6 kinase; IGF, insulin-like growth factor; IRS, insulin receptor substrate; PI3K, phosphoinositide-3-kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate.
1.6 Mechanisms by which metformin can affect radiation response

1.6.1 Metformin increases intrinsic radiosensitivity

With a number of retrospective studies indicating that metformin use can improve outcome following radiation therapy (RT), and preclinical studies corroborating these findings in vivo, a limited number of studies have been carried out to determine whether metformin renders cancer cells more sensitive to radiation toxicity. In these studies, it was observed that metformin increases radiosensitivity in some cell lines; however the effect appeared both cell line and context-dependent. Possible candidates mediating radiosensitization by metformin may include AMPK and p53, which have been implicated in mitigation of genotoxic stress in addition to acting as downstream effectors of metformin. Sanli et al. reported induction of AMPK following IR treatment in lung carcinoma cell lines differing in LKB1 status. AMPK is likely phosphorylated by the DNA damage-sensing kinase ATM following IR. Activation of AMPK by ATM in response to genotoxic stress has been demonstrated using a patient-derived cell line lacking ATM, cells transfected with antisense expression constructs to quench ATM expression, and treatment with a chemical inhibitor specific to ATM\textsuperscript{247,248}. Sanli et al found that metformin treatment enhanced AMPK activation and decreased clonogenic survival following irradiation, while treatment with Compound C, an AMPK inhibitor, increased clonogenicity. Together, AMPK activation by metformin was concluded to contribute to radiosensitivity\textsuperscript{249}. Another study investigated the relationship between AMPK and intrinsic radiosensitivity more closely, using cell lines originating from fibrosarcoma and breast. Both cell lines were radiosensitized in the presence of metformin, and upon siRNA knockdown of AMPK in both cell lines, clonogenic survival was increased following combined metformin and IR treatment. These findings, whereby loss of AMPK promoted activity of mTOR and its downstream effectors to elicit survival and proliferation, were consistent with those reported by Sanli and colleagues\textsuperscript{237}. Thus, loss of negative relation of mTOR and its activation of pathways promoting survival and radiation tolerance may well constitute a mechanism by which cancer cells lacking functional AMPK signaling remain more resistant to IR even in the presence of metformin, and is in line with previously characterized signaling pathways. In line with these data, AMPK has also been shown as necessary to restrain metabolism and promote survival in irradiated cells by our lab,
but only under low-glucose conditions\textsuperscript{250}. Thus, the role of AMPK following irradiation warrants further clarification.

The combination of metformin and IR was also reported to increase DNA damage and cell cycle arrest in cancer cells\textsuperscript{249,251}, prompting Skinner and colleagues to address a possible interaction between radiation response and the cell cycle regulator p53. Using a panel of 38 cell lines derived from patients with head-and-neck squamous cell carcinoma (HNSCC), it was demonstrated that cell lines harbouring \textit{TP53}-disruptive mutations were more intrinsically radioresistant compared to those with wild-type or non-disruptive \textit{TP53} mutations. Interruption of p53 using stably-expressed short-hairpin RNA (shp53) increased clonogenic survival of cell lines with wild-type p53, while it decreased that of cells with disruptive mutations. Furthermore, treatment with metformin in combination with IR was able to selectively increase radiosensitivity both in cell lines harbouring p53-disruptive mutations and in those having lost wild-type p53 through shRNA knockdown. In a corresponding retrospective study of 74 patients with HNSCC, p53-disruptive mutations were also associated with decreased survival and increased rates of loco-regional recurrence (LRR), while metformin use was independently associated with lower rates of LRR. From these results, the authors concluded that p53-disruptive mutations in HNSCC were gain-of-function (GOF) with respect to radiosensitivity, and were associated with poor outcome in patients. Loss of wild-type p53 rendered cell lines more radioresistant, through loss of cell cycle regulation and elimination via apoptosis, and selectively disadvantaged cells in the presence of metformin due to their differential stress response\textsuperscript{181}.

As discussed earlier, it is uncertain whether direct p53 activation is required for metformin to elicit growth arrest and cell death in cancer cells. Following irradiation however, p53 is a critical player in the cellular DNA damage response (DDR) \textit{(illustrated in Figure 1:5)}. To protect against DNA damage, such as that inflicted by IR, the sensor kinase ATM is rapidly activated via auto-phosphorylation at Ser-1981 and is released as an active monomer to initiate cell cycle changes and DDR\textsuperscript{222,252,253}. ATM phosphorylates many targets following IR, including as p53 at serine-15/20 of its N-terminal domain, which interrupts its interaction with MDM2 and thus prolongs its cellular half-life\textsuperscript{254,255}. Phosphorylation of p53 by ATM results in its stabilization as well as modification of its biochemical functions required for increased activity as a transcription factor, leading to an adaptive response that is determined based on the source,
intensity and duration of cellular stress encountered. Induction of apoptosis or cell cycle arrest depends on p53 abundance, specific post-translational modifications, and interaction with different protein partners, such as p21. Activation of p21 by p53 yields inactivation of cyclin-

E-CDK2 complexes and subsequently arrests cells at the G1/S transition. This prevents replication of any damaged DNA before entry into S phase, allows for DNA repair mechanisms to take place, and permits cells with severe DNA damage to be eliminated via apoptosis.

It is interesting that loss or aberration of wild-type p53 signaling was demonstrated by Skinner et al. to increase radiosensitivity by metformin, particularly as p53 loss has generally been associated with an increasingly radioresistant phenotype. Loss of wild-type p53 alone, however, was shown by this group to increase intrinsic radioresistance, which is consistent with characterized mechanisms. It is also worth noting that in some instances, p53 loss has been associated with either no change or an increasingly radiosensitive phenotype. The discrepancy may be attributed to cellular dependency on apoptosis, with p53-mediated radioresistance arising in cells primarily reliant upon apoptosis for cell death, as opposed to necrosis.

Previously, our group was unable to demonstrate the radiosensitizing effect of metformin in HCT116 colon carcinoma cells. Thus, the context in which metformin can affect intrinsic radiosensitivity remains unclear. However, AMPK and p53 have been identified as potential candidates to mediate an interaction between radiation and metformin.
Figure 1:5 A simplified schematic of the DNA damage response. DNA damage inflicted by IR activates ATM, which phosphorylates p53 directly to enhance its transcriptional activity and disrupt its association with MDM2. ATM can also phosphorylate AMPK, which can interact with p53 to induce metabolic arrest at G₁ checkpoint. Induction of p53 can also induce apoptosis. Dependent on the extent of DNA damage, activation of ATM, and possibly AMPK and p53, may promote DNA repair processes to ensure cell survival. These effects constitute an adaptive response likely dependent on cell type and magnitude of cellular stress encountered. Abbreviations: IR, ionizing radiation; ATM, ataxia telangiectasia mutated; MDM2, mouse double minute 2 homolog; AMPK, 5’AMP activated protein kinase.
1.6.2 Metformin alters the tumour microenvironment to abrogate chronic hypoxia

Factors other than intrinsic radiosensitivity can also determine biological outcomes following radiotherapy, to which metformin can contribute favourably. Recently, an alternate mechanism by which metformin can improve radiation response has come to light from our laboratory. In this mechanism, tumour (xenograft) hypoxia was reduced after metformin administration, presumably because reduced cellular oxygen consumption in cancer cells proximal to the tumour vasculature promoted oxygen penetration throughout the tumour. In this study, the ability of metformin to reduce oxygen consumption was confirmed in an *in vitro* setting by directly measuring oxygen consumption rates (OCR) of cancer cells treated with metformin. Increased oxygenation of established xenografts in mice injected with metformin was demonstrated by immunostaining for co-localization of pimonidazole and EF5 (2-(2-nitro-1H-imidazole-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide), which are 2-nitroimidazole probes used to label viable hypoxic cells *in vivo* due to their forming adducts with thiol groups only in hypoxic cells.\(^{266,267}\) Finally, the effect of metformin use upon tumour radiation response was assessed in a growth delay experiment, whereby mice receiving metformin and irradiation in combination experienced significantly increased growth time and survival compared to control mice. However, in contrast to *in vitro* studies outlined above,\(^{181,237,249}\) *ex vivo* clonogenic assays performed by this group provided no evidence indicating that metformin was able to increase radiation sensitivity directly. The authors of this study concluded that improved radiation response following metformin use can be secondary to its inhibition of cellular oxygen consumption and reduced tumour hypoxia.\(^{182}\)

1.6.2.1 Hypoxia as a contributor to radiation resistance

Hypoxia arises when tumour development occurs faster than its supporting vasculature can expand. Furthermore, the tumour vasculature is poorly organized and developed, rendering it unable to provide sufficient nutrients or oxygen. Tumour cells located at a distance greater than approximately 150 μm from blood vessels will usually experience diffusion-limited, or chronic, hypoxia, as supplied oxygen is consumed by cell layers more proximal to the vasculature.\(^{268}\) Hypoxia can also arise due to temporary occlusion of blood vessels by tumour cells, or collapse in regions of high interstitial pressure, which is perfusion-limited, or acute, hypoxia.\(^{269,270}\) Thus,
tumours beyond 1mm in diameter possess hypoxic regions\textsuperscript{271}. While normal tissue have median oxygen concentrations ranging from 40-60 mmHg, fewer than 10\% of all solid tumours have oxygen levels in this range; conversely, half of all solid tumours have concentrations of less than 10 mmHg. Using oxygen electrode measurements, low oxygen levels have been identified in many human solid tumours, including those originating from the brain, head-and-neck, breast, cervix, and soft tissue sarcomas\textsuperscript{270}.

During RT, IR is delivered to the tumour site. Photons excite electrons within cellular molecules and yield an ionization cascade that subsequently deposits energy into macromolecules to form free radicals and inflict cellular damage. Cell kill resulting from IR is caused by damage to DNA. Radiation lesions are generated in DNA following either direct ionization or indirect reaction with hydroxyl radicals, which are produced from radiolysis of surrounding water molecules. These become fixed in the presence of oxygen, as oxygen reacts with the DNA radical to generate a chemically stable organic peroxide and inflict irreversible damage. In the absence of oxygen, DNA radicals can potentially be restored to their original form through reaction with reducing species, such as thiols, leading to cell survival following treatment\textsuperscript{272}. Full restoration of restorable DNA damage lesions occurs when oxygen levels are below 0.2 mmHg, while at oxygen levels between 0.2 and 20 mmHg, competition occurs between fixation and restoration\textsuperscript{273–275}. Anoxic cells within tumours represent those maximally resistant to radiation, as they require an IR dose approximately 3 times higher than that of normoxic cells in order to induce the same level of cell kill\textsuperscript{276}. Clinically, tumour hypoxia is a negative prognostic factor: it is predictive of worse outcome in patients treated for head-and-neck (HN), uterine cervix, prostate, or soft tissue sarcomas treated with RT\textsuperscript{277–280}, though poor outcome has also been demonstrated in other treatment modalities\textsuperscript{281}.

1.6.2.2 Approaches to address hypoxia clinically

Approaches to overcome hypoxia in an effort to improve radiotherapy efficacy have focused on improving tumour oxygenation by increasing oxygen supply. In the past, hyperbaric oxygen therapy (HBO) was used as a method of increasing oxygen supply to tumours, by having patients breathe in air containing high pressures of oxygen\textsuperscript{282}. This practice was abandoned due to inconclusive results in clinical studies, patient discomfort leading to non-compliance, and the unfortunate explosion of a pressure chamber\textsuperscript{282}. Another approach to increase oxygen supply to
tumours is the use of carbogen (95% oxygen and 5% carbon dioxide) during radiation therapy\textsuperscript{283,284}. More recently, carbogen treatment was combined with administration of a vasodilator, nicotinamide, in an effort to improve tumour perfusion. When assessed in a Phase III clinical trial comprising 333 patients with bladder carcinoma, use of carbogen and nicotinamide in conjunction with RT modestly improved cystoscopic control, and significantly improved local relapse-free survival (RFS), urinary and rectal morbidity, and overall survival (OS), compared to use of RT alone\textsuperscript{285}. In another Phase III clinical trial involving 345 laryngeal cancer patients, addition of carbogen and nicotinamide to RT significantly improved regional control, and this benefit was observed particularly in patients with hypoxic tumours\textsuperscript{286}. In a meta-analysis of 32 randomized trials involving almost 5000 head-and-neck squamous cell carcinoma (HNSCC) patients being treated with curative radiotherapy, inclusion of a hypoxic modifier alongside therapy improved loco-regional control and disease-specific survival compared to control cases receiving radiotherapy alone\textsuperscript{287}. This suggests that hypoxia not only correlates with poor outcome following RT, but functionally contributes to it as well.

Mathematical modeling has suggested that increasing oxygen supply is a relatively inefficient method of reducing tumour hypoxia; instead, lowering oxygen demand within tumours should decrease hypoxic fraction substantially and more efficiently\textsuperscript{288}. Thus, use of pharmaceutical agents that target and inhibit cellular respiration, such as metformin, represent feasible approaches to improve response to RT. This has already been explored \textit{in vitro}, using spheroid models containing hypoxic, radioresistant cells in their centers, whereby treatment with various respiratory inhibitors, including oligomycin, rotenone, and several nitrobenzene derivatives, led to radiosensitization\textsuperscript{289–291}. Furthermore, use of respiratory inhibitors arsenic trioxide and meta-iodobenzylguanadine was demonstrated to improve radiation response in preclinical models, and are approved for clinical use\textsuperscript{292,293}. However, their toxicity renders them infrequently prescribed and thus unsuitable for combination with curative radiotherapy. Metformin, meanwhile, is a respiratory inhibitor both widely available and generally well tolerated, and thus represents a strong candidate for repurposing as a hypoxic modifier for future use in the clinic to improve response in patients with hypoxic tumours receiving RT.
1.7 Translational challenges concerning metformin use in cancer

1.7.1 Tumour uptake

In implementing metformin as a potential anticancer agent or adjuvant to current treatment modalities, factors influencing the ability of metformin to act on tumours directly must be evaluated. As the majority of mechanisms detailing its anticancer activity require its presence directly within tumour cells, the efficacy of metformin in cancer treatment likely would be limited by its tumoural uptake.

The ability of metformin to act upon cancer cells may well be affected by their expression of OCT and MATE transporters, which control metformin uptake and excretion on a cellular level and affect its pharmacokinetics on a systemic level. As metformin’s preferential activity upon the liver has been attributed to its high expression of OCT1, it is plausible to speculate that expression of OCTs in particular would greatly contribute to the ability of metformin to exert its effects upon cancer cells directly (Figure 1:6).

1.7.2 Regulation of OCT and MATE expression in cancer

As metformin entry into cancer cells depends upon OCTs, and its excretion is dependent upon MATEs, one limitation facing its use in cancer is the regulation of these transporters during cancer development. Regulation of OCT1 expression in human liver is mediated by hepatocyte nuclear factor-4α (HNF-4α), which interacts with 2 corresponding response elements in the promoter of SLC22A1 to activate transcription\(^{77,294}\). It was also demonstrated that HNF-4α-mediated transcription is inhibited by chenodeoxycholic acid, through the action of a small heteromeric partner (SHP) of the bile-acid-inducible transcriptional repressor. This has been speculated to be the mechanism causing decreased hepatic expression of OCT1 in cholestasis, whereby increased levels of bile acids may inhibit HNF-4α-mediated activation of OCT1 transcription\(^{77,294}\). Similar results have been reported for OCT3, wherein hepatic expression is significantly downregulated in cholestatic patients\(^{115}\), suggesting a similar mechanism of regulation. Furthermore, microarray analyses have indicated that OCT1 protein expression is downregulated in hepatocellular carcinoma (HCC) relative to adjacent normal tissue\(^{295,296}\), and comparison of OCT1 gene expression in healthy liver tissue and human hepatocarcinoma cell lines indicated that its expression was almost completely lost in cancer cell lines\(^{297}\). A further
study by Schaeffeler et al. uncovered a significant inverse correlation between expression of \textit{SLC22A1} at the mRNA level, and DNA methylation of individual CpG sites within the \textit{SLC22A1} promoter region, suggesting transcription of OCT1 is restricted by hypermethylation occurring in cancer development\textsuperscript{298}. Downregulation of OCT2 has been observed in human renal carcinoma cell lines\textsuperscript{297}; as well, it has been reported that DNA methylation of the \textit{SLC22A2} proximal promoter controls kidney-specific expression of OCT2\textsuperscript{299}, perhaps in a manner similar to the epigenetic regulation of OCT1 transcription. No studies have yet investigated factors affecting expression of MATE1 and MATE2.

Apart from downregulation during cancer development, an alternative mechanism of OCT expression has also been described. In studies involving rats, it was demonstrated that the expression of OCT2 is regulated by steroid hormones and is gender dependent. Urakami et al demonstrated that there was greater expression of OCT2 at the mRNA and protein level in male rat kidneys compared to their female counterparts, and that OCT2 transporters in the male rats had higher basolateral uptake of the cationic substrate TEA into proximal tubule cells\textsuperscript{300}. Moreover, treatment with testosterone in female rats yielded increased protein expression of renal OCT2, while treatment with estradiol in male rats produced the opposite effect and downregulated renal OCT2 expression\textsuperscript{301}. In contrast, a subsequent study using rabbits indicated that both male and female rabbits expressed renal OCT2 equally at the protein level, and had equivalent basolateral uptake of TEA into renal proximal tubule cells\textsuperscript{302}. No studies have yet been carried out to confirm whether human OCT2 expression is similarly steroid-dependent; however clinical studies in healthy and diabetic patients do not indicate a gender disparity in OCT2 expression or function\textsuperscript{122,129,303}. Thus the steroid dependency of OCT2 expression is likely species-dependent.

Apart from downregulation during cancer and cholestasis, and possible steroid dependency that is likely species-dependent, no other factors are known to regulate expression of OCTs and MATEs. Nonetheless, evidence of downregulation of these transporters during cancer development is a subject of some concern, particularly as a lack of expression of these transporters in cancer cells can render the direct beneficial effects of metformin moot if it cannot enter its target cells. Online databases such as the Cancer Cell Line Encyclopedia (CCLE)\textsuperscript{304} have made gene expression data for hundreds of different cancer cell lines publicly available, however, and provide an excellent resource for assessing expression levels of these transporters.
in cancer cells. Expression data for these transporters have also been made available for normal human tissue through the Genotype-Tissue Expression (GTEx) project\textsuperscript{305}, facilitating some comparison between expression profiles of normal and cancerous specimens (Figure 1:7). Though direct comparison of gene expression between normal and cancer cells is difficult due to the use of relative expression values arising from different detection methods and normalization techniques, it is evident that cancer cell lines express these transporters at levels at least sufficient for detection through microarray analysis, with some cancer types expressing them at relatively high levels. Though certain tissue types may undergo downregulation of transporter expression in carcinogenesis, such as seen in OCT1 expression in the liver, these data may indicate sufficient expression of these transporters in order to facilitate uptake of metformin into cancer cells in order to exert its direct anticancer effects.

1.7.3 Identification of biomarkers

With metformin entering clinical trials for use as a potential anticancer agent or adjuvant to current treatment modalities, it is important to develop appropriate biomarkers in order to stratify the patient population. In considering the use of metformin as an adjunct to radiotherapy, as has been the subject of focus throughout this literature review, the benefits offered by metformin are varied yet specific. As its use represents a targeted approach to improve patient response, it is particularly important to identify patients most likely to benefit. Identification of appropriate biomarkers may require a deeper understanding of mechanisms behind metformin’s specific anticancer effects in order to define specific characteristics responsible for conferring response.

Its indirect inhibition of tumour cell proliferation through reduction of systemic insulin levels is likely to provide benefit in patients having elevated insulin levels to begin with, or with tumours expressing the insulin receptor. Its direct inhibition of cancer cell proliferation can benefit treatment response irrespective of treatment modality due to reduced regrowth rates, provided that it is able to achieve sufficient intracellular concentrations and suppress necessary growth pathways. Based on current literature, it is possible that impaired AMPK or p53 signaling may negatively impact the ability of metformin to exert its antiproliferative activity, though conflicts in existing data suggest possible involvement of mutations and/or hyperactivation in other signaling pathways that have not been explored in this review. As not all cancer cell types
investigated were responsive to growth arrest following metformin, it is necessary to identify factors that can determine response to this particular effect of metformin, especially in the context of data indicating that metformin use was able to increase proliferation and growth of certain tumours\textsuperscript{306}.

The effect of metformin upon intrinsic radiosensitivity of cancer cells has yet to be clearly characterized, however administering metformin for such a purpose to improve RT response would require identification of factors that contribute to increased radiosensitization. Though much remains to be elucidated about the interaction between metformin and radiation response, previous studies have identified AMPK and p53 as possible contributing candidates.

The recently demonstrated ability of metformin act as a hypoxic modifier through its suppression of oxygen consumption in cancer cells proximal to the tumour vasculature could reasonably improve radiation response in patient tumours having high levels of chronic hypoxia.

For anticancer benefits of metformin that require its intracellular accumulation, such as its direct inhibition of cancer cell proliferation, suppression of oxygen consumption, and possible direct radiosensitization, consideration of factors affecting metformin uptake into tumours is also necessary. As microarray data have indicated that metformin transporter expression is not fully lost in cancer development, these can play an important role in determining the extent of metformin’s direct effects.
**Figure 1:6** OCT and MATE expression control intracellular accumulation of metformin. High net accumulation facilitated by high OCT and low MATE expression may predict for increased sensitivity, while low net accumulation through low OCT and high MATE expression may predict for decreased sensitivity to metformin.
B) 

**SLC22A2 expression (normal tissue)**

**SLC22A2 expression (carcinoma tissue)**
Figure 1:7 Expression of OCTs and MATEs in normal and cancer cells. Comparison of OCT1 (A), OCT2 (B), OCT3 (C), MATE1 (D), and MATE2 (E) expression profiles across normal (top) and cancerous (bottom) tissues. Modified from GTEx\textsuperscript{305} and CCLE\textsuperscript{304} expression data. Abbreviations: RPKM, reads per kilobase transcript per million reads.
2 Objective, Aims & Hypotheses

Recent epidemiological and retrospective studies have indicated metformin as having a preventative or therapeutic effect in cancer. Observations of decreased cancer incidence in diabetic patients receiving metformin compared to the general population \(174,175\), coupled with retrospective studies demonstrating increased pathological response and disease free survival (DFS) in metformin users receiving chemotherapy either alone or in combination with radiation therapy \(178-181\), have spurred interest in repurposing this antidiabetic drug for use in cancer. The ability of metformin to inhibit cancer cell proliferation has been well studied, with numerous laboratory studies demonstrating its inhibition of cancer cell proliferation \textit{in vitro}, and delaying of tumour growth \textit{in vivo}. Its ability to slow cancer cell growth constitutes one mechanism by which it can improve outcome following treatment.

Retrospective data have indicated that patients receiving metformin also exhibit improved outcome following radiation therapy (RT). Increased DFS has been reported in cohorts of head-and-neck \textsuperscript{307} and castrate-resistant prostate \textsuperscript{172,182} cancer patients using metformin. This has been supported by laboratory evidence demonstrating that metformin may delay tumour growth following irradiation \textsuperscript{237,308}, as well as several reports suggesting that it can directly radiosensitize cancer cells \textit{in vitro} \textsuperscript{181,237,249}. Its respiratory inhibition has also been linked to reprogramming of cancer cell metabolism and alteration of tumour microenvironment to abrogate tumour hypoxia \textsuperscript{182}.

Radiation response is largely determined by 3 biological components: intrinsic radiosensitivity, proliferation, and hypoxia. There is evidence indicating that metformin can affect all of these factors and potentially improve radiation response in 3 distinct manners, thus providing a strong rationale for its inclusion in RT. However, it is necessary to identify biomarkers of these specific effects of metformin in order to justify its clinical use, as patients receiving metformin must be appropriately stratified.

Thus, the objective of this thesis is to assess factors that can determine sensitivity to, and act as biomarkers of, metformin’s radiosensitization, inhibition of proliferation, or hypoxic modification.
Specific Aims

Aim 1: Determine whether metformin’s direct radiosensitizing or anti-proliferative effects are mediated by AMPK- or p53-dependent signaling

The ability of metformin to increase radiosensitivity in cancer cells has been reported in several studies. Metformin was able to decrease clonogenic survival following metformin treatment in breast (MCF-7), fibrosarcoma (FSall)\textsuperscript{237}, lung (A549, NCI-H1299)\textsuperscript{249} and patient-derived head-and-neck cancer cell lines\textsuperscript{181}. In contrast, previous experiments in our lab using HCT116 colon carcinoma cells provided no evidence of radiosensitization by metformin\textsuperscript{182}. The ability of metformin to directly radiosensitize cells thus remains unclear.

In the literature, AMPK proficiency was suggested to contribute to mediating metformin’s radiosensitizing effect, as siRNA knockdown of AMPK mitigated the ability of metformin to exacerbate decreased cell survival\textsuperscript{237}. Additionally, p53-disruptive mutation, or loss of wild-type p53, was demonstrated to increase the ability of metformin to radiosensitize HNSCC cell lines\textsuperscript{181}. These data are consistent with a model in which AMPK-dependent p53 activation protects cells against radiation toxicity, analogous to a previously demonstrated metabolic checkpoint. As discussed in the previous chapter, AMPK can limit metabolism and proliferation of cells undergoing metabolic stress, and its activation by metformin has been well characterized. Its requirement was also demonstrated for mediating survival in irradiated cells, indicating that its presence may facilitate an interaction between radiation and metformin. AMPK has been shown to limit cell growth through both induction of a p53-dependent G\textsubscript{1}/S checkpoint and restriction of mTOR activity. Moreover, p53 is highly involved in radiation response, as its activation controls cell cycle arrest or apoptosis during DNA damage response. Cells deficient in p53 were also selectively susceptible to metformin toxicity, suggesting that p53 may also mediate metformin and radiation interaction.

Given that AMPK- and p53-dependent signaling control cell growth, mediate adaptive responses to genotoxic and metabolic stresses conferred by metformin and irradiation, and were reported to modulate radiation sensitivity by metformin in previous studies, we hypothesize that AMPK and p53 are determinants of metformin’s direct radiosensitizing and anti-proliferative effects.
Aim 2: Assess whether expression of OCTs and MATEs can determine sensitivity to metformin’s respiratory-inhibiting or anti-proliferative effects

The primary effect of metformin results in inhibition of cellular oxygen consumption and alteration of cellular redox state\textsuperscript{19,20,67}. These specific effects of metformin may depend upon its intracellular accumulation, which is facilitated by OCT and MATE transporters.

*Given that OCTs and MATEs control metformin uptake and excretion, we hypothesize that they contribute to unique cancer cell sensitivity to the respiratory inhibition and anti-proliferative effect of metformin.*
3 Methods

3.1 Cell culture

In Aim 1, A549 lung carcinoma and HCT116 colon adenocarcinoma cell lines were obtained from American Type Culture Collection (ATCC) and cultured in DMEM:F12 and RPMI 1640 media respectively. Paired ras-transformed mouse embryonic fibroblasts (MEFs) either wild-type or lacking both \(\alpha_1\alpha_2\)-subunits of AMPK were a kind gift from Dr. Keith Laderoute\(^{309}\), and were cultured in DMEM media. Paired HCT116 cells either wild-type or lacking p53 were kindly provided by Dr. Bert Vogelstein\(^{310}\), and were cultured in McCoy’s 5A media. All cells were cultured in media supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

For Aim 2, a panel of carcinoma cell lines representing different sites of origin was assembled. HCT116 colorectal carcinoma and LNCaP prostate carcinoma cells were cultured in RPMI 1640 media. HepG2 hepatocarcinoma and RCC4 renal carcinoma cells were cultured in DMEM media. HeLa and ME-180 cervical carcinoma cell lines were both cultured in alpha-MEM media. A549 lung carcinoma cells were cultured in DMEM:F12 medium. The head-and-neck cell lines FaDu, SCC-16A, SCC-16B, SCC-19A, SCC-19B, SCC-74A, and SCC-74B were all cultured in MEM-F15 medium. All of the aforementioned cell lines were cultured as adherent monolayers kept in exponential growth phase, in growth medium supplemented with 10% fetal bovine serum. POP-092S primary colon adenocarcinoma cells were cultured in suspension in growth media to enrich for stem cells. All cell lines were obtained from ATCC, with the exception of POP-092S, which was established at Princess Margaret Cancer Centre, and the patient-derived head-and-neck cell lines (SCC-16A/B, SCC-19A/b, SCC-74A/B), which were generously provided by Dr. R. Grenman from Turku University Hospital (Finland).

3.2 Oxygen consumption rates

Cells were seeded into 96-well plates and incubated for 24h prior to measurement of oxygen consumption rate (OCR) with the Seahorse XF96 Extracellular Flux Analyzer. Basal OCR was first measured in untreated cells for 30 min; thereafter, inhibition of OCR was monitored following injection of metformin into the wells for up to 8h. OCR measurements were
normalized to cell number, as quantified using the CyQUANT NF Proliferation Assay kit. Inhibition of OCR was measured by normalizing to basal OCR, and comparing against that of an untreated control. This protocol was used for both Aims 1 and 2, with metformin final concentrations of 5mM used in Aim 1, and 0.02mM, 0.5mM, 2mM, and 10mM used in Aim 2.

3.3 Metformin treatment, irradiation and clonogenic assays

Cells were seeded, incubated for 24h, and treated with 5mM metformin. After 24h in metformin, cells were irradiated with 4Gy or 8Gy. 1h post irradiation, cells were isolated for protein analysis. 24h post irradiation, cells were harvested, counted and seeded in single-cell suspension for clonogenic survival. After 10-14 days growth in metformin-free media, colonies were fixed, stained (0.2% methylene blue in 80% ethanol) and counted. Plating efficiencies (PEs) were calculated by dividing the number of colonies counted by the number of cells seeded. Surviving fractions were calculated as the fraction of plating efficiencies for a treated over untreated sample.

3.4 Immunoblotting

Cells were treated with 5mM metformin for 24h prior to exposure to 4Gy ionizing radiation. 1h following irradiation, protein was extracted using RIPA lysis buffer (Tris-HCl: 50mM, pH 7.4; NP-40: 1%; Na-deoxycholate: 0.25%; NaCl: 150mM; EDTA: 1mM), resolved on 10% SDS-PAGE and transferred to a nitro-cellulose membrane. Membranes were probed using antibodies against AMPK (Cell Signaling Technology Inc., #2532), phospho-ACC (Ser-70) (Cell Signalling, #3661), tubulin (Abcam Inc., #6046) and p53 (Santa Cruz Biotechnology Inc., #6243), followed by anti-rabbit-HRP secondary antibody (GE Healthcare #NA931V), all diluted 1:1000. Protein was detected using an enhanced chemiluminescence assay (Thermo Scientific).

3.5 RNA extraction and qPCR

RNA was isolated from cells with TRIzol reagent and reverse transcribed into cDNA. Subsequently, qPCR was performed to measure expression of OCT1, OCT2, OCT3, MATE1 and MATE2, using primers that were independently designed and validated (Appendix 1). All experiments were performed in triplicate and repeated independently 3 times. RPL13a, HPRT, Y-WHAZ and HSP90AB1 were used as reference genes, and the resulting Ct values were
analyzed using the standard curve method. To account for variation in expression of housekeeping genes across the cell panel, the highest- and lowest-expressed reference genes were omitted, and the remaining two were averaged to generate a normalizer value, to which the expression of each target gene was normalized. These transformed expression values were then centered around the average expression of each gene measured across the cell panel, which was set to 1, in order to yield a final relative expression value.

### 3.6 Proliferation assays

Cells were seeded into black, clear-bottom 96-well assay plates and incubated for 24h, prior to treatment with serially increasing concentrations of metformin. Doses of 1mM, 5mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM, and 50mM were used. After 72h exposure to metformin, the cells were then mixed with alamarBlue® fluorescence reagent and incubated for 4h. The resulting fluorescence intensity was read, and inhibition of proliferation was calculated by normalizing to an untreated control.

### 3.7 Statistical analysis

For Aim 1, two-sided $t$ tests were used to calculate statistical significance, which was set at $p<0.05$. In Aim 2, statistical testing was performed using GraphPad Prism software. Pearson’s correlation coefficient $r$ was used to measure linear dependence between the tested variables. Correlations were deemed significant when $p<0.05$. 
4 Results

4.1 Aim 1: Role of AMPK and p53-dependent signaling in response to metformin and radiation

We first sought to determine whether metformin was able to function as a radiosensitizer, as had been described by others in the literature. To this end, we used A549 lung carcinoma cells, which were previously reported to have been radiosensitized by metformin, as well as HCT116 colon carcinoma cells. Our group had previously found no radiosensitization in HCT116 cells after 30 minutes exposure to metformin; therefore, in these experiments we increased exposure time to reproduce protocols that were shown to be radiosensitizing by others. Cells were treated with 5mM metformin 24h prior to irradiation with 4Gy or 8Gy, and 24h thereafter were seeded for colony-forming assays, which detect all cells retaining long-term clonogenic capacity following irradiation, and thus measure cell survival regardless of mechanism of reproductive cell death. Metformin treatment alone decreased clonogenic survival substantially in HCT116 cells, but had no discernable effect upon survival of A549 cells (Figure 4:1A). Contrary to previous reports, we were unable to demonstrate that metformin increases radiosensitivity (Figure 4:1B,C). Instead, our data indicated that metformin caused slight radiation protection in both cell lines, which was statistically significant at some dose points.

Both metformin and radiation elicit activation of AMPK and p53 signaling pathways to mediate cellular response to metabolic stress and DNA damage. Furthermore, other groups have shown that AMPK proficiency and p53 deficiency confer sensitivity to radiation in the presence of metformin in other cell types. We therefore investigated the contributions of both proteins in facilitating an interaction between metformin and radiation response. To determine whether AMPK proficiency was required to mediate an interaction between metformin and radiation, we used ras-transformed mouse embryonic fibroblasts (MEFs) which were either wild-type for AMPK (AMPK-WT) or had both α1 and α2 subunits knocked out (AMPK-KO). Immunoblotting confirmed the loss of AMPK in AMPK-KO MEFs (Figure 4:2A). Furthermore, treatment with metformin activated AMPK, as evidenced by phosphorylation of its downstream target ACC, in AMPK-WT MEFs only (Figure 4:2B). To assess whether p53 disruption was necessary to confer synergism between metformin and radiation, we used HCT116 cells that...
were either wild-type (p53-WT) or knockout for both alleles of p53 (p53-KO). As expected, we did not observe p53 stabilization in irradiated p53-KO cells (Figure 4:2C). Treatment with metformin, however, yielded activation of AMPK in both cell lines, and is hence independent of p53 status (Figure 4:2D) as expected. AMPK activation by radiation has been reported by others, but was negligible in both MEFs and HCT116 cells, at least as measured by ACC phosphorylation (Figure 4:2B,D). Taken together, the results presented in Figure 4:2 validated the isogenic cell models used and confirmed that both metformin and radiation treatments activated their canonical signaling pathways.

The ability of metformin to induce metabolic stress and modulate downstream signaling pathways is thought to depend upon the extent of its cellular uptake and mitochondrial inhibition. We therefore determined if AMPK or p53 proficiency impacted basal oxygen consumption rates (OCR) and respiratory inhibition following metformin treatment in these cell lines. Isogenic MEFs and HCT116 cells were assayed for basal OCR measurement, and immediately thereafter were subjected to acute challenge with 5mM metformin. Changes in OCR were measured up to 9h following metformin exposure. Isogenic MEFs had equivalent basal OCR (Figure 4:3A). In HCT116 cells, lower basal OCR was observed in p53-KO cells relative to p53-WT cells, though the difference did not reach statistical significance (p>0.05) (Figure 4:3B). Both pairs did not differ in their response to acute metformin (Figure 4:3C,D). These data indicate that any differences observed in metformin response between the isogenic cell lines are due to differential stress response rather than magnitude of metabolic stress conferred by metformin itself.

Metformin is known to inhibit cell proliferation and has been shown to induce AMPK- and p53-dependent metabolic cell-cycle checkpoints in breast and prostate cancer cell lines. We therefore assessed the roles of AMPK and p53 in mediating inhibition of proliferation in response to metformin in our cell lines. Treatment with 5mM metformin for 48h resulted in a significant decline in cell number in exponentially-growing MEF and HCT116 cells, compared to their untreated counterparts (Figure 4:4A,B). Interestingly, the decline in cell number was exacerbated in MEFs lacking AMPK (Figure 4:4A), as well as in HCT116 lacking p53 (Figure 4:4B). As loss of cells can reflect either decreased proliferation or direct cell loss, we used colony-forming assays to ascertain whether cells remained equally clonogenic following metformin treatment. Cells were treated with 5mM metformin for 48h, counted and plated for clonogenic survival. Treatment with metformin did not affect long-term viability of wild-type
MEFs or HCT116 cells (Figure 4:4C,D), although some variability in the HCT116 data may have precluded interpretation (Figure 4:4D). AMPK-KO MEFs were equally insensitive to the toxic effects of metformin as their wild-type counterparts were (Figure 4:4C). This suggests that the loss in cell number seen in Figure 3:4A was largely a reflection of decreased proliferation, and that AMPK was not involved in mediating long-term survival following metformin treatment. In HCT116, treatment with metformin substantially reduced survival in p53-KO cells (Figure 4:4D). This observation is consistent with previous data demonstrating that metformin selectively inhibited growth of cells lacking p53\textsuperscript{234}, though this effect is unlikely to be universal, due to use of a knockout model that may not recapitulate p53 mutations in which partial functionality or dominant negative activity are retained.

Finally, to understand the interaction between metformin and response to radiation, we subjected both MEF and HCT116 isogenic pairs to colony-forming assays following irradiation with 4Gy 24h after treatment with metformin. Upon correcting the resulting surviving fractions for metformin toxicity (Figure 4:4C,D), we once again found no evidence demonstrating that metformin increased radiation sensitivity (Figure 4:4E,F), which corroborated the results we obtained using A549 and HCT116 cells (Figure 4:1A,B). Instead, as before, metformin-treated cells trended toward radiation protection, and this was consistent in both MEFs and HCT116, as well as independent of AMPK status in MEFs (Figure 4:4E) and p53 status in HCT116 (Figure 4:4F). Importantly however, is that though metformin treatment induced moderate radiation protection in all 3 cell lines tested (Figure 4:1A,B, 4:4E), this was offset by the toxicity of metformin itself for both cancer cell lines (A549, HCT116), resulting in similar levels of overall cell death following radiation both with and without metformin treatment (Figure 4:5A,B). However, this was not observed in MEFs, which demonstrated increased overall survival following irradiation in the presence of metformin (Figure 4:5C). Taken together, we suggest that the direct toxicity and anti-proliferative activity of metformin likely outweigh its radioprotection, and are more important in determining its clinical utility when combined with radiotherapy\textsuperscript{312}. 

\textsuperscript{234}
Figure 4:1 Metformin confers moderate radiation protection in A549 and HCT116 cells.
(A) Clonogenic survival following 48h of treatment with 5mM metformin prior to plating and growth for 10-14 days, in A549 and HCT116 cells. Surviving fractions have been normalized to the untreated control. Mean ± S.E.M., n=6. *p<0.05.
A549 (B) and HCT116 (C) were treated with 5mM metformin for 24h, and then exposed to ionizing radiation (IR) 24h while still in media containing metformin prior to plating for colony-forming assays. Surviving fractions of IR+metformin-treated cells were normalized to those of metformin alone to correct for toxicity. Mean ± S.E.M, n=3-6. *p<0.05.
Figure 4.2 Validation of genetic models.
Immunoblotting confirmed loss of AMPK in MEF AMPKα1,α2-KO cells (A) and phosphorylation of ACC, an AMPK target, in MEF AMPKα1,α2-WT and KO cells following treatment with 5mM metformin for 24h and irradiation with 4Gy (B). Similar experiments were performed with HCT116 isogenic cells, confirming p53 loss in HCT116 p53-KO cells (C), and phosphorylation of ACC in HCT116 p53-WT and KO cells treated with metformin and radiation (D). Representative blots from 3 independent experiments are shown.
Figure 4:3 Metformin inhibits oxygen consumption independently of AMPK or p53.
Basal oxygen consumption rate (OCR) per cell was plotted for MEF AMPKα1α2-WT and KO (A) and HCT116 p53-WT and KO (B) Mean ± S.E.M, n=4. Observed differences in basal OCR in (B) were not statistically significant (p=0.29).
Average OCR following exposure to 5mM metformin was normalized to basal OCR. Change in OCR over 8h was expressed as a percentage of an untreated control in MEF AMPKα1α2-WT and KO (C), and HCT116 p53-WT and KO (D). Mean ± S.E.M., n=4. No statistically significant differences were observed.
Figure 4: Loss of AMPK or p53 promotes metformin’s anti-proliferative and toxic effects. Average number of cells counted after 48h treatment with 5mM metformin, expressed as a percentage of an untreated control for both MEF AMPKα1α2-WT and KO (A) and HCT116 p53-WT and KO (B). Mean ± S.E.M., n=7. Clonogenic survival following 48h of treatment with 5mM metformin prior to plating and growth for 10-14 days, in MEF AMPKα1α2-WT and KO (C) and HCT116 p53-WT and KO (D). Surviving fractions have been normalized to the untreated control. Mean ± S.E.M., n=3-6. Clonogenic survival following combination of 4Gy irradiation and 5mM metformin 24h prior to plating and growth for 10-14 days, in MEF AMPKα1α2-WT and KO (E) and HCT116 p53-WT and KO (F). Surviving fractions have been normalized to metformin alone to correct for toxicity. Mean ± S.E.M., n=3-6, *p<0.05.
Figure 4:5 Radiation protection of metformin is balanced by toxicity of metformin itself in cancer cell lines. Overall clonogenic survival following combination of 5mM metformin and irradiation in wild-type A549 (A), HCT116 (B) and MEF (C). Surviving fractions have all been normalized to the untreated control. Mean ± S.E.M., n=3-6, *p<0.05.
4.2 Aim 2: Contributions of OCTs and MATEs to cancer cell sensitivity to metformin

The beneficial effects of metformin in cancer cells, by directly inhibiting either mitochondrial oxygen consumption or downstream signaling pathways affecting growth and proliferation, require its tumoural uptake. OCT and MATE transporters mediate metformin transport into and out of cells, and can contribute to its intracellular accumulation. We sought to determine whether differential expression of OCTs and MATEs could confer unique sensitivity or resistance to metformin’s inhibition of oxygen consumption and/or proliferation. To this end, we first assembled a panel of carcinoma cell lines in order to validate metformin transporter expression patterns as indicated by expression data publicly accessible through the Cancer Cell Line Encyclopedia (CCLE)\textsuperscript{304}. We first identified commercially available cell lines highly expressing OCT2 (RCC4, renal carcinoma), OCT3 (LNCaP, prostate carcinoma), MATE1 (HepG2, hepatocarcinoma) and MATE2 (FaDu, upper aerodigestive carcinoma) for inclusion in our panel. Furthermore, we included cell lines from cervical (HeLa and ME-180) and head-and-neck (SCC-16A/B, SCC-19A/B and SCC-74A/B) tumour sites as clinically, these solid tumours present with high levels of hypoxia and represent relevant sites for translation of metformin as a hypoxic modifier. Matched pairs of patient-derived head-and-neck cancer (HNC) cell lines originated from the same patient, where A originated from the primary tumour and B was established from metastatic site. HCT116 and POP-092S colorectal adenocarcinoma cells were also used, as our lab had previously demonstrated that these cell lines both formed xenografts and responded to metformin treatment in mitigating hypoxia and improving radiation response \textit{in vivo}. Finally, using the Genomics of Drug Sensitivity in Cancer (GDSC) database\textsuperscript{313}, we identified A549 lung carcinoma cells as being particularly sensitive to metformin toxicity due to its relatively low IC\textsubscript{50} among the >500 carcinoma cell lines screened, and included it in the panel also. In all, our panel constituted of 15 different carcinoma cell lines ranging in site of origin, expected transporter expression and expected sensitivity to metformin.

We next measured metformin transporter expression across the panel using quantitative PCR (\textit{Figure 4:6A-E}). We observed considerable variation in the ranges of OCT and MATE expression patterns, which was consistent with expression data presented through the CCLE. Expression of OCT1, OCT2 and MATE2 ranged 3-fold between lowest and highest-expressing cells (\textit{Figure 4:6A,B,E}), while expression of OCT3 and MATE1 ranged 8-fold across the panel.
Also consistent was the high expression of individual metformin transporters in cell lines selected from the CCLE for their extreme expression: our results corroborated RCC4 as a high OCT2 expresser (Figure 4:6B), LNCaP as a high OCT3 expresser (Figure 4:6C), HepG2 as a high MATE1 expresser (Figure 4:6D), and FaDu as a high MATE2 expresser (Figure 4:6E). Our results also identified RCC4 as having the highest OCT1 expression across the panel (Figure 4:6A).

For cell lines included in both our panel and in CCLE, we observed significant correlation between relative expression values of OCT3 (Pearson’s correlation $r=0.884, p<0.05$), MATE1 (Pearson’s correlation $r=0.932, p<0.05$) and MATE2 (Pearson’s correlation $r=0.994, p<0.05$) from both sets, however this was not observed for relative expression of OCT1 (Pearson’s correlation $r=0.632, p>0.05$) and OCT2 (Pearson’s correlation $r=0.618, p>0.05$) across datasets (Appendix 2). Differences in expression as measured in our data and in the CCLE may have arisen from the low level of variation seen in OCT1 and OCT2 expression across cell lines, as well as methods used to detect expression. CCLE used a microarray platform to measure gene expression, which may have had limitations in detecting differences in expression at low levels when compared to qPCR, particularly if the probes used did not work well. Differences in normalization and transformation of microarray data may have also contributed to the disparity in relative expression between CCLE and our panel.

We then assembled a composite score to reflect the net expression of OCTs and MATEs in each cell line by subtracting the sum of MATE1 and MATE2 expression values from the sum of those of OCT1, OCT2 and OCT3 (Figure 4:6F, Appendix 3). We chose this algorithm to calculate the composite score, as it was the simplest representation of the contributions of importer and exporter proteins to metformin’s intracellular accumulation, in the absence of any data in the literature indicating functional inequality between transporter paralogs. Using this score, LNCaP would be predicted to be a particularly sensitive cell line, and HepG2 resistant.

We also attempted to generate a weighted risk score using multiple linear regression analysis of individual transporter expression to predict for metformin sensitivity. Data were obtained from publicly available resources, and comprised a dataset of 341 cell lines. However, both univariate analysis and multivariate modeling did not indicate any significant correlation between transporter expression and metformin toxicity as reported in CCLE and...
GDSC, and did not warrant consideration of a weighted risk score (Appendix 4). The unweighted composite score was used hereafter.

We subsequently profiled the ability of metformin to inhibit proliferation across the carcinoma panel. Using the alamarBlue® assay, we measured cellular metabolic activity following 72h treatment with 1-50mM metformin. Metformin inhibited proliferation in a dose-dependent manner in 13 out of 15 cell lines in our panel (Figure 4:7A,B,D). Our data indicated most cell lines, regardless of tumour site, demonstrated nearly complete loss of proliferative activity following treatment with the highest dose of metformin. The clear exceptions were 4 of the 7 HNC cell lines (SCC-16A/B, SCC-19A/B), which exhibited reductions of 0-30% by comparison (Figure 4:7C). Notably, HNC cell lines differing in their response to metformin occurred in pairs that originated from the same patient, presumably with SCC-74A/B representing sensitive tumours, and SCC-16A/B and SCC-19A/B representing resistant tumours. We also observed that cell lines originating from metastases differed in their response to metformin when compared to their matched counterparts originating from primary tumours, though there was no evident trend between these differences (Figure 4:7B,C). Thus, metformin’s anti-proliferative activity may vary between tumour subclones as well as change during tumour progression or metastasis.

We used the area-under-the-curve (AUC) metric to summarize the dose-response curves (Figure 4:7A-D) into a single number reflective of the degree of cellular sensitivity to metformin’s anti-proliferative activity (examples shown in Figure 4:7E). We found this metric to be more accurate than the traditional IC$_{50}$ statistic, as it does not require fitting to a canonical sigmoidal curve. It can also capture differences in dose-dependent responses that cannot be represented by IC$_{50}$ statistics$^{314}$. We then ranked cell lines according to their area-under-dose-response-curve (AUC), with lower values indicating greater sensitivity to metformin’s anti-proliferative activity (Figure 4:7F, Appendix 5). The prostate cell line, LNCaP, was the most sensitive to inhibition of proliferation by metformin, while HNC cell lines trended among the least sensitive. Consistent with data from the GDSC screen, the lung carcinoma cell line A549 was also greatly sensitive; however when comparing cell lines included in both our panel and in the GDSC screen, we did not observe significant correlation ($p>$0.05) between the sensitivities measured (Appendix 6). The disparity may reflect the different methods used to quantify
sensitivity to metformin, as highly normalized IC\textsubscript{50} statistics were used in the GDSC as opposed to raw AUCs used in our panel.

Finally, to determine whether carcinoma cell sensitivity to inhibition of proliferation by metformin was associated with expression of OCTs and MATEs, we correlated the calculated AUCs with OCT-MATE composite scores assigned to each cell line. Our data, however, did not indicate a significant correlation between OCT/MATE expression and inhibition of proliferation by metformin across the panel of cell lines (\textit{Figure 4:7G}), suggesting that factors other than the expression of metformin transporters, possibly genetic alterations in individual cancers (\textit{Appendix 7}), dominate the ability of metformin to affect proliferation.

Thereafter, we investigated the sensitivity of cell lines across the panel to inhibition of oxygen consumption by metformin using the Seahorse Extracellular Flux Analyzer. We measured oxygen consumption rate (OCR) prior to, and up to 8h following, administration of 0mM, 0.02mM, 0.5mM, 2mM and 10mM metformin. Across all cell lines, including those that were resistant to inhibition of proliferation, we observed that metformin inhibited OCR in a dose- and time-dependent manner (\textit{Figure 4:8A-E, Appendix 8}). We plotted dose-response curves using OCR values measured after 8h of metformin, in which individual cell lines exhibited unique response kinetics and sensitivity to metformin (\textit{Figure 4:8F-H}). To quantify this sensitivity, we used AUCs (\textit{examples shown in Figure 4:8I}) to rank cell lines according to their sensitivity to inhibition of oxygen consumption (\textit{Figure 4:8J, Appendix 9}). Once again, LNCaP prostate carcinoma cells and A549 lung carcinoma cells demonstrated the greatest sensitivity to metformin as they exhibited the greatest decline in OCR following metformin treatment (\textit{Appendix 8, Figure 4:8A}). By contrast, HepG2 liver carcinoma cells were the least sensitive to OCR inhibition among the panel, although they were not completely resistant to metformin and remained responsive to OCR inhibition when higher doses were used (\textit{Figure 4:8B}).

As the transporter-mediated uptake of metformin is thought to contribute to its ability to inhibit mitochondrial respiration, we sought to investigate whether cellular sensitivity to OCR inhibition by metformin was associated with net expression of OCTs and MATEs. Upon correlating AUCs with respective OCT-MATE composite scores, we observed a statistically significant correlation between the two variables (Pearson’s correlation coefficient $r=0.679$, \textit{Figure 4:8A}).
$p<0.05$; however, analysis of outliers revealed the correlation was driven by LNCaP, an extremely sensitive cell line that had extremely high net OCT expression (Figure 4:8K). Thus, high net expression of OCTs may be a pattern indicative of extreme sensitivity to inhibition of oxygen consumption by metformin. Importantly however, no cell lines in our panel exhibited a lack of response to OCR inhibition by metformin, suggesting that they all had sufficient OCT expression to enable adequate uptake and intracellular accumulation of metformin to facilitate its mitochondrial inhibition.

To assess whether the effect of metformin upon proliferation was affected by its ability to inhibit cellular oxygen consumption, we aimed to determine whether cellular inhibition of OCR was associated with inhibition of proliferation in response to metformin. We therefore correlated AUCs reflecting sensitivity to OCR inhibition with those reflecting sensitivity to inhibition of proliferation. Once again, though we found a statistically significant correlation between inhibition of proliferation and inhibition of OCR (Pearson’s correlation coefficient $r=0.649$, $p<0.05$), exclusion of the outlier cell line LNCaP rendered the association insignificant (Figure 4:8L). This indicated to us that factors downstream of metformin’s ability to directly inhibit mitochondrial respiration ultimately control the ability to affect cellular proliferation for most cell lines.
Figure 4: OCTs and MATEs are expressed differentially across the cell carcinoma panel. Relative expression values of mRNA plotted for OCT1 (A), OCT2 (B), OCT3 (C), MATE1 (D), and MATE2 (E). Relative abundance for each gene was transformed by normalizing to the mean of 4 housekeeping genes with the highest- and lowest-expressed reference genes omitted, and then normalized to the average expression of each gene measured across the panel. Mean ± S.E.M., n=3.

(F) Carcinoma cell lines ranked according to net OCT and MATE expression, calculated using relative expression values presented in (A-E). Full calculations shown in Appendix 3.
Figure 4:7 Metformin inhibits proliferation in vitro
Cellular proliferation following 72h metformin treatment plotted as a function of the log concentration of metformin added. Cell lines have been grouped by site of origin, such as prostate and cervix (A), head-and-neck (B,C), and remaining sites including colon, liver, kidney and lung (D). Values plotted have been expressed as a percentage of an untreated control. Mean ± S.E.M., n=3. Proliferation data in (A) and for the SCC-74A cell line in (B) were obtained through collaboration with Eric Yung. 

(E) Examples of area-under-the-curve (AUC) from plotted dose response curves. 
(F) Cell lines ranked according to their sensitivity to metformin’s anti-proliferative effect. AUCs were calculated as shown in (E), and have been inversed and normalized to 1 in order to separate values and generate a positive trend. Values are plotted in order of increasing sensitivity to metformin. Full calculations shown in Appendix 5. 
(G) OCT-MATE composite scores presented in (4:6F) were correlated with inverse-AUCs for proliferation inhibition following 72h metformin. Pearson’s coefficient r was used to measure correlative dependence. No significant correlation was observed (p>0.05).
Figure 4: Metformin inhibits mitochondrial oxygen consumption in vitro
Inhibition of OCR measured up to 8h following acute exposure to increasing doses of metformin (0.02mM-10mM). Cell lines represented are A549 (A), HepG2 (B), FaDu (C), RCC4 (D), and 19A (E). Plotted values have been normalized to basal OCR and expressed as a percentage of an untreated control. Mean ± S.E.M., n=3.
OCR traces of remaining cell lines in panel can be found in Appendix 8. Data for HCT116, POP92, LNCaP and SCC-74B cell lines were replotted from previously published data. Data for SCC-74A, SCC-16A/B and SCC-19A/B cell lines were a courtesy of Hala Muaddi. Data for HeLa and ME-180 cell lines were obtained through collaboration with Eric Yung (both members of Koritzinsky laboratory).
Dose-response curves in which OCR values as measured after 8h metformin were plotted as a function of the log concentration of metformin added. All cell lines in the panel were included and organized by site of origin ranging from prostate and cervix (F), head-and-neck (G), and remaining sites colon, liver, kidney and lung (H). Mean ± S.E.M., n=3.
(I) Examples of AUCs from plotted dose response curves
(J) Cell lines ranked according to their sensitivity to OCR inhibition by metformin. Lower AUCs, and higher inverse-AUCs (1/AUCs), indicate greater sensitivity to metformin. Full calculations shown in Appendix 9.
(K) OCT-MATE composite scores calculated in (4:6F) were correlated with inverse-AUCs for OCR inhibition following 8 h metformin.
(L) Inverse-AUCs for OCR inhibition were correlated with those of proliferation inhibition. Pearson’s coefficient r was used to measure correlative dependence in (K) and (L), and significance was set at p<0.05.
5 Discussion

5.1 Factors affecting metformin as a direct radiosensitizer

In Aim 1, we have demonstrated that metformin is mildly radioprotective in the 3 cell lines investigated. These data are in contrast with other studies investigating metformin and radiosensitivity, in which metformin has been reported to elicit either unaltered or increased radiosensitivity. However, more in line with our data, a recent study reported that metformin treatment elicited significant radioprotection in both malignant and nonmalignant murine cell lines, as well as increased protection against normal tissue toxicity in irradiated C3H mice, even when administered 24-48h after irradiation. As such, the effect of metformin upon intrinsic radiosensitivity clearly remains controversial, and may depend on cell type and other factors that have yet to be identified. A possible factor that may account for differences in observed results is the lack of consistency in clonogenic assay protocols that were used to assess the contributions of metformin and radiation among published reports. Even when we used the A549 cell line to reproduce another group’s previous findings, differences in doses and duration of metformin treatment, as well as the length of time allowed for treated cells to form colonies, may have influenced whether the surviving fractions scored at the end of the assay truly reflected cell survival or were measuring proliferation instead. It is possible that treating with a higher dose of metformin may have produced different results, due to the dose-dependent response of cells to metformin’s direct respiratory inhibition observed in Aim 2; however we observed no correlation between inhibition of OCR by metformin and radiation protection by metformin in our cell lines. In particular, the A549 cell line, which was demonstrated in Aim 2 as being particularly sensitive to metformin, was similarly radioprotected by metformin, suggesting that the influence of metformin upon cellular radiosensitivity is independent of the magnitude of its primary respiratory inhibition.

We attempted to assess the role of AMPK and p53 signaling in contributing to radiosensitivity in the presence of metformin, as was implicated in previous studies. Loss of AMPK using siRNA knockdown, as examined by Song and colleagues, was suggested to increase radiation resistance in the presence of metformin. This was demonstrated using 2 different cell lines, both of which responded differently to combined treatment with metformin
and IR. In the breast cancer cell line MCF-7, siRNA knockdown of AMPK increased survival following irradiation in the presence of metformin. In the fibrosarcoma cell line, however, AMPK knockdown contributed to increased radioresistance irrespective of metformin treatment and implies a role for AMPK in mediating an adaptive response following irradiation, rather than an interaction between radiation and metformin in this cell line. These data were in contrast with those obtained in our experiments, whereby loss of AMPK in MEFs trended toward increased radiosensitivity. Correspondingly, our lab has previously demonstrated that AMPK is required for restricting energy metabolism and promoting survival in irradiated cells, particularly in austere environments. In these experiments, loss of AMPK in MEFs decreased survival following irradiation and this was exacerbated in low nutrient conditions. In all, our data are in line with AMPK as a mediator of cell survival following irradiation, and its loss associated with increased radiosensitivity; however, we have not found any evidence to conclude AMPK involvement in facilitating radiosensitization by metformin.

Loss of wild-type p53 signaling using shRNA knockdown, as demonstrated by Skinner and colleagues, increased radiosensitivity following metformin treatment in a panel of head-and-neck cancer cell lines. In these experiments, patient-derived HNSCC cells with p53-disruptive mutations were intrinsically more radioresistant than cells with wild-type or non-disruptive p53 mutations, indicating a role for p53 status in determining radiosensitivity. Metformin treatment radiosensitized cells with p53-disruptive mutations, which was lost with shRNA knockdown. Conversely, shRNA knockdown in cells having wild-type p53 increased radiosensitivity by metformin, which also supported a role for p53 signaling in determining an interaction between metformin and IR. Our data did not corroborate this finding however, as we did not observe a significant difference in clonogenic survival in irradiated HCT116 cells proficient or deficient in p53. Furthermore, metformin treatment resulted in radiation protection in HCT116 cells irrespective of p53 status. The disparity between our results and those of Skinner and colleagues may arise from differences in cell lines used in both studies: HCT116 derives from colon carcinoma and also has been characterized with mutations in KRAS and PIK3CA, comprising oncogenic alterations in survival pathways that may promote radiation resistance and contribute to differences in observed results. Thus, the role of p53 in mediating response to metformin and radiation does not appear to be universal and instead may depend upon genetic alterations in individual cancers and possibly site of tumour origin.
It is important to note that in spite of the mild radiation protection conferred by metformin, this was balanced by the toxicity of metformin itself. In both cancer cell lines investigated, including those lacking p53, irradiation resulted in similar overall levels of cell death irrespective of metformin treatment. Conversely, MEFs were less sensitive to metformin’s direct toxicity and thus were more susceptible to radiation protection by metformin following irradiation, resulting in increased cell survival. This may reflect an enhanced therapeutic ratio for metformin when combined with radiation therapy, whereby it selectively radioprotects normal cells while remaining selectively toxic to cancer cells in the presence of radiation. However, further studies investigating the response of nonmalignant cells or tissues to metformin in combination with radiation are warranted in order to determine a possible therapeutic ratio.

In all, though we observed radioprotection following metformin treatment that reached statistical significance, its clinical relevance is likely outweighed by its direct toxicity. In addition, further benefits of metformin in cancer, such as its ability to inhibit proliferation and reprogram cancer cell metabolism, may also contribute to improving radiation response. Further studies are needed to identify mechanisms underlying possible interactions between metformin and radiation, as well as addressing such interactions \textit{in vivo}. Furthermore, exploration of possible biomarkers to identify patients that would benefit from metformin in combination with radiotherapy is warranted for future clinical implementation.

5.2 Factors determining sensitivity to metformin as an anti-proliferative agent

The anti-proliferative effect of metformin may contribute to improving radiation efficacy from a therapeutic standpoint, as decreased proliferation can elicit lower regrowth rates and slow growth of tumour cells during fractionated radiation. This can improve the probability of curing the tumour by decreasing the number of cells to kill during RT. In both Aim 1 and Aim 2, we assessed different factors for their potential as biomarkers of metformin’s anti-proliferative effect. In Aim 1, we saw that loss of AMPK and p53 exacerbated the anti-proliferative effect of metformin; this may be clinically relevant, as both are frequently inactivated in human cancers due to mutations in tumour suppressors p53 and LKB1\textsuperscript{317,318}. The enhanced inhibition of proliferation in MEFs lacking AMPK was unexpected, as loss of AMPK activity has previously been reported to rescue cells from growth inhibition by metformin\textsuperscript{200,232}. However, recent data
have also indicated a role for AMPK promoting survival in cancer cells by restricting cellular metabolism and protecting against energetic crisis.\textsuperscript{188,250,319,320} Our data are consistent with such studies, and suggest a requirement for AMPK in supporting initial adaptation to metformin. Loss of p53 rendered cells extremely sensitive to metformin’s anti-proliferative and toxic effects; these results indicate a role for p53 in mediating adaptation to and survival with metformin. This is consistent with previous data indicating p53 as necessary for inducing compensatory reduction in energy consumption and upregulation of glycolysis and autophagy in order to adapt to metabolic stress conferred by metformin\textsuperscript{234}. Thus, as p53 and AMPK signaling are frequently impaired in human cancers due to common mutations in \textit{TP53} and the upstream kinase LKB1, p53 and LKB1 mutations represent clinically relevant candidates for exploration as biomarkers of metformin’s anti-proliferative effect.

In Aim 2, we further assessed the anti-proliferative activity of metformin in a panel of carcinoma cell lines and found that it successfully inhibited growth in 13 out of 15 cell lines. We did not observe a correlation between OCT and MATE expression and metformin’s anti-proliferative effect across our panel, however, which indicated to us that factors other than expression of metformin importers and exporters dominate the ability of metformin to affect proliferation. Furthermore, sensitivity to metformin’s inhibition of proliferation was largely independent of its inhibition of oxygen consumption, suggesting that metformin’s effect on proliferation is determined by inhibition of growth pathways and signaling networks, or perhaps its modulation of redox balance\textsuperscript{67}. The effect of metformin upon proliferation should be interpreted with some caution in these experiments however, due to the use of a metabolic assay to reflect cellular proliferation across the panel. Inclusion of another non-metabolic assay to assess proliferation, such as measuring DNA synthesis or population doubling time, would have better distinguished the effect of metformin upon metabolism following its respiratory inhibition, and its effect upon proliferation.

We did not observe much correlation between inhibition of proliferation as measured with our assays, and those published previously in the GDSC database, which used similar metabolic assays to assess proliferation. The disparity may arise from the different statistics used to quantify inhibition, as data reported in the GDSC were in the form of highly normalized IC\textsubscript{50} statistics, and likely differ when compared to inhibition as measured using untransformed AUC values. It is worth noting that toxicity data as reported in the GDSC has faced criticism,
particularly regarding the lack of consistency between it and similar screens performed by CCLE. Moreover, metformin toxicity data were recently retracted from the GDSC due to irreproducibility.

In an attempt to explore other factors that may influence cellular response to metformin’s effect on proliferation, we profiled cells in our panel for common mutations in growth and survival pathways (Appendix 7). Cells lacking LKB1, the upstream kinase of AMPK, have been shown to be particularly susceptible to energetic stress, such as that conferred by metformin, due to their inability to conserve ATP and circumvent energy crisis. Among our panel, the lung cell line A549 and cervix cell line HeLa had LKB1 mutations. Though A549 ranked among the most sensitive cell lines in the panel, HeLa cells did not; thus of the 2 LKB1-mutated cell lines investigated, we did not find a trend toward enhanced sensitivity to metformin. Based on our results from Aim 1, whereby loss of AMPK or p53 signaling exacerbated metformin’s anti-proliferative effect, it was surprising that both cell lines with LKB1 mutations were not particularly sensitive to inhibition of proliferation. HeLa cells, in addition to having an LKB1 mutation, also had inactivated p53 due to human papilloma virus (HPV) infection; on this basis, we would have expected these cells to be especially susceptible to growth inhibition by metformin. Furthermore, the HNSCC cell line FaDu had a TP53 mutation yet was among the most resistant cell lines. It is evident that mutations in p53 and LKB1 may not necessarily result in loss of p53- and AMPK-dependent signaling, which is an important distinction that should be explored prior to consideration of these mutations as biomarkers of metformin’s anti-proliferative effect. Gain-of-function p53 mutations are well documented and their aberrant signaling promotes survival instead of growth arrest and apoptosis; this may account for the increased resistance of FaDu cells to inhibition of proliferation, as they have p53-disruptive mutations. It is also possible that the presence of other background mutations renders LKB1 or p53 mutations poor predictors of sensitivity on their own.

Other mutations in genes involved in cellular growth and survival pathways, such as in PIK3CA and KRAS, did not appear indicative of sensitivity or resistance to metformin either, however as this study was not designed to determine involvement of particular mutations, there were a small number of cell lines in which to explore trends. No genetic data was available for the patient-derived HNSCC cell lines; however due to their response to metformin’s respiratory inhibition, we may speculate that their resistance to metformin’s anti-proliferative effect arises
from possible hyperactivation of downstream effectors of mTOR or inactivation of proteins that restrain cell proliferation in the event of energetic crisis, such as p53 or its downstream cyclin inhibitors. It is also possible that these cells are primarily reliant upon glycolysis for ATP production, and thus experience minimal energetic stress following respiratory inhibition. In all, we found no substantial evidence of a particular mutation that could affect response to metformin’s inhibition of proliferation. Given the small number of cell lines in the panel that had mutations, however, the scope of this observation is limited and requires studies of a larger sample size to verify the influence of these mutations.

In all, our data did not support the use of a composite OCT or MATE expression score as candidate biomarkers for metformin’s anti-proliferative activity. We observed that loss of AMPK and p53 signaling exacerbated inhibition of proliferation in an isogenic system, however increased sensitivity was not seen in cell lines having LKB1 or p53 mutations. This may suggest that LKB1 mutations do not abrogate AMPK-dependent signaling and may not reflect loss of AMPK, though this can be resolved using isogenic cell models. Loss of p53 signaling may be reflected by loss-of-function mutations, which warrants further exploration of TP53 mutations as predictors of sensitivity to metformin’s anti-proliferative effect. Further examination of patient mutations in relation to response will likely uncover other candidates for exploration as biomarkers.

5.3 Factors affecting metformin as a hypoxic modifier

In Aim 2 we assessed expression of OCTs and MATEs as potential biomarkers for sensitivity to the ability of metformin to inhibit cellular oxygen consumption, as it represents the primary mechanism by which it can reprogram cancer cell metabolism, increase tumour oxygenation and potentiate radiation response through hypoxic modification. Here we demonstrated a significant correlation between OCT and MATE expression score and inhibition of oxygen consumption by metformin in cancer cells. Our data indicated a trend toward high net expression of OCTs and increased sensitivity to inhibition of oxygen consumption; this was striking in the outlier prostate cell line LNCaP, which had extremely high OCT and low MATE expression. Thus, we suggest that high net OCT expression may be indicative of extreme sensitivity to inhibition of oxygen consumption by metformin, though this would have to be investigated further using other cell lines with similarly high OCT expression. In a previous
study using 4 preclinical animal xenograft models to characterize metformin’s effect on tumour reoxygenation and potentiation of radiation response, LNCaP xenografts were also most responsive to metformin. It is interesting that a cell line originating from prostate was most responsive to metformin both *in vitro* and *in vivo*, particularly in the context of a retrospective study identifying metformin use as beneficial in prostate cancer patients receiving radiotherapy. However, further studies are warranted to determine whether prostate cancer cells are preferentially sensitive to metformin.

Conversely, no cell lines in our panel were resistant to the inhibition of OCR by metformin, which may suggest that all cell lines investigated had sufficient OCT expression to confer response to metformin’s suppression of oxygen consumption. This was also corroborated previously using preclinical models, in which xenografts established from HCT116 and POP-092S colon adenocarcinoma cells responded to metformin as a hypoxic modifier, in spite of their relatively low OCT expression. Thus, our data indicate OCT expression as a possible candidate for identifying responders to metformin’s inhibition of oxygen consumption, though further studies involving an independent validation cohort are necessary. However, in this same study, xenografts established from the SCC-74B HNSCC cell line were not reoxygenated by metformin, though they expressed OCTs at higher levels than the responsive colon cell lines. SCC-74B xenografts demonstrated high levels of acute hypoxia, however, which may have mitigated the ability of metformin to reduce hypoxia and suggests that high levels of acute hypoxia may contraindicate the use of metformin as a hypoxic modifier *in vivo*.

Considering that cell lines that had low OCT expression and were moderately sensitive to metformin *in vitro* remained responsive to hypoxic modification *in vivo*, it must be determined whether extreme response to metformin *in vitro* is necessary to justify its beneficial use in the clinic as an adjunct to radiotherapy. Certainly, moderate response to metformin *in vitro* was sufficient for tumour reoxygenation and potentiation of radiation response using preliminary animal models, however the same may not be true in the context of clinically approved metformin doses and fractionated treatment schedules. Thus, clinical exploration of OCTs and MATEs as biomarkers for metformin’s hypoxic modification is warranted, in conjunction with assessment of tumour hypoxia. Previous data has also indicated acute hypoxia as a potential contraindication for use of metformin as a hypoxic modifier; this would be difficult to evaluate.
clinically, though perhaps could be addressed through use of pimonidazole and measurement of its distance from blood vessels.

The use of OCT and MATE expression as possible candidate biomarkers for cancer cell response to metformin’s inhibitory effect on oxygen consumption may also be balanced against other factors that can potentially affect cancer cell sensitivity to metformin. OCT and MATE transporters are highly polymorphic, and specific SNPs in OCT1, OCT2, MATE1 and MATE2 have previously been linked to reduced function, altered disposition and pharmacokinetics, and reduced glucose-lowering effects in diabetic patients. Functional variants occurring among these transporters likely would affect the ability of metformin to act upon cancer cells in patients. Thus, SNPs affecting function represent another factor that should be investigated when considering the predictive value of OCT and MATE expression upon cancer cell response to metformin.

A recent study using DNA-barcoded competitive proliferation assays and pooled RNAi screens identified cancer cell lines possessing glucose utilization defects and mitochondrially-encoded core complex I mutations as particularly sensitive to low-glucose conditions and biguanides, due to impaired adaptation of oxidative phosphorylation and glycolysis in response to metabolic stress. In particular, these cell lines exhibited substantially reduced proliferation following low-glucose conditions and treatment with the biguanide phenformin. On the basis of these data, it is likely that the signature of mitochondrially-encoded ETC mutations, or low expression of glucose transporters and glycolytic enzymes, can also define cell lines that adapt poorly to metformin and are susceptible to its inhibition of proliferation, and potentially of oxygen consumption. These signatures should therefore be investigated as further possible determinants of sensitivity to metformin’s inhibition of oxygen consumption and proliferation.

In all, OCT and MATE expression may serve as a possible biomarker of sensitivity to metformin’s inhibition of cellular oxygen consumption, which facilitates its reprogramming of cancer cell metabolism, reoxygenation of tumours and potentiation of radiation response. Further validation is necessary, however, in order to determine whether expression of these transporters can predict, or are required for response to metformin. It must also be noted that these scores represent expression of OCTs and MATEs at the mRNA level, which may not reflect their abundance at the plasma membrane, or their function. This may require further experiments
interrogating whether protein levels of OCTs and MATEs can more robustly predict for metformin sensitivity. OCT and MATE expression may also be incorporated alongside other factors that can potentially influence sensitivity to metformin, such as SNPs affecting function, mitochondrially-encoded ETC mutations, glucose utilization defects, and tumoural acute hypoxia levels, in order to generate a more comprehensive predictive score for patient stratification in future clinical trials.
6  Future Directions, Limitations and Conclusions

6.1  Future Directions

6.1.1  Chronic exposure to metformin

With metformin entering clinical trials as a potential anticancer agent, it is necessary to optimize its dosage for safe use within current treatment schedules. Observations surrounding the kinetics by which metformin was able to decrease respiratory rate in vitro have led to the speculation that metformin can accumulate within the mitochondrial matrix and achieve concentrations in excess of those measured in the plasma\(^9\). If true, this would affect considerations for optimizing dosage for use in cancer treatment. Thus, whether metformin is able to accumulate intracellularly at higher concentrations than measured in the plasma remains an important question to be addressed, particularly if chronic exposure to higher mitochondrial levels of metformin alters cellular sensitivity to its inhibitory effects.

We sought to characterize the effect of long-term exposure to low doses of metformin upon cancer cells, and determine whether chronic metformin treatment resulted in sensitization or resistance to its mitochondrial inhibition. From data obtained in Aim 2, we selected cell lines that were highly sensitive to inhibition of mitochondrial oxygen consumption (LNCaP) and proliferation (SCC-74B), as well as cell lines that were more resistant to these effects (HCT116, HepG2). These were cultured as adherent monolayers kept in exponential growth phase within their respective growth media, which was also supplemented with metformin at concentrations of either 0.02mM or 0.5mM. The former concentration of metformin was used to recapitulate plasma levels reported in diabetic patients, while the latter represented the minimum dose at which we were able to observe an inhibitory response following acute administration (Figure 4:3A-E, Appendix 8). These cell lines were grown in metformin for 5 weeks, which was replenished twice weekly upon passaging. After 1, 4 and 5 weeks of metformin treatment, cells were assayed for mitochondrial function using the Seahorse XF96 Extracellular Flux Analyzer and XF Cell Mito Stress Test kit (Seahorse Bioscience, #101706-100). This was performed in 3 independent experiments.

Data obtained from these experiments were inconclusive, however, with high variability between replicates precluding interpretation in all cell lines tested (Appendix 10). We then
attempted to discern the margin of technical error inherent in the assay used. We first assayed cells plated at identical densities in parallel from a common parental culture (*Appendix 11*). We next used cells from a single culture seeded at slightly increasing densities, in order to test for sensitivity (*Appendix 12*). In both instances, we again observed great variability, and determined that the high level of technical error inherent in this assay due to independent trypsinization and cell counting limited our success in addressing the research question. Protocols would have to be optimized to minimize these errors and circumvent this technical limitation.

Further experiments in our lab are underway to address this question *in vivo*, measuring the hypoxic fraction in xenografts with mice treated with metformin for various periods of time. This approach may be both more feasible and relevant for translation into clinical trials.

### 6.1.2 Expansion of the *in vitro* cell panel

In Aim 2, we demonstrated evidence of an association between expression of metformin importers and response to metformin’s inhibition of oxygen consumption in a panel comprising 15 human carcinoma cell lines. In this panel, we did not observe a lack of response to inhibition of oxygen consumption in any cell lines tested, which may indicate that all cell lines expressed OCTs at sufficient levels to facilitate intracellular accumulation of metformin. We also observed that one cell line expressing OCTs at extremely high levels also demonstrated dramatic sensitivity to this inhibition by metformin. In Aim 1, we demonstrated that cells lacking AMPK- and p53-dependent signaling were particularly susceptible to inhibition of proliferation by metformin, and absence of these signaling pathways may constitute clinically relevant alterations that can possibly predict for response to metformin’s anti-proliferative activity. We explored other genetic alterations occurring among cell lines in our panel in an effort to identify other mutations that may portend increased sensitivity or resistance to inhibition of proliferation by metformin; however, this was constrained by the small number of cell lines bearing such mutations. On the basis of these observations, further characterization of the effect of extremely high OCT expression upon sensitivity to metformin’s inhibition of oxygen consumption, and frequently mutated genes upon sensitivity to inhibition of proliferation, are warranted. Measuring OCT expression and sensitivity to metformin’s inhibition of oxygen consumption and proliferation across a significantly larger cell panel would be necessary to validate the correlations uncovered in Aim 2 *in vitro*. Furthermore, use of an expanded cell panel would
allow inclusion of greater number of cell lines with mutations of interest, which may facilitate characterization of these mutations upon sensitivity to metformin’s anti-proliferative effects and identification of additional candidates for exploration as biomarkers.

6.1.3 Clinical trials

Based on the translational focus of this project, further assessment of potential biomarkers identified in Aims 1 and 2 may be justified in the context of future clinical trials investigating metformin and radiotherapy. Our data indicated that high OCT expression may predict for extreme sensitivity to inhibition of cellular oxygen consumption by metformin, which was consistent both in vitro and in vivo. From these results, investigating high OCT expression as a biomarker of extreme response to hypoxic modification by metformin is warranted. However, in our panel, no cell lines were resistant to metformin’s inhibitory effects in vitro, and this was also seen in vivo, as xenografts established from moderately sensitive cell lines with low OCT expression still responded to hypoxic modification by metformin. This may suggest that response to metformin as a hypoxic modifier in vivo is not limited to cells with extreme response to respiratory inhibition by metformin, but can also include cells with an in vitro response threshold that has yet to be defined. However, response to hypoxic modification by metformin in vivo was demonstrated in preliminary animal models that received IR at a single subcurative dose, and was not reflective of patient response following metformin treatment in a fractionated treatment schedule. Thus, further studies are warranted in order to define sufficient patient response to metformin’s hypoxic modification in order to justify its clinical incorporation. In the context of these future studies, exploration of OCTs as biomarkers of metformin’s hypoxic modification may be superiorly assessed in a prospective clinical setting. Such a setting would also facilitate evaluation of tumour hypoxia levels as well as reduced-function SNPs in OCTs occurring in patients, and their impact upon response to metformin, as this would be difficult to recapitulate accurately in vitro and in vivo. Furthermore, acute hypoxia levels as a contraindication to metformin use in radiotherapy could also be explored.

Our data in Aim 2 indicated that metformin was able to inhibit proliferation in the majority of cell lines tested. Based on our data from Aim 1, we found that loss of p53 and AMPK signaling rendered cells more susceptible to inhibition of proliferation by metformin. As p53 and LKB1 mutations frequently occur in human cancers, these data indicate that these
mutations may be clinically explored as biomarkers. However, it must be determined whether LKB1 or p53 mutation necessarily result in loss of AMPK and p53 signaling; this may also result in further stratification, as inactivating mutations in these tumour suppressors may affect their downstream signaling pathways, and response to metformin, differently from gain-of-function mutations. Characterization of LKB1 and TP53 mutations upon proliferation by metformin in future clinical trials may be warranted, with inhibition of proliferation feasibly assessed through Ki67 staining.

Assessing the effect of metformin upon proliferation of tumour cells in a clinical setting would also facilitate the identification of other patient mutations that may contribute to differential response to metformin. In particular, mitochondrially-encoded ETC mutations or glucose-utilization defect signatures were indicative of greater sensitivity to inhibition of proliferation following phenformin treatment, as discussed previously\textsuperscript{324}. Exploration of these mutations or gene signatures may be justified in patients within a prospective clinical setting in combination with Ki67 staining to assess proliferation. This would facilitate further identification of clinically relevant biomarkers for use of metformin as an anti-proliferative agent.

6.2 Limitations

This study was designed as an exploratory project to evaluate hypothesized factors as predictors for response to metformin as a radiosensitizer, anti-proliferative agent, or hypoxic modifier. The methodologies used in these experiments were selected to best address the research aims; nonetheless they present some drawbacks that should be acknowledged.

6.2.1 Isogenic cell model

Experimentation from Aim 1 relied upon the use of isogenic cell lines to elucidate the contributions of p53- and AMPK-dependent signaling in response to metformin and radiation. The use of isogenic cell lines accounted only for differences in AMPK and p53 between pairs, while the presence of other background mutations were not considered. In the case of HCT116 colon adenocarcinoma cells, which have numerous mutations in critical signaling pathways such as in \textit{KRAS} and \textit{PIK3CA}, this is especially important and may have contributed to differences in radiosensitivity than that reported by previous groups\textsuperscript{181}. To confirm that phenotypic differences observed between isogenic pairs were truly due to loss of AMPK or p53, reintroducing wild-type
AMPK into AMPK-deficient MEFs and wild-type p53 into p53-deficient HCT116 cells would be a more robust method. Furthermore, this study used cell lines exclusively, and would benefit from examining the effects of metformin in combination with radiation using 3-D environments and animal models in order to recapitulate patient tumours more faithfully and ensure that changes in intrinsic radiosensitivity translate into improved response to radiation \textit{in vivo}.

6.2.2 Cell culture models

The studies from Aim 2 characterized the response to metformin’s respiratory inhibition and anti-proliferative effects in a panel of human carcinoma cell lines, which do not recapitulate the heterogeneity of human tumours faithfully. Nonetheless, for the purposes of this project, which was to explore the possibility of OCTs and MATEs as predictive biomarkers for response to metformin, the use of a multitude of cell lines provided a concise approach to assess sensitivity to metformin across a variety of tumour types. Further studies assessing these transporters as predictive biomarkers for metformin’s hypoxic modification are warranted clinically, or at least using animal models.

Experiments from both aims were conducted in an \textit{in vitro} setting and thus were subjected to the use of supra-physiological doses of metformin to elicit its effects upon radiation sensitivity, proliferation, and respiratory inhibition. As was discussed previously, cell culture models are optimized for maximal growth and thus may require higher doses of metformin than would be required in a clinical setting to affect its desired endpoint. The use of high glucose growth medium has been demonstrated to blunt the effects of metformin upon proliferation in previous studies\textsuperscript{206,245,246}; furthermore, treatment with metformin can enhance lactate production in its target cells due to its inhibition of oxidative phosphorylation and promotion of ATP synthesis via glycolysis\textsuperscript{250}. These conditions differ from those of the tumour microenvironment, where glucose and oxygen concentrations are even lower than physiological. These factors may contribute to a higher dose of metformin required to affect respiration, proliferation or radiosensitivity using cell models in these experiments, and necessitate further studies in a preclinical or clinical setting to ascertain whether these effects can be achieved using clinically relevant doses.
6.2.3 Normalization methods

In Aim 2, certain assumptions were made when compiling a composite score to represent net expression of OCTs and MATEs across cell lines. The algorithm used in the calculation of this score was the simplest in which net movement of metformin across cells could be reflected. As stated previously, transporters were given equal weight in calculation of the composite score as there is no evidence to suggest that OCT and MATE homologs are functionally inequivalent in transporting metformin. Multivariate analysis of publicly available transporter expression and metformin toxicity data also did not indicate preferential association of a particular transporter to metformin sensitivity, nor necessitate the use of a weighted score (Appendix 4). As these data have since been retracted, investigation of OCTs and MATEs as independent predictors of metformin sensitivity is currently underway in our lab, with correlations suggesting OCT3 and MATE2 as independent predictors of sensitivity to metformin’s inhibition of OCR and proliferation respectively (Appendix 13).

Additionally, the values used in the calculation of these composite scores were relative expression values that had been highly normalized, due to the high variation of expression of housekeeping genes among cell lines. The normalization reflects an inherent bias, as transformed expression values reflect relative differences among individual transporters. The relative expression values used however do not account for differences in absolute expression levels between transporters. Due to the large variation seen in expression of housekeeping genes across the panel, however, the use of absolute values to measure expression of these genes was ruled out. Furthermore, these expression values were only at the mRNA level, and thus may not reflect protein expression or transporter function at the plasma membrane. Further studies are warranted to interrogate correlation between mRNA expression and protein abundance of these transporters.

6.2.4 Correlative associations

The associations uncovered in Aim 2 are correlative, and would have been strengthened by addressing possible mechanisms between OCT and MATE expression and susceptibility to respiratory inhibition by metformin. Establishing a cell line having OCT1, OCT2 and OCT3 knocked out or down, and assessing whether respiratory inhibition by metformin was abrogated in these triple-knockout cells would have strengthened the observation that OCT expression
across the panel was sufficient to confer response to metformin. Reintroduction of individual OCT transporters within triple-knockout cells in parallel and assaying for differences in their OCR inhibition by metformin would also assess if OCT homologs are truly functionally equivalent, while lending credence to a possible mechanistic link between transporter expression and metformin activity. Studies addressing functional involvement of OCTs in OCR inhibition by metformin are currently underway in our lab.

6.3 Conclusions

Throughout the course of this thesis, potential biomarkers for 3 specific effects of metformin, which have been reported to improve radiation response, were explored. First, we assessed AMPK and p53 signaling as contributors to metformin’s radiosensitizing effect. While we found no evidence supporting metformin as a radiosensitizer, our data indicated that loss of AMPK and p53 exacerbated its anti-proliferative effect. As p53 and the upstream kinase of AMPK, LKB1, are frequently mutated in human cancer, the loss of either signaling pathway represents a potential biomarker for the anti-proliferative effect of metformin that warrants clinical exploration. Second, we evaluated the expression of OCTs and MATEs as predictors of sensitivity to metformin’s inhibition of proliferation or oxygen consumption. OCT and MATE expression did not correlate with inhibition of cellular proliferation, suggesting that downstream targets such as AMPK or p53 mediate this effect. The number of cell lines in our panel limited identification of other potential mutations that confer increased sensitivity to inhibition of proliferation; nonetheless exploration of commonly mutated genes in aberrant growth pathways may uncover further targets. OCT expression trended toward increased sensitivity to metformin’s respiratory inhibition, which correlated with its previously reported hypoxic modification in preclinical models. More importantly, we observed response to respiratory inhibition in all cell lines investigated, suggesting that all had sufficient OCT expression to confer response. This warrants clinical investigation of OCTs as potential biomarkers of metformin’s hypoxic modification, but also further exploration of response to hypoxic modification necessary for justified use of metformin in the clinic. In all, both studies identify 2 putative biomarkers of response to metformin for further clinical exploration: loss of p53 and AMPK signaling as determinants of sensitivity to inhibition of proliferation, and OCT expression as predictors of sensitivity to inhibition of cellular oxygen consumption and hypoxic modification.
References


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307. Sandulache, V. C. *et al.* Residual nodal disease in patients with advanced-stage oropharyngeal squamous cell carcinoma treated with definitive radiation therapy and


## Appendices

**Appendix 1: OCT and MATE primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC22A1</td>
<td>5'-TTATCTCACCTGACCTGCACTGG</td>
<td>5'-TGGTGATTCCCATTCGGCCAAC</td>
</tr>
<tr>
<td>(OCT1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC22A2</td>
<td>5'-CTGGAGTCTGTCAGAGGAAC</td>
<td>5'-GTTCCAGTCCACCTCGTAGC</td>
</tr>
<tr>
<td>(OCT2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC22A3</td>
<td>5'-GCCCTGTCCAGCAATAAGA</td>
<td>5'-GAGAGCCAAAATGTCCCAA</td>
</tr>
<tr>
<td>(OCT3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC47A1</td>
<td>5'-TTCATAAGCTCCGTGTCTGT</td>
<td>5'-AGTGACATTGATAACCGCGAT</td>
</tr>
<tr>
<td>(MATE1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC47A2</td>
<td>5'-GCAGGGCCAGTTTTCATTTA</td>
<td>5'-TGGGAGATGATGTTGGCATA</td>
</tr>
<tr>
<td>(MATE2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 1:* Independently designed and validated primer sequences used for quantitative PCR in Aim 2.
Appendix 2: Comparison of expression data from CCLE and Aim 2

Correlations between relative expression values of OCT1, OCT2, OCT3, MATE1, and MATE2 as reported by the CCLE™ using microarray data, and as measured in our data using quantitative PCR.
### Appendix 3: Calculation of composite score

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Σ[OCT]</th>
<th>Σ[MATE]</th>
<th>[OCT]-[MATE]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>3.086</td>
<td>8.372</td>
<td>-5.286</td>
</tr>
<tr>
<td>19B</td>
<td>0.456</td>
<td>2.938</td>
<td>-2.482</td>
</tr>
<tr>
<td>19A</td>
<td>0.665</td>
<td>2.503</td>
<td>-1.838</td>
</tr>
<tr>
<td>74B</td>
<td>1.795</td>
<td>2.796</td>
<td>-1.001</td>
</tr>
<tr>
<td>FaDu</td>
<td>2.937</td>
<td>3.045</td>
<td>-0.107</td>
</tr>
<tr>
<td>ME-180</td>
<td>1.162</td>
<td>0.948</td>
<td>0.214</td>
</tr>
<tr>
<td>74A</td>
<td>1.163</td>
<td>0.527</td>
<td>0.637</td>
</tr>
<tr>
<td>HeLa</td>
<td>3.185</td>
<td>2.280</td>
<td>0.906</td>
</tr>
<tr>
<td>HCT116</td>
<td>0.962</td>
<td>0.001</td>
<td>0.960</td>
</tr>
<tr>
<td>POP92</td>
<td>1.112</td>
<td>0.003</td>
<td>1.109</td>
</tr>
<tr>
<td>16A</td>
<td>1.543</td>
<td>0.209</td>
<td>1.334</td>
</tr>
<tr>
<td>A549</td>
<td>5.710</td>
<td>3.457</td>
<td>2.253</td>
</tr>
<tr>
<td>16B</td>
<td>6.355</td>
<td>2.142</td>
<td>4.212</td>
</tr>
<tr>
<td>RCC4</td>
<td>5.426</td>
<td>0.756</td>
<td>4.671</td>
</tr>
<tr>
<td>LNCaP</td>
<td>9.442</td>
<td>0.024</td>
<td>9.418</td>
</tr>
</tbody>
</table>

*Table 2: Calculation of composite [OCT]-[MATE] scores using summed expression values for OCTs and MATEs*
Appendix 4: Predictive modeling using publicly available data

Univariate analyses of metformin toxicity and relative expression of OCT1, OCT2, OCT3, MATE1, and MATE2. Data for 341 cell lines were obtained from GDSC \(^\text{313}\) and CCLE \(^\text{304}\) online databases, and split into testing and training sets. Spearman’s coefficient was used to determine correlation between variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT1</td>
<td>-0.076</td>
<td>0.103</td>
<td>0.469</td>
</tr>
<tr>
<td>OCT2</td>
<td>0.078</td>
<td>0.104</td>
<td>0.467</td>
</tr>
<tr>
<td>OCT3</td>
<td>-0.079</td>
<td>0.107</td>
<td>0.462</td>
</tr>
<tr>
<td>MATE1</td>
<td>-0.107</td>
<td>0.105</td>
<td>0.311</td>
</tr>
<tr>
<td>MATE2</td>
<td>-0.016</td>
<td>0.110</td>
<td>0.885</td>
</tr>
</tbody>
</table>

*Table 3*: Multiple linear regression using metformin sensitivity as the outcome variable. The multivariate model was unable to predict for metformin sensitivity (p=0.75). These data were obtained in collaboration with Jenna Sykes.
Appendix 5: Ranking cell lines by sensitivity to anti-proliferative effect of metformin

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>AUC</th>
<th>1/AUC</th>
<th>Normalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>60.03</td>
<td>0.017</td>
<td>1.743</td>
</tr>
<tr>
<td>A549</td>
<td>69.11</td>
<td>0.014</td>
<td>1.514</td>
</tr>
<tr>
<td>74A</td>
<td>79.33</td>
<td>0.013</td>
<td>1.319</td>
</tr>
<tr>
<td>HepG2</td>
<td>84.25</td>
<td>0.012</td>
<td>1.242</td>
</tr>
<tr>
<td>POP92</td>
<td>88.37</td>
<td>0.011</td>
<td>1.184</td>
</tr>
<tr>
<td>ME-180</td>
<td>90.02</td>
<td>0.011</td>
<td>1.162</td>
</tr>
<tr>
<td>RCC4</td>
<td>110.6</td>
<td>0.009</td>
<td>0.946</td>
</tr>
<tr>
<td>HeLa</td>
<td>116.7</td>
<td>0.009</td>
<td>0.897</td>
</tr>
<tr>
<td>HCT116</td>
<td>119.7</td>
<td>0.008</td>
<td>0.874</td>
</tr>
<tr>
<td>74B</td>
<td>131.0</td>
<td>0.008</td>
<td>0.799</td>
</tr>
<tr>
<td>FaDu</td>
<td>133.5</td>
<td>0.007</td>
<td>0.784</td>
</tr>
<tr>
<td>16B</td>
<td>152.4</td>
<td>0.007</td>
<td>0.687</td>
</tr>
<tr>
<td>19B</td>
<td>162.6</td>
<td>0.006</td>
<td>0.643</td>
</tr>
<tr>
<td>19A</td>
<td>170.5</td>
<td>0.006</td>
<td>0.614</td>
</tr>
<tr>
<td>16A</td>
<td>176.4</td>
<td>0.006</td>
<td>0.593</td>
</tr>
</tbody>
</table>

Table 4: Cell lines ranked according to their sensitivity to metformin’s anti-proliferative effect. Inverse-AUCs (1/AUCs) were used to generate a positive trend, and normalized to 1 to separate values. Lower AUCs, or higher normalized values, are indicative of increased sensitivity to metformin.
Appendix 6: Comparison of proliferation inhibition values from GDSC and Aim 2

Correlation of sensitivity to inhibition of proliferation by metformin as reported in the GDSC screen (IC$_{50}$) and as measured in our data (AUC).

Correlation of sensitivity to inhibition of proliferation by metformin as reported in the GDSC screen (IC$_{50}$) and as measured in our data (AUC).
### Appendix 7: Common mutations occurring in cell panel

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mutation</th>
<th>Ranked sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td><strong>NOTCH1</strong> (T865N, intron)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>PTEN</em> (K6fs, Q1665fs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>NF1</em> (A431V, A690V)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>APC</em> (R2714C)</td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td><strong>STK11</strong> (Q37*)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>KRAS</em> (G12S)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>SMARCA4</em> (L728fs, Q729fs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>APC</em> (3'UTR)</td>
<td></td>
</tr>
<tr>
<td>74A</td>
<td><strong>UNKNOWN</strong></td>
<td>3</td>
</tr>
<tr>
<td>HepG2</td>
<td><strong>KRAS</strong> (5'UTR)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><strong>NRAS</strong> (Q61L)</td>
<td></td>
</tr>
<tr>
<td>POP92</td>
<td><strong>UNKNOWN</strong></td>
<td>5</td>
</tr>
<tr>
<td>ME-180</td>
<td><strong>PIK3CA</strong> (E545K)</td>
<td>6</td>
</tr>
<tr>
<td>RCC4</td>
<td><strong>VHL</strong></td>
<td>7</td>
</tr>
<tr>
<td>HeLa</td>
<td><strong>STK11</strong> (p.0?)</td>
<td>8</td>
</tr>
<tr>
<td>HCT116</td>
<td><strong>NOTCH1</strong> (P915L, G1195R)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td><em>PIK3CA</em> (H1047R)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>KRAS</em> (G13D)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>PTEN</em> (3'UTR)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>NF1</em> (P388T, T676fs, P678fs, I679fs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>SMARCA4</em> (L1163P, S122fs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>KDM6A</em> (3'UTR)</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>Source</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>74B</td>
<td>FaDu</td>
<td>TP53 (R248L, V225_splice)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDKN2A (p.?)</td>
</tr>
<tr>
<td>16B</td>
<td>UNKNOWN</td>
<td></td>
</tr>
<tr>
<td>19B</td>
<td>UNKNOWN</td>
<td></td>
</tr>
<tr>
<td>19A</td>
<td>UNKNOWN</td>
<td></td>
</tr>
<tr>
<td>16A</td>
<td>UNKNOWN</td>
<td></td>
</tr>
</tbody>
</table>

*Table 5: Mutations that may affect cellular growth or metformin response in cell lines arranged according to sensitivity to inhibition of proliferation. Mutation data compiled in collaboration with Eric Yung.*
Appendix 8: OCR inhibition by metformin across the cell panel

A)
Changes in oxygen consumption rate (OCR) measured up to 8h following addition of 0.02mM, 0.5mM, 2mM or 10mM metformin. OCR traces for cell lines in (A) were replotted from previously published data\cite{182}, or performed by Eric Yung (B) and Hala Muaddi (C).
**Table 6**: Cell lines ranked according to their sensitivity to OCR inhibition by metformin. Lower AUCs, and higher inverse-AUCs (1/AUCs), indicate greater sensitivity to metformin. Inverse-AUCs were then normalized to 1 in order to separate values.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>AUC</th>
<th>1/AUC</th>
<th>Normalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>102.8</td>
<td>0.010</td>
<td>1.782</td>
</tr>
<tr>
<td>A549</td>
<td>137.1</td>
<td>0.007</td>
<td>1.336</td>
</tr>
<tr>
<td>HeLa</td>
<td>178.4</td>
<td>0.006</td>
<td>1.027</td>
</tr>
<tr>
<td>RCC4</td>
<td>191.2</td>
<td>0.006</td>
<td>0.958</td>
</tr>
<tr>
<td>19A</td>
<td>191.5</td>
<td>0.005</td>
<td>0.957</td>
</tr>
<tr>
<td>74B</td>
<td>192.4</td>
<td>0.005</td>
<td>0.952</td>
</tr>
<tr>
<td>FaDu</td>
<td>193.9</td>
<td>0.005</td>
<td>0.945</td>
</tr>
<tr>
<td>16A</td>
<td>194.3</td>
<td>0.005</td>
<td>0.943</td>
</tr>
<tr>
<td>19B</td>
<td>198.7</td>
<td>0.005</td>
<td>0.922</td>
</tr>
<tr>
<td>ME-180</td>
<td>199.0</td>
<td>0.005</td>
<td>0.920</td>
</tr>
<tr>
<td>74A</td>
<td>200.0</td>
<td>0.005</td>
<td>0.916</td>
</tr>
<tr>
<td>HCT116</td>
<td>211.2</td>
<td>0.005</td>
<td>0.867</td>
</tr>
<tr>
<td>POP92</td>
<td>213.0</td>
<td>0.005</td>
<td>0.860</td>
</tr>
<tr>
<td>16B</td>
<td>226.3</td>
<td>0.004</td>
<td>0.809</td>
</tr>
<tr>
<td>HepG2</td>
<td>227.2</td>
<td>0.004</td>
<td>0.806</td>
</tr>
</tbody>
</table>
Appendix 10: Effect of chronic metformin exposure upon mitochondrial bioenergetics

A) 74B (1 week)

B) HCT116 (1 week)

A) 74B (4 weeks)

B) HCT116 (4 weeks)

A) 74B (5 weeks)

B) HCT116 (5 weeks)
Mitochondrial stress tests performed for SCC-74B (A), HCT116 (B) and HepG2 (C) cell lines grown in 0-0.5 mM metformin at 1, 4 and 5 weeks metformin (LNCaP data not shown). Mean ± S.E.M., n=3. High variation between replicates precluded interpretation or statistical analysis of the data.
Appendix 11: Reproducibility within chronic metformin OCR assay measurements

Mitochondrial stress test performed on untreated SCC-74B cells. Cells from the same parental culture were seeded into 8 separate plates at identical densities and grown in parallel, prior to trypsinization, counting and seeding for OCR assays at the same cell density. The expectation for this experiment was for each of 8 replicates to have identical OCR and response to mitochondrial stress test as they were treated identically. This was not observed, as variation inherent in independent trypsinization, counting and seeding limited the technical accuracy of this assay.
Appendix 12: Sensitivity of chronic metformin OCR assay measurements

(A) Mitochondrial stress test performed on untreated SCC-74B cells originating from the same parental culture seeded at slight incremental densities (17,000-24,000 cells/well) for OCR measurement. These densities range about the optimized cell density at which these experiments are performed (20,000 cells/well). The purpose of this experiment was to determine the sensitivity of the assay in detecting OCR changes over a small range of densities. The expectation was for serially increasing densities of cells to correspond to serially increasing OCR measurements: this was not observed. Normalization of measured OCR to cell density as quantified following the assay did not result in similar basal OCR/cell either (B). Finally, no correlation between initial and final seeding density was observed (C), indicating great variation in absolute OCR measurements that limited its sensitivity in detecting small differences in cell number.
Appendix 13: Individual transporter correlations

A)
Expression values for OCT1, OCT2, OCT3, MATE1 and MATE2 were individually correlated with sensitivity to inhibition of OCR (A) and proliferation (B). OCT3 was significantly correlated with inhibition of both OCR and proliferation, though this was driven by inclusion of the extreme responder, LNCaP. MATE2 was negatively associated with inhibition of proliferation. Pearson’s coefficient $r$ was used to measure correlative dependence in (A) and (B), and correlations were deemed significant when $p<0.05$. 