

Metformin abolishes increased tumor ^{18}F -2-fluoro-2-deoxy-D-glucose uptake associated with a high energy diet

Haider Mashhedi,^{1,3} Marie-José Blouin,³ Mahvash Zakikhani,³ Stéphanie David,³ Yunhua Zhao,³ Miguel Bazile,³ Elena Birman,³ Carolyn Algire,^{1,3} Antonio Aliaga,⁴ Barry J. Bedell⁴ and Michael Pollak^{2,3,*}

¹Departments of Experimental Medicine and ²Oncology; McGill University; ³Cancer Prevention Center; Jewish General Hospital;

⁴McConnell Brain Imaging Centre; Montreal Neurological Institute; McGill University; Montreal, QC Canada

Key words: FDG, PET, metformin, hyperinsulinemia, colon cancer

Insulin regulates glucose uptake by normal tissues. Although there is evidence that certain cancers are growth-stimulated by insulin, the possibility that insulin influences tumor glucose uptake as assessed by ^{18}F -2-Fluoro-2-Deoxy-D-Glucose Positron Emission Tomography (FDG-PET) has not been studied in detail. We present a model of diet-induced hyperinsulinemia associated with increased insulin receptor activation in neoplastic tissue and with increased tumor FDG-PET image intensity. Metformin abolished the diet-induced increases in serum insulin level, tumor insulin receptor activation and tumor FDG uptake associated with the high energy diet but had no effect on these measurements in mice on a control diet. These findings provide the first functional imaging correlate of the well-known adverse effect of caloric excess on cancer outcome. They demonstrate that, for a subset of neoplasms, diet and insulin are variables that affect tumor FDG uptake and have implications for design of clinical trials of metformin as an antineoplastic agent.

Introduction

^{18}F -2-Fluoro-2-Deoxy-D-Glucose Positron Emission Tomography (FDG-PET) exploits the increased glucose uptake rate of tumors that was first described by Warburg¹ to image neoplastic disease.² FDG is transported into cells by the same mechanisms as glucose and is phosphorylated by hexokinase, effectively trapping it within the cell as ^{18}F -2-Fluoro-2-Deoxy-D-Glucose-6-Phosphate (FDG6P). Due to the absence of a 2'-hydroxyl group, low levels of glucose-6-phosphatase in the tumor cell and the high rate of glucose transport by neoplastic tissue, FDG6P accumulates in the neoplasm at a higher rate than in normal tissues.² Clinical studies have examined ways to optimize the utility of FDG-PET for imaging malignant disease by defining the most favorable systemic metabolic conditions to maximize sensitivity. For example, it is well documented that hyperglycemia decreases FDG uptake by neoplastic tissue due to dilution of the labeled analog by the elevated levels of unlabeled glucose. Although it is not common practice to increase the amount of administered tracer to avoid the loss of sensitivity associated with hyperglycemia in diabetics, patients often are asked to fast prior to a PET scan in order to minimize dilution of the radiolabeled agent by hyperglycemia.³ It has been proposed that insulin treatment of diabetics could increase imaging sensitivity by increasing the ratio of labeled to unlabeled glucose, and this has been demonstrated experimentally.⁴ Although these technical issues influence FDG-PET

image intensity by affecting the relative concentrations of FDG to glucose, it is of interest that there is no evidence that variation in circulating glucose concentration is in itself an important determinant of glucose uptake by tumors. This is likely due to the fact that tumors have sufficient glucose uptake capacity to meet their requirements at all physiologically relevant glucose concentrations.⁵

Despite unprecedented interest in cancer metabolism,⁶ much clinical and experimental research involving FDG-PET has used the method simply for imaging and as an end-point for assessing the efficacy of various experimental therapeutic agents rather than as a tool to specifically measure glucose uptake in the context of host and tumor metabolism. While insulin-responsive tumor models have been described in references 7–9, and population studies (for example, refs. 10 and 11) are consistent with the hypothesis that certain tumors are insulin-sensitive, there have been no studies of the effects of hyperinsulinemia on FDG-PET imaging.

Metformin is one of the most frequently prescribed drugs used to treat type II diabetes, and there is evidence from population (reviewed in refs. 12–14) and laboratory (reviewed in refs. 7 and 15–19) studies that it has antineoplastic activity (reviewed in ref. 20). Metformin and other biguanides exert their antidiabetic effects in large part by decreasing hepatic gluconeogenesis, resulting in reduced circulating glucose concentrations,²¹ with a secondary reduction in circulating insulin levels. It is possible

that this reduction is sufficient to attenuate the growth of the subset of tumors that are insulin-responsive.²² In mice with diet-induced hyperinsulinemia, metformin indeed attenuates tumor growth.^{7,15} There is also evidence for separate direct effects of metformin on neoplastic cells.^{23,24} These effects result from decreased mitochondrial ATP production due to the inhibitory action of metformin on respiratory complex I,²⁵ resulting in activation of AMP-activated kinase (AMPK), which downregulates energy-consuming processes, such as protein synthesis and proliferation, and upregulates processes that lead to ATP synthesis, including glucose uptake.²⁶ Therefore, the effects of metformin or other AMPK activators on FDG-PET image intensity are difficult to predict, as drugs with anti-proliferative activity generally decrease FDG uptake, but agents capable of activating AMPK in tumors would be expected to increase their FDG uptake.

MC38 colon cancer cells are known to form aggressive tumors in vivo. This finding is especially true in animals on a high energy diet,^{16,27} and there is strong prior evidence to implicate diet-induced hyperinsulinemia as a major mediator of the adverse effect of obesity on neoplasia, since a high energy diet is associated with increased circulating insulin levels and increased insulin receptor (IR) activation in tumors.⁹ Our present results provide an example of a tumor for which FDG uptake varies with diet and metformin administration in a manner that suggests that FDG-PET imaging is influenced by insulin. These results have important implications for interpreting FDG-PET images and, also, for understanding the influences of host metabolism and metformin on tumor behavior.

Results

Insulin receptor expression and effects of metformin or insulin on MC38 cell proliferation. We confirmed expression of the insulin receptor- β subunit (IR- β) by MC38 cells (Fig. 1B). Furthermore, these cells were significantly responsive to in vitro growth stimulation by physiologically relevant concentrations of insulin (Fig. 1C) (control vs. insulin treatment conditions, $n = 4$, $p = 0.0059$).

We observed significant inhibition of MC38 cell proliferation in vitro with increasing doses of metformin (Fig. 1D) (control vs. metformin treatment conditions, $n = 4$, $p = 0.0056$). Under these in vitro conditions, metformin cannot be acting as an insulin-lowering agent, so we hypothesized that this growth inhibition was related to that previously described in reference 23, a direct action of metformin as an AMPK-dependent growth inhibitor.

Insulin and metformin stimulate glucose uptake in vitro. The in vitro effect of insulin or metformin on glucose uptake by MC38 cells was determined using a colorimetric assay. Treatment with physiologically relevant concentrations of insulin significantly increased glucose uptake in MC38 cells (Fig. 2A) (control vs. insulin treatment conditions, $n = 3$, $p = 0.0127$). This provides evidence that MC38 is a tumor cell line where glucose uptake is insulin-stimulated rather than constitutive. As expected, this finding was correlated with increased IR^{Tyr972} and Akt^{Ser473} phosphorylation (Fig. 2C). We also observed significant stimulation of glucose uptake by metformin in vitro (Fig. 2B) (control vs.

metformin treatment conditions, $n = 3$, $p = 0.0056$). On exposure to metformin, AMPK^{Thr172} phosphorylation was elevated in MC38 cells (Fig. 2C), which is consistent with prior reports.²³ As AMPK signaling leads to alterations in cellular metabolism to increase ATP levels by increasing ATP generation and decreasing ATP consumption,²⁶ the finding that metformin increases glucose uptake while decreasing proliferation is plausible.

Metformin treatment reduces serum insulin in the setting of diet-induced hyperinsulinemia. We observed a significant increase in serum insulin levels in mice on the high energy diet prior to metformin treatment vs. mice on the control diet (Fig. 3) (4.355 ± 0.7956 ng/ml vs. 1.523 ± 0.2365 ng/ml, $n = 5$, $p = 0.004$). There was a significant decrease in serum insulin levels following metformin treatment for mice on the high energy diet (pre-treatment vs. post-treatment, 4.355 ± 0.7956 ng/ml vs. 2.334 ± 0.5131 ng/ml, $n = 5$, $p = 0.05$). In contrast, there was no effect of metformin on insulin levels in mice on the control diet (pre-treatment vs. post-treatment insulin levels, 1.523 ± 0.2365 ng/ml vs. 1.21 ± 0.1039 ng/ml, $n = 5$, $p = 1$).

Influence of diet and metformin on blood glucose levels. Blood glucose levels were measured in mice prior to FDG-PET scanning, while animals were under anesthesia following an overnight fast and prior to FDG infusion. Between Day 0 and Day 3, metformin had no significant effect on fasting blood glucose levels of mice on the high energy diet (Day 0 vs. Day 3, 8.25 ± 0.86 vs. 8.73 ± 0.55 , $n = 6$, $p = 1$). This observation is important as this diet-induced obesity model did not show fasting hyperglycemia, thereby mitigating any potential confounding of FDG-PET analysis due to alterations in the ratio of labeled to unlabeled glucose. Similarly, metformin did not have any effect on fasting blood glucose levels of mice on the control diets between Day 0 and Day 3 (Day 0 vs. Day 3, 10.30 ± 0.87 vs. 11.38 ± 0.76 , $n = 6$, $p = 1$).

There was no significant difference in blood glucose levels between mice on the high energy diet and mice on the control diet on Day 0 of FDG-PET imaging (Day 0 high energy diet vs. Day 0 control diet, 8.25 ± 0.86 vs. 10.30 ± 0.87 , $n = 6$, $p = 0.4118$). Similarly, there was no significant difference in blood glucose levels between the dietary groups on Day 3 (Day 3 high energy diet vs. Day 3 control diet, 8.73 ± 0.55 vs. 11.38 ± 0.76 , $n = 6$, $p = 0.1812$).

Effect of diet and metformin on tumor FDG uptake. Fasted mice had glycemia measured prior to FDG infusion while under 1.5% isoflurane, in order to ensure similar experimental conditions between dietary groups. Glucose levels were not significantly different between groups either at baseline or following metformin treatment. Tumors from mice on control diets and high energy diets were visualized by FDG-PET (Fig. 4A). On the left are axial, coronal and sagittal views of MC38 tumors on Day 0 from the same mouse on the control diet for 12 weeks prior to metformin treatment (baseline) and post-metformin treatment. On the right are views of a mouse on the high energy diet for 12 weeks prior to metformin treatment (baseline) and post-metformin treatment. Tumor FDG uptake is represented as a color scale, with high uptake represented by red and decreasing uptake represented by yellow, green and blue. There is a visually apparent increase in tumor FDG uptake with the high energy diet and

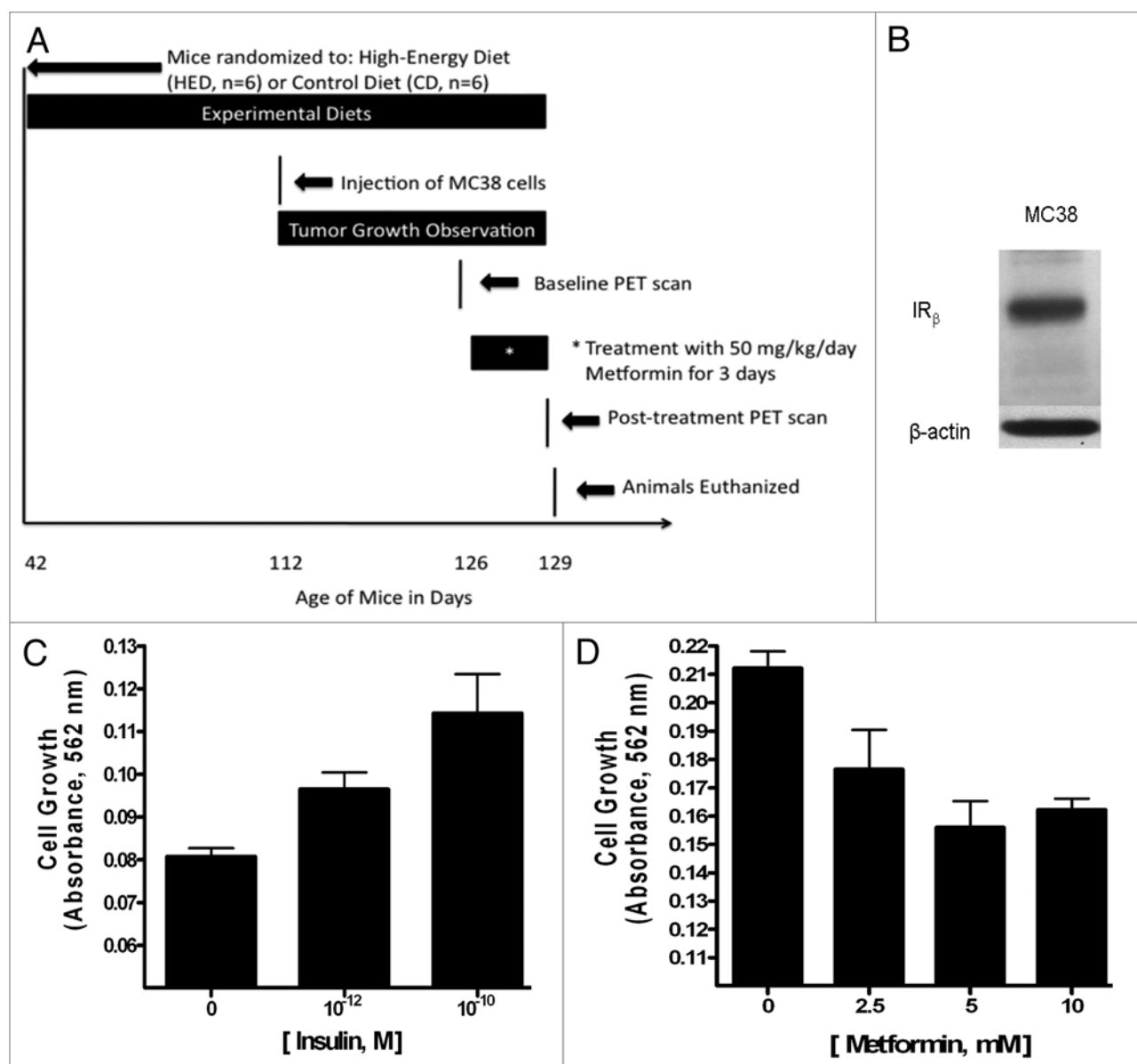


Figure 1. Experimental design, insulin receptor (IR) expression and effects of insulin or metformin on MC38 cell proliferation. (A) Six week-old mice were fed either a high energy diet or a control diet for 10 weeks. They were then injected with 5×10^5 MC38 cells. Two weeks after tumor implant, mice underwent baseline FDG-PET scans. They then received metformin (50 mg/kg/day for three days) and underwent a follow-up FDG-PET scan. (B) MC38 cells were plated in culture medium containing 10% FBS, and cell lysates were analyzed by western blot for insulin receptor (IR) subunit β (95 kDa). MC38 cells were seeded onto 96-well plates in the presence of DMEM media with 10% FBS and, after 24 h, treated with different concentrations of insulin (C; $p = 0.0059$) or metformin (D; $p = 0.0056$) with 0.1% FBS. Cell growth in each well was measured by MTT dye reduction after 72 h.

a decrease in tumor FDG uptake in mice on the high energy diet following metformin treatment.

FDG tumor uptake was quantified as a standardized uptake value (SUV) using three-dimensional regions of interest (ROIs) in tumors. Baseline SUV was higher in mice on the high energy diet compared with mice on the control diet (Fig. 4B) (2.84 ± 0.59 vs. 1.86 ± 0.58 , respectively, $n = 6$, $p = 0.0292$). Following metformin treatment, SUV dropped in the high energy diet group (from 2.84 ± 0.59 to 1.87 ± 0.51 , $n = 6$, $p = 0.0309$) and remained largely unchanged in the control diet group (from 1.86 ± 0.58 to 1.83 ± 0.37 , $n = 6$, $p = 0.9676$).

Effects of diet and metformin treatment on signaling pathways. As shown in Figure 5, IR^{Tyr972} phosphorylation in MC38 tissue was greater in tumors from mice on the high energy diet as compared with mice on the control diet, indicating an effect of host metabolic status on signal transduction within neoplastic cells. IR^{Tyr972} phosphorylation was reduced in tumors from high energy diet mice that were treated with metformin, reaching levels similar to IR^{Tyr972} receptor phosphorylation in MC38 tumors from mice on the control diet. Metformin had no effect on insulin receptor activation of tumors of animals on the control diet. Akt^{Ser473} phosphorylation was consistent with the IR^{Tyr972} data.

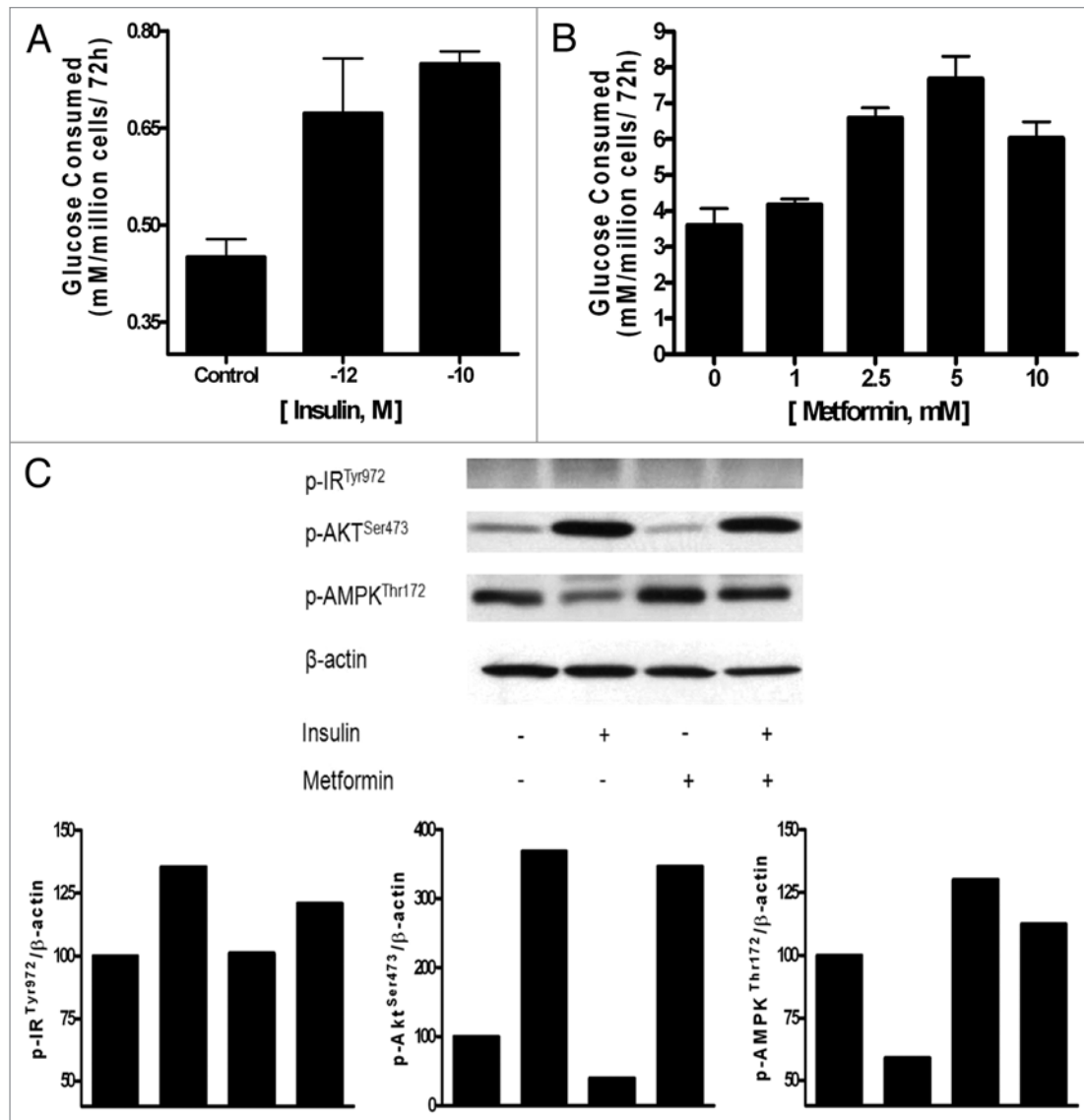


Figure 2. Effects of insulin and metformin on glucose uptake, insulin receptor activation and AMPK activation in vitro. Cells were plated in 6-well plates in the presence of DMEM media with 10% FBS. Twenty-four hours later, culture medium with different concentrations of (A) insulin ($p = 0.0127$) or (B) metformin ($p = 0.0056$) with 0.1% FBS was added. Culture medium was collected from each well and was analyzed by a glucose consumption assay. (C) Cells were plated in Petri dishes in the presence of DMEM media with 10% FBS. Twenty-four hours later, culture medium containing 2% FBS was added with insulin (1 μ M), metformin (5 mM) or both. Fort-eight hours later, cells were lysed and analyzed by western blot. Antibodies specific for phospho-IR^{Tyr972}, phospho-AKT^{Ser473}, phospho-AMPK^{Thr172} and β -actin were used as described in the Material and Methods section. Data shown are representative of at least two independent experiments.

AMPK^{Thr172} phosphorylation in tumors was not influenced by metformin, regardless of diet. This data contrasts with AMPK activation in MC38 cells by metformin in vitro, suggesting that metformin accumulation in liver tissue was sufficient to inhibit gluconeogenesis and secondarily lower insulin levels, but levels in tumor tissue were inadequate to activate AMPK, as noted in a prior model system.²⁸

Discussion

Neoplastic tissue is often regarded as having a high level of glucose uptake that is constitutive in contrast to tissues, such as liver,

muscle and fat, where glucose uptake is regulated by insulin. Older²⁹ and more recent^{8,9} evidence suggests that the growth of some cancers may be insulin-stimulated. However, the possibility that tumor glucose uptake and FDG-PET imaging results may vary with insulin level for a subset of tumors has not been examined in vivo.

Metformin, which lowers both glucose and insulin levels in the setting of type II diabetes, has recently been shown to have antineoplastic activity in some experimental models,^{7,15-19,23} and pharmacoepidemiological research (reviewed in refs. 14 and 20) provides evidence for decreased cancer burden in patients using metformin. In view of increasing interest in the roles of insulin

and metformin in neoplasia,²² we sought to examine their influence on tumor glucose uptake measured by FDG-PET in an experimental system. In order to model the clinical situation, we used a diet-induced hyperinsulinemic model rather than a transgenic or insulin-clamp model.

We recognized that there were several plausible hypotheses: (1) with respect to insulin, increased insulin might increase the FDG-PET signal for an insulin sensitive tumor and (2) with respect to metformin, this agent might either decrease the FDG-PET signal by reducing circulating insulin levels or increase FDG tumor uptake secondary to activation of AMPK.

Our results reveal that in the MC38 colon cancer model, the FDG-PET signal is, indeed, increased with a high energy diet that leads to hyperinsulinemia and increased insulin receptor activation in neoplastic tissue. This observation suggests that, at least for a subset of human cancers, host metabolic state influences tumor glucose uptake. While our results do not conclusively demonstrate that hyperinsulinemia is the mediator of the effect of host diet on the FDG-PET signal, circumstantial evidence is consistent with this possibility. In any case, our data provide the first functional imaging correlate of the well-known adverse effect of caloric excess or hyperinsulinemia on cancer outcome.^{10,11,30}

Metformin exposure had no effect on insulin levels or tumor FDG uptake in normoinsulinemic mice but decreased insulin levels and FDG uptake in hyperinsulinemic mice. This suggests that the decline in insulin levels induced by this compound is more important than any metformin-related AMPK activation in neoplastic tissue in this model. Importantly, while we previously showed activity of metformin regardless of degree of hyperinsulinemia for LKB1^{-/-} neoplasms,¹⁵ the MC38 cells employed in the present work were LKB1 wild-type.¹⁵ Although metformin increased glucose uptake in vitro, as expected with activation of AMPK, this phenomenon was not observed in vivo.

We speculate that prior clinical reports of increased FDG uptake in the gastrointestinal tract^{31,32} associated with oral metformin administration is due to AMPK activation in tissue of the gut wall, which are directly exposed to high luminal metformin concentrations. Furthermore, recent studies that describe the ability of orally administered metformin to activate AMPK in a colorectal carcinogenesis model provide encouraging evidence that this drug has chemopreventative activity for this type of cancer.³³ In contrast, in our in vivo model, direct AMPK activation did not occur in mouse tumor tissue, a finding consistent with some (reviewed in ref. 28), but not all, prior reports. It is important to mention that the ability of metformin to decrease serum insulin levels may contribute to its chemopreventative activity in a lung carcinogenesis model.²⁸

Interestingly, life span-extending mutations or caloric restriction in model organisms reduces hyperglycemia and hyperinsulinemia, which are similar to the effects of metformin in type II diabetic patients.³⁴⁻³⁶ Other studies have pointed to the apoptosis-promoting effects of metformin in aggressive triple-negative breast cancer models³⁷ in the absence of hyperglycemia or hyperinsulinemia.

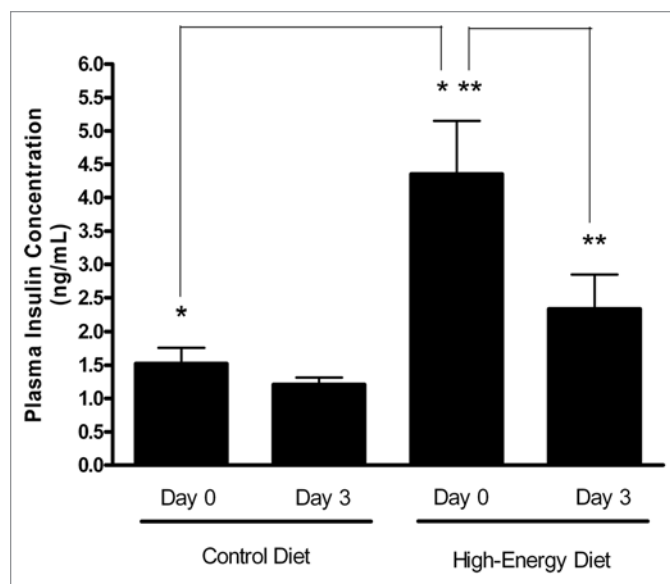


Figure 3. Acute metformin treatment reduces serum insulin. Non-fasting plasma insulin levels were determined in mice following 12 weeks on the respective diets (Day 0; * $p = 0.004$). Mice were then injected intraperitoneally with 50 mg/kg/day of metformin for three consecutive days, and non-fasting plasma insulin levels were determined again (Day 3; ** $p = 0.05$).

There are important gaps in knowledge regarding whole organism and cellular pharmacokinetics of biguanides, such as metformin. The OCT1 and OCT2 transporters are known to import metformin into cells,³⁸ but, to date, have been studied mainly in the context of drug distribution of metformin relevant to diabetes treatment;³⁹ it is possible that tumors with high levels of expression of these transporters may achieve metformin levels sufficient to cause AMPK activation, leading to a secondary decrease in proliferation and increase in glucose transport and hence FDG uptake.

Our observations provide an example of an insulin-responsive tumor, where diet-induced increases in insulin level are associated with increased FDG uptake on PET images, and where attenuation of diet-induced hyperinsulinemia by metformin is associated with decreased FDG uptake, raising the possibility that a subset of human cancers may exhibit similar behavior. However, our findings do not imply that such behavior is universal. Tumors with constitutive activation of PI3-kinase, for example, would not be expected to be insulin-sensitive with respect to growth or FDG uptake signal intensity.

In the context of increasing interest in clinical trials of metformin in cancer treatment, our model suggests that, if this agent has antineoplastic activity related to its effect on insulin levels, such activity would be confined to hyperinsulinemic subjects with insulin sensitive neoplasms.

Materials and Methods

Cell lines and cultures. The mouse colon adenocarcinoma MC38 cells (kindly donated by Pnina Brodt's lab, McGill

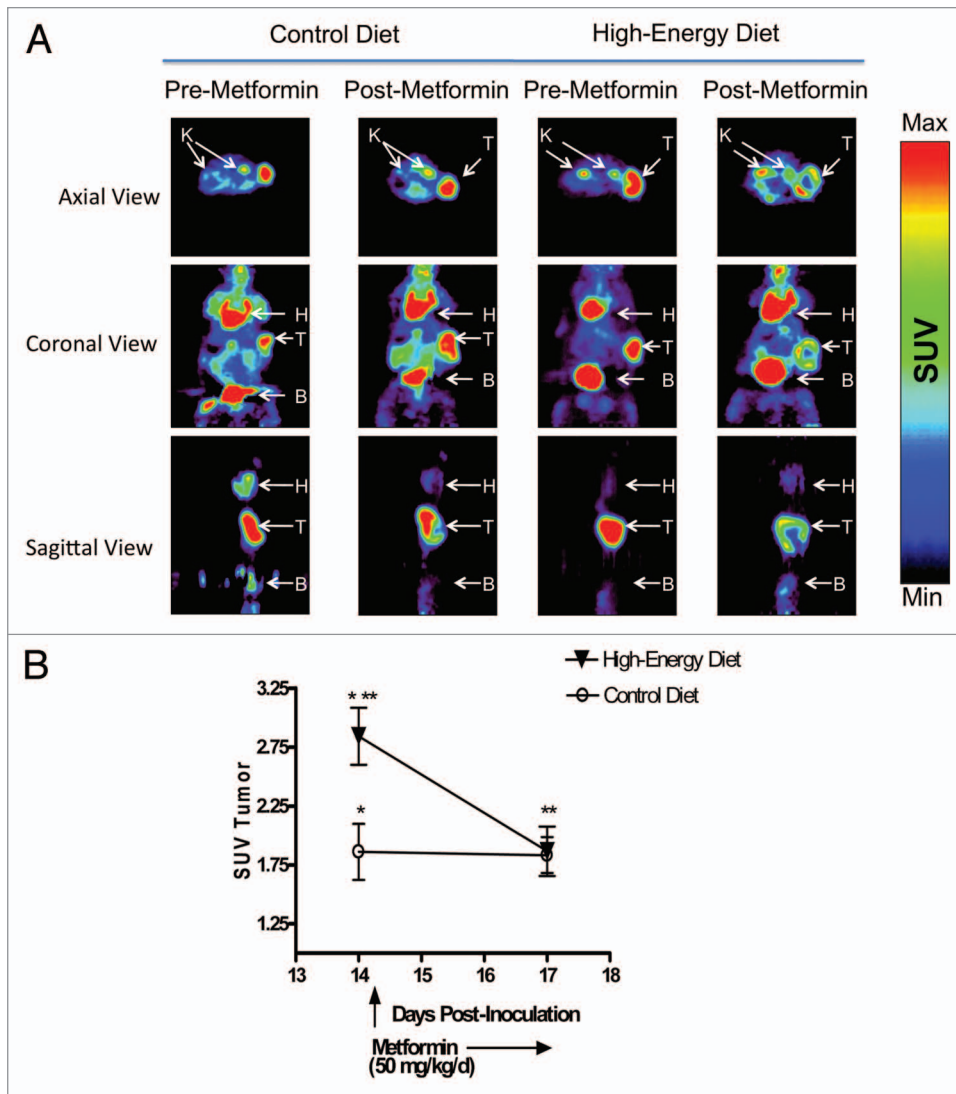


Figure 4. FDG uptake by MC38 tumors is increased by a high energy diet, and this effect is blocked by metformin. (A) Representative images of tumors from mice on control diets and high energy diets were visualized by FDG-PET. Left shows axial, coronal and sagittal views of MC38 tumors from the same mouse on control diet pre-metformin treatment (baseline) and post-metformin treatment (50 mg/kg/day for three days). Right shows the same views of a mouse on the high energy diet for 12 weeks pre-metformin treatment (baseline) and post-metformin treatment (50 mg/kg/day for three days), displaying a reduction in tumor FDG uptake. K, kidney; T, tumor; B, bladder; SUV, standardized uptake values. (B) Quantitative FDG tumor uptake was calculated using a 3D ROI in the tumors by performing sequential FDG-PET scans on six mice on the control diet and six mice on the high energy diet at baseline and again following metformin treatment (* $p = 0.0292$; ** $p = 0.0309$). ROI, region of interest.

University, Montreal, QC Canada) were maintained in DMEM (Wisent, St. Bruno, QC Canada) and supplemented with 10% FBS (Wisent) and gentamicin (Sandoz, Boucherville, QC Canada).

Cell proliferation assay. The effect of insulin on MC38 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical, St. Louis, MO). Two thousand cells per well were plated in 96-well plates and incubated in medium containing 10% FBS. After 24 h, the complete medium was replaced with medium containing 0.1% FBS and different concentrations of metformin (Sigma) or with serum-free

medium containing various doses of insulin (Feldan, Montreal, QC Canada) for a period of 72 h. Proliferation assay was measured with an MTT assay as described in reference 40.

Glucose consumption. Media samples were collected, and glucose consumption was measured using a colorimetric assay as described previously in references 40 and 41. Briefly, on the day of the assay, 10 μ l of medium samples were mixed with 435 μ l of H_2O , 500 μ l of the buffer/chromophore reagent [0.8 M sodium Phosphate buffer, pH 6.0, 10 mM 4-aminoantipyrine (Sigma) and 10 mM N-ethyl-N-sulfofpropyl-m-toluidine (Sigma)], 5 μ l of 540 U/ml horseradish peroxidase (Sigma) and 50 μ l of 310 U/ml glucose oxidase (Sigma). A standard curve was made using D-glucose (Sigma) at concentrations varying between 0.05 and 25 mM. As a negative control, glucose oxidase was omitted and replaced by H_2O . The reactions were incubated for 45 min in the dark, and then the absorbance was read at 562 nm. Each condition was in duplicate, and the experiment was performed three times. We previously found that results of this assay were completely consistent with results obtained with a tritiated glucose uptake assay.⁴⁰

Animals. All experiments performed were approved by the McGill University Animal Care and Handling Committee. Six week-old C57BL/6J-mice (Jackson Laboratory, Ann Arbor, ME) were randomized into four groups of mice: control diet, control diet + metformin (50 mg/kg/day for three days),

high energy diet and high energy diet + metformin (50 mg/kg/day for three days). Food and water were given ad libitum. Diets were purchased from OpenSource Diets (New Brunswick, NJ). The high energy diet (D12492) consisted of 20% protein, 20% carbohydrate, 60% fat and provided 5.24 kcal/g consumed. The control diet (D12450B) consisted of 20% protein, 70% carbohydrate, 10% fat and provided 3.85 kcal/g consumed. The animals were on the respective diets for 12 weeks, as shown in our experimental design (Fig. 1A).

Metabolic assays. Hormone serum levels were measured using rodent insulin immunoassay (Millipore, Etobicoke, ON Canada)

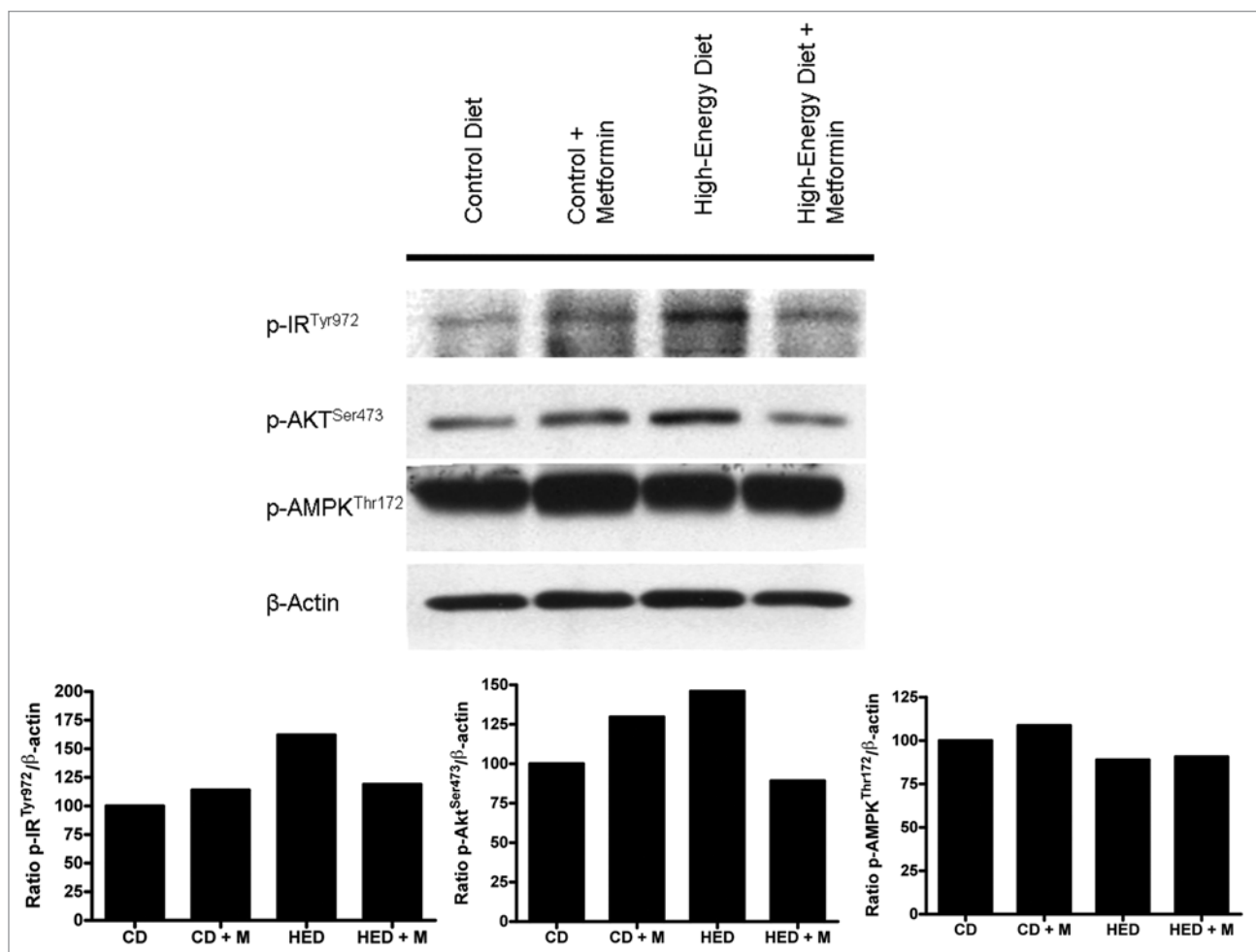


Figure 5. Effects of diet and acute metformin treatment on tumor signaling in vivo. MC38 tumor tissue from mice on different diets receiving metformin (50 mg/kg/day for three days) or no metformin was analyzed by western blot. Antibodies specific for phospho-IR^{Tyr972}, phospho-AKT^{Ser473}, phospho-AMPK^{Thr172} and β-actin were used as described in Material and Methods section. Data shown are representative of at least two independent experiments. CD, control diet; HED, high energy diet; M, metformin.

according to the manufacturer's protocols. Blood was taken from non-fasting animals after 12 weeks on the experimental diets.

Syngeneic tumor model. Mice were injected s.c. with 5×10^5 MC38 cells, following 10 weeks on the experimental diets. Tumors were palpable 7 d after injection and were grown for two weeks until baseline FDG-PET and post-treatment FDG-PET imaging.

Metformin. Six mice on the control diet and six mice on the high energy diet underwent acute metformin treatment following day 14 tumor growth baseline PET scans. Mice were treated with 50 mg/kg metformin (Sigma Chemical) i.p. daily for three days until the post-treatment day 17 tumor growth PET scan. This dose is in the same order of magnitude as the dose of metformin employed clinically, where 2 g per day is a typical dose.

PET imaging. PET images were acquired immediately prior to the initiation of treatment (Day 14 post-tumor implantation) and upon completion of the treatment regimen (Day 17 post-tumor implantation). The animals were fasted overnight and had their

blood glucose measured before FDG injection and imaging. The mice were prepared for imaging while under isoflurane (Abbott Laboratories, Montreal, QC Canada) anesthesia delivered through a nose cone at a concentration of 1.5% in medical air. A bolus injection of 60 to 90 μCi FDG in 50 μl of normal saline into the tail vein was performed. Images were acquired using a CT1 Concorde R4 microPET scanner (Siemens/CTI Concorde, Knoxville, TN). Imaging studies were performed while the animal was anesthetized and placed in the supine position on the bed of the scanner at the center of the field of view. Imaging was initiated exactly 45 minutes after FDG injection and lasted for 20 min. Mice were kept warm with a heating lamp, while physiologic parameters (cardiac rate, respiration and temperature) were continuously monitored (Biopac Systems, Goleta, CA). All images were reconstructed using the filtered back projection after applying normalization and scatter correction. The PET images were analyzed using ASIpro software (Concorde MicroSystems, Inc.) with 3D regions of interest (ROI) drawn around areas of increased tracer accumulation. To quantify the uptake of FDG

in the tumor, standardized uptake values (SUV) were calculated as average tissue concentration (MBq/mL) divided by the ratio of injected dose (MBq) over animal weight.^{42,43}

Protein extraction and western blot analysis. Cells were washed thrice with ice-cold PBS and lysed in lysis buffer [20 mM TRIS-HCl (pH 7.5)], 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, 1 mM Na_3VO_4 , 1 mM EGTA, 1% Triton and Roche Complete protease inhibitor tablet (Roche, Laval, QC Canada). Cellular debris was removed by centrifugation. Following assay for total protein (Bio-Rad, Mississauga, ON Canada), clarified protein lysates from each experimental condition were boiled for 5 min and subjected to western blot analysis.

MC38 tumor was dissected from the mice at the time of euthanasia, washed in PBS and frozen in liquid nitrogen. The tissue samples were homogenized and sonicated in lysis buffer [50 mM Hepes, 1% Triton-X 100, 150 mM NaCl, 0.02% sodium azide, 60 mM β -glycerophosphate, 1 mM DTT, Roche Complete protease inhibitor cocktail (Roche) and 5 μM pepstatin A (Sigma), pH 7.2]. Tissue debris was removed by centrifugation and following assay for total protein (Bio-Rad), clarified protein lysates from each experimental condition were pooled, boiled and subjected to western blot analysis.

Proteins (50 μg) were resolved electrophoretically on denaturing SDS-polyacrylamide gels (10%), transferred to nitrocellulose membranes, and probed with the following antibodies overnight at 4°C: anti-phospho-IR^{Tyr972} (Millipore), anti-phospho-Akt^{Ser473}, anti-phospho-AMPK^{Thr172} and anti- β -actin (Cell Signaling,

Danvers, MA). The position of the proteins was visualized with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin antibodies (ECL, Amersham, Baie d'Urfé, QC, Canada).

Experiments were repeated at least twice. Band intensity was evaluated by densitometry using Scion Corporation software (version Alpha 4.0.3.2, Frederick, MD).

Statistical analysis. All values are expressed as means $\pm$ SEM. Prior to statistical analysis, data were square-root transformed to satisfy the assumptions of analysis. Statistical significance was evaluated using GLM Procedure. Two-way (blood glucose, insulin ELISA and SUV) and one-way (cell proliferation, glucose consumption) analysis of variance (ANOVA) was used to determine whether there was significant difference among all treatment groups. Additionally, least squares means post hoc for multiple unpairwise comparisons of means (LSMEANS statement with Bonferroni correction) was applied. All statistical analyses were performed using Statistical Analysis System software, version 9.2 (SAS Institute, Cary, NC), with p values ≤ 0.05 considered significant.

Acknowledgments

The authors wish to acknowledge Dr. Pnina Brodt for generously providing us with the MC38 cells. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. Haider Mashhedi is supported through the McGill Integrated Cancer Research Training Program.

References

- Warburg O. On the origin of cancer cells. *Science* 1956; 123:309-14; PMID:13298683; DOI:10.1126/science.123.3191.309.
- Gambhir SS. Molecular imaging of cancer with positron emission tomography. *Nat Rev Cancer* 2002; 2:683-93; PMID:12209157; DOI:10.1038/nrc882.
- Roy FN, Beaulieu S, Boucher L, Bourdeau I, Cohade C. Impact of intravenous insulin on ^{18}F -FDG PET in diabetic cancer patients. *J Nucl Med* 2009; 50:178-83; PMID:19164226; DOI:10.2967/jnumed.108.056283.
- Torizuka T, Fisher SJ, Brown RS, Wahl RL. Effect of insulin on uptake of FDG by experimental mammary carcinoma in diabetic rats. *Radiology* 1998; 208:499-504; PMID:9680582.
- Johnson JA, Pollak M. Insulin, glucose and the increased risk of cancer in patients with type 2 diabetes. *Diabetologia* 2010; 53:2086-8; PMID:20645073; DOI:10.1007/s00125-010-1855-0.
- Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009; 324:1029-33; PMID:19460998; DOI:10.1126/science.1160809.
- Algire C, Zakikhani M, Blouin MJ, Shuai JH, Pollak M. Metformin attenuates the stimulatory effect of a high energy diet on in vivo H59 carcinoma growth. *Endocr Relat Cancer* 2008; 15:833-9; PMID:18469156; DOI:10.1677/ERC-08-0038.
- Novosyadly R, Lann DE, Vijayakumar A, Rowzee A, Lazzarino DA, Fierz Y, et al. Insulin-mediated acceleration of breast cancer development and progression in a nonobese model of type 2 diabetes. *Cancer Res* 2010; 70:741-51; PMID:20068149; DOI:10.1158/0008-5472.CAN-09-2141.
- Venkateswaran V, Haddad AQ, Flesher NE, Fan R, Sugar LM, Nam R, et al. Association of diet-induced hyperinsulinemia with accelerated growth of prostate cancer (LNCaP) xenografts. *J Natl Cancer Inst* 2007; 99:1793-800; PMID:18042933; DOI:10.1093/jnci/djm231.
- Ma J, Li H, Giovannucci E, Mucci L, Qiu W, Nguyen P, et al. Prediagnostic body-mass index, plasma C-peptide concentration and prostate cancer-specific mortality in men with prostate cancer: a long-term survival analysis. *Lancet Oncol* 2008; 9:1039-47; PMID:18835745; DOI:10.1016/S1470-2045(08)70235-3.
- Wolpin BM, Meyerhardt JA, Chan AT, Ng K, Chan JA, Wu K, et al. Insulin, the insulin-like growth factor axis and mortality in patients with nonmetastatic colorectal cancer. *J Clin Oncol* 2009; 27:176-85; PMID:19064975; DOI:10.1200/JCO.2008.17.9945.
- Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* 2005; 330:1304-5; PMID:15849206; DOI:10.1136/bmj.38415.708634.F7.
- Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD, Evans JM. New users of metformin are at low risk of incident cancer: A cohort study among people with type 2 diabetes. *Diabetes Care* 2009; 32:1620-5; PMID:19564453; DOI:10.2337/dc08-2175.
- Lee MS, Hsu CC, Wahlqvist ML, Tsai HN, Chang YH, Huang YC. Type 2 diabetes increases and metformin reduces total, colorectal, liver and pancreatic cancer incidences in Taiwanese: a representative population prospective cohort study of 800,000 individuals. *BMC Cancer* 2011; 11:20; PMID:21241523; DOI:10.1186/1471-2407-11-20.
- Algire C, Amrein L, Bazile M, David S, Zakikhani M, Pollak M. Diet and tumor LKB1 expression interact to determine sensitivity to antineoplastic effects of metformin in vivo. *Oncogene* 2011; 30:1174-82; PMID:21102522; DOI:10.1038/onc.2010.483.
- Algire C, Amrein L, Zakikhani M, Panasci L, Pollak M. Metformin blocks the stimulative effect of a high energy diet on colon carcinoma growth in vivo and is associated with reduced expression of fatty acid synthase. *Endocr Relat Cancer* 2010; 17:351-60; PMID:20228137; DOI:10.1677/ERC-09-0252.
- Hirsch HA, Iliopoulos D, Tschlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res* 2009; 69:7507-11; PMID:19752085; DOI:10.1158/0008-5472.CAN-09-2994.
- Huang X, Wulschleger S, Shpiro N, McGuire VA, Sakamoto K, Woods YL, et al. Important role of the LKB1-AMPK pathway in suppressing tumorigenesis in PTEN deficient mice. *Biochem J* 2008; 412:211-21; PMID:18387000; DOI:10.1042/BJ20080557.
- Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, et al. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer Res* 2007; 67:6745-52; PMID:17638885; DOI:10.1158/0008-5472.CAN-06-4447.
- Pollak M. Metformin and other biguanides in oncology: advancing the research agenda. *Cancer Prev Res (Phila)* 2010; 3:1060-5; PMID:20810670; DOI:10.1158/1940-6207.CAPR-10-0175.
- Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* 2005; 310:1642-6; PMID:16308421; DOI:10.1126/science.1120781.
- Pollak M. Insulin and insulin-like growth factor signaling in neoplasia. *Nat Rev Cancer* 2008; 8:915-28; PMID:19029956; DOI:10.1038/nrc2536.

23. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Res* 2006; 66:10269-73; PMID:17062558; DOI:10.1158/0008-5472.CAN-06-1500.
24. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer Res* 2007; 67:10804-12; PMID:18006825; DOI:10.1158/0008-5472.CAN-07-2310.
25. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* 2000; 275:223-8; PMID:10617608; DOI:10.1074/jbc.275.1.223.
26. Hardie DG. AMP-activated protein kinase: a cellular energy sensor with a key role in metabolic disorders and in cancer. *Biochem Soc Trans* 2011; 39:1-13; PMID:21265739; DOI:10.1042/BST0390001.
27. Yakar S, Nunez NP, Pennisi P, Brodt P, Sun H, Fallavollita L, et al. Increased tumor growth in mice with diet-induced obesity: impact of ovarian hormones. *Endocrinology* 2006; 147:5826-34; PMID:16959846; DOI:10.1210/en.2006-0311.
28. Memmott RM, Mercado JR, Maier CR, Kawabata S, Fox SD, Dennis PA. Metformin prevents tobacco carcinogen-induced lung tumorigenesis. *Cancer Prev Res (Phila)* 2010; 3:1066-76; PMID:20810672; DOI:10.1158/1940-6207.CAPR-10-0055.
29. Heuson JC, Coune A, Heimann R. Cell proliferation induced by insulin in organ culture of rat mammary carcinoma. *Exp Cell Res* 1967; 45:351-60; PMID:6021927; DOI:10.1016/0014-4827(67)90185-1.
30. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity and mortality from cancer in a prospectively studied cohort of US adults. *N Engl J Med* 2003; 348:1625-38; PMID:12711737; DOI:10.1056/NEJMoa021423.
31. Gontier E, Fourme E, Wartski M, Blondet C, Bonardel G, Le Stanc E, et al. High and typical ¹⁸F-FDG bowel uptake in patients treated with metformin. *Eur J Nucl Med Mol Imaging* 2008; 35:95-9; PMID:17786437; DOI:10.1007/s00259-007-0563-6.
32. Ozilker T, Ozulker F, Mert M, Ozpacaci T. Clearance of the high intestinal (18)F-FDG uptake associated with metformin after stopping the drug. *Eur J Nucl Med Mol Imaging* 2010; 37:1011-7; PMID:20107796; DOI:10.1007/s00259-009-1330-7.
33. Hosono K, Endo H, Takahashi H, Sugiyama M, Uchiyama T, Suzuki K, et al. Metformin suppresses azoxymethane-induced colorectal aberrant crypt foci by activating AMP-activated protein kinase. *Mol Carcinog* 2010; 49:662-71; PMID:20564343; DOI:10.1002/mc.20637.
34. Anisimov VN, Piskunova TS, Popovich IG, Zabezhinski MA, Tyndyk ML, Egormin PA, et al. Gender differences in metformin effect on aging, life span and spontaneous tumorigenesis in 129/Sv mice. *Aging (Albany NY)* 2010; 2:945-58; PMID:21164223.
35. Anisimov VN, Berstein LM, Popovich IG, Zabezhinski MA, Egormin PA, Piskunova TS, et al. If started early in life, metformin treatment increases life span and postpones tumors in female SHR mice. *Aging (Albany NY)* 2011; 3:148-57; PMID:21386129.
36. Anisimov VN. Metformin for aging and cancer prevention. *Aging (Albany NY)* 2010; 2:760-74; PMID:21084729.
37. Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE, et al. Metformin induces unique biological and molecular responses in triple negative breast cancer cells. *Cell Cycle* 2009; 8:2031-40; PMID:19440038; DOI:10.4161/cc.8.13.8814.
38. Kimura N, Masuda S, Tanihara Y, Ueo H, Okuda M, Katsura T, et al. Metformin is a superior substrate for renal organic cation transporter OCT2 rather than hepatic OCT1. *Drug Metab Pharmacokinet* 2005; 20:379-86; PMID:16272756; DOI:10.2133/dmpk.20.379.
39. Graham GG, Punt J, Arora M, Day RO, Doogue MP, Duong JK, et al. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet* 2011; 50:81-98; PMID:21241070; DOI:10.2165/11534750-000000000-00000.
40. Blouin MJ, Zhao Y, Zakikhani M, Algire C, Piura E, Pollak M. Loss of function of PTEN alters the relationship between glucose concentration and cell proliferation, increases glycolysis and sensitizes cells to 2-deoxyglucose. *Cancer Lett* 2010; 289:246-53; PMID:19744772; DOI:10.1016/j.canlet.2009.08.021.
41. Blake DA, McLean NV. A colorimetric assay for the measurement of D-glucose consumption by cultured cells. *Anal Biochem* 1989; 177:156-60; PMID:2742145; DOI:10.1016/0003-2697(89)90031-6.
42. Scimille Y, Benard F, Rousseau J, Pepin E, Aliaga A, Tessier G, et al. Impact on estrogen receptor binding and target tissue uptake of [¹⁸F]fluorine substitution at the 16alpha-position of fulvestrant (faslodex; ICI 182,780). *Nucl Med Biol* 2004; 31:691-8; PMID:15246359; DOI:10.1016/j.nucmed-bio.2004.02.010.
43. Aliaga A, Rousseau JA, Cadorette J, Croteau E, van Lier JE, Lecomte R, et al. A small animal positron emission tomography study of the effect of chemotherapy and hormonal therapy on the uptake of 2-deoxy-2-[¹⁸F] fluoro-D-glucose in murine models of breast cancer. *Mol Imaging Biol* 2007; 9:144-50; PMID:17334852; DOI:10.1007/s11307-007-0091-6.