Window of Opportunity Studies: Investigating the Bioactivity of Metformin in Cancer

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Abstract
Window of opportunity study trial designs utilise the short period of time prior to starting definitive therapy for cancer to test the bioactivity of drugs. The repurposing of metformin as a cancer drug has been hampered by the lack of knowledge regarding its mechanism of action in cancer. To address this problem eight window studies have been published to date mainly exploring metformin’s effects on intratumoral immunohistochemical and host serum metabolic markers. Although there is much discrepancy between these studies, overall the findings have been that metformin’s anti-cancer effects are most likely driven by its modulation of the insulin axis and that future trials should focus on obese cancer patients and those with the metabolic syndrome. In this review we discuss the design, key findings and flaws of these trials.

ABBREVIATIONS
AMP: Adenosine Monophosphate; ATP: Adenosine Triphosphate; AMPK: Activated Protein Kinase; CBP: Coactivator CREB Binding Protein; DM: Diabetes Mellitus; mTOR: Mammalian Target of Rapamycin; IPSC: Induced Pleuripotent Stem Cells; P38: Phosphoinositide 3-Kinase; MAPK: Mitogen-Activated Protein Kinase; IR: Insulin Receptor; IGF-1: Insulin-like Growth Factor 1; HOMA: Homeostasis Model Assessment; BMI: Body Mass Index; HER2: Human Epidermal Receptor Growth factor receptor 2; TUNEL: Terminal Deoxynucleotidyltransferased UTP Nick End Labelling; ACC: Acetyl-Co-A Carboxylase; ERK1/2: Extracellular-signal-Regulated Kinase ½; CRP: C-Reactive Protein; IGFBP-1: Insulin Growth Factor Binding Protein 1; IGFBP-3: Insulin Growth Factor Binding Protein-3; TCA cycle: Tri Carboxylic Acid Cycle; p4EBP-1: Phosphorylated 4E Binding Protein 1; PSA: Prostate Specific Antigen

INTRODUCTION
There are now over 100 trials worldwide examining the potential of metformin as a drug in the cancer setting (ref: clinical trials.gov). Metformin is the most commonly prescribed drug for the treatment of non-insulin dependent diabetes and is generally well tolerated. The interest in metformin’s anti-cancer effects grew out of a series of epidemiological and observational studies that showed that diabetic patients treated with metformin have a reduced risk of developing cancer [1-4]. The most recent meta-analysis summarising 47 independent epidemiological studies reported a 31% relative risk reduction in cancer incidence and 34% reduction in cancer mortality for diabetic patients treated with metformin compared to those on other anti-diabetic agents [4]. However the evidence is not unequivocal, as a meta-analysis of clinical trials comparing the safety and efficacy of metformin to sulfonylurea’s and rosiglitazone, did not show a significant difference in cancer incidence [5]. Epidemiological evidence also suggests that when combined with standard chemotherapy or radiotherapy metformin may improve outcomes in several different tumour types suggesting of a possible synergistic effect [6-12]. Furthermore, many in vitro and in vivo studies using cancer cell line models have demonstrated a growth inhibitory effect of metformin including breast [13-15], endometrial [16], colorectal [17], pancreatic [18] and lung cancer cell lines [19]. Nevertheless, it is unclear whether these results can be extrapolated to the clinical setting as the concentrations of metformin used to see effect in these laboratory studies are up to a thousand fold higher than the peak plasma level in humans at standard clinical dosing [20]. Notably, one recent study has shown that metformin can inhibit mitophagy in vitro at clinically equivalent concentrations suggesting a novel mode of action [21]. This could explain metformin’s reported synergistic effect with chemotherapy and radiotherapy given that a cell that cannot remove dysfunctional mitochondria is likely to be more susceptible to reactive oxygen species.

Insulin-dependent versus insulin-independent mechanisms of action

In the context of diabetes metformin is understood to lower circulating glucose and insulin levels via the inhibition of hepatic gluconeogenesis, although increased glucose uptake in muscle cells may also play a role [22]. The mechanism on a molecular level is still subject to discussion and investigation. However the...
widely accepted view is that metformin directly inhibits complex I of the mitochondrial respiratory chain resulting in reduced glucose oxidation hence interfering with energy homeostasis. This leads to an increase in the intracellular adenosine monophosphate (AMP) to adenosine triphosphate (ATP) ratio. Subsequent activation of AMP-activated protein kinase (AMPK), a serine/threonine protein kinase that is a key regulator of cellular energy metabolism and in liver cells results in the inactivation of gluconeogenesis [23]. Other, possibly direct, mechanisms for the inhibition of gluconeogenesis have been proposed including the inhibition of the mitochondrial enzyme glycerol phosphate dehydrogenase [24] and phosphorylation of the transcriptional co activator CREB binding protein (CBP) [25].

With regard to the metformin’s anti-cancer mechanism of action, two hypotheses predominate (Table 1). The direct effect proposes that metformin inhibits glucose oxidation in cancer cells, leading to activation of AMPK resulting in the activation of a myriad of downstream catabolic pathways and inhibition of anabolic pathways in order to conserve ATP levels. These downstream effects would be expected to include the down regulation of several pathways that are already therapeutic anti-cancer targets in their own right including mammalian target of rapamycin (mTOR) signalling and fatty acid synthesis (via inhibition of fatty acid synthase) [24,26,27]. It is possible that AMPK’s role in inactivating the tumour suppressor gene p53 may also play a role in metformin’s anti-cancer effect [25, 28]. AMPK independent pathways inhibiting mTOR signalling have also been proposed; for example, metformin suppresses RAG GTases that are involved in mTOR signalling in cells lacking AMPK [29]. There have been reports on the ability of metformin to prevent reprogramming of somatic cells into pleuripotent stem cells (iPSC) or tumour-propagating cells and that this maybe AMPK dependent [30,31] and to preferentially kill cancer stem cells [12,32].

In contrast the indirect mechanism of action proposes that metformin alters host metabolism by lowering blood glucose and insulin and insulin growth factor serum levels resulting in the decreased stimulation of the phosphoinositide 3-kinase (PI3K) and Ras/mitogen-activated protein kinase (MAPK) pathway in cancer cells [33-35]. The insulin receptor (IR) and insulin-like growth factor 1 receptor (IGFR) are expressed on many cancer cells including breast, liver, colon, pancreas and skin cancer cells [36]. Insulin and IGFs have been shown to stimulate the growth of cancer cell lines and raised circulating insulin levels have been shown to correlate adversely with cancer outcomes [37]. Hence the rationale for the ‘indirect’ mechanism is well described and if defined would suggest a role for metformin in the treatment of cancer patients with the metabolic syndrome or type 2 diabetes.

In summary, despite the wealth of in vitro and in vivo studies examining metformin’s activity in cancer cells a definitive understanding of its mechanism of action, especially at clinical doses has not been established from laboratory studies. Unravelling the effect of metformin on cancer cell metabolism and defining whether one or both of the insulin-dependent and independent pathways drives its effects will help us to select groups of patients for future trials and help identify potential drug combinations. One clinical trial design that is especially suited to understanding the bioactivity of drugs in cancer is the ‘window of opportunity’ study in which drugs are given to cancer patients often prior to any other therapy for a short window (usually 2-4 weeks) and typically prior to any other therapy. Blood sampling, biopsies and/or imaging can then be carried out either side of this window of treatment to determine the pharmacodynamic effects of the drug. This trial design has been used in the investigation of metformin and has already given some insight into metformin’s bioactivity in cancer. Here we summarise the eight studies published to date - an overview of the design and key findings of all the studies is given in (Table 2).

### Breast cancer

Four window studies have been published in breast cancer to date. All these studies compared tumour tissue taken at biopsy with a surgical sample in non-diabetic breast cancer patients. All 4 trials also assessed the effects of metformin on serum metabolic markers. Two of the studies were randomised placebo controlled trials [38,39] whilst one compared their results to matched historical controls [40] and another had no control arm [41].

### Immunohistochemical endpoints

Change in the immunohistochemical marker of proliferation, Ki67, was used as the primary outcome measure in all 4 trials. Ki67 is a well validated predictive marker of clinical outcome and pathological response in the neo-adjuvant treatment of breast cancer and for several other tumour types [42,43]. However the effect of metformin on Ki67 in an unselected breast cancer population remains unclear. Two out of the four studies (Hadad

| Indirect effect on tumour cells (insulin-dependent) | Lowering of glucose, insulin and insulin growth factor levels via AMPK dependent inhibition of hepatic gluconeogenesis | Reduced activation of PI3K pathway in cancer cell | Reduced activation of Ras/MAPK pathway in cancer cell |
| Direct effect on tumour cells (insulin-independent) | Activation of AMPK secondary to TCA cycle disruption within cancer cell | Reduced mTOR signalling | Reduced fatty acid synthesis via inhibition of ACC activity | Phosphorylation of tumour suppressor gene p53 | Modulation of programming of iPSC (may or may not be AMPK independent) |
| | AMPK independent mechanisms | Suppression of Rag GTases and inhibition of mTOR |

**Abbreviations:** AMPK: AMP-Activated Protein Kinase; PI3K: Phosphoinositide 3-Kinase; MAPK: Mitogen Activated Protein Kinase; MTOR: Mammalian Target of Rapamycin; ACC: Acetyl-CoA; iPSC: Induced Pleuripotent Stem Cells

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**Table 1:** Direct and Indirect effects of metformin.
et al and Niraula et al) demonstrated a significant fall in Ki67 staining after treatment with metformin [38,40]. The largest trial showed a decrease in Ki67 solely in the subgroup of patients with insulin resistance (defined by a homeostasis model assessment [HOMA] score greater than 2.8) [39]. Niraula et al demonstrated a significant decrease in IR expression and that higher baseline glucose levels were correlated to greater falls in Ki67 after treatment [40]. However, the study by Kalinsky et al, which only selected for overweight patients (body mass index [BMI]>25) who might be expected to have features of the metabolic syndrome showed no change in Ki67 overall [41].

The baseline Ki67 staining percentages varied widely amongst the studies, reflecting differences in the degree of tumour proliferation in the breast cancer population recruited. This might be due to differences in the cohorts with regard to hormone receptor and human epidermal receptor growth factor receptor 2 (HER2) expressions. No correlation between metformin’s effect on proliferation and tumour estrogen and progesterone receptor status has been demonstrated. One study did find a significant decrease in proliferation markers after treatment in the subgroup of patients with HER2 over expression [44]. This is supported by the results from an in vivo model in which metformin inhibited tumour proliferation in ErbB2 transgenic mice [45]. It is important to note that given the intrinsic variability of Ki67, analysis of studies that have used Ki67 as an endpoint for response to endocrine therapy in breast cancer have suggested that a change in Ki67 of between 32% -
50% should be considered significant for an individual patient [46]. Hence, it is not clear whether the small differences in Ki67 staining seen in the metformin studies is relevant.

Much like Ki67 the effect of metformin on markers of apoptosis has contrasted between studies. Terminal deoxynucleotidyl transferased UTP nick end labelling (TUNEL) was increased in the study by Niraula et al [40] but in the largest study there was no change overall [39].

The canonical view is that any direct effect of metformin on cancer cells would be driven via activation of AMPK and this pathway was assessed in two of the studies. Hadad et al reported a significant increase in the phosphorylation status of AMPK [38] whilst Dowling et al unexpectedly demonstrated a reduction in phosphorylation of both AMPK and its downstream target acetyl-CoA carboxylase (ACC) [47]. Both trials also assessed the effect of metformin on insulin receptor signalling. The Ras/MAPK pathway (of which extracellular-signal-regulated kinase 1/2 [ERK1/2] is a member) and PI3K pathway (of which AKT is a member) are both downstream of the insulin receptor. Here, there was consistency between the 2 studies with a fall in phosphorylated AKT and insulin receptor expression and one of the studies also demonstrated a fall in phosphorylation of ERK1/2 [38,47]. These findings support the hypothesis that metformin predominantly drives its effects through modulation of host metabolism.

**Effect of metformin on serum markers of host metabolism**

All 4 breast cancer studies measured the effect of metformin on serum insulin levels. There was variability in the insulin response with 3 of the studies demonstrating a decrease in levels but another actually showing an increase (however this may be due to poor study design, the patients having received a dextrose infusion prior to the post-metformin level being taken). Niraula et al demonstrated a significant decrease in HOMA index for all women [40] and Bonanni et al. a significant decrease in glucose and insulin levels in the subgroup of obese women [39] and as discussed above it was this subgroup that had a significant fall in Ki67. Other metabolic biomarkers have also been examined. A subgroup analysis of the Bonnani study explored the interaction between the HOMA index, C-reactive protein (CRP), Insulin Growth Factor Binding Protein-1 (IGFBP-1) and Insulin Growth Factor Binding Protein-3 (IGFBP-3) with change in Ki67. Here, compared to placebo, metformin significantly decreased Ki67 in women with baseline HOMA >2.8, those in the lowest baseline IGFBP-1 quintile, the highest IGF-BP3 quartile, low baseline free IGF-1 and in the top CRP tertile. They also showed that serum adiponectin levels (a hormone that plays a key role in regulating glucose and fatty acid oxidation) decreased after metformin treatment [48]. Circulating adiponectin levels have been linked to an increased risk of developing obesity related malignancies [49].

**Endometrial Cancer**

There is particular interest in the potential of metformin as a cancer therapeutic for Type 1 endometrial cancer due to the strong association between this disease with obesity and the metabolic syndrome. Type 1 endometrial cancer has characteristic endometrioid histology, is typically low grade, is associated with a good prognosis and typically occurs in peri- or post-menopausal women with obesity or type 2 diabetes. In contrast Type 2 endometrial cancer has a more aggressive phenotype and no association with the metabolic syndrome [50].

Three window studies have been carried out in endometrial cancer, all comparing a pre-treatment endometrial biopsy with a post-metformin surgical specimen. Two of the studies had control arms and one of the studies selected only obese women with endometrial cancer (BMI >30) [51-53].

**Immunohistochemical endpoints**

All three window studies reported a significant reduction in Ki67 (mean proportional decrease in studies; 9.7% - 20.7%) [51-53]. Surprisingly, the one study that compared the effect of metformin on Ki67 staining demonstrated lower proliferation not only in Type 1, as might be expected given the association with type 2 diabetes and obesity, but also in Type 2 endometrial cancer [51].

There was also consistent evidence of mTOR inhibition with a statistically significant decrease in phosphorylation of S6 in all three studies. Activation of ERK1/2 and AKT once again indicated that metformin treatment was leading to reduced insulin receptor signalling [51-53]. Only in the trial by Mitsuhashi et al. was increased AMPK activity seen [52].

**Serum and intra tumoral metabolic endpoints**

Schuler et al in their study analysed metabolomic profiling of the tumour samples pre- and post-metformin using mass spectrometry techniques. This revealed an elevation in intra tumoral glucose levels and glycogen metabolites in the responders as defined by those patients that had a decrease in Ki67 post-metformin. The study also showed an increase in the intra tumoral levels of the ketone body 3-hydroxybutyrate in responders and it was postulated that this might indicate increased fatty acid oxidation in response to metformin [although in our opinion it more probably suggests accumulation of acetyl-CoA due to disruption of the tricarboxylic acid (TCA) cycle] [53].

Serum glucose levels post-metformin decreased, but this fall was only significant in the subgroup with decreased proliferation after metformin exposure. Additionally, elevations of several serum free fatty acids and glycerol was suggestive of an increase in adipose tissue lipolysis in response to metformin. Again this effect was more pronounced in the responders [53]. The other two studies by Mitsuhashi and Laskov showed significantly lower levels of insulin and IGF-1 after metformin exposure [51,52].

**Prostate cancer**

Previous pre-clinical studies have shown that metformin can reduce proliferation of prostate cancer cell lines [54] and epidemiological evidence has suggested that metformin reduces prostate cancer-related mortality [55,56]. The single window trial performed in patients with prostate cancer by Joshua et al recruited 24 patients and compared Ki67 staining in the diagnostic biopsy core to the surgical specimen taken after 4-12 weeks of metformin. Although this study did not meet its recruitment target a statistically significant 30% decrease in the Ki67 index was observed. There was no evidence of activation of
AMPK (no change in phosphorylated ACC or AMPK) but there was a significant decrease in phosphorylated 4E binding protein 1 (4EBP1) suggestive of mTOR inhibition. A statistically significant fall in fasting glucose levels, IGF-1 levels and BMI was observed and there was also a trend toward a decrease in prostate specific antigen (PSA) [57].

CONCLUSION

The repurposing of metformin as an anti-cancer drug has been extensively investigated using 'window of opportunity' trial designs and lessons from this experience will inform future studies. The window studies to date have had contrasting results and it is not possible to draw an overriding conclusion but overall the theme has been that metformin's effects on Ki67 have occurred most strongly in patients with features of the metabolic syndrome. It is interesting that compared to the breast cancer studies, all 3 studies of metformin's effects in endometrial cancer showed a significant and often strong reduction in Ki67. This may well reflect the association of this disease with altered host metabolism and also adds weight to the theory that metformin's clinical anti-cancer effects are derived from its modulation of the insulin axis rather than a direct effect on the cancer cell. On this basis future phase 3 clinical trials should look to select patients with cancers that have an association with obesity and the metabolic syndrome such as endometrial cancer.

Several criticisms can be levelled at these studies. Commonly biopsy samples have been compared to surgical samples in these studies and there has been significant concern that differences in sample collection may alter Ki67 and other marker expression [58]. The immune staining and scoring of Ki67 has varied between the different studies (Table 3).

To exemplify the importance of attention to detail it should be noted that one study took the post-metformin blood samples to determine changes in host metabolic markers after a 5% dextrose infusion and then compared this to a pre-metformin fasting sample, clearly compromising the validity of this paired assay [38]. In most of the above studies there was no randomised placebo controlled group.

The authors recognise that the cost of running an exploratory 'window study' with controls may be prohibitive and that the feasibility of asking patients to go through a series of extra tests when they may not be receiving the drug of interest can be

<table>
<thead>
<tr>
<th>Study</th>
<th>Ki67 index</th>
<th>Comments</th>
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<tbody>
<tr>
<td><strong>Breast cancer</strong></td>
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<td>Hadad 2011 / Hadad 2015 (RCT)</td>
<td>Metformin group: 23.56% baseline vs. 20.15% pre-op (P=0.027) Control group: 14.57% baseline vs. 14.54 pre-op (P=0.455)</td>
<td>Ki67 scored using image analysis. Immunostaining technique not described in detail. At least 1000 nuclei assessed for each sample.</td>
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<tr>
<td>Bonnani 2012 / Cazzaniga 2013 / DeCensi 2014 (RCT)</td>
<td>Metformin group: 19% baseline vs. 21% pre-op Control group: 18% baseline vs. 20% pre-op (P=0.4)</td>
<td>Ki67 manually scored. Immunostaining using an autostainer. At least 2000 nuclei assessed for each sample.</td>
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<tr>
<td>Niraula 2012 / Dowling 2015 (Single arm)</td>
<td>36.5% baseline vs. 33.5% pre-op (P=0.016)</td>
<td>Ki67 manually scored. Immunostaining using an autostainer. At least 1000 nuclei assessed for each sample.</td>
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<tr>
<td>Kalinsky 2014 (historical controls)</td>
<td>Metformin group: 24% baseline vs. 21% pre-op Control group: 29% baseline vs. 20% pre-op (P=0.045)</td>
<td>Ki67 manually scored. Immunostaining technique not described in detail. At least 1000 nuclei assessed for each sample.</td>
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<td><strong>Endometrial cancer</strong></td>
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<td>Laskov 2014 (historical controls)</td>
<td>Metformin group: 5.3 baseline vs. 2.2 pre-op (P=0.03) Control group: 3.2 baseline vs. 3.0 pre-op (P=0.7)</td>
<td>Ki67 scored using image analysis. Ki67 expressed as intensity (range 0-3) x percentage of distribution.</td>
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<td>Mitsuhashi 2014 (historical controls)</td>
<td>Metformin group: 51% baseline vs. 30.3% pre-op (P=0.001) Control group: 45.2% baseline vs. 46.4% pre-op (P=0.12)</td>
<td>Ki67 manually scored. Immunostaining using an autostainer. At least 500 nuclei assessed for each sample.</td>
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<td>Schuler 2015 (Single arm)</td>
<td>Overall: -11.75% (P=0.008) Pre-treatment Ki67 index 47.3% in responders vs. 24.9% in non-responders</td>
<td>Ki67 scored using image analysis. Immunostaining technique not described in detail. Number of nuclei assessed not stated.</td>
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<td><strong>Prostate cancer</strong></td>
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<tr>
<td>Joshua 2014 (Single arm)</td>
<td>4.69% baseline vs. 2.84% pre-op (P=0.0018)</td>
<td>Ki67 scored using image analysis. Immunostaining manually. Number of nuclei assessed not stated.</td>
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problematic. However, further efforts to understand the effects of a prior biopsy on the post treatment result and the differences than can occur in assay results if different techniques are used (e.g. breast core biopsy versus surgical breast biopsy) need to be explored. In several studies the duration of metformin treatment would vary widely between patients (in the most extreme case by 1 to 10 weeks). Lastly, the metformin was stopped up 24 or 48 hours prior to surgery in several of the studies which might have impacted on very dynamic markers such as AMPK although Bonanni et al found no relationship between any biomarker change and the interval from last drug intake to surgery [39].

The assays used in these studies were relatively limited (immunohistochemistry and serum metabolic markers) and we believe that the integration of several pharmacodynamic assays such as imaging, functional genomics, metabolomics along with immunohistochemical and serum assays has the potential to allow a much more detailed assessment of pharmacodynamic effects. Indeed we have recently completed recruitment to a ‘window trial’ (results unpublished to date) that used all these modalities to assess the bioactivity of metformin [59] (Figure 1). This approach is more likely to tease out whether metformin’s effects toward cancer metabolism are direct or an indirect host effect. In particular, an understanding of metformin’s in vivo effect on mitochondrial function needs to be better understood. For example [F-18] FMISO PET-CT could determine whether metformin modulated oxygen consumption in tumours.

In conclusion, the metformin repurposing story has already shown the power of window studies to aid drug development, understand better how to select patients for future clinical trials and potentially inform drug combination.

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