

# A pilot ‘window of opportunity’ neoadjuvant study of metformin in localised prostate cancer

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**BACKGROUND:** Metformin is an inhibitor of complex 1 in the respiratory chain, and is widely used to reduce insulin resistance. It has also been described to have pleotropic effects including via AMPK on inhibiting the mTOR kinase. Pre-clinical and epidemiological studies suggest an ability to modulate disease evolution in prostate cancer. In this study, we aimed to (i) demonstrate safety and tolerability of neoadjuvant metformin administration and (ii) document changes in proliferative (Ki67) and AMPK-related signalling indices between matching biopsies and prostatectomies

**METHODS:** Men were treated in a single-arm ‘window of opportunity’ study between their decision to undergo radical prostatectomy and the operation itself. Forty patients were planned but only 24 patients were enrolled owing to slow accrual. Twenty-one patients were evaluable for pathological outcomes and 22 for serum metabolic indices. Metformin was given at doses to 500 mg t.i.d. Ki67 index was calculated using the Aperio-positive pixel count algorithm, whereas immunohistochemical measurements were by consensus H-Score. Comparative statistics were analysed by students *t*-tests and/or Wilcoxon matched pairs signed rank test.

**RESULTS:** Baseline characteristics included median PSA 6 ng ml<sup>-1</sup> (3.22–36.11 ng ml<sup>-1</sup>). Median duration of drug treatment was 41 days (18–81). Treatment was well tolerated with only three patients developing G3/4 toxicities. In a per patient and per tumour analyses, metformin reduced the Ki67 index by relative amounts of 29.5 and 28.6 % (*P* = 0.0064 and *P* = 0.0042) respectively. There was also a significant decrease in P-4EBP1 staining (*P* < 0.001) but no change in P-AMPK or P-ACC. There were no correlations between any metabolic, morphometric or cancer-related serum indices. There was a trend towards PSA reduction (*P* = 0.08). The study is limited by small patient numbers and tumour heterogeneity.

**CONCLUSIONS:** Neoadjuvant metformin is well tolerated prior to radical prostatectomy. Data to date indicate promising effects on metabolic and tissue proliferation and signalling parameters.

## INTRODUCTION

Prostate cancer remains the second most commonly diagnosed cancer in men in the western world.<sup>1</sup> Although advances in the treatment of advanced castrate refractory disease are extending overall survival in the setting of metastatic disease, only hormonal therapy has been shown to increase contemporary cure rates in the setting of definitive curative therapy (radiation). Further advances are critically required in this area to reduce the morbidity and mortality from the disease.

Metformin is the most commonly prescribed normoglycaemic agent worldwide for non-insulin-dependent diabetes mellitus. Its canonical mechanism of action involves inhibition of the type 1 respiratory complex and subsequent AMPK activation resulting in mTOR inhibition. Importantly, pre-clinical studies have shown a significant effect on several prostate cancer cell lines’ proliferation and accompanying xenograft growth.<sup>2</sup>

In addition, metformin reduces circulating insulin levels in non-diabetics,<sup>3</sup> relevant because higher insulin and C-peptide levels have been associated with poor outcomes in prostate cancer patients.<sup>4–6</sup> Furthermore, the overexpression of insulin receptors

(IRs) in prostate cancer and the association between IR expression or IR/IGF1R activation has been noted.<sup>7</sup>

Taken together with the larger epidemiological studies that have suggested a lack of effect on prostate cancer incidence<sup>8–10</sup> but a beneficial effect on prostate cancer-related mortality,<sup>11,12</sup> we undertook a phase 2 ‘window of opportunity’ study with metformin in men scheduled to undergo radical prostatectomy to assess the effects of metformin on cellular indices relevant to cancer to further understand and quantify its utility in this disease.

## MATERIALS AND METHODS

### Patients

From July 2009 to Feb 2011, we accrued 24 patients in a window of opportunity study prior to radical prostatectomy from the Urologic Oncology clinics at the Princess Margaret Cancer Centre, Toronto, ON, Canada. The study was approved by the University Health Network Research Ethics Board and registered on clinicaltrials.gov (NCT NCT00881725). Relevant inclusion criteria included (i) biopsy-proven, localised prostate cancer (as defined below); (ii) histologically confirmed prostate cancer

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involving at least 20% of one or more unfragmented biopsy cores in available archived biopsy cores; (iii) transrectal ultrasound within the last 3 months and biopsy tissue available for study. Relevant exclusion criteria included (i) patients medicated with any drug used for the treatment of any form of diabetes, or patients that begin treatment for any form of diabetes during the course of the study; (ii) patients receiving any other investigational, herbal or anticancer agents while on study; (iii) a current history of alcohol intake (>2 standard drinks per day) or binge drinking (five or more drinks) in one session of 1–3 h; (iv) past history of lactic acidosis or risk factors for lactic acidosis, such as congestive heart failure (NYHA Class 3 or greater), hypoxia (resting PO<sub>2</sub><91%) or renal insufficiency (eGFR<60 ml min<sup>-1</sup>). Patients were identified by participating urologists and screened by a research assistant.

After informed consent and study enrolment, patients were instructed to take metformin at doses increasing from 500 mg daily to 500 mg t.i.d. over 5 days (500 mg daily day 1–2; 500 mg b.i.d. day 2–4; 500 mg t.i.d. day 5+) for 4–12 weeks preceding their prostatectomy. Metformin was continued until the patients were nil per oral for surgery. Toxicity assessment was based on CTCAE v3. Dose reductions were permitted for select grade 3,4 toxicities.

### Baseline measurements

Baseline measurements included fasting weight, height, fasting blood glucose/insulin, 2 h postprandial glucose, lipid profile (cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides), insulin indices (insulin-like growth factor 1, IGFBP3, C-peptide), PSA and serum metformin levels. Serum levels of insulin-like growth factor 1, IGFBP3 and C-peptide were quantified using standard enzyme-linked immunosorbent assay methods, with reagents from IDS Ltd (London, UK). Serum and tissue levels of metformin were quantified using high-performance liquid chromatography. Serum metformin levels were taken from fasting patients in the week before surgery.

Pathological assessments of the baseline tumours were acquired from the original pathology reports to determine eligibility and subsequently confirmed by the study pathologists. Radical prostatectomy specimens were injected with formalin immediately *ex vivo* by the study pathologists to ensure optimal tissue preservation. Sections at 5 µm thickness were cut from formalin-fixed paraffin-embedded diagnostic core biopsy and surgical specimens, and slides were stained for each of the following antibodies with appropriate positive and negative controls: Ki67—Novus Biologicals (Littleton, CO, USA) NB100-90592 1/1500 Citrate 1 h; P-4ebp1—Cell Signaling (Beverly, MA, USA) 2855 1/100 Citrate 1 h; P-AMPK—Cell Signaling 2535 1/300 Tris-EDTA overnight; P-ACC—Cell Signaling 3661 1/400 Tris-EDTA overnight; P-GSK3B—Life Span (Seattle, WA, USA) LS-0105395 1/200 citrate overnight; P-AKT—Cell Signaling 4060 1/20 citrate overnight; ERG—Biocare Medical (Concord, CA, USA) cm421 AP Tris-EDTA pH 9.0 1/300 overnight. The 'Ki67 index' (percentage of nuclei showing nuclear immunoreactivity of any intensity) was determined by automated counting from the positive pixel count algorithm on the Aperio system after encircling all visible tumour foci in a region of interest. Other immunohistochemical assessments were performed by two pathologists (JS, MRD), who rated each sample separately before reconciling differences to reach a consensus.

### Statistical plan

There were two primary statistical end points—safety (prospectively analysed) and scientific (retrospectively analysed). Originally, an optimal 2-stage design was used to test the null hypothesis that the toxicity rate of ≥20% vs the alternative toxicity rate is <10%, with interim analyses after 19 patients, to accrue a total of 37 patients. Toxicities were defined as serious adverse events or peri-operative morbidity/mortality possibly related to study drug. Alternatively, in the event of one episode of lactic acidosis the study would have been terminated. Similarly, the primary scientific end point was the quantitation of the Ki67-proliferation index. The sample size of 37 was originally designed to provide an ~85% power to detect a 0.5 s.d. difference between the original Ki67 or the biopsy and the subsequent prostatectomy. The power is calculated based on paired *t*-test with a significance level of 0.05. Assuming 10% drop-out rate, a total sample size of 40 patients was recommended to fulfil both aims of the study, with ~2–3 patients per month over 18 months.

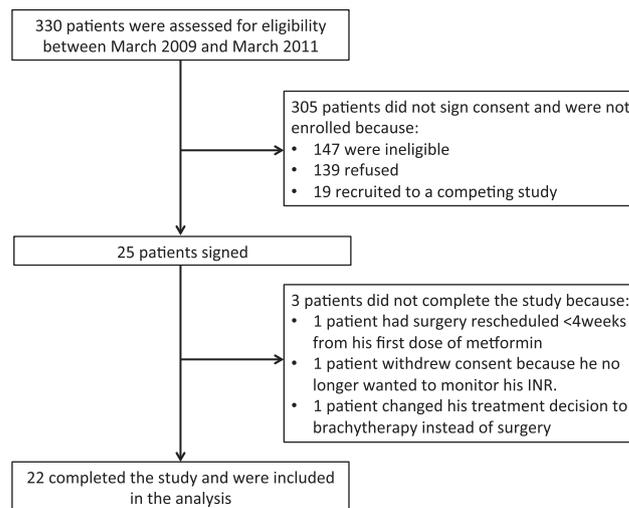
Statistical analysis was carried out after tabulation of results in Microsoft Excel. Statistical tests included students *t*-tests and Wilcoxon matched pairs signed rank test (Advanced Analytics, LLC, Gaithersburg, MD, USA) and

standard correlation coefficients. The homeostatic model assessment calculations were carried out using HOMA2 calculator online (Oxford Centre for Diabetes, Endocrinology and Metabolism, Oxford, UK).

## RESULTS

Despite intense screening, slow patient accrual necessitated early trial closure. The CONSORT diagram is shown in Figure 1. Patient characteristics of those enrolled are summarised in Table 1. Toxicities were generally minor and easily managed. These are summarised in Table 2. Notable grade 3 toxicities occurred in three patients only. These included pelvic abscess (two patients), dizziness (one patient), abdominal pain (one patient), urticaria (one patient). None of these grade 3 toxicities was thought to be related to metformin. Thus, there was no evidence of peri-operative morbidities related to metformin.

As a result of the early cessation of the trial, the power of the primary scientific end point was reduced to 65%, nevertheless, the Ki67 index per tumour decreased from a median of 4.696% Ki67 +ve nuclei to 2.84 ( $P=0.0018$ ), whereas the Ki67 index per patient (with four patients having >1 tumour identified) reduced from a median of 4.78% Ki67 +ve nuclei to 2.76 ( $P=0.0064$ )



**Figure 1.** CONSORT diagram for the study indicating patient screening and accrual patterns during the course of the study.

**Table 1.** Baseline characteristics of patients enrolled in the study

Variable	Median	Range
<i>Baseline characteristics<sup>a</sup></i>		
Age	64	53–73
PSA	6 µg l <sup>-1</sup>	3.7–36.11
Duration of metformin treatment	41 days	18–81
Number of positive biopsy cores	4	2–8
Percentage maximal core involvement	50	30–90
Primary Gleason	3	3–4
Secondary Gleason	4	3–4
<i>Post intervention characteristics</i>		
Gleason score	7	6–8
Percentage tissue involvement	4	2–20
Pathological staging	N/A	T2a–T3b

<sup>a</sup>Of the 24 evaluable patients enrolled, 22 were evaluable for assessment and 21 for pathology assessment.

(Figure 2). This represents a relative decrease of Ki67 of 29.5% (95% CI: (- 2.51, - 0.32),  $P = 0.0064$ ) for the former and a relative decrease of 28.6% (95% CI: (- 2.375, - 0.5036),  $P = 0.0042$ ) for the

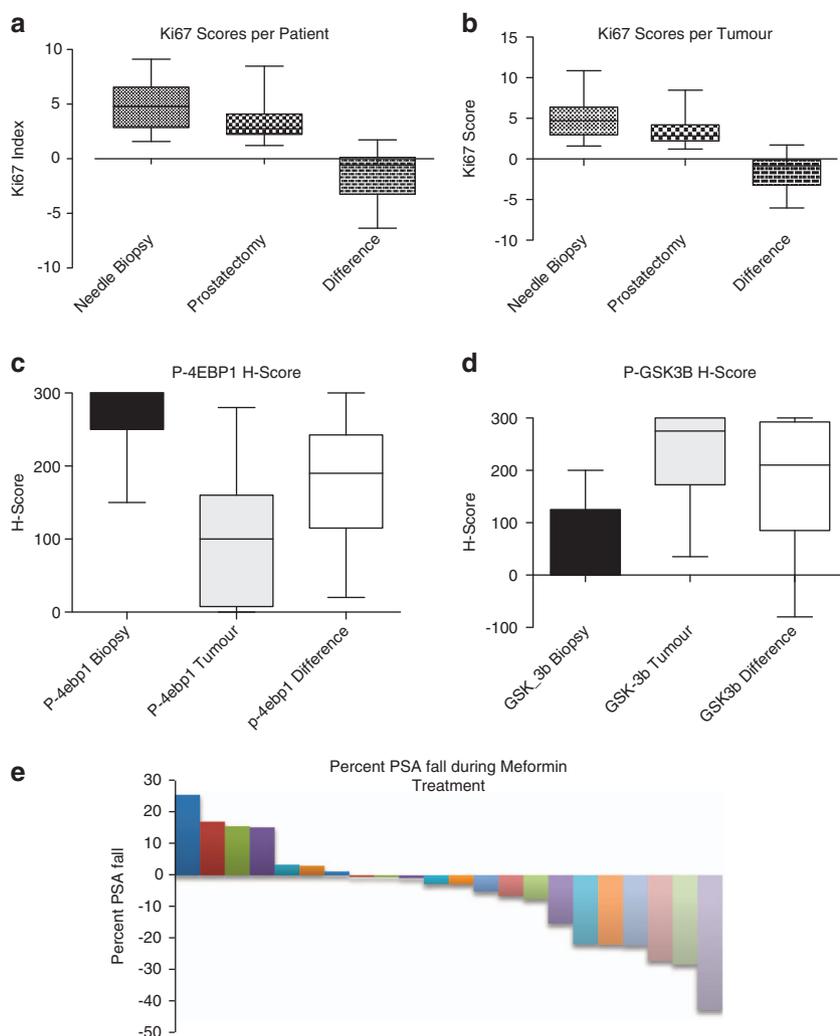
	n	Percent
Number that received at least one dose of metformin	24	100
Number that developed any AE	23	96
Patients with G3-4 AE	3 <sup>a</sup>	12.5
<i>AE present in &gt; 15% of patients</i>		
Diarrhoea (any grade)	8	33
Nausea (any grade)	4	17

Abbreviation: AE, adverse events.  
<sup>a</sup>Total number of G3-4 AE was five.

latter. Accompanying H-score assessments for immunohistochemical pathways of interest are summarised in Table 3 and depicted in Figure 2. This showed a significant decrease in P-4EBP1 staining and an increase in P-GSK3B staining, whereas no significant difference was noted in P-AMPK or P-ACC. To further clarify these findings, we carried out immunohistochemistry for P-AKT; however, we were unable to demonstrate evidence of increased P-AKT signalling after metformin administration (see Table 3).

Serum assays as outlined in the protocol are summarised in Table 4. These demonstrate significant reductions in insulin-like growth factor 1, fasting glucose and body mass index. In an exploratory analysis, we examined the associations between Ki67 index change (per patients and per tumour) or change in P-4EBP1 staining and baseline factors (listed in Table 1) as well as serum, anthropological or immunohistochemical indices, however, no associations were found (data not shown).

In addition, owing to the multifocal nature of prostate cancer, as a quality control measure, we undertook ERG immunohistochemistry on both the biopsy and correlated section of the radical prostatectomy to ensure the sections represented similar tumour



**Figure 2.** (a) Metformin reduces the Ki67 proliferation index by 1.44% (relative decrease of 29.5%) (95% CI: (- 2.51, - 0.32),  $P = 0.0064$ ). Box and whisker plots represent median value (middle line) and 25th and 75th percentile, with whiskers representing minimum and maximum values, respectively. Shading is illustrative only. (b) Metformin reduces the Ki67 proliferation index by 1.44% (relative decrease of 28.6%) (95% CI: (- 2.375, - 0.5036),  $P = 0.0042$ ). Box plot description as above. (c) and (d) Metformin reduces the P-4EBP1 staining and increases the P-GSK3B as measured by H-Score ( $P < 0.001$ ). Values have been divided by 100 to facilitate visual assessment. Box plot description as above. (e) Percent PSA fall arranged by extent of fall.

foci. This analysis revealed that of the 22 matched samples obtained (biopsy/tumour), the majority had concurrent ERG status (12 consistently negative, 6 consistently positive, 3 mixed, 1 ineligible) suggesting that we were likely to have assessed the same tumour focus in both the biopsy and the radical prostatectomy specimen. To further refine the analyses, we undertook an exploratory analysis of tumours with the same ERG status, reducing the dataset to 18 samples. Herein, the change in Ki67 index remained significant for on a per patient ( $P = 0.0063$ ) and a per tumour basis ( $P = 0.0016$ ).

## DISCUSSION

To our knowledge, this is the first neoadjuvant intervention study with metformin in prostate cancer, although others are in progress (NCT01433913). We have demonstrated a reduction in a calculated Ki67 index between the baseline biopsies and the radical prostatectomy tissue both per patient and per tumour. These results do mirror recent findings in breast cancer,<sup>13–16</sup> and are consistent with pre-clinical findings *in vitro* and *in vivo*.<sup>2,17,18</sup> Our findings of decreased P-4EBP1 may indicate a mechanistic insight into the primary end point as its role in increasing cap-dependent translation that ultimately leads to cell growth.<sup>19</sup> A perplexing finding of the study was the apparent increase in GSK3B staining, despite the absence of any increase in P-AKT staining that would have been expected given the known feedback between S6K and IRS1.<sup>20</sup> This apparent paradox may have been confounded by issues with tissue and immunohistochemical heterogeneity (in both the biopsies and prostatectomies) and phospho-protein preservation (in the prostatectomies), which may have hampered the interpretation. In an exploratory analysis we did not see any stratification by ERG status in these immunohistochemical markers (data not shown).

Strengths of our study included the assessment of serum, anthropometric and physiologic measurements as surrogate for

metformin's indirect mechanism of action, as well as tumour tissue pre- and post-metformin, use of a commonly prescribed dose of metformin and continuation of metformin to the evening prior to surgery in all subjects. Although the dose of metformin is not the maximum dose commonly prescribed to diabetic patients (2250 mg), it was felt that the dose used (1500 mg) offered the best balance of efficacy and toxicity/ compliance in this otherwise well population and is similar to doses used in other interventional studies.

Weaknesses of the study included a small sample size and the absence of a control group. With regards to the latter, we have previously stained 10 matched historical biopsies and prostatectomies from our institution from the same period as this study but were unable to demonstrate a difference in Ki67 index (data not shown). Reasons for the slow accrual to this trial are uncertain—as outlined, a small number of patients were enrolled on a competing trial (19 patients) but the majority of patients were not eligible because of either refusal or ineligibility. Refusal was most commonly related to; unable/unwilling to commit the time (46%), not interested in participating in research (26%); concern about the possible side-effects of metformin; (7%) and feeling overwhelmed by their diagnosis (7%), whereas ineligibility was most commonly due to the requirement for an adequate amount of tumour tissue in their biopsy sample (112 of 147 patients) to ensure robust immunohistochemical assessment (initially 50% but ultimately reduced to 20% of any core with sequential protocol amendments). An additional weakness is that metformin was administered for a relatively short period of time and the optimal duration of exposure to metformin necessary for any antineoplastic action is unknown.

An earlier study by Goodwin *et al.*<sup>3</sup> identified a larger decrease in fasting insulin levels (22%) when metformin was administered to non-diabetic breast cancer patients for 6 months than was seen or C-peptide in this study (no change between baseline and surgical samples). It is possible that this reflects differences in the study populations; it is also possible that administration of metformin for 2 weeks is insufficient for a full effect on fasting insulin. Our study did benefit from measuring metformin concentration in blood, with average levels of metformin being  $6.7 \text{ nmol ml}^{-1}$ , a concentration generally lower than  $20.9 \text{ nmol ml}^{-1}$  reported from large databases. Importantly, there was evidence of a direct effect on tumour signalling pathways at this concentration, despite concerns that such effects would not be recapitulated *in vivo*,<sup>21</sup> possibly because metformin concentrates in the mitochondria at up to 1000 fold its circulating concentration.<sup>22</sup> We also measured the metformin concentration in prostate tissue in five samples suggesting that metformin does reach prostate tissue, albeit in nanogram  $\text{mg}^{-1}$  quantities. Mechanisms respon-

**Table 3.** H-scores for immunohistochemical stains associated with prostate biopsies and radical prostatectomy tissues

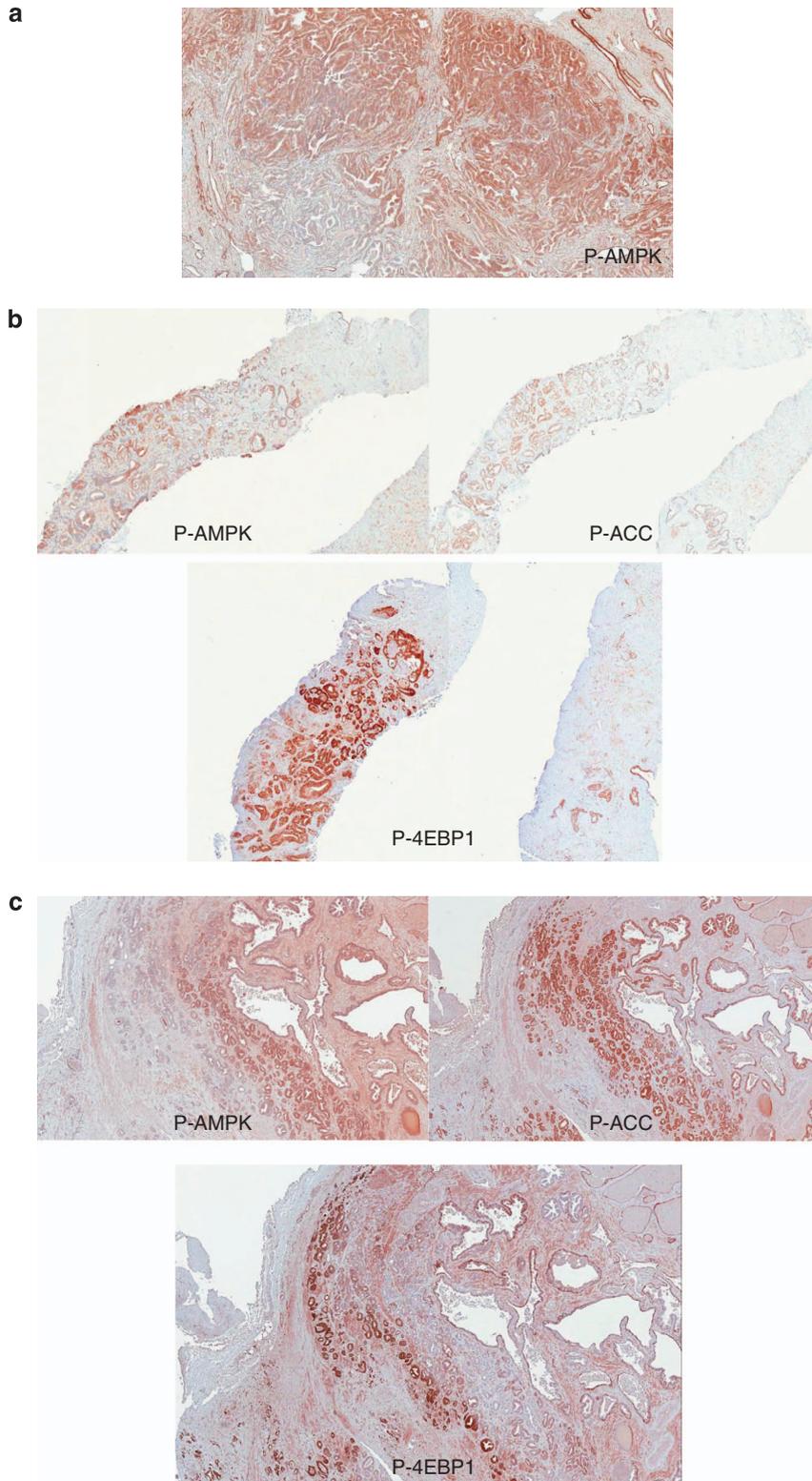
Stain	N (matched samples)	Difference H-score (mean)	Significance (t-test)
P-AMPK	22	46.6	0.07
P-ACC	22	2.73	0.92
P-4EBP1	22	170.6	<0.01
P-GSK3B	18	174.7	<0.01
P-AKT	14	31.2	0.12

**Table 4.** Serum and morphological measurements for patients enrolled on study

Assay	Median pre-metformin ( $\pm$ s.d.)	Median post-metformin ( $\pm$ s.d.)	P
IGF1 ( $\text{ng ml}^{-1}$ )	143.5 ( $\pm$ 25)	134.3 ( $\pm$ 28)	0.02
IGFBP3 ( $\text{ng}^{-1} \text{ml}^{-1}$ )	3856.3 ( $\pm$ 614)	3658.8 ( $\pm$ 730)	0.93
C-peptide ( $\text{ng ml}^{-1}$ )	2 ( $\pm$ 0.6)	2.1 ( $\pm$ 0.84)	0.91
HOMA-IR	1.6 ( $\pm$ 0.43)	1.6 ( $\pm$ 0.54)	0.55
PSA ( $\text{ng dl}^{-1}$ )	5.9 ( $\pm$ 7.9)	5.3 ( $\pm$ 7.6)	0.08
Fasting glucose ( $\text{mmol l}^{-1}$ )	5.5 ( $\pm$ 0.87)	5.3 ( $\pm$ 0.55)	0.03
Postprandial glucose ( $\text{mmol l}^{-1}$ )	6.5 ( $\pm$ 2.52)	6.7 ( $\pm$ 2.57)	0.167
Body mass index	28.1 ( $\pm$ 3.6)	26.8 ( $\pm$ 3.2)	<0.01
Serum metformin ( $\text{ng ml}^{-1}$ )	N/A	6.7 (range 1.62–16.04)	N/A
Tissue metformin <sup>a</sup> ( $\text{nmol mg}^{-1}$ )	N/A	0.293 (range 0.218–1.732)	N/A

Abbreviations: HOMA-IR, homeostasis model assessment-estimated insulin resistance; IGF1, insulin-like growth factor 1; IGFBP3, insulin-like growth factor-binding protein 3.

<sup>a</sup>Five samples only were tested.



**Figure 3.** Illustration of staining patterns on study specimens, according to methods outlined above. **(a)** Illustration of radical prostatectomy section demonstrating heterogeneity in P-AMPK staining in tumour sections, with a component of the tumour (bottom left) staining negative for P-AMPK. **(b)** Biopsy specimen demonstrating consistency with known signalling pathways with P-4EBP1 appearing positive in tumour tissue otherwise negative for P-AMPK and P-ACC. Antibody concentrations and conditions described in methods section. **(c)** Radical prostatectomy specimen demonstrating consistency with known signalling pathways as above. Antibody concentrations and conditions described in Materials and Methods section.

sible for uptake into prostate tissue are unclear, but staining for OCT1/2 are documented in prostate cancer tissue.<sup>23,24</sup>

The use of Ki67 index as a marker of tumour activity is less established in prostate cancer compared with breast cancer. However, it does have prognostic value in a variety of settings including post-radiation,<sup>25</sup> surgery<sup>26,27</sup> and biopsy.<sup>28</sup> In this setting, we felt it was a more appropriate marker to assess the effect of metformin as its mechanism of action is predominantly metabolic inhibition rather than cytotoxic. However, we did not see an association between a change in Ki67 index and other markers of tumour proliferative ability such as PSA; however, both the numbers of patients are limited and the PSA values in early stage disease can fluctuate. Assessment of some of the secondary end points were exacerbated by challenges with tissue fixation and preservation of epitopes in radical prostatectomy sections, despite our efforts with prompt injection of formalin. Indeed, we found numerous instances of heterogeneity in phospho-epitope staining despite adequate fixation, whereas in other instances the phospho-epitope stains were consistent with known signalling pathways (Figure 3).

Our results, together with both epidemiological and pre-clinical data support the further investigation of metformin in prostate cancer. We are currently undertaking a randomised phase 3 clinical trial of metformin vs placebo in the active surveillance setting (NCT01864096). However, notable from our data, albeit limited by small patient numbers, is the lack of association of benefit with any baseline factor. A number of hypotheses have recently been raised regarding subgroups in which metformin may have an additive benefit, in this setting an analyses of the Physicians Health study<sup>29</sup> is revealing in suggesting that obesity is linked with poorer prostate cancer prognosis primarily in men harbouring the TMPRSS2-ERG fusion suggesting a possible target for metformin intervention. We did not find any corroborative evidence in our dataset that only TMPRSS2-ERG patients benefitted, as there were greater PSA falls in TMPRSS2-ERG-negative patients (median fall 11.4% vs 0.5% rise) ( $P=0.02$ ). Indeed, evidence of PSA decrease on metformin is intriguing itself, and the phenomena has been verified to occur in a recently completed phase 2 trial in castrate resistant disease,<sup>30</sup> suggesting there may be some validity to our findings.

Delivering interventions to men in a window of opportunity prior to radical prostatectomy is feasible and in particular metformin, a normoglycaemic drug, does not appear to cause significant adverse events when given to non-diabetic non-obese men. In this setting, we have demonstrated that metformin may affect cellular proliferation and signalling indices that may underlie recent evidence to suggest benefit in reducing prostate cancer progression and mortality. Further investigation is warranted, in particular addressing the weakness of the study herein.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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