Anti-diabetic doses of metformin decrease proliferation markers in tumors of patients with endometrial cancer

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HIGHLIGHTS

- Pilot 'window of opportunity' trial of metformin, administered from diagnostic biopsy to surgery for endometrial cancer
- Significant reduction in Ki-67 and pS6 in the endometrial cancer cells *in vivo*
- Significant decrease of plasma insulin, IGF-1 and IGFBP-7

ABSTRACT

Background. Metformin has been associated with reduced cancer risk. The mechanisms underlying this cancer protective effect remain unknown.

Methods. "Window of opportunity" study of metformin in women with operable endometrial cancer (EC). Eleven newly diagnosed, untreated, non-diabetic patients with EC received metformin 500 mg tid from diagnostic biopsy to surgery. Fasting plasma insulin, insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 1 (IGFBP-1) and insulin-like growth factor binding protein 7 (IGFBP-7) measurements were taken before and after metformin treatment. Ki-67, pAMPK, and pS6 immunohistochemistry staining was performed on the endometrial cancer before and after metformin treatment and was compared to a control group of 10 women with EC who did not receive metformin.

Results. Metformin was administered for a mean of 36.6 days. None of the patients suffered side effects requiring withdrawal from the study. The study group comprised 8 patients with endometrioid EC, and 3 non-endometrioid EC, with a mean follow-up time of 57 months. Mean plasma insulin ($p = 0.0005$), IGF-1 ($p = 0.001$), and IGFBP-7 ($p = 0.0098$) were significantly reduced after metformin treatment. A clear reduction in ki-67 and pS6 expression was observed by both conventional light microscope analysis and digital image analysis with a significant mean reduction in percentage of cells staining for ki-67 (9.7%, $P = 0.02$) and pS6 (31%, $P = 0.03$). In the non-treated control group expression was similar between the biopsy and the surgical specimens.

Conclusions. This pilot trial presents biological evidence consistent with anti-proliferative effects of metformin in women with EC in the clinical setting.

Introduction

In Western countries, endometrial cancer is the most common gynecological malignancy [1,2]. Death rate from this disease has alarmingly increased over the past ten years, paralleling the rise in the obesity epidemic [3].

Endometrial cancers are classified into two major groups, Type I and Type II. The first type comprises tumors of endometrioid histology representing 70–80% of cases and is associated with unopposed estrogen stimulation, either endogenous or exogenous. Women who develop these tumors are typically peri- or post-menopausal and often have risk factors such as obesity or diabetes mellitus. Obesity is a well-established risk factor for the development of type I endometrial cancer and has been estimated to account for up to 40–90% of type I endometrial cancer cases [3,4]. Diabetes and insulin resistance have also emerged as independent risk factors for endometrial cancer [5–7] and have been

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associated to a 2–3 fold increased risk of developing this disease. Evidence of an increased risk of cancer with diabetes and obesity has led to great concern given the worldwide epidemic of obesity and diabetes. Type I tumors are usually low-grade neoplasms, with an endometrioid, well-differentiated morphology, and are generally associated with a relatively good prognosis. On the other hand, Type II non-endometrioid tumors are mostly diagnosed at an advanced stage, do not correlate with exposure to estrogens and display a more aggressive phenotype.

Metformin is a well-tolerated biguanide drug used for decades to treat type 2 diabetes. Epidemiological evidence suggests that metformin lowers cancer risk and reduces cancer incidence and deaths among diabetic patients, but some of the retrospective studies are controversial with respect to methodology [8–10]. One retrospective epidemiological study has demonstrated a protective effect of metformin on endometrial cancer risk [11], and there have been no prospective studies.

The mechanisms underlying the possible cancer protective effects of metformin are unknown, but there are two general hypotheses [12,13]. One is that metformin favorably alters the endocrine milieu of the host (for example by reducing hyperinsulinemia) secondary to its actions on the liver, where it acts to inhibit gluconeogenesis due to an inhibitory action on oxidative phosphorylation [14–16]. Another proposed mechanism is that the drug accumulates in the tissues where carcinogenesis is occurring to sufficient concentrations to have direct local effects, such as activation of AMPK [17]. AMPK regulates energy metabolism and is activated in response to cellular stresses that deplete cellular energy levels and increase the AMP/ATP ratio [15,18]. AMPK mediates its effect on cell growth through inhibition of the phosphatidylinositol 3-kinase (PI3K)-AKT/mammalian target of rapamycin (mTOR) pathway. Metformin may behave as a novel mTOR inhibitor and has been shown to dramatically decrease proliferation in a number of different human cancer cell lines *in vitro* [17,19–22]. Previous work in endometrial cancer cell lines has shown that metformin-mediated AMPK activation decreases cell growth and translation through inhibition of mTOR and a decrease in phosphorylation of its downstream target, S6 [23]. However, in certain contexts, AMPK activation can have pro-survival as well as anti proliferative effects [24].

The potential clinical anti-cancer effect of metformin in EC merits further investigation and is the subject of ongoing trials. Only few small "window-of-opportunity" biomarker trials have been completed in breast cancer [25–27], and results have been inconsistent [12].

This pilot window-of-opportunity trial was aimed at assessing the anti-proliferative activity of metformin in non-diabetic women with EC who were candidates to surgery. The primary outcome measures after metformin treatment were Ki-67, pS6 and pAMPK.

Methods

Patients

This prospective study was approved by the local institutional review board and conducted in a tertiary cancer center. Patients diagnosed with endometrial cancer in the division of Gynecologic Oncology, who were candidate for elective surgery were enrolled in this pilot clinical trial. After obtaining written informed consent, patients received metformin for 3 to 6 weeks until surgery. Duration of treatment was solely influenced by operating room availability and waiting times in this Canadian government supported academic center.

Control patients were retrospectively, randomly selected from our tumor bank provided that their biopsy and surgical specimens were available for analysis at the pathology archives. Eligibility criteria were: histologically confirmed EC without prior treatment; normal liver and renal function tests; and signed informed consent for biobanking. Exclusion criteria included prior invasive malignancy within 5 years, diabetes mellitus and treatment with metformin.

Collected data are presented in Table 1: age, BMI, smoking, age at menopause, use of HRT, histologic confirmation of endometrial cancer, stage and grade, lymphovascular space invasion (LVSI), lymph node (LN) metastasis, treatment, recurrence and 5 year survival. The tumor stage is presented according to the 2009 FIGO stage and histologically classified and graded according to WHO.

Table 1
Clinicopathological characteristics of study and control groups.

Endometrial cancer patients		Study group *n* (%)	Control group *n* (%)	*P* value
Number		**11**	**10**	–
Days from biopsy to surgery, median (range)		44.5 (27–57)	50 (26–70)	0.4
Metformin treatment days, median (range)		38 (21–50)	–	–
Age (y), median (range)		60 (49–75)	70 (57–76)	0.009
Median BMI (range)		28.6 (20.5–34.9)	28.8 (25–40)	0.4
Smokers		0 (%)	1 (10%)	0.28
Parity	0	3 (27%)	2 (20%)	0.6
	>1	8 (73%)	8 (80%)	0.69
Menopause	Pre	0 (%)	0 (%)	–
	Post	11 (100%)	10 (100%)	–
HRT		3 (27%)	1 (10%)	0.3
Histology	Endometroid	8 (73%)	9 (90%)	0.3
	Non–endometroid	3 (27%)	1 (10%)	0.3
Stage	I	8 (73%)	9 (90%)	0.3
	II	1 (9%)	0 (%)	0.32
	III	2 (18%)	1 (10%)	0.59
	IV	0 (%)	0 (%)	–
Grade	1	2 (18%)	5 (50%)	0.12
	2	5 (45%)	2 (20%)	0.21
	3	4 (36%)	3 (30%)	0.75
Neo adjuvant tr.		–	–	–
Residual disease		1 (9%)	0 (%)	0.32
LVSI		2 (18%)	3 (30%)	0.52
Positive metastatic LN's		1 (9%)	0 (%)	0.32
Adjuvant tr.		4 (36%)	3 (30%)	0.7
Recurrence		3 (27%)	2 (20%)	0.69
	Median time, month (range)	20 (11–27)	24 (14–34)	0.68
Deceased		3 (27%)	1 (10%)	0.31
	Median time, month (range)	20 (11–44)	16 (16)	0.6
Follow-up	Median time, month (range)	51 (24–76)	52 (36–61)	0.3

Table 2
Baseline and peri-operative fasting plasma levels of insulin, IGF-1, IGFBP-1 and IGFBP-7 in relation to body mass index.

Patient[a]	BMI	Baseline insulin mU/l	Peri-operative insulin mU/l	Baseline IGF-1 ng/ml	Peri-operative IGF-1 ng/ml	Baseline IGFBP-1 ug/l	Peri-operative IGFBP-1 ug/l	Baseline IGFBP-7 ng/ml	Peri-operative IGFBP-7 ng/ml
1	31.6	8.66	1.17	146.32	84.18	1.52	5.38	87.21	73.63
2	26.3	5.03	3.25	108.09	99.91	3.50	2.48	76.47	58.92
3	20.5	4.02	2.92	164.50	135.82	2.09	4.44	80.37	78.65
4	29.3	4.55	2.33	86.02	91.13	0.80	1.65	66.59	58.81
5	27.8	11.44	1.93	100.63	81.13	0.90	2.11	94.45	78.37
6	28.6	10.27	7.72	131.05	85.26	0.63	3.83	66.91	47.18
7	31.7	8.35	3.39	105.15	78.57	1.70	5.28	82.06	73.39
8	34.9	60.23	22.32	71.43	47.14	24.33	15.86	161.19	167.31
9	27.7	10.19	3.54	133.23	104.03	0.31	2.82	61.29	55.41
10	32.5	12.43	5.47	92.75	72.62	0.27	1.33	84.79	82.89
11	23.9	11.84	3.25	119.45	101.32	3.47	2.54	70.77	69.40
Mean	28.65	13.36	5.20	114.4	89.19	3.59	4.33	84.74	76.72
SD	4.1	15.83	5.94	27.6	22.18	6.97	4.0	27.26	32.08
P-Value[b]	–	0.0005		0.001		0.07		0.0098	

BMI, body mass index; SD, standard deviation.
[a] Patients number 5, 6 and 9 are type 2 endometrial cancer patients.
[b] Wilconxon matched pairs test.

Treatment

Baseline endometrial biopsy of tumor tissue (pre-treatment) was obtained at study entry and the surgical specimen (post-treatment) at the time of definitive surgery. Patients uniformly received metformin 500 mg tablets TID; this dose was chosen due to the fact that endometrial cancer patients tend to be overweight. To increase compliance, patients were handed in advance metformin pills and did not need to purchase it by themselves. Patients were also encouraged to report any difficulty they had adhering to the treatment plan. Treatment was suspended 48 h before anesthesia to avoid risk of lactic acidosis in compliance with US Food and Drug Administration prescribing indications [28].

Insulin, IGF-1, IGFBP-1 and IGFBP-7 measurements

For the study group, fasting venous blood was withdrawn for insulin, insulin-like growth factor (IGF-1), insulin-like growth factor binding protein 1 (IGFBP-1) and insulin-like growth factor binding protein 7 (IGFBP-7) level determinations at the morning after the enrollment and

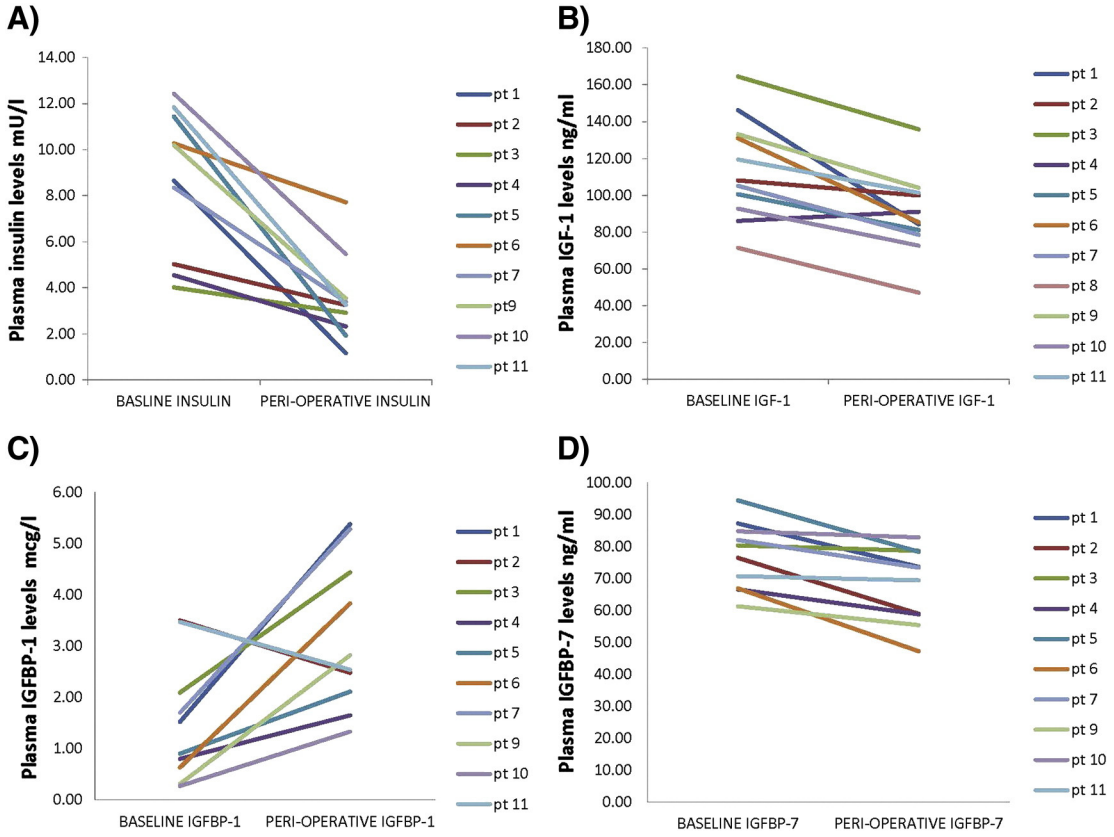


Fig. 1. Individual paired patient data ($n = 11$) of fasting plasma insulin (**A**), IGF-1 (**B**), IGFBP-1(**C**) and IGFBP-7 (**D**) levels at baseline and peri-operative (post-metformin treatment) in the study group.

at surgery following induction of anesthesia. Plasma aliquots were measured on frozen samples stored at -80 °C until assayed by the Insulin Elisa assay (Mercodia Inc., Uppsala, Sweden), IGF-1 Elisa assay (Immunodiagnostic Systems (IDS Ltd), Boldon, UK), IGFBP-1 Elisa assay (Alpco, Salem, NH, USA) and the IGFBP-7 Elisa assay (Antigenix America, Inc. Huntington Station, NY, USA).

Pathology and immunohistochemistry measures

Endometrial biopsies and surgical specimens were formalin-fixed as per routine. Immunohistochemistry staining was performed in the Cancer Center Research Pathology Facility. Briefly, tissue samples were cut at 4-μm, placed on SuperFrost/Plus slides (Fisher), and dried overnight at 37 °C. The slides were then loaded onto the Discovery XT Autostainer (Ventana Medical System). All solutions used for automated immunohistochemistry were from Ventana Medical System unless otherwise specified. Slides underwent de-paraffinization with the EZ PREP solution (Ref# 950-100), heat-induced epitope retrieval with Cell Conditioning solution CC1 pH 8.0 (Ref# 950-224) at standard condition (60 min at 95 °C). Immunostaining for ki-67 and pS6 was performed online using a heat protocol. Rabbit polyclonal anti ki-67 (Novus Biologicals # NB 110-89717, Oakville, ON), rabbit monoclonal anti-pS6 (Ser240/244) (Cell Signaling # 5364, clone D68F8) and rabbit monoclonal anti-pAMPK (Thr 172) (Cell Signaling, # 2535) diluted respectively at 1:100, 1:1000 and 1:50 in the antibody diluent (Ref# 251-018) were manually applied for 32 min at 37 °C then followed by the detection kit (Omnimap anti-Rabbit HRP Ref# 760-4311 and ChromoMap-DAB Ref: 760-159). A negative control was performed by the omission of the primary antibody. Slides were counterstained with hematoxylin for 4 min; blued with Bluing Reagent for 4 min, removed from the autostainer, washed in warm soapy water (Dawn) dehydrated through graded alcohols, cleared in xylene, and mounted with Permount.

In order to compare pre/post-treatment tissues among each other, sections were first analyzed by light microscopy and scored by two independent examiners blinded to the clinical data. Slides were assessed according to a schema based on conventional descriptors of endometrial histology using a recognized and validated scoring [29,30]. Briefly, each examiner randomly selected five fields of view containing endometrial gland tumor. Field views (20× objective) were scored for Intensity (0 = no staining, 1 = mild staining, 2 = moderate staining, 3 = strong staining) and for percentage of distribution (0 = 0%, 1 < 25%, 2 = 25–50%, 3 = 50–75%, 4 > 75%). Scores for intensity & percentage of distribution were averaged and then multiplied by each other to reach a final number (*intensity x distribution*) for comparison analysis.

Following light microscopy review, slides were scanned into a virtual microscopy format using ScanScope Digital Slide scanner (Aprio, Vista, CA, USA) at 20× magnification. Quality control of the scanned images and all further analysis were performed using ImageScope v11.2.0.780 (Aprio) using standard compression methodology [31–33]; only invasive tumor cells were assessed. Great care was taken to exclude normal epithelial, in situ epithelial, stromal and inflammatory elements. Slides were analyzed by using one of the following algorithms: Nuclear Count v9 or Cytoplasmic Count v2. The nuclear algorithm quantifies nuclei by

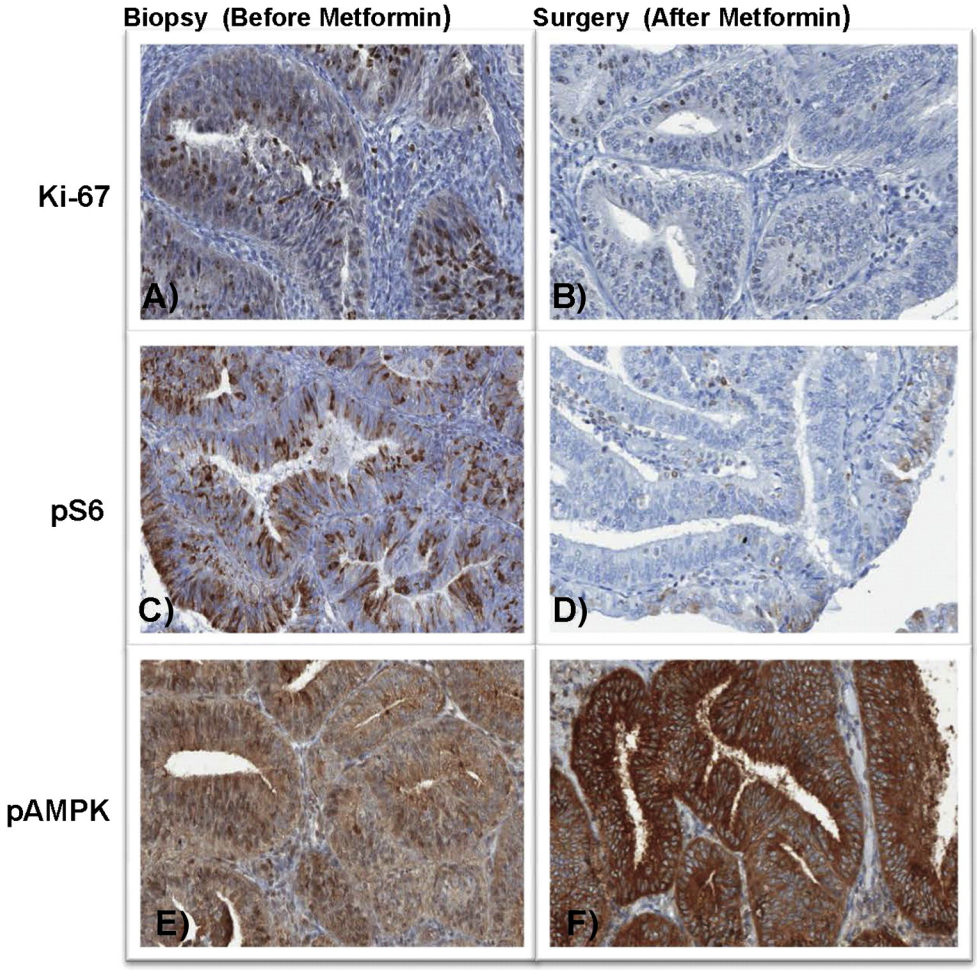


Fig. 2. Representative immunohistochemistry expression for ki-67, pS6 and pAMPK. Samples were obtained from the same patient (endometroid type endometrial cancer, stage IIa, grade 2) at the time of diagnostic endometrial biopsy and following definitive surgery. **2A**–**B**: ki-67, **2C**–**D**: pS6 and, **2E**–**F**: pAMPK.

staining intensity and provides automatic cytoplasmic stain removal (Algorithm User Guide: Nuclear Quantification. Human Tissue Resource Center: the University of Chicago, 2012); this algorithm was chosen to quantify ki-67. The cytoplasmic algorithm is set to analyze staining intensity and provide the percentage of cells containing stain within the nucleus and the cytoplasm (Algorithm User Guide: Cytoplasmic. Human Tissue Resource Center: the University of Chicago, 2012); this algorithm was chosen to quantify pAMPK and pS6.

Statistical analysis

All values are expressed as mean ± SD. Staining levels of each parameter in the biopsy and surgical samples were compared using a two-tailed paired Student's *t*-test; $P < 0.05$ was considered significant. Data were analyzed using Prism (GraphPad Software version 3.00, La Jolla, USA).

Results

Patients

Table 1 summarizes patient's clinic-pathological characteristics.

Eleven non-diabetic women who met the inclusion criteria consented to the trial and were assigned to metformin treatment for the indicated time period. The median age of our study cohort was 60, range 49–75. All of the women were menopausal or peri-menopausal, nearly 80% were overweight or obese, and 3 women were using hormone replacement therapy. The median duration of metformin use was 38 days (range 21–50 days).

No patient withdrew from the trial due to toxicity. None of the patients had a notable change on physical examination during the 3–6 weeks of metformin therapy. The study group comprised 8 patients with endometrioid endometrial cancers, 2 serous papillary endometrial cancers and 1 carcinosarcoma. Eight patients had stage I, 1 had stage II and 2 had stage III. At the time of last follow-up, recurrence had occurred in 3 of 11 patients in the study cohort (27.2%). Patients were evaluated for survival analysis and the median follow-up time of surviving patients was 51 months (range 24–76) compared to 52 months (range 36–61) in the control group ($P = 0.3$); 3 patients died in the study group (27.2%) vs. 1 (10%) in the control group during the follow-up period.

Effects of metformin on plasma insulin, IGF-1, IGFBP-1, IGFBP-7 levels

Fasting baseline venous blood was obtained the morning after enrolment to the study and, for comparison, peri-operative fasting blood was obtained in 11/11 patients of the study group (Table 2, Fig. 1, study group patients 5, 6 and 9 are type 2 endometrial cancer patients). Fasting insulin levels were reduced from baseline to peri-operative measurements in all patients (mean baseline 13.36 vs. mean peri-operative 5.2, normal fasting values 2–25 (mU/l) ($p = 0.0005$)). Plasma IGF-1 and IGFBP-7 levels were reduced in all patients but one ($p = 0.001$ and $p = 0.0098$ respectively). Plasma IGFBP-1 levels were increased in all but 3 patients ($p = 0.07$).

Immunohistochemical analysis

Representative immunohistochemical expression of ki-67, pS6 and pAMPK from the same patient is presented in Fig. 2. As shown on Fig. 2, ki-67 expression (Fig. 2A–B) was mainly observed in the nucleus while pS6 (Fig. 2C–D) and pAMPK (Fig. 2E–F) expression was observed in the tumor cell cytoplasm, with pS6 demonstrating also weak nuclear staining.

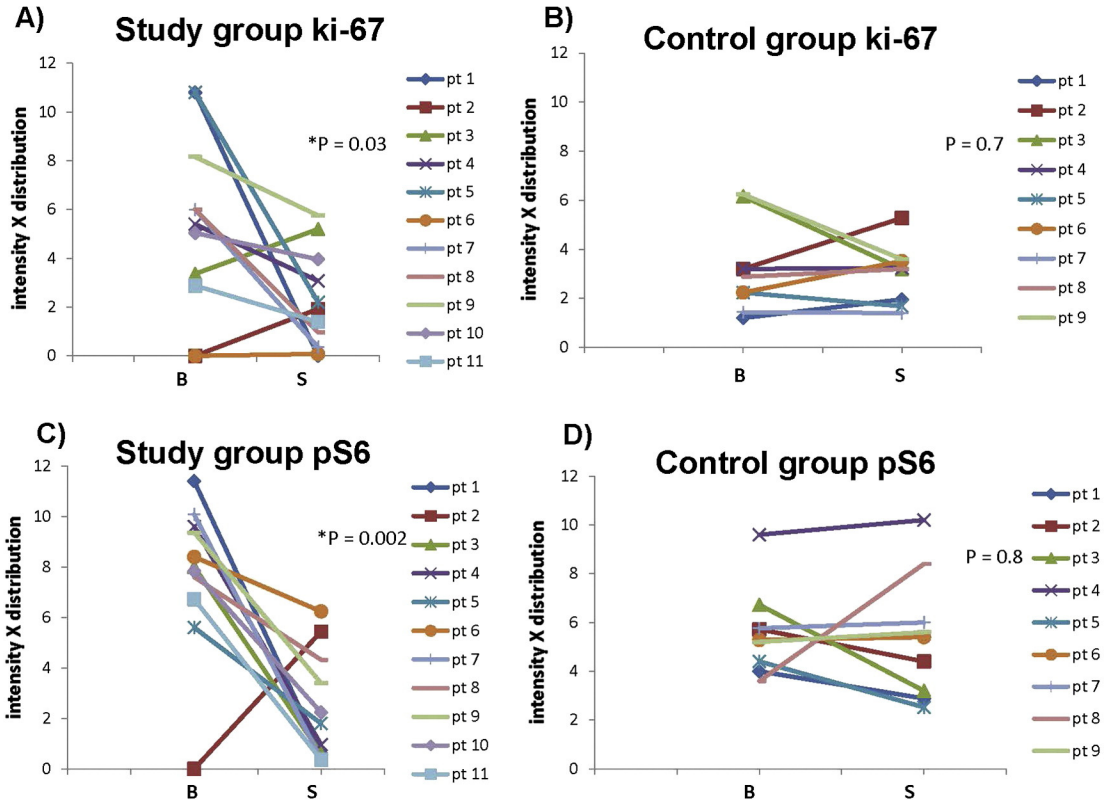
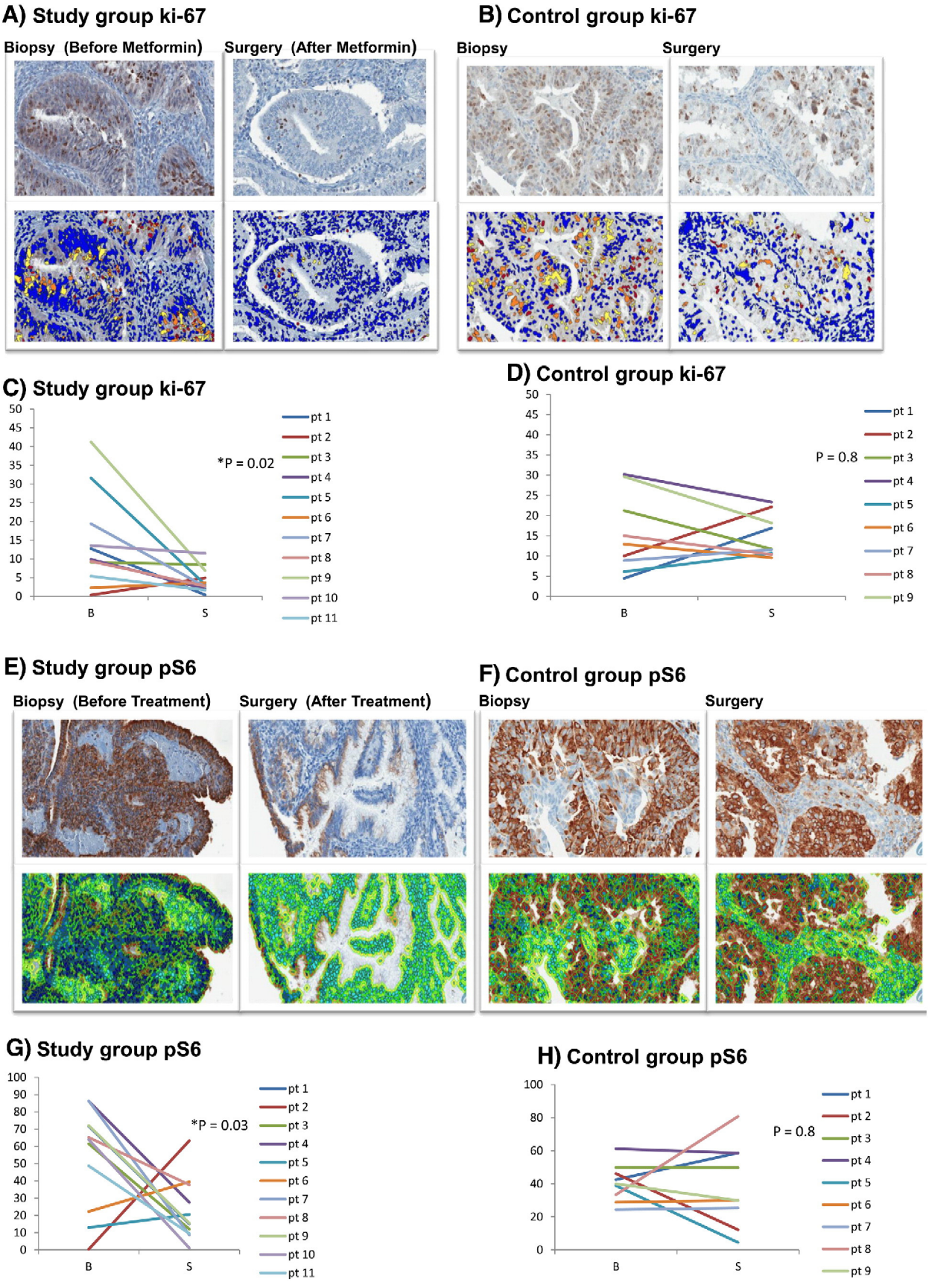


Fig. 3. Light microscopy visual score, following immunohistochemistry for ki-67 (**3A–B**) and pS6 (**3C–D**) for study and control groups. Study group patients 5, 6 and 9 are type 2 endometrial cancer patients.

Light microscopy visual score by two independent examiners had a positive correlation coefficient for the variation of ki-67, pS6 and pAMPK at 0.78, 0.91, and 0.8 respectively. We observed a reduction in the ki-67 expression in response to metformin between the biopsy and the surgery in 8 out of 11 patients (see Fig. 3A). The mean *intensity* × *distribution* for ki-67 fell after metformin treatment from



5.3 ± 3.6 in the biopsy specimens to 2.2 ± 2.0 in the surgical specimens ($P = 0.03$) for the first examiner and fell from 5.4 ± 2.4 to 3.0 ± 1.4 in the surgical specimens ($P = 0.02$) for the second examiner. However, this difference was not observed in control patients in whom ki-67 *intensity* × *distribution* score remained stable (Fig. 3B) (biopsies 3.2 ± 1.8, surgical specimens 3.0 ± 1.19 ($p = 0.7$)), suggesting that the observed ki-67 reduction was due to metformin exposure rather than to tissue processing differences between endometrial biopsies and surgical specimens.

Similarly, the reduction of pS6 expression was evident in all except 1 patient between the biopsy and post-metformin surgery specimen. For pS6, the mean *intensity* × *distribution* fell after metformin from 7.69 ± 3.0 in the biopsy specimens to 2.39 ± 2.1 in the surgical specimens ($p = 0.002$) for the first examiner and fell from 6.3 ± 1.5 in the biopsy specimens to 2.8 ± 1.6 in the surgical specimens ($p = 0.006$) for the second examiner. Again, pS6 did not vary significantly during the time period between the biopsy and surgical specimen in control patients: biopsies 5.6 ± 1.7 and surgical specimens 5.4 ± 2.5 ($p = 0.8$) (data is presented for pS6 score on Fig. 3C–D), suggesting that metformin is responsible for the decreased pS6 in the study group.

On the other hand, both independent examiners reported that on both pre-metformin biopsies and post-metformin surgical specimens pAMPK staining was similarly highly distributed with strong intensity.

Representative pictures of immunohistochemistry digital image analysis output images are presented in Fig. 4A–B. The red, orange and yellow colors represent what is classified as positively stained by the nuclear count algorithm. A minimum of 2000 invasive tumor nuclei were examined with the exception for one pre-treatment (patient #2 with 795 nuclei assessed). Individual ki-67 values fell in all but two patients following metformin intake (patient #2 and #6). The mean percentage of cells stained positive for ki-67 was 14.0 ± 12% for the biopsy specimens and 4.3 ± 3.3% for the post-metformin surgical specimens. The mean percentage of nuclear staining for ki-67 fell after metformin by a mean of 9.7% ($P = 0.02$) but remained stable in control patients (Fig. 4C–D): biopsies 15.3 ± 9.6% and surgical specimens 14.9 ± 5.3% ($p = 0.8$).

The mean percentage of cells stained positive for pS6 (Fig. 4E–H, cytoplasmic count algorithm) was 53.7 ± 29% for the biopsy specimens and 22.7 ± 18% for the surgical post-metformin specimens, with a mean reduction after metformin treatment of 31% ($P = 0.03$). Individual pS6 values was reduced in all but 3 patients following metformin intake (patient #2, #5 and #6) (Fig. 5G), results similar to our conventional microscopy analysis (Fig. 3C). A mean of 3900 cells (range 3100–4300) per tumor specimen was assessed to obtain results. Here also pS6 staining values remained stable in control patients (Fig. 5G–H): biopsies 40.5 ± 11% and surgical specimens 38.8 ± 24% ($p = 0.8$). The mean percentage of cells staining positive for pAMPK was high in both pre-treatment biopsy (91.9 ± 15.9%) and post-treatment surgical (87 ± 18.1%) specimens. The positive pixel count and the cytoplasmic count algorithms showed no significant changes between the groups (data not presented).

Finally, we evaluated if the duration of metformin treatment could impact the extent of tumor response in terms of pS6 and ki-67 reduction and found no correlation between the two parameters.

Discussion

Abnormalities in glucose, insulin and estrogen metabolism are strongly associated with endometrial cancer [5–7]. *In vitro* studies and retrospective epidemiologic evidence suggest the hypothesis that metformin may have a therapeutic value against endometrial cancer [11,23,34–36], but there is presently insufficient data available to justify use of metformin as a preventive or as an anti-cancer agent in EC [11,37].

This two-stage window-of-opportunity, prospective pilot trial supports the potential anti-cancer effect of metformin in non-diabetic women with EC, by showing a reduction of cancer cell proliferation using ki-67 biomarker, as well as a reduction of pS6 expression, a protein downstream of the mTOR pathway. Based on *in vitro* studies [34,35], we attempted to determine if AMPK phosphorylation increased in tumors following metformin administration. However, because of the high average basal levels of expression of pAMPK in the biopsies (92 ± 16%) we could not detect any additional increase following metformin intake by immunohistochemistry.

To eliminate the potential bias that the differences observed resulted from sequential sampling, we analyzed biopsies and surgical specimen from a control group of patients who did not receive metformin. We found no modulation of pS6 or ki-67 expression in this control group, indicating that the observed effect was due to metformin and not to the way or timing of sampling the endometrium (Fig. 4).

To improve reproducibility of our findings, we evaluated protein expression using both light microscopy visual scoring and automated digital image analysis. We used a validated method for manual interpretation of immunohistochemistry [29,30]. We then confirmed our readings using automated digital image analysis for the interpretation of immunohistochemistry to present robust, reproducible, objective and quantitative measurements. Despite the powerful advantages mentioned above, digital image analysis has not been widely applied to diagnostic work-up apart from breast cancer studies [38].

Since insulin resistance and hyperinsulinemia are associated with a higher risk of developing Type 1 EC [5–7], one would expect metformin to have its greatest impact on those subtypes of tumors. In one study on breast cancer, metformin decreased ki-67 in patients with insulin resistance, while there was an inverse trend in women with normal insulin sensitivity [26]. In EC however, epidemiologic studies on diabetic patients who used metformin showed an improved survival in those with Type 2 cancers [11]. Although Type 2 cancers are less common than Type 1 cancers, they account for a disproportionate number of recurrences and deaths. Metformin as shown here, although only on a few cases, appeared to decrease proliferation markers, similarly in both types.

This study provides evidence for an effect of metformin in reducing circulating insulin and IGF-1 levels in non-diabetic patients. The expected increase in IGFBP-1 was observed but did not reach statistical significance. This data supports the first hypothesis that indirect, insulin- and glucose-mediated effects may be the main mechanism of anticancer effect of metformin in endometrial cancer. However, this will require further confirmation as we cannot exclude the possibility that the perioperative state at the time of second blood sampling contributed to the observed change in insulin levels. It would have been interesting to see that there was no change in insulin levels in the control group compared to the study group but since the control group in this pilot study was recruited retrospectively from randomly selected samples of our tumor bank based on the availability of both biopsy performed in our centre and surgical specimens, there were no plasma samples available for this comparison. Nevertheless, the trend for a greater decline in subjects with highest basal insulin levels is what would be expected from a metformin effect. IGFBP-7 binds to the unoccupied IGF-1 receptor and suppresses downstream signaling. The abundance of IGFBP-7 correlates with tumor progression [39] and endometrial cancer in Chinese women

Fig. 4. Digital image analysis, following immunohistochemistry for ki-67 (**A–D**, nuclear count algorithm) and pS6 (**E–H**, cytoplasmic count algorithm). Output image of endometrioid endometrial cancer, stage IIa, grade 2 (patient # 7, study group, **4A**), and stage Ia, grade 2 (patient # 3, control group, **4B**) at biopsy and at surgery, before and after applying the nuclear count algorithm. Red, orange and yellow colors represent positive staining. **4C**: The mean study group percentage of nuclear staining for ki-67 fell after metformin treatment ($P = 0.02$, paired *t*-test) but remained stable in control patients (**4D**). **4E–F**: Output image of endometrioid endometrial cancer, stage Ib, grade 1 (patient # 11, study group) and stage Ia, grade 2 (patient # 4, control group) at biopsy and at surgery. After applying the cytoplasmic count algorithm red and blue colors represent positive staining. **4G**: The mean study group percentage of cytoplasmic staining for pS6 fell after metformin treatment ($P = 0.03$, paired *t*-test) but remained stable in control patients (**4H**). Study group patients 5, 6 and 9 are type 2 endometrial cancer patients. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[40]. The available literature does not explain how metformin affects IGFBP-7. Our preliminary data show a decrease in IGFBP-7 levels following metformin treatment.

In conclusion, this small study has demonstrated the clinical effects of metformin at the conventional anti-diabetic doses (500 mg every 8 h) on the tumors of women with endometrial cancer, including the downregulation of the tumor proliferation marker ki-67 and the downregulation of pS6, a critical downstream target of mTOR. These findings add to prior retrospective pharmacoepidemiologic evidence regarding a benefit of metformin in EC.

Our study involved a limited number of patients, so caution is required in interpreting the data. A larger cohort might further allow us to determine if metformin-induced changes in systemic markers of insulin resistance are correlated with effects on Ki67 and pS6, which could provide more clues regarding the relative importance of "direct" and "indirect" mechanisms. However, the findings do represent a prospective clinical biomarker evidence for activity of metformin in EC, and contribute to the justification of formal clinical trials to further investigate whether metformin has clinical activity on specific subsets of endometrial cancers.

Note added in proof

An article published online in the journal Cancer a few days ago by Mitsuhashi et al. reports similar findings in a study of 31 patients with endometrial cancer treated with metformin for 4 to 6 weeks prior to surgery. Cancer. 2014 Jun 10. http://dx.doi.org/10.1002/cncr.28853. [Epub ahead of print].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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