Phase II clinical trial of metformin as a cancer stem cell-targeting agent in ovarian cancer

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ABSTRACT

**Background:** Epidemiologic studies suggest that metformin has antitumor effects. Laboratory studies indicate metformin impacts cancer stem-like cells (CSCs). As part of a phase II trial, we evaluated the impact of metformin on CSC number, and carcinoma associated mesenchymal stem cells (CA-MSC), and clinical outcomes in non-diabetic patients with advanced stage epithelial ovarian cancer (EOC).

**Methods:** Thirty-eight patients with stage IIC(n=1)/III(n=25)/IV(n=12) EOC were treated with either (i) neoadjuvant metformin, debulking surgery and adjuvant chemotherapy + metformin, or (ii) neoadjuvant chemotherapy and metformin, interval debulking surgery, and adjuvant chemotherapy + metformin. Metformin treated tumors, compared to historical controls, were evaluated for CSC number and chemotherapy response. Primary endpoints were (i) a greater than 2-fold reduction in ALDH+CD133+ CSC and (ii) a relapse free survival at 18 months of >50%.

**Results:** Metformin was well-tolerated. Median progression-free survival was 18.0 months (95% CI 14.0-21.6) with relapse-free survival at 18 months of 59.3% (95% CI 38.6-70.5). Median overall survival was 57.9 months (95% CI 28.0 – Not Estimable). Tumors treated with metformin had a 2.4-fold decrease in ALDH+/CD133+ CSC and increased sensitivity to cisplatin ex-vivo. Furthermore, metformin altered the methylation signature in CA-MSC which prevented CA-MSC driven chemoresistance in-vitro.

**Conclusions:** Translational studies confirm an impact of metformin on EOC CSC and suggest epigenetic change in the tumor stroma may drive the platinum sensitivity ex-vivo. Consistent with this, metformin therapy was associated with better than expected overall survival, supporting the use of metformin in phase-III studies.
INTRODUCTION

It is estimated that in 2020, 21,750 women will be diagnosed with epithelial ovarian cancer (EOC) and 13,940 will die of ovarian cancer (1). While antiangiogenic therapy has offered improvement in progression-free survival (PFS), a significant overall survival (OS) advantage is unclear (2, 3). Similarly, Poly-ADP Ribose Polymerase (PARP) inhibitors have demonstrated an impressive increase in PFS in BRCA mutation carriers, but OS data is not mature (4). Furthermore, these therapeutic approaches are extremely costly (5-9). Thus new, cost-effective approaches are needed to reduce relapse rates for EOC patients and improve OS. Metformin, a low-cost modulator of cellular metabolism, represents one potential approach.

Pre-clinically, metformin has demonstrated antitumor effects in several cancers (10, 11). Epidemiologic studies, while not in complete agreement, have indicated that patients with ovarian cancer taking metformin compared to patients not taking metformin, have a significantly longer OS (12-16).

Many mechanisms of metformin’s anti-cancer activity have been proposed. Several studies have suggested metformin modulates AMPK signaling, AKT activity, and the induction of apoptosis (17, 18). Metabolic actions have been proposed related to gluconeogenesis, mitochondrial function, and cellular metabolism (19, 20). Metformin has been reported to inhibit epithelial-mesenchymal transition (EMT), IGF signaling, and selectively suppress cancer stem-like cell (CSC) growth (21-25). In ovarian cancer, metformin is reported to reverse chemotherapy resistance, reduce cancer cell migration and metastasis, and prevent EMT (17, 20, 26-28). We reported that metformin targets ALDH+ ovarian CSCs (29, 30) and enhances response to chemotherapy (31).
Currently, at least 55 clinical trials are currently evaluating metformin as a cancer treatment (32). Here, we present results of a non-randomized phase-II study of metformin administered in combination with chemotherapy for non-diabetic patients with advanced-stage EOC. The primary objective of this study was a translation endpoint to evaluate the impact of metformin on CSCs and 18 month RFS.

RESULTS

Patient Population: 91 patients were enrolled in the study between October 28, 2011 and March 8, 2016. Study design is as indicated (Figure 1). Five patients withdrew consent prior to treatment initiation related to timing/location of surgery. Eighty-six patients were treated with metformin prior to surgery. Of these patients, 37 were excluded due to ineligible pathology (24 had benign disease or non-ovarian carcinoma, and 13 had stage I/IIA ovarian carcinoma). An additional 10 were non-evaluable due to withdrawn consent for either intolerance of metformin; personal issues (e.g., moving to other cities); or non-compliance (Figure 2). Target accrual was 50 evaluable patients; however—due to the unexpectedly high number of patients with early-stage disease who were enrolled but ultimately not eligible; an unexpectedly high number of non-ovarian malignancies; and limited financial support—the trial closed early.

Table 1 summarizes patient characteristics. 25 (65.8%) had stage III disease, and 12 (31.6%) had stage IV disease. Twenty-three patients (60.5%) received neoadjuvant chemotherapy; 15 (39.5%) received adjuvant therapy. Thirty (78.9%) achieved optimal debulking status (<1cm residual disease). Twenty-seven (71.1%) had platinum-sensitive tumor. Two of the 23 (8.7%) patients receiving neoadjuvant chemotherapy and metformin achieved pathologic complete responses
with no evidence of residual disease at interval debulking surgery. Thirty-two patients (84.2%) completed at least six cycles of chemotherapy with metformin. Two patients chose to continue metformin therapy (off-label, provided by their internist) after completion of the study.

**Safety:** Table 2 details toxicities potentially related to metformin. A severe dermatologic reaction was the only grade 4, non-hematologic toxicity. One patient had grade-3 diarrhea. The anticipated metformin-associated diarrhea and nausea were the most common side-effects. Five of 16 patients treated at 1000mg BID (twice daily) withdrew from the trial due to metformin-related GI side-effects. Subsequently, patients were enrolled with target doses of 500mg twice daily. Three patients were dose-reduced to 500mg/daily secondary to GI side-effects. All patients who experienced nausea were taking 1000mg BID. Hepatologic adverse effects included mild elevation in AST and ALT. Anticipated rates of hematologic and neurologic toxicity were observed.

**Efficacy:** The data cutoff for primary analysis was January 22, 2018. With a median follow-up of 45 months, the median PFS for the entire population was 18.0 months (95% CI 14.0-21.6) (Figure 3A). For the twenty-seven patients with non-persistent disease after therapy, RFS at 18 months was 59.3% (95% CI 38.6-70.5). Median OS was 57.9 months (95% CI 28.0 – Not Estimable; Figure 3B), with a 3-year OS of 65.7% (95% CI 48.3-78.4) (Figure 3B). Comparing by stage, those with stage IIc/III disease had a median PFS of 18.3 months (95% CI 5.8-21.7), while patients with stage IV disease had a median PFS of 14.8 months (95% CI 7.6-23.4) (Figure
Median OS by stage was 58.0 months (95% CI 44-NE) for patients with stage IIc/III disease and 22 months (95% CI 7-NE) for patients with stage IV disease (Figure 3D).

**TRANSLATIONAL STUDIES**

**Metformin Treatment Reduces CSC:** Prior studies suggest metformin can reduce cancer ‘stemness’ (21, 25, 31). We have shown that ALDH+CD133+ ovarian cancer cells are enriched for ovarian CSCs (29). Thus, a primary endpoint of this study was to evaluate ALDH+CD133+ CSC in the metformin-treated specimens and matched non-metformin-treated control patients. For homogeneity, we only evaluated samples from 22 patients with stage III/IV high-grade serous cancer. Controls met the eligibility criteria set forth for the trial and were consented for tissue collection via an IRB-approved tumor banking protocol. Selecting from a bank of >200 patient samples, 22 controls were matched to have identical stage, histology, and chemotherapy (including adjuvant vs. neoadjuvant). Average age of controls at time of surgery was similar at 61.1 years (range 42-76). 75% of control patients underwent optimal debulking (15 of 20; debulking status unavailable for 2). Flow cytometry revealed metformin-treated patients exhibited an average 2.4-fold reduction in percentage of ALDH+CD133+ cells compared to non-metformin-treated ovarian cancer controls (p<0.0001, Figure 4A).

To further evaluate the stemness of these tumors, tumor cells from six metformin-treated patients and seven controls were grown in hanging-drop suspensions in serum-free media and serially passaged. Spheroids were then analyzed for response to cisplatin therapy over time and expression of ALDH or CD133. In line with a potential reduction in stemness, metformin-treated cells were more sensitive to platinum treatment, unlike controls, and appeared to not develop therapeutic resistance with passaging (p<0.001, Figure 4B). Consistent with the initial analysis,
spheroids from metformin-treated patients initially demonstrated reduced levels of both ALDH and CD133 (Figure 4C). Furthermore, these levels stayed lower over time and, in the case of ALDH, appear to amplify less over time.

**Metformin Impacts DNA Methylation of Cancer-Associated Mesenchymal Stem Cells in the Tumor Microenvironment (TME):** Metformin use has been associated with an immediate impact on blood cell DNA methylation (33). Metformin has also been reported to modify cancer cell DNA methylation (34-36). Given the reduction in CSC numbers and the persistence of platinum response ex-vivo, we hypothesized a potential epigenetic change in cancer cells and performed DNA methylation analysis on tumor cells from control and metformin-treated tumors using the Illumina Infinium MethylationEPIC DNA methylation array. To eliminate changes associated with chemotherapy, we only used tumors that were treated with metformin neoadjuvantly in the absence of chemotherapy. Uniform Manifold Approximation and Projection (UMAP), using all CpG sites, did not separate metformin-treated tumor cells and non-treated control tumors (Figure 5A). After correction for multiple comparisons, we were unable to identify statistically significant differentially methylated loci, using either supervised or unsupervised hierarchical clustering with the top 5% most variable sites (Figure 5B).

We previously demonstrated that mesenchymal stem cells (MSCs) are important members of the ovarian TME (37, 38) that assume a unique pro-tumorigenic cancer-associated MSC (CA-MSC) phenotype (39). One of the pro-tumorigenic aspects of the CA-MSCs is to enhance stemness and chemoresistance. We therefore similarly analyzed the impact of metformin on CA-MSC DNA methylation status. We profiled three groups of MSC samples, including MSCs derived from normal omental adipose tissue; CA-MSCs derived from high-
grade serous ovarian cancer (HGSOC) omental metastasis (control-CA-MSCs); and CA-MSCs derived from metformin-treated HGSOC omental metastasis (metformin-CA-MSCs). A similar UMAP analysis as done with the tumors demonstrated that normal MSCs and control CA-MSCs segregated into two distinct groups. Metformin-CA-MSCs separated into two groups, one overlapping control-CA-MSCs and the other creating an intermediate group between CA-MSCs and normal MSCs (Figure 6A). An unsupervised hierarchical clustering analysis on the top 5% CpG sites, selected based on variance, recapitulated the global UMAP comparison (Figure 6B). Five of the 11 metformin-CA-MSCs (Group II) clustered with the control-CA-MSCs, while 6 (Group I) demonstrated a distinct methylation pattern. Even with multiple comparison correction and small sample size, we were able to identify 14,791 differentially methylated CpG sites (within 4,986 differentially methylated regions (Figure 6C) at the 5% FDR level. Group-I metformin-CA-MSCs segregated with normal MSCs.

To determine whether CA-MSC methylation profiles correlated with outcome, we compared the OS of patients whose CA-MSC sorted into Groups I and II. All five patients in Group II (CA-MSC profile more like non-metformin-treated controls) were deceased. In contrast, only three of six patients in Group I (patients whose CA-MSCs sorted with normal MSCs) were deceased.

To determine if the metformin response of CA-MSCs could cause better patient outcomes, we performed in vitro chemosensitivity assays with CAOV3 ovarian cancer cell lines co-cultured with control CA-MSCs (n=3); Group-I metformin-CA-MSCs (n=3); and Group-II metformin-CA-MSCs (n=2). As we previously reported (37), non-metformin-treated CA-MSCs significantly increase chemoresistance of ovarian cancer cells (Figure 6D). However, ovarian cancer cells cultured with Group-I metformin-treated CA-MSCs demonstrated no increase in
chemoresistance. Group-II metformin-CA-MSCs had an intermediate phenotype. Taken together these data suggest metformin’s impact on CA-MSCs may prevent CA-MSC-driven chemoresistance. Supporting the idea that metformin may help to maintain platinum sensitivity, of 21 platinum-sensitive patients (patients who experience recurrence ≥6 months after completion of adjuvant chemotherapy) for whom data on response to second-line therapy were available, 18 (82%) demonstrated a response to second-line therapy (11 CR, 7 PR, 3 PD). This compares favorably to historical controls, in which a response of 50-65% has been observed (40, 41).

**DISCUSSION**

This is the first prospective study to evaluate metformin as a treatment in non-diabetic ovarian cancer patients. The trial met the primary endpoint of ≥2-fold reduction of ALDH+CD133+ CSC and ≥50% RFS at 18 months, however the RFS endpoint has a wide confidence interval. The median PFS of 18 months and OS of 57.9 months compare favorably to other clinical trials with similar patient populations and historical expectations (2, 42).

The metformin dose of 500mg BID was well tolerated. However, doses of 1000mg BID were not tolerated due to gastrointestinal adverse effects. This may be unique to the ovarian cancer population as patients found the GI side-effects mentally distressing; they were reminiscent of patients’ presenting ovarian cancer symptoms and provoked anxiety. Consequently, many patients would not consider dose-reduction of the metformin, withdrawing from the trial.
Outcome results, while encouraging, are limited as this was a non-randomized trial. While cross-trial comparisons are obviously problematic, median PFS and OS compare favorably to the outcomes in recent landmark clinical trials with similar patient populations, such as GOG218 (the majority of patients were stage III with optimally debulked disease) and GOG262 (2, 43-48). While our study had a higher rate of optimal debulking than these two studies, this likely reflects the fact that 60% of the patients in the current study received neoadjunct chemotherapy which increases optimal debulking rates, but does not impact overall survival (49). Of note, as bevacizumab was not approved for adjuvant therapy at the time of this study, no patients were treated with bevacizumab.

Selective targeting of CSCs is a proposed mechanism of action of metformin (27, 32, 50-52). We previously found metformin was associated with a ~2-fold reduction in ovarian CSCs in animal studies and increased in chemotherapy response (31). Exactly how metformin reduced CSC numbers was unclear. Translational studies completed as part of this clinical trial observed a very similar 2.4-fold CSC reduction in metformin-treated patients. Consistent with reduced stemness, metformin-treated patient cells demonstrated an increased sensitivity to platinum ex-vivo, and unlike controls, resistance to platinum did not increase with passage. Sensitivity to platinum maintained over time could explain the modest PFS yet excellent OS of the metformin-treated population. Our study did not include metformin as a maintenance therapy for financial reasons; however, if metformin works to reduce chemotherapy resistance, continued use may further improve patient outcomes. This is being tested in an ongoing randomized phase-II trial of metformin as chemotherapy adjuvant (NCT02122185).

Metformin may also impact stemness indirectly via an impact on TME. We observed strong metformin-related DNA methylation changes in CA-MSCs of patients with good
outcomes. Unlike non-metformin-treated CA-MSCs, CA-MSCs from metformin-treated tumors did not have the ability to drive chemoresistance ex-vivo. As MSCs are well established as regulators of CSCs (37, 53, 54), we speculate metformin may, in part, affect CSCs indirectly via its effect on MSCs. As MSCs are known to be potent regulators of the immune response, a metformin impact on MSCs would be consistent with its reported immunomodulatory effects (24, 55). Improved OS without significant impact on PFS could also be attributable to an improvement in anti-tumor immune response.

While our translational findings are of interest, they are limited in interpretation as this was not a randomized trial. However, control tumors were well matched contemporaries, from the same institution. Our studies are consistent with a randomized phase-II in lung cancer of an EGFR inhibitor +/- metformin, which demonstrated statistically significant PFS improvement with an impressive 14-month improvement in OS (1). In contrast, clinical trials of metformin in pancreatic cancer did not show a survival benefit (56). If metformin acts to prevent the development of chemoresistance, it is unlikely to have an impact on pancreatic cancer which, unlike ovarian and lung cancer, is rather therapy resistant on primary presentation. Further, if metformin acts via conversion of MSCs to CA-MSCs, studies of metformin in pancreatic cancer, where the tumor stroma is well established, are less likely to be positive. Consistent with this, for patients with stage IV ovarian cancer, we observed OS consistent with historical controls whereas, for stage III patients, we observed a better-than-expected OS.

In conclusion, we demonstrated that metformin is well-tolerated in non-diabetic patients. Metformin treatment resulted in a significant reduction the CSC population and alteration of DNA methylation of CA-MSCs, which eliminated CA-MSC-driven increases in chemoresistance. This was associated with a better-than-expected median OS, particularly for
patients with stage II-III disease. This study strongly supports the use of metformin in phase-III clinical trials for adjuvant treatment of EOC.

METHODS

Study Design and Eligibility Criteria

This was a single-center open-label phase-II trial of patients with a new diagnosis of confirmed advanced-stage EOC. All patients gave written informed consent before participation in the study. Progression was defined using GCIG-RECIST criteria (57). Primary endpoints were an 18-month median relapse-free survival (RFS) and 2-fold reduction in CSC versus non-metformin-treated historical control samples. PFS and OS were secondary endpoints. Survival intervals were defined from the date of diagnosis to the date of first evidence of progression or death from any cause.

Eligible patients had evidence of malignancy consistent with stage IIC, III, or IV ovarian, fallopian, or primary peritoneal cancer (58), ECOG performance status 0-2, age 18-80 years, and intact renal (serum creatinine ≤1.4mg/dL) and hepatic function (bilirubin ≤1.5 times the upper limit of normal and AST and ALT ≤2.5 times the upper limit of normal). Patients having a diagnosis of diabetes mellitus, metformin use in the preceding 6 months, hypersensitivity to metformin, or history of metabolic acidosis were excluded, as were those with a history of other active malignancies.

Procedures

Patients were initially treated with oral metformin 500mg BID for 7 days and then increased to 1000mg twice daily. However, after an initial high dropout rate (n=5 of the first 16) due to metformin-related GI side-effects, the remaining patients were enrolled with target dose of
500mg twice daily. Patients either received 7-10 days of metformin prior to primary debulking surgery, followed by at least six cycles of adjuvant metformin and platinum/taxane chemotherapy or three cycles of neoadjuvant metformin + platinum/taxane chemotherapy, followed by interval debulking and 3-6 cycles of adjuvant platinum/taxane chemotherapy with metformin (Figure 1). Patients were consented and started metformin the same day they were evaluated for surgery to assure receiving metformin neoadjuvantly did not delay patients undergoing surgery. Patients were treated with either standard Q3week carboplatin (AUC=6) and paclitaxel (175mg/m2) or Q3week carboplatin (AUC=6) and weekly taxol (80mg/m2) per physician’s preference. The decision to treat patients with initial surgery or neoadjuvant chemotherapy was up to the treating surgeon, based on perceived ability to perform optimal debulking. Pill counts and patient journals were assessed after every cycle to confirm treatment compliance. Metformin was discontinued after completion of the study.

Safety and Toxicity Monitoring

Patients were evaluated for toxicity at enrollment and with each therapeutic cycle. Adverse events were graded according to CTCAE criteria (version 4.0) (59). The number and proportion of the highest-graded toxicity for each category were reported. Patients were withdrawn from study for serious metformin-related non-hematologic adverse events (grade ≥3). Dose reduction was allowed for low-grade toxicity.

Statistical Analysis

The study was powered for two objectives, CSC number and PFS at 18 months. Based on prior analysis, we assumed an average CSC number of 3% and hypothesized, from preliminary data,
that metformin would reduce CSC number to 1.5%. To calculate power, the scale of the measurements was transformed to angular equivalents, using an adaptation of the arcsine transformation of the square root of the proportion, in order to normalize the otherwise skewed distribution. Assuming mild variability, an assumption formalized by setting the standard deviation equal to half the mean for each group, with 50 treated and 50 control cases, using a two-sample, two-sided, t-test would yield over 90% power to detect the hypothesized difference significantly, with at most 5% type I error.

PFS at 18 months was powered using a one-sided, one-sample exact binomial test (DSTPLAN, Version 4.2). Allowing at most 2.5% type I error, 49 patients were needed to achieve 80% power to detect 20% change in PFS. OS was a secondary endpoint. Time-to-event endpoints, OS and PFS, were estimated using the product-limit method of Kaplan and Meier, with overall median study-wide follow-up time estimated using reverse censoring for survival.

Cancer Stem Cell Studies

Tumor samples were processed into live single-cell suspensions (60) and frozen for batch analysis. The proportion of ALDH1+/CD133+ CSCs was evaluated via flow-cytometry as previously described (61-64).

Hanging-Drop Spheroids

Tumor cell suspensions from six neoadjuvant metformin-treated patients and seven control patients were gown as previously described in five replicate plates (61). After 7 days, spheroids were harvested, disaggregated, and single cells counted and re-plated on a new hanging-drop plate as drops of 100 cells, to form passage-1. This process was repeated weekly for 6-passges. Live-cell phase-microscopy images of the spheroids were collected (Olympus IX81, and CellSens software) on Days 1, 3, and 7 of culture for each passage to monitor spheroid
formation/proliferation (61-64). At the time of disaggregation, a portion of cells was used for flow-cytometry analysis of ALDH and CD133 as above. Seven-day-old spheroids were treated with cisplatin at a concentration of 50uM. The effect of drug treatment was determined at 72 hours, using the alamarBlue assay as described (61-63).

DNA Methylation Profiling

Tumor cell suspensions were washed x3 to eliminate dead cells and DNA collected using the DNeasy column-based purification kit (Qiagen). Patient-derived CA-MSCs and normal omental MSCs were cultured as described (37-39), then FACS-sorted for the cell-surface markers CD105, CD90, and CD73 and genomic DNA isolated as above. DNA methylation profiling was performed using the Infinium MethylationEPIC BeadChip kit (Illumina). Raw IDAT files were processed using R package SeSAMe (65) with noob background correction (66), non-linear dye-bias correction, and non-detection masking. A beta value was calculated for each locus as the ratio of methylated signal intensity to the sum of unmethylated and methylated signal intensities, with a range from 0 to 1, corresponding to the fraction of methylated allele in the assayed sample at this locus. We masked measurements from sub-optimally designed probes due to overlapping with SNPs and mapping issues (67). Dimensionality reduction for visualizing sample similarities was achieved by (UMAP), [arXiv:1802.03426] using all CpG sites assayed. Hierarchical clustering was done with the R function hclust (68), based on top 5% of most variable CpG probes and performed alongside the UMAPs. Differential methylation analysis was conducted using R package DMRcate (69), version v1.8.6, with default significance cutoff settings (false-discovery rate controlled at 5% with the Benjamini-Hochberg procedure for multiple correction).

Translational Studies Statistics
Spheroid studies were repeated five times, with at least 40 spheroids (technical replicates) interrogated for each analysis at each time-point and at least 6 patient samples. Spheroid proliferation at Day 7 was normalized to Day 1 for each specimen. Normalized viability is expressed as percentage of untreated controls. Statistical significance between passages was assessed with student’s t-tests and comparisons made between metformin and non-metformin samples at each passage, using one/two-way ANOVA and Tukey's post-hoc analysis to determine specific significant differences (p<0.05). Three independent flow-cytometry analyses were performed to identify an average percentage of ALDH+ or CD133+ populations. All data are expressed as mean ± standard error. Statistical analyses were performed using Prism 7 (GraphPad) and SAS, version 9.4.

**MSC–Cancer Cell Co-Culture Chemosensitivity Assay**

Assays were performed as previously described (37). Briefly, GFP-labeled CAOV3 cells (ATCC, Manassas VA) were co-cultured with patient derived MSCs, 1:1 ratio for 24 hours (10,000 cancer cells:10,000 MSCs), in a 1:1 mixture of DMEM-10% FBS and complete MSC media, then treated with 1µg or 2µg of cisplatin for 48 hours. Cells were harvested and viable (DAPI-negative) CAOV3-GFP cells were counted, using flow cytometry with constant time and volume across samples.

Study Approval: The study (protocol HUM00047900, clinicaltrials.gov identifier: NCT01579812) received approval of the University of Michigan Comprehensive Cancer Center IRB.
Author Contributions: Data Acquisition: JB, JS, LC, Patient enrollment, tissue acquisition, patient care: RKR, CJ, KM, SU, RL, LC, LGC, Biostatistical and Bioinformatic Analysis: KG, HF, HS, Laboratory experimentation: DC, ST, LGC, RS, KY, GM, RJB, Study Design and Manuscript Preparation: JB, DC, LT, LGC, RJB.

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Table 1: Clinical Characteristics of Enrolled Patients

| Characteristic                      | N (%)     |
|------------------------------------|-----------|
| **Median Age**                     |           |
| Years (Range)                      | 59.5 (31-76) |
| **Sex**                            |           |
| Female                             | 38 (100)  |
| **Stage**                          |           |
| II                                 | 1 (2.6)   |
| III                                | 25 (65.8) |
| IV                                 | 12 (31.6) |
| **Histology**                      |           |
| High grade, serous                 | 29 (76.3) |
| High grade, endometrioid           | 1 (2.6)   |
| Low grade, mucinous                | 0 (0)     |
| Low grade, serous                  | 4 (10.5)  |
| Other                              | 5 (13.2)  |
| **Optimal Debulking Status**       |           |
| Yes                                | 30 (78.9) |
| No                                 | 7 (18.4)  |
| N/A (No Surgery)                   | 1 (2.6)   |
| **Metformin Total Daily Dose**     |           |
| 500 mg                             | 3 (7.9)   |
| 1000 mg (500 mg BID)               | 19 (50.0) |
| 2000 mg (1000 mg BID)              | 16 (42.1) |
| **Neoadjuvant Chemotherapy**       |           |
| Yes                                | 23 (60.5) |
| No                                 | 15 (39.5) |
| **Platinum Sensitive**             |           |
| Yes                                | 27 (71.1) |
| No                                 | 11 (28.9) |
| **ECOG Performance Status**        |           |
| 0                                  | 29 (76.3) |
| 1                                  | 8 (21.1)  |
| 2                                  | 1 (2.6)   |
Table 2 Metformin Attributed Adverse Events and Grade 3-4 Hematologic Toxicities (any attribution).

| Adverse Event          | Metformin Attributed Toxicities |
|------------------------|--------------------------------|
|                        | Any Grade | Grade 3 or 4 |
| Any adverse event      | 20 (52.3) | 1 (2.6)      |
| Diarrhea               | 7 (18.4)  | 0            |
| Nausea                 | 6 (15.8)  | 0            |
| Elevated ALT           | 5 (13.2)  | 0            |
| Abdominal Pain         | 4 (10.6)  | 0            |
| Elevated AST           | 3 (7.9)   | 0            |
| Vomiting               | 3 (7.9)   | 0            |
| Skin Rash              | 2 (5.3)   | 1 (2.6)      |
| Weight Loss            | 2 (5.3)   | 0            |
| Alkalosis              | 1 (2.6)   | 0            |
| Dizziness              | 1 (2.6)   | 0            |
| Dyspnea                | 1 (2.6)   | 0            |
| Elevated Creatinine    | 1 (2.6)   | 0            |
| Gagging                | 1 (2.6)   | 0            |
| Ileus                  | 1 (2.6)   | 0            |

| Adverse Event          | Grade 3-4 Hematologic Toxicity |
|------------------------|--------------------------------|
|                        | Any Attribution                |
| Anemia                 | 12 (32)                         |
| Neutropenia            | 9 (24)                          |
| Thrombocytopenia       | 6 (16)                          |
| Thromboembolic event   | 2 (5)                           |
Figure 1. Clinical Study Trial Design. Patients were treated with Metformin prior to debulking surgery either for 2 weeks or along with 3 cycles of neoadjuvant chemotherapy. Surgical specimens were used for later CSC studies. Metformin was continued along with adjuvant chemotherapy for a total of 6 cycles.
**Figure 2. Enrollment and Exclusion in Clinical Trial.** Of 91 patients enrolled, 38 were evaluable.

Excluded patients included those who withdrew prior to trial initiation, were ineligible due to benign or lower stage pathology, or who withdrew after treatment was initiated but before receiving chemotherapy either due to intolerance of metformin, non-compliance, or other reasons such as moving/choosing to receive adjuvant treatment at another institution.
Figure 3. Kaplan-Meier Estimates of Progression Free and Overall Survival. (A and B) Kaplan-Meier estimates of PFS and OS for the entire population. Median PFS was 18.0 months (14.0-21.6). Median OS was 57.9 months (28.0 – Not Estimable). (C and D) Kaplan-Meier estimates of PFS and OS by stage.
Figure 4. Tumors Treated with Metformin Have Decreased Cancer ‘Stemness’. A. Summary of FACS analysis of ALDH+/CD133+ cancer stem like cells (CSC) in metformin-treated (n=22) and matched control ovarian cancers (n=22) demonstrating a 2.4-fold decrease in CSC in metformin treated tumors. B. Cell viability of tumor cells from metformin-treated patients (n=6) or control patients (n=7) grown in suspension, passaged weekly and treated with Cisplatin (5 replicates each). Tumor cells from metformin treated patients maintain platinum sensitivity with serial passage, while control tumor cells increase platinum resistance over time. C. Evaluation of ALDH and CD133 expression in Metformin-treated and control tumor cells grown in suspension and after serial passages. Metformin treated samples start at a lower baseline and amplify less
over time relative to controls. Lines in boxes represent averages. Statistical significance between passages was assessed with student’s t-tests and comparisons made between metformin and non-metformin samples at each passage, using one/two-way ANOVA and Tukey's post-hoc analysis to determine specific significant differences (p<0.05). All data are expressed as mean ± standard error.

**Figure 5. Metformin does not Impact DNA Methylation of Bulk Cancer Cells.** A. Primary two-dimensional UMAP analysis showing intermixed metformin-treated tumors cells (mtTumor, red, n=10) and non-treated control tumor cells (ctrlTumor, blue, n=6). B. Dendrogram of hierarchical clustering using top 5% most variable CpGs in tumor cells from control and metformin treated patients.
Figure 6. Metformin Impacts DNA Methylation of Host Cancer Associated-Mesenchymal Stem Cells and Prevents CA-MSC Induced Chemoresistance. A. Primary two dimensional UMAP
analysis for the MSC groups, including metformin-CA-MSC (mtCAMSC, red, n=11), control-CA-MSC (CA-MSC, blue, n=9) and normal adipose MSC (MSC, green, n=6). B. Dendrogram from hierarchical clustering using top 5% most variable CpGs for the MSCs. Metformin-CA-MSC samples split into two groups as indicated. C. DNA methylation heatmap of 14,791 probes differentially methylated between Group I and Group II. MIR200C promoter DNA methylation level (unmethylated in epithelial cells and methylated in mesenchymal cells) is included as a measure of CA-MSC purity. Group-I metformin treated CA-MSCs segregate with normal MSCs, distinct from control CA-MSC. D. Viable cancer cell number following co-culture with the indicated MSC types and the indicated doses of cisplatin. A student’s two-tailed T-test was used for comparison.