Alterations of choline phospholipid metabolism in endometrial cancer are caused by choline kinase alpha overexpression and a hyperactivated deacylation pathway

Sebastian Trousil¹, Patrizia Lee²,³, David J. Pinato³, James K. Ellis², Roberto Dina⁴, Eric Aboagye¹, Hector Keun², Rohini Sharma³

¹Comprehensive Cancer Imaging Centre at Imperial College, Faculty of Medicine, Imperial College London, United Kingdom
² Department of Surgery and Cancer, Imperial College London, United Kingdom
³ Department of Experimental Medicine, Imperial College London, United Kingdom
⁴ Department of Pathology, Imperial College NHS Trust, London, United Kingdom

Corresponding Author: Dr Rohini Sharma FRACP PhD, Senior Lecturer Medical Oncology and Clinical Pharmacology, Imperial College London, Hammersmith Campus, Du Cane Road, W12 0HS, London, United Kingdom, Tel: +44 208 3833720, E-mail: r.sharma@imperial.ac.uk

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Abstract

Metabolic rearrangements subsequent to malignant transformation are not well characterized in endometrial cancer (EC). Identification of altered metabolites could facilitate imaging-guided diagnosis, treatment surveillance and help to identify new therapeutic options. Here we employed high resolution-magic angle spinning magnetic resonance mass spectroscopy on EC surgical specimens and normal endometrial tissue to investigate key modulators that might explain metabolic changes, incorporating additional investigations using qRT-PCR, Western blotting, tissue microarrays and uptake assays of [3H]-labeled choline. Lipid metabolism was severely dysregulated in EC with various amino acids, inositols, nucleobases and glutathione also altered. Among the most important lipid-related alterations was increased phosphocholine levels (increased 70% in EC). Mechanistic investigations revealed that changes were not due to altered choline transporter expression, but rather increased expression of choline kinase α (CHKA) and an activated deacylation pathway, as indicated by upregulated expression of the catabolic enzymes LYPLA1, LYPLA2 and GPCPD1. We confirmed the significance of CHKA overexpression on a tissue microarray including a large series of endometrial hyperplasia, atypical hyperplasia and adenocarcinoma tissues, supporting a role for CHKA in malignant transformation. Lastly, we documented several-fold increases in the uptake of [3H]choline in endometrial cancer cell lines compared to normal endometrial stromal cells. Our results validate deregulated choline biochemistry as an important source of non-invasive imaging biomarkers for EC.
Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy in North America and Europe, and despite research efforts the 5-year overall survival in patients with metastatic disease remains less than 20%. The main risk factor in the development of EC is unopposed estrogen, a complication of obesity, hence the incidence of EC is projected to dramatically increase in the next decade (1).

Early stage EC can be effectively managed with surgery with 5-year survival >80%. While surgery is curative in patients with disease confined to the uterus, lymphovascular invasion and spread to the regional lymph nodes is most important prognostic factor in EC, and will dictate the choice of adjuvant therapy (2). Currently, patients deemed to be at “high risk” of extrauterine spread undergo staging pelvic lymphadenectomy such that a certain number of patients will undergo an unnecessary surgery while others will be undertreated. Reliable staging therefore is critical to both the management and prognostic stratification of patients. Conventional imaging techniques have limited sensitivity in the assessment of lymph node status and more effective imaging modalities are urgently needed (3). Understanding the metabolic and biochemical changes that underlie the malignant progression of the disease would allow utilization of non-invasive molecular imaging techniques for clinical staging and follow-up. Furthermore, as an increasing number of novel targeted therapies are entering clinical trial, more effective imaging can facilitate drug development efforts in this tumor type, aid patient stratification and thereby improving clinical outcome.

Choline (Cho) is an essential nutrient that is phosphorylated to phosphocholine (PCho) by choline kinase and then incorporated into the cell membrane via the
Kennedy pathway (4). Choline kinase exists in at least three isoforms – $\alpha_1$, $\alpha_2$ (CHKA) and $\beta$ (CHKB) – of which the alpha but not beta isoforms have been associated with malignancy (5). CHKA overexpression, elevated Cho uptake and PCho formation have been shown in a wide array of human cancers, including breast, ovarian, lung and prostate cancer (6-10). These changes in choline handling have most commonly been studied using magnetic resonance spectroscopy (MRS). $^1$H MRS studies report alterations in the spectral profile in the region of 3.20 to 3.24 ppm that are indicative of PCho, glycerophosphocholine (GPC) and free Cho. In the majority of tumor models an increased PCho:GPC ratio has been reported compared with normal tissues (11). More recently, the enzyme endometrial differential 3 (EDI3) has been identified as cleaving GPC to form glycerol-3-phosphate and choline, which may also contribute to the increased PCho:GPC ratio (12). Mechanisms of PCho accumulation within tumor cells can occur via a number of mechanisms including enhanced choline transport, CHKA dedicated phosphorylation, and activation of phosphatidylcholine specific phospholipases. Furthermore, over-expression of CHKA (6-8) and abundance of choline metabolites (13-15) have been shown to be prognostic in a number of cancers including prostate, breast, lung and ovarian cancer. More recently inhibition of CHKA, both by shRNA and small molecule inhibitors, has been shown to have both an anti-mitogenic and anti-proliferative effects in vitro and in vivo (16-21).

Celik and colleagues identified an abnormal choline signal in proliferating endometrium compared with EC by spectroscopy (7). However, neither alterations in choline phospholipid metabolism in the development of EC nor alterations in CHKA expression and activity has been considered in EC, and may represent both a novel therapeutic target and imaging strategy. The aim of this study was therefore to
confirm an altered choline profile in EC compared with normal endometrial tissue from patients using high resolution-magic angle spinning (HR-MAS) $^1$H nuclear magnetic resonance (NMR) technique. We then further investigated alterations in the enzymes responsible for choline homeostasis and choline uptake in EC using comparative mRNA analysis of relevant genes. We then considered whether the expression of CHKA correlates with progressive histologic changes from a normal endometrial tissue, hyperplasia and EC using a tissue microarray (TMA) approach. Furthermore, we investigated differential activity of CHKA using radioactivity uptake studies.
Materials and Methods

Clinical Specimens

Fresh frozen endometrial tissue (~5 mm$^3$) was obtained following approval from the Hammersmith Hospital tissue bank. Informed consent to use leftover biologic material for investigative purposes was obtained from all patients. Samples were taken at the time of initial surgery from 20 patients, 10 with histologically confirmed grade III endometrioid EC and 10 with normal endometrium. The median age of the sample set with normal tissue was 47.8 years while with the cancer set was 65.8 years. Samples were immediately frozen in liquid nitrogen and stored within the tissue bank at –80°C until use. The natural morphology in this tumor type is that of discrete lesions rather than diffuse infiltrate; contamination by non-tumor tissue is deemed negligible.

$^1$H Nuclear Magnetic Resonance (NMR) spectral acquisition and analysis

Tissue preparation and NMR acquisition were undertaken as per previously published (22, 23). Briefly, for High Resolution-Magic Angle Spinning (HR-MAS) ~5 mm$^3$ (5–18.5 mg) endometrial tissue was thawed and rinsed with a small volume of 0.9% saline (90 mg NaCl in 10 ml D$_2$O) before being placed into a zirconia rotor (Bruker Biospin). Prior to spectral acquisition using the Bruker DRX600 spectrometer operating at 600.13-MHz $^1$H NMR, sufficient time was allowed for the rotor to reach a steady spin rate of 5 kHz and a temperature of 283 K. Standard 1D Nuclear Overhauser Effect Spectroscopy (NOESY)-presaturation pulse sequence [RD-90°-t1-
90°-tm-90°-ACQ] was used to manually determine the receiver gain, water saturation power, 90° pulse length and shimming for each sample. Each spectrum was acquired with 8 dummy scans (to allow sufficient time for the nuclei spin to reach steady-state) and 32 scans (2.73 seconds per scan). Field homogeneity was optimized by measuring the $v_{1/2}$ (ca. 1.5-2.2Hz) for L-lactate (δ1.33) prior to the final acquisition and manual adjustment of the shim coils (24). In order to suppress signals arising from macromolecules, 1D Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence [RD-90°-{-180°-)n-ACQ] was employed with $\tau = 400$ uS and $n=300$. For 1D CPMG experiments, 16 dummy scans followed by 256 transients were collected. A line-broadening factor of 0.3 Hz was multiplied to all FIDs prior to Fourier transformation. For peak assignment, 2D J-Resolved (J-Res) and Correlation spectroscopy (COSY) experiments were also performed on a selected sample. Spectra were calibrated to the L-alanine resonance (δ1.48), phasing and baseline correction were done manually using TopSpin software (v.3.1, Bruker Biospin). Subsequent data processing was carried out using an in-house scripts written in MATLAB (R2011a, Mathworks) written by Dr. T.M.D. Ebbels, Dr. H.C. Keun, Dr. J.T. Pearce, Dr. K. Veselkov and Dr. O. Cloarec. Ethanol resonances (δ1.06–1.31, δ3.56–3.75), introduced during sample collection, and the residual water resonance (δ4.68–δ5.16) were removed. Regions at δ<0.6 and δ>9.0 were also removed to reduce effects of noise at baseline. Median fold-change normalization (mfc) was performed on all reduced spectra to compensate for effects arising from variation in tissue weight or any systematic preparation error (25). Multivariate analysis (Principal Component Analysis and Partial Least Squares Discriminant Analysis (PLS-DA)) was applied to the reduced and normalized data using SIMCA P+ software (v.12.0.1,
Umetrics, Sweden). Seven-fold cross-validation and permutation tests were carried out to validate the robustness of our models and to assess their predictability (26). Receiver operating characteristic curve (ROC) analysis was employed to indicate the model’s ability to accurately discriminate the presence of disease. Non-parametric Mann-Whitney U test and Wilcoxon signed-rank tests were performed using Excel (Microsoft, USA). The Benjamini-and-Hochberg multiple testing correction (27) was used to assess false discovery rate (FDR) and counteract multiple hypothesis testing. Specifically for more accurate estimation of ratios between PCho, GPC and choline, the relative abundance of these metabolites was estimated using manual spectral deconvolution via Profiler (Chenomx, Canada) software. Cell samples were collected and the aqueous metabolites extracted as previously described (28). Briefly, cold methanol was used to quench metabolism and lysates extracted in a dual phase process. Chloroform/methanol (300 μL, 2:1, v/v) was added to the dried methanol quenched samples, vortexed (30 seconds) and centrifuged (16000×g, 10 minutes). Ultrapure water (300 μL) was added and samples vortexed and centrifuged as before, the aqueous and organic phases were then aspirated to fresh sample tubes. To maximize metabolite recovery the extraction was repeated and the samples pooled and dried. Blank samples were also generated to account for any contamination from the extraction solvents. All 1D CPMG experiments were conducted in solution state at 600 MHz 1H and 300K using a Bruker AVANCE DRX600C spectrometer fitted with a 5 mm broadband-inverse tube probehead (Bruker Biospin). The spectral data were processed, digitized and imported into MATLAB using in-house scripts (see above). The area under the peak was measured
for the PCho and Cho resonances at approximately 3.2 ppm and the ratio calculated for each of the three EC cell lines.

**Tissue microarray and immunohistochemistry**

Three types of tissue microarrays (TMA) were constructed comprising of normal endometrium (n=17), endometrial hyperplasia (n=15) and EC (n=28). Briefly, formalin-fixed, paraffin-embedded specimen and corresponding haematoxylin and eosin (H&E) slides were retrieved from the local pathology archive. After review of H&E stained sections, three 1 mm cores were identified from the most representative areas of the tumor tissue, then re-embedded into recipient TMA blocks using an MTA-1 Manual Tissue Microarrayer (Beecher Instruments, USA). Consecutive 4 μm thick sections of the TMA blocks were cut and stained for CHKA using an automated protocol on a Bond Max Autostainer (Leica Microsystems, Wetzlar, Germany) using the Polymer-HRP system (BioGenex, USA) with subsequent development in diaminobenzidine and Mayer’s Haematoxylin counterstaining. CHKA rabbit polyclonal antibody (Sigma-Aldrich, HPA024153) was used at a 1:25 dilution in Dako REAL antibody diluent (Dako, UK) and the Dako EnVision+ System-Peroxidase/DAB+ Rabbit kit (Dako) secondary antibody was used to visualize antibody binding. Slides were counterstained with haematoxylin. Normal bronchial tissue was used as positive control to confirm reaction specificity. Omission of the primary antibody was used as negative control reaction. Tissue samples were scored manually using the immunohistochemical score method (IHS), as described previously (29). Briefly, each sample was assigned an IHS ranging between 0 to 300, based on the product of the percentage of cells showing immunohistochemical
expression (0–100) and the intensity of the signal (graded 1–3). Every core was assessed individually. A composite score of 0–300 was generated from the product of intensity and percentage. A final score on a scale of 0 to 3 was obtained; 0: no expression; 1: weak expression (composite score <100); 2: moderate expression (composite score 101–200) and 3: strong expression (composite score 201–300). For statistical purposes immunoeexpression patterns were divided into two groups: reduced phenotype with a staining scored 0 or 1 and preserved phenotype with a staining scored 2 or 3. The pattern of staining (cytoplasmic, membranous, nuclear, or diffuse) was also described in each case. Two observers (RD and RS) scored all the cases independently, blindly from clinical data and results were found to be consistent.

Real-time PCR

RNA was extracted from cells using RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions and 1 µg total RNA reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen). Gene expression was analyzed by quantitative real-time PCR (qRT-PCR) using SYBR green method and Platinum SYBR Green qPCR SuperMixUDG (Invitrogen) master mix according to manufacturer’s instructions. The reaction was run in a total volume of 20 µL using 25 ng cDNA at a final primer concentration of 400 nM. The following custom designed primers were supplied by Invitrogen: CHKA (Fwd. CGGAAGTATCCACCAAGAA; Rev. TCCCCAGAGGAATGAGATG), CHKB (Fwd. TGGTGCTAGAAAGCGTGATG; Rev. GCCGACTTGGGATGTACTGT), PCYT1A (Fwd. GCAACCAGCTCCTTTTCTG; Rev. GCAAACTCCACCAATGAGGT). The following primers were custom designed by
PrimerDesign Ltd: PCYT1B (Fwd. TCTAAGTGTTTCTGCTGAGTTG; Rev. CATACCCATACAAATAACCCCAAGAG), CHPT1 (Fwd. TCTGCTCTTTTATTGGGGATGTTTG; Rev. CAACACAAGACAATCACTAAAGC), SLC44A4 (Fwd. GGCATACGGGATCTCTACTGC; Rev. TGTTAGGCACTGAGGTTGG), SLC22A3 (Fwd. TAGGACTTCTCGGTCTCTGC; Rev. CATGCTCTTTGTCTCCTC), GPCD1 (Fwd. CCTGGAACCTCTCTCTGTC; Rev. GAAGTTCCAAGGGCTCAG), LYPLA1 (Fwd. AGAAACTGGGCAAGGTCAC; Rev. GTGGCAGTGGAATAAGGATATC), LYPLA2 (Fwd. AAGAAGGCAGCAGGACACATC; Rev. CTCCAGGAGCATCTCAGTG), SLC5A7 (Fwd. CCTCAGCTCAACTTTCACC; Rev. GATGGGGTACTGAAATTATCTTC), PLD1 (Fwd. CTCTGACTGATGGGTGCTG; Rev. GATATAGATATAGTGCTGCTGTT).

Gene expression assays were performed on an ABI 7900HT Fast Real-Time PCR machine (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Data were analyzed using comparative Ct method as previously described with PPIA (Fwd. CTGCACTGCCAAGACTGA; Rev. CATTCCTGGACCCAAA) as an internal control (30).

**Western blotting in tissues**

Tissue samples were homogenized in RIPA buffer containing protease and phosphatase inhibitors (all Sigma-Aldrich) using a Precellys®24 homogenizer with CK14 beads. Homogenates were cleared of debris by centrifugation at 5000xg at 4°C for 5 minutes and supernatants recovered. 20 µg protein were resolved on a 4–15% Mini-PROTEAN TGX gel (Bio-Rad) and transferred to a PVDF membrane using Trans-Blot Turbo Transfer Pack (Bio-Rad). Membranes were blocked for 1 hour in 5% milk in Tris-buffered saline containing 0.1% v/v tween (TBST, Cell Signaling) and incubated
with the following antibodies in 5% milk overnight at 4°C: CHKA (Sigma-Aldrich, HPA024153) and β-actin (Abcam, ab6276). Secondary HRP conjugated rabbit and mouse antibodies (Santa Cruz Biotechnology, sc-2004 and sc-2005) were applied for one hour at room temperature. Signals were visualized using Amersham ECL Western Blotting Detection Reagent (GE Healthcare) and Amersham Hyperfilm (GE Healthcare).

**Cell Culture**

Ishikawa (Health Protection Agency Culture Collections) and HEC-1B (ATCC) were maintained in DMEM (Invitrogen). Endometrial stromal cells, St-T1B, were a kind gift of Professor Jan Brosens and cultured in DMEM/F12. All media were supplemented with 10% fetal calf serum, glutamine, penicillin and streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Commercially available cell lines were authenticated by provider by short-tandem repeat analysis and immediately expanded. Low-passage stocks were used for studies.

**[³H]-choline uptake assay**

St-T1B, HEC-1B and Ishikawa cells were plated into 6-well plates at a density of 5×10⁵ cells per well. The following day, cells were pulsed with 0.1 μCi/mL [³H]choline (Perkin Elmer) for one hour then washed once with PBS, trypsinized and centrifuged. The cell pellet was washed three times with PBS and lysed in RIPA buffer (Sigma-Aldrich). Radioactivity was measured on a scintillation counter after addition Ultima
Gold scintillation cocktail (PerkinElmer). Counts were normalized to protein content as determined by BCA assay (Pierce).

**Statistical analysis**

Unless stated otherwise, data were analyzed using either GraphPad Prism software version 5.01 or SPSS version 5.01. Statistical significance of differences was determined as specified and a *P*-value of <0.05 deemed significant.
Results

MAS analysis illustrates unique EC spectra compared with normal endometrial tissue, and increased levels of choline-containing metabolites in EC tissue

CPMG-derived $^1$H NMR spectra analysis of the tissue samples revealed prominent differences in concentration of multiple metabolites (Fig. 1A). In total 18 samples (10 normal endometrial tissue and 8 EC) were analyzed. Two samples were excluded from further analysis due to poor spectral quality. To model all these differences, a total of 68 spectral regions covering most distinguishable resonances across the spectra were integrated for multivariate analysis. Unsupervised Principal Component Analysis (PCA) with unit variance scaling was initially employed to look for visible separation between the metabolic profiles of different classes. This revealed a clear division between the normal endometrial tissue and cancer groups in model scores (Fig. 1B). To examine further the relationships between cancer and normal endometrial tissue PLS-DA was exploited to model specifically the covariance between the class membership of the samples and spectral variables. The resulting 2-component model explained 95% of the class variance ($R^2_Y$), which fell to 75.7% with cross-validation ($Q^2_Y$). A permutation test where models were generated on randomly classified data indicated that these model statistics were significant (Fig. 1C). The ROC curve, based on fitted Y values, gave an AUC of 0.9875 indicative of the ability of the model to positively identify EC from normal endometrial tissue (Fig. 1D).

Following the establishment of a valid and significant model, we began to deconvolute the metabolic fingerprint of EC in order to identify the metabolites
contributing to the classification of normal endometrial tissue and EC observed in the PLS-DA model. Figure 2 illustrates a variable importance for the projection (VIP) plot, which summarizes in order of the overall importance individual metabolite/regions that correlates to our model. The metabolites observed to be contributing most to the classification model were PCho, myo-inositol, nucleosides, glutathione, several amino acids, lipids and glucose, suggesting generally deregulated metabolism in EC. To confirm which metabolite changes were statistically significant, we compared the integrals of individual metabolites of cancer against control using Mann-Whitney U test, which provided a semi-quantitative analysis in terms of relative metabolite concentration change. In the case where one metabolite has more than one signal across the spectrum, the one with the least signal overlapping was selected; for metabolites of low abundance (e.g. phenylalanine), the signal with the highest contrast from the baseline was selected based on visual inspection, otherwise the metabolite with negative integrals were excluded (e.g. uracil), as signal-to-noise ratio tends to be relatively poor upfield of the NMR spectrum. Out of the 44 metabolites tested, metabolites showing statistical significance were similar to those observed from our multivariate model (Table 1; a list of all metabolites is summarized in Supplementary Table 1). In EC, significantly elevated metabolites included a number of amino acids – leucine, valine, alanine, proline, tyrosine – in addition to PCho glutathione, inositols, Inosine/adenosine and unsaturated lipids and triglycerides. Importantly, on superimposing spectra from normal endometrial tissue and EC, a significantly increased PCho:Cho ratio (1.59 fold) was detected in EC compared with normal endometrial tissue, indicative of activated choline kinase (Fig. 3). No significant differences were observed in GPC
and Cho content, while an increase in the PCho:GPC ratio (1.29 fold) approached but did not reach significance ($P=0.08$).

**Expression of enzymes responsible for phosphocholine synthesis is increased in EC**

Increased levels of PCho can be attributed to increased Cho uptake and CHKA activity or increased activity of phospholipid catabolic pathways. In order to establish which aspects of the choline biochemistry are deregulated in EC we investigated the mRNA expression of enzymes involved in differing aspects of choline metabolism using tissues from normal endometrial tissue ($n=10$) and grade III endometrioid EC ($n=10$, Fig. 4A). We observed a significant increase in the expression of CHKA ($P=0.02$), GPCPD1 ($P=0.01$), LYPLA1 ($P<0.01$) and LYPLA2 ($P=0.01$) in EC compared with normal endometrial tissue, but found no alterations in expression of the organic cation transporters or choline transporter-like proteins. These results suggest that the deacylation pathway may be activated in clinical specimens of EC and the generated Cho is consequently converted by CHKA to PCho, which results in the high PCho accumulation in the metabolic profile.

**Choline kinase is overexpressed in EC compared with normal endometrial tissue and endometrial hyperplasia**

Due to the high intracellular accumulation of PCho, we hypothesized that CHKA is a key driver for deregulated choline biochemistry and further investigated the role of CHKA in EC. In an initial study, we performed western blot analysis on the same sample set we used for mRNA analysis and found an increased expression of CHKA in EC compared to normal endometrial tissue ($P<0.001$, Fig. 4B–C). To confirm this we
employed a TMA approach in order to assess whether the immunohistochemical expression of CHKA changes between normal endometrial tissue, typical and atypical hyperplasia of endometrium and EC. The expression of CHKA was cytoplasmic in all samples, and a significantly increased CHKA expression was observed in EC and atypical hyperplasia compared to normal endometrial tissue (chi-square test, \( P < 0.02 \)). However, no difference was observed between atypical endometrial hyperplasia and EC (Fig. 5A).

**Choline Kinase activity in endometrial cell lines**

Finally, in order to investigate whether upregulated CHKA expression and activity would allow utilization of choline-based positron emission tomography (PET) examinations we measured \([^{3}\text{H}]\)choline uptake in HEC-1B and Ishikawa EC cells compared to that of St-T1b endometrial stromal cells. Incorporated radioactivity was increased 7 and 12-fold in the two EC cell lines, respectively, which indicates good differential uptake to stromal tissue (\( P < 0.001 \), Fig. 5B). Increased CHKA activity was further confirmed by MRS as denoted by increased PCho/Cho ratios in cancer cell lines versus normal control (Fig. 5C). Elevated flux was caused by higher baseline expression of choline transporters SLC44A, SLC22A3 and CHKA. In addition we found that – comparably to the patient-derived samples – PCYT1B and LYPLA1 and 2 were significantly higher expressed in cancer-derived cell lines compared to St-T1b (Fig. 5D).
Discussion

Abnormal NMR-detectable metabolic profiles have been reported in a number of tumor types, including breast, cervical and colorectal cancers (9, 31-34). However, this has not been studied in EC. In this study, we investigated metabolic alterations in EC using complementary approaches with the aim of identifying novel potential therapeutic and molecular imaging targets. We reveal an increase in the PCho pool in EC compared with normal endometrial tissue that can be attributed to an increase in CHKA activity and up-regulation of enzymes involved in the deacylation pathway. Alterations in choline handling directly impacts radiolabelled choline uptake allowing PET imaging to be used to assess tumor stage. Furthermore, as choline biochemistry is intimately involved with cellular proliferation, deregulated transporters or enzymes involved in choline handling may act as potential therapeutic targets. To our knowledge this is the first study to show altered choline handling in EC, and moreover this study uses the novel technique of HR-MAS MRS to illustrate specific alterations in choline metabolites in primary tissue specimens of endometrial carcinoma.

A number of studies have investigated the utility of HR-MAS MRS in cancer where the metabonomic phenotype was correlated with recognized prognostic features (13, 33, 35, 36). Overall these studies illustrate increased total Cho resonance signal at ~3.2 ppm in cancer compared with normal tissue consistent with our findings. PCho, GPC and Cho all contribute to the total choline resonance and we report relative levels of PCho, GPC and Cho consistent with previously reported values, with PCho being the main contributor to the total choline resonance. We reported a
~70% increase in PCho concentration in cancer compared to normal tissue. Consistent with previous publications in ovarian and breast cancer, we report a significant increase in the PCho:Cho ratio (11, 37) and non-significant increase in the PCho:GPC ratio in EC. We hypothesize that the lack of a distinct PCho:GPC switch, which is associated with oncogenic transformation in breast cancer (11), may not occur in EC and that overexpression of GPCPD1 identified in EC converts GPC via the deacylation pathway into free choline, which is again converted to PCho by CHKA (38, 39).

Considering the changes observed by MAS MRS we investigated the gene expression of enzymes responsible for phosphatidylcholine anabolism (Kennedy pathway), catabolism (deacylation pathway) and choline substrate transporters in order to ascertain if the changes in the choline peak in EC observed on MAS resulted from the deregulated intracellular metabolism of choline or transport of exogenous choline into the cells. Choline flux can only be measured accurately in vitro, and as we utilized clinical samples, qRT-PCR was used to assess the expression of transporters. The changes in relative gene expression of enzymes involved in choline handling have been studied in a number of tumor types including breast and ovarian cancer, and these studies are consistent in reporting an increase in the expression and activity of CHKA in cancer cells compared to normal epithelial cells (9, 38). Activation of CHKA results in an increase in the product, PCho, and product:substrate ratio, PCho:Cho. Furthermore, we report an increase in the expression of CHKA in EC compared with normal endometrial tissue by IHC. Of interest, we noted increase in CHKA expression not only in EC but also in hyperplastic endometrial tissue. Our TMA included both simple and complex atypical hyperplasia, the latter being
premalignant and this may account for the increase in CHKA in these specimens. While our approach illustrates tissue heterogeneity, the use of microdissection techniques and larger samples size may reduce the variation observed in the presented results. In a number of studies CHKA has been shown to mediate cellular proliferation, transformation and carcinogenesis, and mediation of this target using siRNA and small molecule inhibitors suggest inhibition of tumor growth (11, 16, 18, 40, 41).

As indicated, we further observed in clinical samples and EC cell lines a significant increase in the mRNA expression of enzymes of the phosphatidylcholine deacylation pathway; GPCPD1, LYPLA1 and LYPLA2. Alterations of the deacylation pathway have been previously investigated for its contribution to the relative PCho pool (38, 42). Iorio and colleagues reported a 2–4 fold increased levels of GPC in a number of ovarian cell lines (38), but found no change in the activity of LYPLA1 and 2. Gene expression analysis conducted in breast cancer cell lines illustrated a reduction in the expression of LYPLA1 in cancerous cell lines compared to normal controls (9). Discrepancies may arise from the use of whole tissue extracts of endometrial tissue rather than cell lines. Furthermore, as we used whole tissue we were only able to consider gene expression rather than enzyme activity. It is unclear from the literature whether any post-translational modifications occur for these enzymes and in future studies it would important to assess the functional alterations of these enzymes in EC. Nonetheless, on MAS the levels of GPC were not significantly altered; despite the increased expression of enzymes in the anabolic arm of choline handling the complementary activities of choline phosphorylation and deacylation could contribute the unique NMR profile observed in EC.
The increase in the expression of CHKA in human EC compared to normal endometrial tissue provides further support for the use of choline radiotracers in the staging of EC, particularly in patients at high risk of lymph node involvement where the use of choline PET imaging may alleviate the need for staging lymphadenectomy and its accompanied adverse effects. A number of studies have investigated the utility of $^{18}$F-fluorodeoxyglucose (FDG) PET in EC (3, 43). These studies are limited by poor sensitivity because of the rapid excretion and accumulation of FDG within the bladder, the largest study reporting 74% sensitivity in detecting lymph node involvement (3). The use of MRS in assessing EC is limited by poor spectral signal to noise ratio (7, 44). We have recently shown the feasibility of imaging using $^{11}$C-choline PET-CT in detecting lymph node involvement in high-risk prostate cancer. Furthermore, we suggest a correlation between the expression of CHKA within prostate cancer specimens with $^{11}$C-Choline uptake (6). To this end we showed differential uptake of $^3$H-choline by EC cells compared with normal stromal by 7 to 12-fold, highlighting the potential utility of choline radiotracers in imaging EC. The utility of $^{11}$C-choline PET/CT was demonstrated in a small study of 22 patients, where the accuracy of detecting lymph node involvement was 86%, increasing to 96% with both $^{11}$C-choline PET/CT and MRI. However, the use of $^{11}$C-choline is limited to institutions with onsite cyclotron given the short half-life of $^{11}$C (20.38 minutes). The use of $^{18}$F-labeled choline tracers is felt to be limited by bladder uptake, however, this could be alleviated through the use of delayed phase imaging, with the novel, more metabolically stable $^{18}$F-Fluoromethyl-[1,2-$^2$H$_4$]-choline or through the application of filtering techniques (45). Choline imaging by PET or MRS
has become an established tool of preclinical and clinical research and small-molecule inhibitors of CHKA are currently in development.

Using an unbiased HR-MAS MRS approach, we identified additional metabolic patterns that illustrate a severely altered phenotype in EC. The majority of these metabolites can be classed into proliferation-associated markers (e.g. various amino acids and the nucleobase uracil, which are indicative of high transcriptional and proliferative activity), maintenance of intracellular redox homeostasis (glutathione), osmoregulation (inositols, taurine) and glucose and lipid metabolism (lactate, acetate, free Cho, PCho, GPC, fatty acids, unsaturated lipids) (46). Glucose and lipid metabolism are tightly connected in cancer. Because of the Warburg effect, less glucose-derived metabolites are fed into the TCA cycle. Consequently, the required intermediates for generation of lipids need to be restored and acetate, lactate, ketone bodies and glutamine serve as substitutes (47). Interestingly, these metabolites were not increased in our data set, but the end products, unsaturated lipids and fatty acids (summarized as lipids in Table 1), were among the most severely up-regulated metabolites.

A limitation of this study is that the menopausal status of the patients studied was not known, however, based on the median age of the patients in the control cohort and those in the cancer cohort, it can be inferred that patients in the control cohort were likely to be partially premenopausal whilst those in the cancer cohort were likely to be predominately post-menopausal. Changes in MRS spectra caused by cyclical changes of the endometrial tissue or with menopause remains an important question for further investigation.
In conclusion, using a multi-modality approach, we have shown that the expression and activity of CHKA is increased in human EC specimens compared to normal endometrium. These findings suggest that deregulated choline metabolism may represent a source of molecular targets with important diagnostic and therapeutic implications for the management of patients with EC.
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Author Contributions:

Conception and design: RS, EA, HK, ST
Development of methodology: RS, ST, HK
Acquisition of data: PL, DJP, ST, RD, JKE
Analysis and interpretation of data: RS, RD, ST, PL, JKE
Writing, review and/or revision of the manuscript: RS, ST, RD, DJP, HK, EA
Study supervision: EA, RS
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Tables

Table 1

<table>
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<th>Multiplicity</th>
<th>Direction of change</th>
<th>% Change to Control</th>
<th>p-value</th>
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<tr>
<td>Valine</td>
<td>0.98 - 1.00</td>
<td>d</td>
<td>↑</td>
<td>58.88</td>
<td>0.0019</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.95 - 0.98</td>
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<td>44.63</td>
<td>0.0059</td>
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<tr>
<td>Alanine</td>
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<td>d</td>
<td>↑</td>
<td>40.26</td>
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<tr>
<td>Proline</td>
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<td>54.97</td>
<td>0.0019</td>
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<tr>
<td>Glutathione</td>
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<td>-43.86</td>
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<tr>
<td>PCho</td>
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<td>69.99</td>
<td>0.0100</td>
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<tr>
<td>Scyllo-inositol</td>
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<td>s</td>
<td>↓</td>
<td>-60.12</td>
<td>0.0019</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>3.52 - 3.55</td>
<td>dd</td>
<td>↓</td>
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<td>0.0005</td>
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<tr>
<td>Tyrosine</td>
<td>6.87 - 6.92</td>
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<tr>
<td>Inosine/adenosine</td>
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<td>s</td>
<td>↓</td>
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<tr>
<td>Lipids</td>
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<td>↑</td>
<td>3419.99</td>
<td>0.0034</td>
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Statistically significant metabolites after Benjamini-and-Hochberg multiple testing correction (FDR of 5%); P-values are derived from Mann-Whitney U test. The integral region indicates the signal that was selected for univariate testing. Fold changes are based on mean values in each tissue type. Abbreviations: s-singlet; d-doublet; dd-double doublet; m-multiplet.
Table and Figure legends

Table 1. Metabolic alterations in endometrial cancer detected by HR MAS ¹H NMR

Figure 1. ¹H HR MAS NMR spectral analysis of endometrial tissue reveals metabolic perturbations in EC. A, Mean spectra (1D-CPMG experiments) for tumour (n=8) and normal tissue (n=10); GPC-glycerophosphocholine; PCho-phosphocholine; Cho-choline; BCAA-branched chain amino acids. B-D, Multivariate analysis of endometrial tissue based on integrals of metabolite data extracted from HR MAS ¹H NMR CPMG spectra. B. Unsupervised PCA scores plot (UV scaled) of the first two principal components reveal discrimination between control (black squares) and cancer (red diamonds) groups, with each point representing a patient’s metabolic profile. C, Validation of the two-component PLS-DA model by permuting 999 random combinations of the class matrix, giving P~0.001. R²Y and Q²Y represent cumulative values for the two-component model, measuring “goodness of fit” and “goodness of prediction” respectively. D, The receiver operating characteristic (ROC) curve summarises the diagnostic accuracy in classifying endometrial cancer and controls.

Figure 2. Variable importance for projection (VIP) plot for PLS-DA model discriminating EC from normal endometrial tissue. Error bars are computed as the ‘jack-knife’ estimate of the 95% confidence interval. X: unassigned resonance.

Figure 3: Superimposition of mean HR MAS ¹H NMR spectra for EC and normal endometrial tissue revealing regions for choline-containing metabolites (3.20–3.24 ppm). Black lines represent controls (n=10), red lines represent EC (n=8). Table below indicates ratios of different choline metabolites. PCho-phosphocholine; GPC-glycerophosphocholine.
**Figure 4:** Determinants of deregulated choline metabolism. A, Relative fold difference in mRNA expression of enzymes in EC samples (n=10) compared with control tissue (n=10). Bar graph represents mean of three, independent qRT-PCR experiments. For each gene, the mean value±SD of the analysed samples is reported, Students T-test, *P*<0.05. B-C, western blot analysis of same EC samples against CHKA and corresponding densitometry results (*P*=0.0025).

**Figure 5.** CHKA is overexpressed in EC and causes increased uptake of radiolabelled choline. A, Representative sections of tissue microarray showing CHKA expression in normal, hyperplastic or endometrial cancer samples. Bar=100 μm. B, Cells were pulsed with [³H]choline for 1 hour and retained activity measured. Uptake of [³H]choline in human endometrial cancer cell lines HEC-1B and Ishikawa was compared to the human endometrial stroma cell line St-T1b (error: SEM; **P*<0.01, ***P*<0.001). C, The aqueous metabolites were extracted from the three EC cell lines, analysed by MRS and the choline metabolites measured. Data expressed as PCho/Cho ratio±SEM and significant differences to the St-T1b cell line indicated (*P*<0.05, **P*<0.01). D, Relative mRNA expression of indicated enzymes involved in choline handling. Expression is shown relative to normal cell line (St-T1b) and statistical significance was determined by comparison of the cancer cell lines to St-T1b (error: SD; *P*<0.05, **P*<0.01).
**Figure 3**

![Graph showing 
$^1$H Chemical Shift (ppm) vs. Concentration with peaks labeled PCho, GPC, and Cho.](image)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCho:GPC</td>
<td>1.69 ± 0.53</td>
<td>2.18 ± 0.57</td>
<td>0.08</td>
</tr>
<tr>
<td>PCho:choline</td>
<td>2.25 ± 0.83</td>
<td>3.57 ± 1.65</td>
<td>0.04</td>
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<tr>
<td>GPC:choline</td>
<td>1.63 ± 1.18</td>
<td>1.73 ± 0.89</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Figure 4

A

Fold change in EC compared to normal

CHKA, CHKB, PCYTIA, PCYT1B, CHPT1, GPDP1, LYP2A1, LYP2A2, PLD1, SLC44A, SLC22A3, SLC5A7

Kennedy pathway  Anabolic pathway  Transporter

B

OD CHKA / OD b-actin

Normal  EC

C

EEC  normal  EEC  normal

CHKA  β-actin
Figure 5

A

Normal

Hyperplasia

EC

B

Relative normalized [H]choline uptake

***

**

St-T1b

HEC-1B

Ishikawa

C

PCho/Cho ratio

**

St-T1b

HEC-1B

Ishikawa

D

mRNA relative expression

Kennedy pathway

Anabolic pathway

Transporter

CHKA

CHKB

PCYT1A

PCYT1B

CHPT1

GPCPD1

LYPLA1

LYPLA2

PLD1

SLC44A

SLC22A3

Notes:

- All data are presented as mean ± SEM.
- Statistical significance is indicated as follows:
  - * p < 0.05
  - ** p < 0.01
  - *** p < 0.001

- Images in panel A show histological staining of tissue samples from different conditions.
- Panels B, C, and D display quantitative results of choline uptake, PCho/Cho ratio, and mRNA expression levels, respectively.

In the Kennedy pathway, CHKA and CHKB are key enzymes involved in the formation of phosphatidylcholine (PC).

In the anabolic pathway, PC is further metabolized to phosphocholine (PCho) and choline (Cho).

In the transporter category, SLC44A and SLC22A3 are implicated in the transport of PCho/Cho across cell membranes.
Alterations of choline phospholipid metabolism in endometrial cancer are caused by choline kinase alpha overexpression and a hyperactivated deacylation pathway


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