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Up-regulation of genes involved in the Insulin signaling pathway (*IGF1, PTEN* and *IGFBP1*) in the endometrium may link Polycystic Ovarian Syndrome and Endometrial Cancer

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Running title: PCOS and EC: Gene related in insulin pathways

Abstract

Background: Endometrial cancer (EC) is the most common gynaecological cancer amongst women in the UK. Although previous studies have found that women with polycystic ovary syndrome (PCOS) have at least a three-fold increase in endometrial cancer (EC) risk compared to women without PCOS, the precise molecular mechanisms which link between PCOS and EC remain unclear. It has been suggested that insulin resistance may contribute to the increased risk of EC in PCOS. The specific expression of genes related to the insulin-signalling pathway including the IGF system in the endometrium of women with PCOS has however never been measured and compared to that in women with EC without PCOS and control women without EC or PCOS. .

Objectives: To test the hypothesis that insulin signaling plays a key role in the development of EC in women with PCOS by measuring and comparing the expression of three key genes involved in the insulin signaling pathway (*IGF1, PTEN* and *IGFBP1*) in endometrial tissue obtained from three groups of women; PCOS without EC, women with EC without PCOS and non-PCOS women without EC (controls). We also aimed to determine the correlation between the gene expressions to various clinical variables among participants.

Methods: This was a cross-sectional study of 102 women in 3 groups (PCOS, EC and controls) at a University teaching hospital in the United Kingdom. Clinical assessment (blood pressure, body mass index (BMI) and waist-hip-circumference ratio), venepuntures (fasting blood sugar, insulin, lipid profile, hormones) and endometrial tissue biopsies were taken in all participants. Endometrial tissue RNA extraction was performed before real time polymerase-chain-reaction for the genes of interest *(IGF1, IGFBP1* and *PTEN)* was carried out. To compare the baseline characteristics of the study population, One-Way-ANOVA test or the Independent t-test was used. For variables that were not normally distributed, the Spearman correlation test was used to calculate the r value. A “p” value of <0.05 was considered statistically significant.

Results: *IGF1*, *IGFBP1* and *PTEN* gene expression were significantly up-regulated in the endometrium of PCOS and EC women compared to controls. However there was no significant difference in the expression of these genes in PCOS compared to EC endometrium. The BMI of women with PCOS and controls, were not significantly different (29.28 (±2.91) vs 28.58 (±2.62) kg/m^2) respectively, women with EC however had a higher mean BMI (32.22 (±5.70) kg/m^2). PCOS women were younger (31.8 (±5.97) years) than women with EC (63.44 (±10.07) years) and controls (43.68 (±13.12) years). The changes in gene expression were independent of BMI, waist hip ratio, estradiol and androgen levels. Protein validation test in the serum samples in the three groups were consistent with the gene findings.

Conclusion: Women with PCOS and EC have an increased endometrial expression of genes (*IGF1, IGFBP1* and *PTEN*) involved in the insulin signaling pathway compared with control women. This may explain the increased risk of EC in PCOS women. This study provides a strong basis for clinical trials aiming to prevent EC in women with PCOS by investigating drugs targeting the insulin signaling pathway. This panel of genes may also serve as clinically useful early biomarkers which predict which women with PCOS will go on to develop EC

Key words: Endometrial cancer, *IGF1, IGFBP1,* PCOS*, PTEN*

Introduction

Endometrial cancer (EC) is the most common gynaecological cancer amongst women in the UK. The incidence has increased by about 50% since the 1990s (National Statistics, UK) because of rising obesity rates. Approximately 99,000 new cases were reported in Europe in 2012 (National Statistics, UK) and the most recent UK data in 2011, revealed a total of 8475 new cases reported that year (National Statistics, UK). It is postulated that every day, 23 women are newly diagnosed with EC in the UK. EC not only causes physical and psychological morbidity but, it is also a huge economic burden. The cost of EC treatment in the USA for example, is over USD14, 000 per case (Fanning, 1999).

Screening, prevention strategies and survival rates also remain poor in comparison to other endocrine cancers where prognosis has improved. Traditionally, chronic exposure to unopposed estrogen, hyperinsulinaemia and obesity are thought to be key predisposing factors to developing EC (Arem et al, 2013; Burzawa et al, 2011). These risk factors are also present in women with polycystic ovary syndrome (PCOS) which affects approximately 10% (Shafiee et al, 2013) of women in the general population and who are three times more likely to develop EC (Barry et al, 2014). A study reported that the prevalence of EC in PCOS women was 20-37% (Navaratnarajah et al, 2008). The precise molecular mechanisms which increase EC risk in women with PCOS are however, unclear (Hardiman et al, 2003; Shafiee et al, 2013).

It has been hypothesized that insulin resistance plays the key role in the development of EC in women with PCOS and that measures which improve insulin sensitivity such as metformin and weight loss might play a key role in preventing the development of EC in women with PCOS (Shafiee et al, 2014). The specific expression of genes related to the insulin signalling pathway including the IGF system in the endometrium of women with PCOS has however never been measured and compared to that in women with EC without PCOS and control women without EC or PCOS.

Evidence in support for the hypothesis that insulin resistance plays the key role in the development of EC in women with PCOS is underpinned by the following studies. Diabetes mellitus is linked with EC and even in women receiving exogenous oestrogens, women with diabetes are at higher risk (Harding et al, 2014); higher insulin levels have been found in postmenopausal women (who have low oestrogen levels) with EC (Ayabe et al, 1997, Nagamami and Stuart, 1998); insulin and insulin like growth factor (IGF) receptors have been identified in both normal and malignant (Ayabe et al, 1997) endometrium; Insulin, IGF 1 and IGF 2 have been shown to have a mitogenic effect on endometrial cells in-vitro with type I IGF receptor mRNA over-expressed in EC (Nagamani 1991) and the number of type I IGF receptors present has been shown to positively correlate with the histological grade of EC (Nagamani *et al* 1991, Talavera *et al* 1990). Genomic (Pillay et al, 2005) and proteomic (Galazis et al, 2013) approaches have also identified potential new genes and proteins which might explain link between PCOS and EC and could up-regulate genes with the insulin signaling pathway in PCOS. A review by Shafiee et al, evaluating possible mechanisms underpinning the molecular links between

PCOS and EC (Shafiee et al, 2013) suggested that the mitogenic effect of high levels of insulin may be mediated through activation of the phosphatidylinositide 3-kinases (PI3K) /Protein kinase B (Akt) and Ras/microtubule-associated protein kinase (MAPK) signaling pathways and that triggering these pathways results in the exaggeration of *IGF1* and IGF binding protein (IGFBP) expression which leads to promotion of cell growth. Measuring the specific expression of genes related to the insulin signaling pathway in the endometrium of women with PCOS compared to that in women with EC and control women without EC or PCOS is however a necessary first step to investigating how these pathways, genes and proteins interact with the insulin signaling pathways in the endometrium of women with PCOS and how this might lead to EC.

Phosphatase and tensin homolog (*PTEN*) is a tumour suppressor gene located at chromosome 10q23 that suppress cell proliferation and differentiation and is involved in the insulin signaling pathway. The protein encoded by this gene has similar protein tyrosine phosphatase property that negatively regulates *PI3K/ Akt* signaling pathway involved in carcinogenesis (Scully et al, 2014) Dysfunction of this gene has been implicated in the development of various cancers in the human including breast, ovarian and EC. In type 1 EC, mutation of *PTEN* occurs in 25-83% of tumours (Melissa et al, 2010). Interestingly, it has been shown that in EC, *PTEN* inactivation almost always occurs at an early cancer stage whereas, in other neoplasms *PTEN* is implicated at the later stages or during metastasis (Melissa et al, 2010). *PTEN* gene expression has however never previously been measured in endometrial tissue from women with PCOS in human as far as we know, although an animal study on a rat model of PCOS (using human chorionic gonadotrophine and norethindrone injection) showed that the expression of *PTEN* gene and protein was significantly increased in ovarian tissue (Ouyang et al, 2013).

The aim of this study was to test the hypothesis that insulin signaling plays a key role in the development of EC in women with PCOS by measuring and comparing the expression of three key genes involved in the insulin signaling pathway (*IGF1, PTEN* and *IGFBP1*) in endometrial tissue obtained from three groups of women; PCOS without EC, women with EC without PCOS and non-PCOS women without EC (controls). This was considered clinically important, because establishing a clear role for the insulin signaling pathway in the mechanisms leading to EC in PCOS would justify clinical trials investigating the use of Metformin and other insulin sensitizers for the prevention of EC in PCOS as well as act as the molecular basis to inform future personalized medicine approaches to match patient's therapies as we currently do not know which subgroups of women with PCOS will go on to develop EC.

Methods

Study Design and ethics (institutional review board) approval.

This was a cross-sectional study conducted within the division of Obstetrics and Gynaecology and Child Health, at Nottingham University Hospital in the United Kingdom. Participants were prospectively recruited from July 2013 to February 2014. Research Ethics (institutional review board) approval was obtained from the National Research Ethics Service, East Midlands-Northampton committee (13/EM/0119) prior to commencement of recruitment.

Participant selection and recruitment

A total of 102 participants were recruited in this study and grouped into three arms: PCOS, EC and control with 34 participants recruited into each arm. PCOS was defined using the Rotterdam European Society for Human Reproduction and Embryology (ESHRE) and the American Society of Reproductive Medicine (ASRM) criteria when two out of the following three criteria were met; oligoovulation and/or anovulation, clinical and/or biochemical evidence of androgen excess and polycystic appearance of ovaries, with the absence of other endocrine causes of oligo/anovulation excluded). The participants were between 18 to 45 years of age and not on any hormonal treatment. Pregnancy was excluded prior to the recruitment. The EC group consisted of women with endometroid (type 1) adenocarcinoma of the endometrium (confirmed by histopathology) undergoing total hysterectomy (laparotomy or laparoscopically). The control groups comprised healthy women without EC or PCOS, not on any hormonal therapy, and are undergoing pelvic operative procedures for benign causes. In the PCOS group, the inclusion criteria were, age between 18 to 45 years old and established diagnosis of PCOS using the Rotterdam European Society for Human Reproduction and Embryology (ESHRE) and the American Society of Reproductive Medicine (ASRM) criteria. Exclusion criteria included women with on infertility treatment, hormone usage (e.g. combined oral contraceptives or progesterone), pregnancy and never been sexually active. In the EC group, the inclusion criteria were a diagnosis of endometroid (type 1) endometrial cancer (all grade and stage) and no previous neo-adjuvant therapy e.g. chemotherapy or radiotherapy. Exclusion criteria were women with cancers other than endometriod endometrial cancer for example papillary serous adenocarcinoma and women with metachronous cancer (ovary, endometrium and cervix cancer. Inclusion criteria in the control group were women aged 18 to 45 years, with a benign disease (non-malignant) undergoing a hysterectomy or a gynaecological procedure (for example hysteroscopy or laparoscopy) where uterine instrumentation was required. Exclusion criteria included women on any hormonal drugs or Metformin and undergoing gynaecological procedures not requiring uterine instrumentation.

Participants with PCOS were identified in the Gynaecology clinics at Nottingham University Hospitals based on the criteria mentioned above. Following verbal and written informed consent, a separate appointment was arranged for a clinical interview, physical examination and endometrial biopsy. At the clinical interview, baseline demographic details were obtained, including age, race, reproductive history, medical history and family history. Physical examination included measurement of sitting blood pressure in millimeter mercury, using a Welch Allyn® electronic sphygmomanometer. Height

(metres) and weight (kilograms) and a body mass index (BMI) calculated (kg/m^2). A Ferriman Galway score for hirsutism was performed and waist and hip circumference measured in centimeter, with participants wearing indoor clothes. Venepunctures were then performed for fasting blood sugar (FBS), low density lipo-protein (LDL), high density lipoprotein (HDL), triglycerides testosterone, sex hormone binding globulin (SHBG), follicle stimulating hormone (FSH), luteinising hormone (LH), prolactin, 17-hydroxy-progesterone and thyroid function tests. The assays were labelled using standard National Health Service (NHS) templates and biochemical request forms were completed and samples sent to the chemical pathology laboratory for routine processing. A baseline urine pregnancy test was also done to exclude pregnancy. Following this, an endometrial biopsy was obtained for histology and gene profiling using a Pipelle® endometrial catheter. Samples were snap frozen in liquid nitrogen at minus 80 degrees centigrade and then transported to the laboratory for storage in freezer at minus 80 degrees centigrade for future analysis. The samples for pathology review were immersed into formalin pot and labelled using the hospital's standard histology request form with an additional request for research purpose. A designated pathologist (SD) took charge of the specimen.

Participants with endometrial cancer were identified from newly diagnosed women with endometroid adenocarcinoma of the endometrium seen in the gynaecology oncology clinic at the City Hospital Nottingham already scheduled to come into the hospital to have a hysterectomy. Following verbal and written informed consent, a clinical assessment was performed and venepunctures performed for samples for endocrine and metabolic assays. Samples were transported and stored for future analysis as was done in women with PCOS. Standard procedures for the planned hysterectomy were performed by the designated gynaecological oncologist. On retrieval of the uterus, the hysterectomy (uterus) specimen was taken from the operating theatre to the histology laboratory where a designated a pathologist (whom was alerted before the procedure) immediately performed a cut-section and gross inspection, before some tissue samples were provided to the researcher for snap freezing in liquid nitrogen at minus 80 degrees centigrade. Experimental samples were then transported to the laboratory for storage in freezer at minus 80 degrees centigrade for future analysis.

Women in the control group were identified and recruited from the gynaecology clinics. Following verbal and written informed consent, a clinical assessment was performed and venepunctures performed for endocrine and metabolic assays. Samples were transported and stored for future analysis as was done in women with PCOS. Endometrial samples were taken during the planned operative pelvic procedure by the researcher using a Pipelle® endometrial sample, uterine curette or by direct excision from the uterine specimen as indicated by the type of gynaecological procedure being performed. Samples were snap frozen in liquid nitrogen at minus 80 degrees centigrade and then transported to the laboratory for storage in freezer at minus 80 degrees centigrade for future analysis.

Endocrine and Metabolic Assays

The assays for the endocrine and metabolic study were performed in the clinical chemical laboratory. Cobas 8000® System (Roche) was utilized for fasting blood sugar, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides levels.

An immunoassay method utilized for follicular stimulating hormone (FSH), leutinizing hormone (LH), estradiol and testosterone levels using the Architect 12000SR® equipment (Abbott). Serial verification methods namely precision, accuracy, linearity and method comparison studies was performed to validate the results, with intra-assay CVs 2.3–4.1%, 2.2–2.6%; 4.4–10%, and 2.9– 5.0%, respectively; and inter-assay CVs 5.3–6.6% and 4.9– 6.3%, 8.8–12.4%, and 9.1–10.9%, respectively.

Histology

Microscopic examination was performed using a standard histology protocol. The tissue samples were fixed in 10% buffered formalin before they were cut into thin sections. The sections were then paraffin-embedded following graded ethanol dehydration and paraffin infiltration. Sections 3 to 10 microns in thickness were cut from paraffin block using microtome. The sections were stained with hematoxylin and eosin (H&E) before mounted on a glass slide and covered with a thin cover glass to seal the preparation. The sections were examined using a light microscope (Carl Zeiss).

Quantitative real-time polymerase chain reaction (qPCR)

RNA extraction from endometrial tissue was performed using the QIAshredder kit (Qiagen, Valencia, CA) and further purified using the QIAamp® Mini-kit (Qiagen, Valencia, CA), after the samples were measured to ensure similar volume. The RNA concentration and quality was checked using Nanodrop®.. Reverse transcription were performed using the Superscript® III First-Strand Synthesis System (Invitrogen ^{TM}). A total RNA of 60ng was prepared for the reaction. Reverse transcription was carried out at 37℃ for 60 minutes, 95℃ for 10 min utes and 4℃ for 5 minutes. The cDNA was stored at -20 degree centigrade until further PCR work. The PCR reactions were undertaken using Taqman® qPCR Gene Expression Master Mix (Life Technologies, USA). The PCR conditions consist of 15 min step at 95℃, 40 cycles at 95℃ for 15 seconds each and 1 minute at 60℃. Life cycler 480® was used to perform the qPCR cycles. To minimize confounders we performed PCR of each gene from all three groups of sample (PCOS, EC and controls) at the same batch. A housekeeping gene beta-actin (*ACTB:* 5'- AGGTGACAGCAGTCGGTTGGA-3'/5'-CCTTAGAGA GAAGCGGGGTGG -3') was used as an internal control to correct differences in the amount of RNA in each sample. The primer and probe used for gene of interest were *IGF1* (5'- GTGCGGAGACAGGGGCTTT-3'/5'- ACTTGGCGGGC TTGAGAGG-3'), *IGFBP1* (5'- CACTTGATGGCCGAGTCCA-3'/5'- CCTCCAGCGACGTCTCACA-3') *and PTEN (*5′-CAGAAGAAGCCCCGCCACCAG-3′/5′- AGAGGAGCAGCCGCAGAAATG-3′). Each experiment was performed in triplicate to minimize errors. Relative gene expression analysis calculation was carried out by using the ratio= (E target) $^{\Delta Ct\ target\ (control\text{-}treated)}$/ (E ref) $^{\Delta Ct\ ref\ (control\text{-}treated)}$

Enzyme-linked Immunosorbent Assay (ELISA) for validation of specific proteins

The ELISA protein detection and quantification for IGF1, IGFBP1 and PTEN proteins was performed following PCR analysis, using serum samples from the PCOS and EC participants. The ELISA kit was purchased from MyBiosource.com ELISA kit®, implementing sandwich enzyme immunoassay. The microtiter plate in this kit was pre-coated with antibody specific to each protein. A total of 36 serum samples from the groups (12 samples each group) and standards (with different concentrations i.e. 0.19ng/mL, 0.38ng/mL, 0.75ng/mL, 1.5ng/mL, 3ng/mL, 6ng/mL and 12ng/mL) were analysed in duplicate. The intra-assay CV for IGF1, IGFBP1 and PTEN were 3.0–4.0%, 7.5–8.5% and 2.5-3.6% respectively; inter-assay CV 8.3–8.8%, 9.5–21% and 4.6-9.7% respectively.

The samples were left at room temperature (18-25 degree celcius) before use. A detail map of the microtiter plate was constructed with an amount of 100 uL of each sample; standard and blank were performed in duplicate. The plate was covered with a sealer before incubated being for 2 hours at 37 degree celcius. The liquid in each well was aspirated using a pipette. An amount of 100uL of Detection Reagent A solution was added to each well. A plate sealer was used to cover the plate before incubated for 1 hour at 37 degree celcius. The solution was aspirated with pipette and each well was washed with 350uL of 1x wash solution. The wash procedure was repeated for three times and the remaining liquid in each well was removed by snapping the plate onto the absorbent paper. An amount of 100 uL of detection reagent B solution was added to each well. The plate was then covered with plate sealer before incubated for 30 minutes at 37 degree celcius. The liquid in each well was aspirated and washed with 350uL of 1x wash solution for five times. The remaining liquid was removed by inverting the plate and blotted it against absorbent paper. An amount of 90uL of substrate solution was added to each well. The plate was covered with a new sealer before incubated for 15 minutes at 37 degree celcius. The mixture was protected from light. An amount of 50uL of stop solution was added to each well. The liquid was mixed well by gently tapping the side of the plate. The microplate reader was run and conducted measurement at 450nm after ensuring no bubble in the well and any fingerprint or liquid at the bottom of plate been cleared. The optical density value was plotted against the standard protein concentration to create a standard curve. The protein quantification in the groups was determined. The data was analysed using Prism GraphPad 6.

Sample size and Statistics

The sample size was calculated to detect a 40% difference in *IGF1* gene expression in women with PCOS compared with controls and women with PCOS compared with endometrial cancer. Thirty four women were required in each arm for a study with a power of 80% at a 5% significance level. A difference of 40% was chosen based on a study (Gloria *et al,* 2003) in which *IGF1* gene levels in women with endometrial cancer were compared were compared with controls. Sixty seven percent of the endometrial cancer samples expressed *IGF1* gene compared with 27% in the control group (a 40% difference). A 5% significance level was chosen to allow for 2 comparisons; PCOS versus controls and PCOS versus endometrial cancer.

Data were summarized on SPSS version 21 for IBM and Microsoft Excel 2010. Data was tested for normality of distribution using a Shapiro normality test and for continuous variables, summarized as means (± standard deviation) if normally distributed or median (± range) if not normally distributed. Categorical variables were summarized as proportions/percentages. Comparison between groups depended on the number of independent variables, and a One-Way-ANOVA test was used for comparing differences in more than 2 groups with an independent T-test used for comparing mean differences between two groups. A Spearman correlation coefficient test was used to check for association between variables, in non-parametric variables. A p-value of less than 0.05 was considered statistically significant. Age and BMI-adjusted analysis was performed due to the significant difference mean in the groups.

Results

Participants' characteristic data

A summary of the participants' characteristic data is presented in table 1. Although the BMI of women with PCOS and controls were not significantly different (29.28 (±2.91) vs 28.58 (±2.62) kg/m^2) respectively, women with EC had a higher mean BMI (32.22(±5.70) kg/m^2). PCOS women were however younger (31.8 (±5.97) years) than women with EC (63.44 (±10.07) years) and controls (43.68 (±13.12) years). Women with PCOS were recruited during their proliferative menstrual phase (based on their menstrual histories). Histologically, two women had secretory phase endometrium, eight samples were inadequate but majority (24 women) was in the proliferative phase.

Among 34 participants with EC, four of them were premenopausal, 13 were perimenopausal and 17 were postmenopausal. Grade 2 endometroid adenocarcinoma was more prevalence (44.1%), followed by grade 3 (29.4%) and grade 1 (26.5%) in the EC group. In controls group, 20 were premenopausal and 14 were postmenopausal, and all were confirmed to have benign diseases.

Gene expression in PCOS, endometrial cancer and control

IGF1 expression was significantly increased in both PCOS ($p<0.001$) and EC ($p=0.0006$) compared to controls. However there was no significant difference between PCOS and EC ($p=0.220$). *IGFBP1* expression was also significantly increased in PCOS ($p<0.001$) and EC ($p=0.001$) compared to controls. However there was no difference between PCOS and EC ($p=0.462$). *PTEN* expression was significantly increased in both PCOS ($p=0.007$) and EC ($p<0.001$) compared to controls. But, again the *PTEN* expression was not statistically significant difference among PCOS and EC ($p=0.607$) Figure 1-3 illustrate the gene expression findings.

Correlation between gene expression and BMI

Spearman correlation test was used to determine the correlation of gene expression to BMI. *IGF1* expression was not significantly correlated with BMI in PCOS (r=0.083, 95% CI=-0.419 to 0.272,

p=0.639) and EC (r=0.081, 95% CI=-0.417 to 0.274, p=0.648). *IGFBP1* expression was also not significantly correlated with BMI in PCOS (r=0.139, 95% CI=-0.464 to 0.219, p=0.433) and EC (r=0.128, 95% CI=-0.455 to 0.229, p=0.471). *PTEN* expression was inversely correlated with BMI in PCOS (r=-0.189, 95% CI=-0.504 to 0.169, p=0.284), but this was not statistically significant. In EC, *PTEN* expression was also not significantly correlated with BMI (r=-0.003, 95% CI=-0.349 to 0.345, p=0.988).

Correlation between gene expression and waist-hip-ratio (WHR)

As WHR was normally distributed but the gene expression was a continuous not normally distributed variable, Spearman correlation coefficient test was used to determine the correlation. *IGF1* expression was not correlated with WHR in both PCOS (r=-0.125, 95% CI=-0.453 to 0.233, p=0.482) and EC (r=0.068, 95% CI=-0.286 to 0.406, p=0.702). *IGFBP1* expression also was not correlated with WHR in both PCOS (r=0.107, 95% CI=-0.250 to 0.438, p=0.549) and EC (r=0.257, 95% CI=-0.099 to 0.554, p=0.143). Similarly, *PTEN* was not correlated with WHR in PCOS (r=-0.016, 95% CI=-0.362 to 0.333, p=0.927) and EC (r=-0.029, 95% CI=-0.373 to 0.321, p=0.867).

Correlation between gene expression and insulin resistance (HOMA-IR)

IGF1 expression was not correlated with HOMA-IR in both PCOS (r=0.159, 95% CI=-0.199 to 0.4797, p=0.365) and EC (r=0.008, 95% CI=-0.339 to 0.355, p=0.962). Similarly, *IGFBP1* expression was not significantly correlated with HOMA-IR in PCOS (r=0.093, 95% CI=-0.263 to 0.426, p=0.602) and EC (r=0.142, 95% CI=-0.216 to 0.467, p=0.422). *PTEN* expression was also not correlated with HOMA-IR in EC (r=-0.321, 95% CI=-0.029 to 0.601, p=0.064). In PCOS, *PTEN* was not significantly correlated with HOMA-IR (r=-0.146, 95% CI=-0.469 to 0.212, p=0.410).

Correlation between gene expression and steroid hormones (estradiol and testosterone)

IGF1 expression was not correlated with estradiol in PCOS (r= 0.251, 95% CI=-0.245 to 0.609, p=0.521) and EC (r=0.059, 95% CI=0.321 to 0.872, p=0.876). Similarly, testosterone level was also not correlated to the *IGF1* expression in both groups (p>0.05). The *IGFBP1* expression in both PCOS and EC was not significantly correlated with estradiol level with r=0.091, 95% CI=0.023 to 0.754, p=0.241 and r=0.125, 95% CI=0.065 to 0.875, p=0.853, respectively. The *PTEN* expression was also not significantly correlated with estradiol and testosterone in PCOS, r=0.431, 95% CI=0.192 to 0.053, p=0.065; r=0.481, 95% CI=0.211 to 0.874, p=0.067, respectively. In EC group, the PTEN expression was also not correlated with estradiol and testosterone (p>0.05).

ELISA specific protein validation in serum

Figure 1 illustrates the concentration of IGF1 protein in the serum of PCOS and EC compared to control. IGF1 level was significantly higher in PCOS compared to control (p=0.023). A similar trend

was observed in EC compared to control ($p<0.001$). In addition, serum IGF1 levels was significantly higher in EC compared with PCOS women EC ($p=0.005$). Figure 3 illustrates the concentration of PTEN protein in the serum of PCOS and EC compared to control. PTEN levels were significantly higher in PCOS compared to control ($p=0.025$). A similar trend was observed in EC compared to controls ($p=0.042$). However, there was no significantly difference in serum PTEN levels between PCOS and EC ($p=0.753$).

Discussion

As far as we know from our literature search, this is the first study that has investigated the expression of genes involved in the insulin signaling pathway in the human PCOS endometrium compared to expression in endometrial biopsies from women with endometrial cancer (EC). The genes investigated, included *IGF1, IGFBP1* and *PTEN*. All three genes were significantly expressed in both PCOS and EC endometrium compared to endometrial biopsies from control women without PCOS and without EC. Furthermore, there was no significant difference in the expression of *IGF1, IGFBP1* and *PTEN* genes in women with PCOS compared with EC ($P>0.05$). These findings were reflected in the serum protein levels. The changes in gene expression were independent of body mass index, waist hip ratio or systemic measurements of insulin resistance using the homeostatic model assessment (HOMA-IR). The changes were also independent of systemic estradiol and androgen levels as estradiol levels were lowest in women with EC compared to the levels in PCOS and control women and androgen levels similar in EC and controls despite the up-regulation of the *IGF1*, *PTEN* and *IGFBP1* genes in EC. This could reflect that the reproductive stages of the participants may not influence the endometrial gene expression. The null hypothesis that there would be no difference in the expression levels of these three key genes involved in the insulin signaling pathway (*IGF1*, *PTEN* and *IGFBP1*) in endometrial tissue obtained from three groups of women; PCOS without EC, women with EC without PCOS and non-PCOS women without EC (controls) was therefore rejected.

These findings are consistent with previous studies which have suggested that insulin resistance may link PCOS with EC (Gunter et al, 2008; Pillay et al, 2005). For example, in one study, EC grade was found to be positively correlated with the expression of IGF1 receptors (Pillay et al, 2005, Nagamani et al, 1998). Knocking down IGF1 receptors was found in another study to suppress cancer growth (Kashima et al, 2009), and in a further study, metformin which is an insulin sensitizing agent was clinically proven to overcome the neoplastic changes in cancer cells (Engelman and Cantley, 2010; Dowling et al, 2011; Wang et al, 2011). The up-regulation of the three genes investigated may also indicate a stepwise progression from changes at molecular level, to systemic insulin resistance and subsequent diabetes, as young women with PCOS show evidence of insulin resistance and not overt diabetes, but then go on to develop overt type 2 diabetes at a later age compared with women without PCOS (Carreau and Baillargeon, 2015).

In addition, our findings were inconsistent with previous studies that investigated a link between obesity and *IGF* expression, obesity and serum IGFBP protein, and obesity to serum IGF1 protein level (Kelly et al, 2011). We found in our study that *IGF1* up-regulation was independent of BMI. *IGFBP1* and *IGF1* genes were similarly up-regulated and that up-regulation of *PTEN* gene was present in EC. A population study in a tertiary centre for example, found that the mean weight of PCOS women was 37.3 +/- 9.9 kg/m^2 (obese) (Yildiz et al, 2008), and out of 3,947 EC patients in a retrospective cohort study, 62% were obese and morbidly obese (Mahdi et al, 2014). Hence it has been suggested that obesity might link PCOS with EC. The results from our study which found that the up-regulation of *IGF1, IGFBP1* and *PTEN* genes in PCOS women were independent of obesity (as PCOS women had a similar mean BMI to controls), however suggests that the women with PCOS might be at higher risk of EC independent of BMI.

Although increased *IGF1* protein levels in circulation are associated with lower levels of *IGFBP* this was not reflected at the molecular level in our study as both genes were up-regulated in PCOS and EC endometrium. However, it is plausible that both genes may independently modulate EC risk in PCOS at the cellular level. It has been for example been suggested that *IGFBP1* could increase the concentration of ligands in the tumour microenvironment which then modulates the Wnt signaling pathways (Declercq et al, 2008). The Wnt pathway is involved in embryonic development, including cell division and migration and it has a strong association with cancer development. In breast cancer, increased expression of beta-catenin (an indicator for Wnt activation) correlated with poor prognosis. (Vadnais et al, 2014).

PTEN is tumor suppressor gene. Mutation or loss of function of this gene is associated with higher risks of cancer and poorer prognosis (Fernández et al, 2014). However in our study, the expression of *PTEN* was up-regulated in both PCOS and EC. Seemingly, with this finding, the potential risk of EC is supposed to be reduced in PCOS. In EC women, hypothetically the prognosis of EC would also be expected to be improved with up-regulation of *PTEN* as found in our study. However, it also plausible that *PTEN* up-regulation as found in both women with PCOS and EC in our study may be linked with increased cancer risk. A recent study investigated the functional level of *PTEN* in astrocyte growth and found that a 'gain of function mutation' could reverse the tumour suppressor role of *PTEN* (Fernández et al, 2014). It was suggested that this gain of function mutation contributes to atypical cell growth in part through up-regulation of the mitogenic *IGF-1/PI3K/Akt*, (Fernández et al, 2014). However, it is inconclusive whether the 'gain of function mutation' theory could fit with our findings where *IGF1* and *PTEN* genes, were similarly increased in expression in PCOS and EC. This 'gain of function mutation', converting *PTEN* into a tumour promoter involving the *IGF1* pathway is relative a novel hypothesis that requires further assessment especially in the context of EC and PCOS.

The strengths of our study were in its importance (EC is the commonest gynaecological cancer) originality (first study to measure *IGF1, IGFBP1* and *PTEN* genes in women with PCOS, EC and controls), rigorous methodology, including participant recruitment, baseline sample size

considerations, the detailed and high quality endocrine/metabolic assays and experiments investigating gene expression, protein validation and the statistical analysis including investigating the impact of potential compounding factors, by performing sub-analysis on clinical and metabolic (HOMA-IR) variables and correlating them to gene expression. It would have been ideal to match all three groups for BMI and age, but this was not considered practical especially with women with EC. Women with PCOS were however of a similar BMI to controls. The other issue was the non homogenousity of the reproductive stage in the participants across the three groups. Most women in the EC group were menopausal and PCOS group were in their reproductive age. This however, indicates the actual prevalence of the specific disease in that particular reproductive stage. As a cross sectional study, this research served a baseline characteristic of the gene of interest activity among the studied population. It is deemed unnecessary to study the gene activity at menopausal stage in women with previous history with PCOS, and very limited number of women diagnosed with EC below 45 years of age. In addition, the gene expression *(IGF1*, *PTEN* and *IGFBP1)* in this study was independent of systemic estradiol and androgen levels. This could reflect that the reproductive stages of the participants may not influence the endometrial gene expression. However, based from the finding in this study, we postulated that it good to conduct a prospective study in the future-following up those women with PCOS with increased IGF signaling gene expression to determine the risk of developing EC in this cohort. However, technically is very challenging due to long interval.

Conclusion

This study found that the expression of *IGF1, IGFBP1* and *PTEN* genes were significantly up-regulated in the endometrium of women with PCOS and women with EC compared with controls This up-regulation was independent of systemic estradiol and androgen levels and estradiol levels BMI, HOMA-IR, WHR. Up-regulation of genes involved in the insulin signaling pathway may therefore be the molecular basis for the increased risk of EC in women with PCOS independent of BMI, serum estradiol, androgen and HOMA-IR. The potential translational implications of these findings include a firmer mechanistic basis for strategies aiming to reduce morbidity and mortality form EC. These include studies investigating drugs targeting endometrial expression of genes involved in the insulin signaling pathway in clinical trials to prevent EC in women with and without PCOS. As the findings at the molecular level were not yet reflected in systemic measures of insulin resistance using the HOMA test, the findings also justify further studies aiming to identify clinically useful early biomarkers which might predict which women with and without PCOS will go on to develop EC. Further studies are required to validate these findings. The gain of function mutation hypothesis, converting *PTEN* into a tumour promoter gene involving the *IGF1* pathway is a relatively novel hypothesis that requires further investigation in women with PCOS.

Contributors

WUA was the principal investigator and academic supervisor for MNS (PhD research). WUA and MNS designed the study, prepared the research proposal and submitted it to the Research Ethics Committee, Northampton, UK. MNS was the PhD student who worked on the project and recruited research participants (with the help of WUA and JA), kept all the confidential data, analysed the samples, conducted experiments and performed the statistical analysis. CS, CC and NM helped to train and supervised MNS for the laboratory experiments. NM assisted MNS with gene data analysis. SD was responsible for the histological aspects of the study. MNS prepared the manuscript and WUA reviewed the first draft. Other co-authors reviewed and approved the final draft before submission.

Declaration of interests

All authors declared no conflict of interest.

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Figure legends

Table 1: Participants' characteristic, biochemical and hormonal data
Figure 1: *IGF1* expression and protein in PCOS and EC compared to control
Figure 2: *IGFBP1* expression in PCOS and EC compared to control
Figure 3: *PTEN* expression and protein in PCOS and EC compared to control

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Group/ Variables	**PCOS**	**EC**	**Control**	**P-value**
Age (Years); Mean (SD)	31.8 (±5.97)	63.44 (±10.07)	43.68 (±13.12)	<0.001*

BMI (Kg/m2); Mean (SD)	29.28 (±2.91)	32.22(±5.70)	28.58 (±2.62)	0.001*
WHC Ratio; Mean (SD)	0.88 (±0.03)	0.91 (±0.04)	0.85 (±0.02)	<0.001*
Systolic BP (mmHg); Mean (SD)	133.4 (±7.09)	146.7 (±10.7)	134.5 (±8.4)	<0.001*
Diastolic BP (mmHg); Mean (SD	82.2 (±7.95)	87.5 (±6.9)	80.56 (±7.0)	0.159
Fasting Insulin; Mean (SD)	19.05 (±28.19)	16.84 (±14.26)	13.22 (±5.52)	0.427
Fasting Glucose; Mean (SD)	5.16 (±0.78)	6.3 (±1.5)	4.9 (±0.5)	<0.001*
HOMA-IR; Mean (SD)	0.25 (±0.39)	0.28 (±0.33)	0.17 (±0.10)	0.281
LDL; Mean (SD)	2.74 (±0.77)	2.57 (±1.07)	2.75 (±0.80)	0.664
HDL; Mean (SD)	1.44 (±0.33)	1.63 (±0.34)	1.47 (±0.33)	0.044*
TG; Mean (SD)	1.43 (±0.51)	1.51 (±0.54)	1.38 (±0.60)	0.598
Total Cholesterol; Mean (SD)	4.73 (±0.91)	4.59 (±1.39)	4.94 (±0.98)	0.418
FSH; Mean (SD)	4.97 (±2.55)	50.78 (±19.45)	15.59 (±21.80)	0.001*
LH; Mean (SD)	11.67 (±10.39)	30.74 (±12.43)	12.33 (±11.30)	0.001*
Testosterone; Mean (SD)	2.82 (±0.66)	1.5 (±0.78)	1.44 (±0.59)	0.001*
Estradiol; Mean (SD)	291.62 (±243.11)	92.61 (±48.45)	336.56 (±576.35)	0.015*
Progesterone;	12.04 (±15.69)	1.21 (±0.47)	6.28 (±13.66)	0.020*

Mean (SD)				
SHBG; Mean (SD)	34.97 (±15.71)	51.94 (±16.7)	51.68 (±34.57)	0.005*

Table 1: Participants' characteristic, biochemical and hormonal data. One way ANOVA test was used to determine the difference between the groups. *P value<0.5 is significant

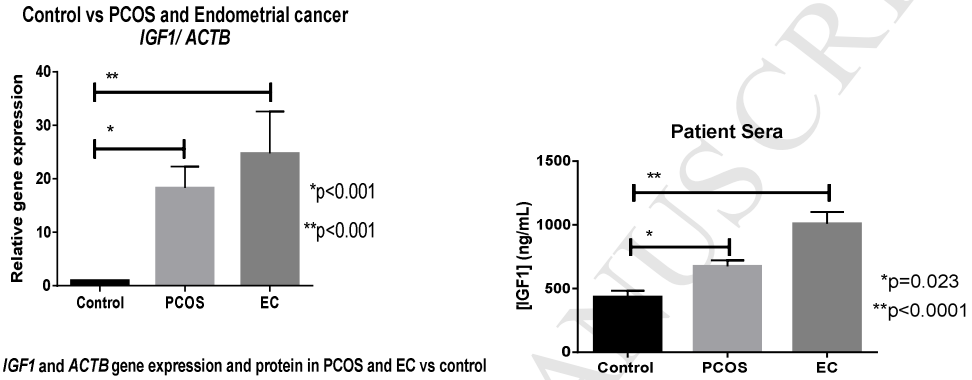

Figure 1: Relative *IGF1* and *ACTB* gene expression and protein in PCOS and EC vs control

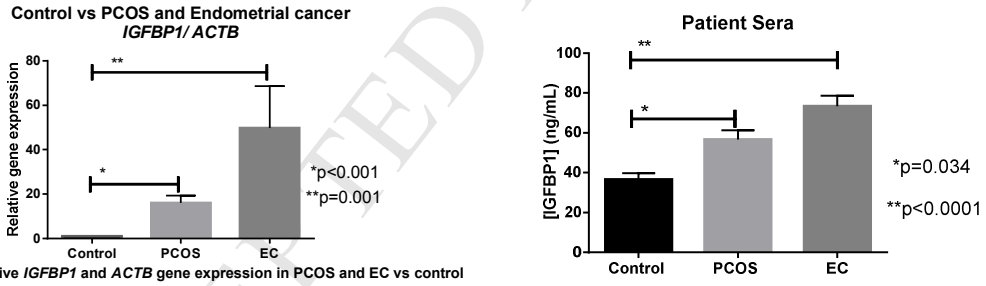

Figure 2: Relative *IGFBP1* and *ACTB* gene expression in PCOS and EC vs control

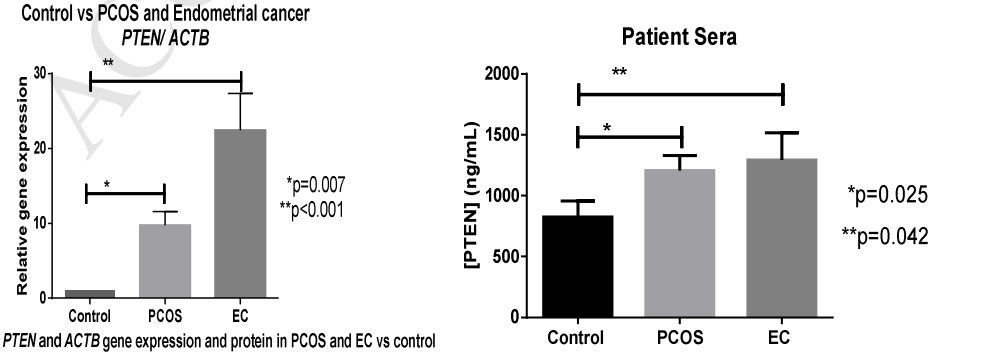

Figure 3: Relative *PTEN* and *ACTB* gene expression and protein in PCOS and EC vs control

Highlights

- Hyperinsulinaemia is postulated to be a major cause in the pathogenesis of both PCOS and EC
- The genes related to insulin signalling pathways (*IGF1*, *IGFBP1* and *PTEN*) were similarly up-regulated in both PCOS and EC