Cannabinoid receptor expression in estrogen-dependent and estrogen-independent endometrial cancer

by

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Abstract

The lack of good diagnostic/prognostic biomarkers and the often late presentation of endometrial cancer (EC), hinders the amelioration of the morbidity and mortality rates associated with this primarily estrogen-driven disease, a disease that is becoming more prevalent in the population. Previous studies on the expression of the classical cannabinoid receptors, CB1 and CB2, suggest these could provide good diagnostic/prognostic biomarkers for EC but those observations have been contradictory. In this study, we sought to resolve the inconsistency of CB1 and CB2 expression levels in different EC studies. To that end, we used qRT-PCR and immunohistochemistry (IHC) for CB1 and CB2 in endometrial biopsies from women with or without EC and found that transcript levels for both CB1 and CB2 were significantly decreased by 90% and 80% respectively in EC. These observations were supported by histomorphometric studies where CB1 and CB2 staining intensity was decreased in all types of EC. These data suggest that the loss of both types of CB receptors is potentially involved in the development of or progression of EC and that CB1 and CB2 receptor expression could serve as useful histological markers and therapeutic targets in the treatment of or prevention of EC.

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Introduction

The natural progression of endometrial cancer (EC) is complex and difficult to understand because in comparison to other estrogen-driven cancers (such as breast, colon and prostate cancer) it is relatively less-well studied. What is known is that the use of unopposed estrogen therapy for peri-menopausal symptoms [1], the presence of postmenopausal estrogen metabolites [2] or adjuvant therapy of breast cancer with tamoxifen (anti-estrogen in the breast, but a pro-estrogen in the uterus [3]), all increase the risk for EC. In these women, the type of EC that results is estrogen-dependent endometrioid (type 1) cancer [4], which tends to present early and has a good prognosis. On the other hand, estrogen-independent non-endometrioid (type 2) cancer has a delayed presentation and is an aggressive disease with a poorer prognosis [5]. The prognosis of EC based solely on hormone receptor status (as is routinely performed for breast cancer) is therefore inefficient [6], although one report suggests hormone receptor status may be of diagnostic and prognostic value [7]. The relative lack of robust serological and histological biomarkers for EC [8] when compared with those available for other tumours suggest novel markers would be of clinical value.

Recently, Guida and colleagues [9] examined the expression of the classical cannabinoid receptors CB1 (CNR1) and CB2 (CNR2) in EC and found that CB2 receptor expression was increased in EC whilst that of CB1 was unaffected, suggesting that CB2 could be used as an inmmunohistological marker for the disease. They also showed that the natural ligand for the CB2 receptor, 2-arachidonyolglycerol (2-AG) was also increased in the EC tissue and suggested that activation of the CB2 receptor by 2-AG may result in disease. Previously, we examined the expression of CB1 and CB2 through the normal menstrual cycle using immunohistochemistry and demonstrated that CB1 receptor expression was significantly decreased and CB2 expression significantly increased in the glandular epithelium in the estrogen-dominated mid to late proliferative phases of the cycle, suggesting estrogen could regulate the expression of both receptor isoforms. These data support the CB2 observations of Guida and colleagues [9], whose samples were primarily estrogen-dependent EC, but do not support their data for CB1. Furthermore, more recent studies [10] demonstrated that when activated by either CB1- or CB2-specific agonists the growth of Ishikawa (a model of human EC type 1) and Hec50co (a model of human EC type 2) cells were both inhibited through an apoptotic

mechanism, suggesting that a loss of either receptor could be an initiating event in both endometrioid and non-endometrioid cancer development. We also reported in a pilot study, that identified the perfect set of reference genes for the study of transcripts in qRT-PCR studies for EC [11], that transcript levels for CB1 (which we used as a test gene), were significantly decreased in EC, especially when 3 reference genes were used to normalise the data [11].

Although these observations support the notion that estrogen regulates CB1 receptor expression and partially contradict the findings of Guida and colleagues [9], we acknowledge that our sample size in that pilot study was small (n=3). Nevertheless, data from a pivotal microarray study by Risinger and colleagues [12] examining transcript levels in type 1 and type 2 EC (http://home.ccr.cancer.gov/risingerdata1102) showed that CB1 receptor expression was decreased in both types of EC, whilst CB2 transcripts were unaffected. These data support our CB1 findings, but not those of Guida and colleagues [9], and prompted us to re-examine CB1 and CB2 expression further in a larger cohort of type 1 and type 2 EC and in more detail.

Materials and methods

Patients and sample collection

Tissues (uterine) were obtained from women undergoing surgical treatment (hysterectomy and bilateral salpingoophorectomy) for endometrial cancer (EC group) or benign conditions, such as uterine prolapse (control group) at the University Hospitals of Leicester National Health Service Trust. All women gave signed written informed consent to take part in the study, which was approved and conducted according to the guidelines of the Leicestershire and Rutland Ethics Committee. The exclusion criteria were hormonal treatment [e.g. hormone replacement therapy or the levonorgestrel intrauterine system (Mirena® Coil)]; on prescription or recreational drugs; suffering from chronic medical conditions or any other form of cancer; being a smoker. The tissues obtained for the EC group were categorised according to the preliminary histology of endometrial biopsies obtained at standard hysteroscopy and then confirmed after hysterectomy. Final histopathological categorization into type and grade was made by the hospital Histopathology Department based on FIGO criteria. Surgery was performed within 2 weeks of the diagnosis.

In all cases, fresh uteri were immediately transported on ice to the Histopathology Department where endometrial biopsies from both normal and malignant tissues were dissected free by a consultant gynaecology histopathologist. All biopsies were divided into two pieces; one to be used for histological confirmation of diagnosis and immunohistochemistry (IHC) and one for the measurement of transcript levels. Both malignant and normal tissue biopsies were washed with phosphate buffered saline (PBS) to remove excess blood and immediately stored in either RNAlater® (Life Technologies, Paisley, UK) at -80°C for RNA extraction or in 10% formalin at 4°C for diagnosis and IHC. After routine fixing and embedding in paraffin wax, 4 μm sections were cut using a microtome, placed onto saline-coated slides and allowed to dry. After drying, representative sections were first subjected to haematoxylin and eosin (H & E) staining for histological confirmation of disease and additional sections stored for later IHC studies. Control tissues (normal human endometrial biopsies and spleen) from the histopathology archive were used for optimisation studies of CB1 and CB2 antibodies and as experimental controls.

RNA extraction and cDNA synthesis

Endometrial tissues biopsies (100 mg) were removed from the RNAlater and placed into lysis/binding buffer (1 ml lysis/binding buffer solution per 100 mg of tissues (miRNA Isolation Kit) and completely homogenised using a TissueRuptor (Qiagen Crawley, UK) at medium speed for 60 seconds on ice. Total cellular RNA was extracted using the mirVana™ miRNA isolation kit (Life Technologies, Paisley, UK) according to the manufacturer's protocol and quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Detroit, MI, USA). After standardising the RNA concentration to 10 μg/100 μl, genomic DNA was digested with a TURBO-DNAse (Life Technologies, Paisley, UK) at 37°C for 30 minutes, the DNAse inactivated with 10 μl of inactivation buffer and the solution centrifuged for 90 seconds at 10000 x g. Supernatants were subjected to first strand synthesis using the high capacity cDNA MultiScribe™ Reverse Transcriptase Kit ((Life Technologies, Paisley, UK) according to the manufacturer's protocol; incubation at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and then cooled to 4°C. The cDNA was stored at -20°C.

Quantitative Real-Time PCR

Quantitative Real-Time PCR experiments were performed as previously described [11] using the validated Taqman endogenous control reference genes MRPL19 (Hs00608519_m1), PPIA (Hs99999904_m1) and IPO8 (Hs00183533_m1) as VIC/TAMARA dye labelled primers and probes, purchased from Applied Biosystems (Life Technologies, Paisley, Scotland, UK). The TaqMan® genes expression assay human CNR1 (CB1: Hs00275634_m1) and CNR2 (CB2: Hs00275635_m1) primers and probes were purchased from Applied Biosystems, as FAM/MGB dye-labelled primers and probes. RT-minus and no template controls (NTC) containing DNAse-free water instead of template mRNA were included in each run. No product was synthesised in the NTC and RT-minus controls confirming the absence of contamination with exogenous DNA. All reactions were performed in the final volume reaction of 20 µl consisting of 2 µl of cDNA, 8 µl of DNAse-free water and 10 µl of TaqMan® universal PCR Master Mix. The plates were run on a StepOne Plus instrument (Applied Biosystems) and the thermal cycler profile used was: 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. All the reactions for the reference and test genes were performed in triplicate (both biological and technical).

Optimisation of antibodies

The CB1 (catalogue number C1108) and CB2 (catalogue number C1358) antibodies were both purchased from Sigma Life Science (Poole, Dorset, UK) and used within standard immunohistochemistry protocols as described [13, 14]. Cross-over studies, pre-incubation of the respective antibodies with authentic proteins expressed in CHO cells (Bakali et al., 2013) and comparison of staining with other commercially available antibodies indicated antigen-antibody specificity (data not shown). The optimal antibody dilutions were determined using normal late proliferative phase endometrium (for CB1) and normal human spleen (for CB2) control tissues [14, 15] and were found to be 1 in 500 for CB1 (Supplemental Figure 1) and 1 in 150 for CB2 (Supplemental Figure 2). An additional test for antibody specificity was determined using equivalent concentrations of non-immune rabbit immunoglobulin G (IgG; Bio-Rad, Hemel Hempstead, Hertfordshire, UK). The lack of any 3,3'–diaminobenzidine (DAB) staining in the IgG (negative) controls indicates that the antibodies are specific for CB1 (Supplemental Figure 1) and CB2 (Supplemental Figure 2).

Identification, localisation and histomorphometric analysis of CB1 and CB2 protein expression

Immunolocalisation was performed using the optimal antibody dilutions (1 in 500 for CB1 and 1 in 150 for CB2). A positive control (late proliferative phase endometrium incubated with either CB1 or CB2 antibodies) and a negative control (late proliferative phase endometrium incubated with rabbit IgG diluted to the same concentrations as the primary antibody) were included in each experiment with each experiment containing all samples to prevent inter-assay variability.

Image capture and histomorphometric analyses were performed as described [14, 16]. Briefly, photomicroscopy images were taken on an Axioplan transmission microscope (Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK) equipped with a Sony DXC-151P analogue camera (Sony Inc., Tokyo, Japan) connected to a computer, running Axiovision image capture and processing software (Axiovision version 4.4; Carl Zeiss Ltd.). All images were captured at 200x magnification and analysed using image analysis software (ImageScope version 10.2.2.2319; Aperio Technologies, Inc., Vista, CA, USA) as previously described [14]. Immunoreactivity (unbiased histoscore, H-score) was assessed semi-quantitatively by assigning scores as 0 (no staining), 100 (weak staining), 200 (moderately stained) and 300 (strong staining) as determined by the software algorithm. The H-score values for the glands (G) and stroma (S) were determined independently and then combined to provide an overall H-score for the entire tissue (G+S).

Statistical analysis

Statistical analysis of the data was performed using Prism version 7.00 for Windows (GraphPad Software, San Diego CA, USA, www.graphpad.com). Data that did not follow a Gaussian distribution (transcript measurements) were expressed as medians and inter-quartile range (IQR) and comparison between groups performed using one-way analysis of variance (ANOVA) followed by the appropriate post hoc analysis. Conversely, data that were consistent with a normal distribution (histomorphometric) were analysed by the parametric one–way ANOVA with Dunnett's post hoc analysis. In all cases a $p<0.05$ was considered to be significant. Relationships between the transcript and protein data were performed using Pearson correlation analyses.

Results

CB1 Expression Type 1 and Type 2 Endometrial Cancer

To investigate CB1 receptor transcript levels in endometrial tissues, quantitative RT-PCR was used (Figure 1). CB1 transcript levels decreased significantly from 2.048 (0.984 - 7.234); [median (IQR)]) in atrophic endometrial samples to 0.093 (0.051 - 1.056) in type 1 EC (n=15) and 0.069 (0.034 – 0.354)] in type 2 EC (n=6) (Figure 1A).

To investigate CB1 protein levels in endometrial tissues immunohistochemistry was used and showed that the staining pattern for immunoreactivity CB1 antibodies was complex and diverse in the different tissue types (Supplemental Figure 3). The rabbit IgG control (Supplemental Figure 3A) was devoid of any brown staining, whilst that of the late proliferative phase sample (Supplemental Figure 3B) showed moderate staining of the glands and scant staining of the stroma, indicating antibody specificity. In atrophic tissue (Supplemental Figure 3C), immunoreactive CB1 protein was expressed very strongly in the glands and concentrated more towards the apical region. In grade 1, grade 2 and grade 3 EC (Supplemental Figure 3D, 3E and 3F, respectively) CB1 immunoreactivity was also confined to glandular epithelial cells and was at a lower intensity when compared to that of the control. The CB1 staining intensity in serous carcinoma (Supplemental Figure 3G) and in carcinosarcoma (Supplemental Figure 3H) was low and very low, respectively when compared to the staining in the atrophic endometrium (control) and involved both the glands and the stroma. Histomorphometric quantification of immunoreactive CB1 protein indicated a variable level of staining in the various tissues, with the levels significantly decreased in the malignant tissue compared to the control tissue in all cases (Supplemental Figure 3I).

Further examination of the histomorphometric analyses for the entire tissue (glands and stroma (G+S) combined) and with the data grouped into type 1 or type 2 EC (Figure 1B), showed a significant reduction in CB1 immunoreactivity in the type 1 EC, but not in type 2 EC. Separation of the gland and stroma data revealed the reason why. The staining in the glands was significantly lower in both type 1 and type 2 EC (Figure 1C), but not affected in the stroma of type 1 EC, whilst significantly elevated in type 2 (Figure 1D).

When all the data were combined to examine the effect of EC on CB1 expression (Figure 2), the data showed that CB1 transcript levels were significantly ($p=0.0004$) lower (~90%) in the EC ($n=21$) tissue compared to that of atrophic ($n=6$) tissues (Figure 2A). Furthermore, immunoreactive CB1 protein levels (Figure 2B) were also significantly decreased from an H-score of 291.0 ± 5.67 (mean ± SEM) in the atrophic endometrium to 191.3 ± 13.79 in the EC tissue. Spearman correlation (Figure 2C) revealed that the expression of total CB1 protein (G + S) and transcripts were significantly related with a Pearson r-coefficient of 0.3826 ($p=0.0489$).

CB2 Expression in Type 1 and Type 2 Endometrial Cancer

To investigate CB2 receptor transcript levels in endometrial tissues, quantitative RT-PCR was used. CB2 transcript levels significantly decreased from 1.079 (0.834 - 1.364) [median (IQR)] in atrophic endometrial samples to 0.180 (0.081 - 0.583) in type 1 EC ($n=15$) and 0.055 (0.022 - 0.149) in type 2 EC ($n=6$) (Figure 1E).

To investigate CB2 protein levels in endometrial tissues immunohistochemistry was used and showed that the staining patterns for immunoreactive CB2 antibodies was less complex than that for CB1 antibodies (Supplemental Figure 4) and that it was clearly decreased in all grades and types of EC (Supplemental Figure 4I). The rabbit IgG control (Supplemental Figure 4A) was devoid of any brown staining, whilst that of the late proliferative phase sample (Supplemental Figure 4B) showed intense staining of the glands and moderate staining of the stroma, demonstrating the specificity of the antibodies. In atrophic tissue (Supplemental Figure 4C), immunoreactive CB2 protein was expressed in the glands with concentrated staining along the apical region with decreased but visible staining in the stroma when compared with the late proliferative phase endometrial sample. The CB2 immunoreactivity gradually decreased in intensity with more progressive disease: grade 1 > grade 2 > grade 3 EC samples (Supplemental Figure 4D, 4E and 4F, respectively). CB2 immunoreactivity was similarly very low in serous carcinoma (Supplemental Figure 4G) and was of low intensity in carcinosarcoma (Supplemental Figure 4H) and was very low when compared to that of the atrophic endometrium and was essentially absent from both the glands and the stroma. Histomorphometric

quantification of immunoreactive CB2 protein indicated a significantly lower amount in the malignant tissue in all cases compared to the control tissue (Supplemental Figure 4I).

Further examination of the histomorphometric analyses for the entire tissue (glands and stroma (G+S) combined) with the data grouped into type 1 or type 2 EC (Figure 1F), showed a significant reduction in CB2 immunoreactivity in both type 1 EC and type 2 EC. Separation of the gland and stroma data revealed that the staining in the glands was significantly lower in the glands of both type 1 and type 2 EC (Figure 1G) and also in the stroma of type 1 EC and type 2 EC (Figure 1H).

When all the data were combined to examine the effect of EC on CB2 expression (Figure 2D), the data showed that CB2 transcript levels were significantly ($p<0.0001$) lower (by ~80%) in EC (n=21) tissue when compared to that of atrophic (n=6) tissue and that immunoreactive CB2 protein levels (Figure 2E) were also significantly decreased from an H-score of 264.3 ± 3.72 (mean ± SEM) in the atrophic endometrium to 30.80 ± 2.81 in the EC tissue. Spearman correlation (Figure 2F) revealed that the expression of CB2 protein and transcripts were closely related with a Pearson correlation coefficient of 0.7632 ($p<0.0001$).

Discussion

Our findings suggest that the expression levels of the classical cannabinoid receptors, CB1 and CB2, are altered in both estrogen-dependent and the estrogen-independent endometrial cancers. The finding of a good correlation between transcript and protein expression levels (Figure 2) makes our observations more robust and valid than preceding studies [9]. These data are, however, at variance with the immunohistochemical findings of Guida and colleagues [9] and the microarray studies of Risinger and colleagues [12]. The reason for this is not apparent, but could be related to methodological differences or differences in sample size; the number of tumour samples we examined herein are similar to [9] or exceed previous studies [11,12] suggesting that while sample size is relatively small, it would be reasonable to draw conclusions from the resulting data which are contrary to both previous studies [9,12] and our pilot study [11], where only one technique was used, whilst here we used two complementary techniques to examine the usefulness, or otherwise, of the data generated. Since the data confirm not only our previous observation [11] and that of

Risinger and colleagues [12] at the transcript level, we believe that these observations are accurate and that CB1 expression is decreased in EC. There is, however, a case for stating here, that more studies would most certainly enhance the conclusions arising from this study.

The lack of effect on CB1 expression in the study by Guida and colleagues is difficult to explain, but our observation that CB1 expression is elevated in the stroma of grade 3, type 1 EC and in the stroma of carcinosracoma tissues points to potential tissue and cellularity issues in immunohistochemical studies related to how the biopsies were collected and processed. We ensured that any apparently 'normal' tissue was excluded from the tissue that was used for both the qRT-PCR and immunohistochemical studies, thus ensuring that only malignant tissue was examined, whereas Guida and colleagues stated that their malignancy biopsies were 'contaminated with normal tissue'. This observation supports our conclusion that only malignant tissue should be used in studies such as those presented herein.

An alternative explanation may arise from patient selection; patients described herein had different types and grades of EC, whereas the patients in the Italian cohort were from a more confined group, mainly type 1 (endometrioid) grade 1 and 2. It is also known that ethnicity can play a role in the production and levels of plasma anandamide (the natural ligand for CB1), but that is unlikely that ethnicity is responsible for the discrepancy in CB1 expression, because the patients in the Italian study were all Caucasian, whilst 95% of our patients were also Caucasian. A difference in EC incidence and mortality due to geographical locale has been reported [17] suggesting a lifestyle or clinical access effect might be responsible for the discrepancy that we have observed.

In our studies, several optimisation steps were performed to ensure that correct controls were used. These are essential, since it is known that antibodies prepared for endocannabinoid research can be notoriously difficult to use and may detect unexpected antigens [18]. For example, some types of CB1 receptor antibodies are reported to co-react with stomatin-like protein 2 in the mouse brain [19]. The fact that we first optimised the antibody concentration, performed cross-over studies with authentic proteins expressed in CHO cells and also used non-immune rabbit IgG in excess and found no non-specific binding issues favours the likely robustness

231 of our data. Furthermore, staining of normal menstrual cycle samples were performed (see Figures 2 and 4)
232 and compared favourably with previously published data [14], where antibodies from several commercial
233 sources were compared 'side-by-side' indicating that the antibodies used herein are indeed CB1-receptor
234 specific. Only once those important control steps had been completed did we perform immunohistochemistry
235 of the study slides.

236 Guida et al., previously demonstrated that CB2 protein increased in EC, whilst Risinger et al. demonstrated
237 that it was unchanged. Since we had not previously examined this, we developed our own strategy for
238 analysis. Using previously validated and robust Taqman primers and probes [11] we demonstrated that
239 transcripts were decreased in both types of EC, supporting the observations of Risinger et al.
240 (http://home.ccr.cancer.gov/risingerdata1102) but not that of Guida et al. [9]. Using CB2 antibodies that had
241 previously been validated by others [20, 21, 22] and after confirmation with established menstrual cycle
242 staining patterns [14], we showed that immunoreactive CB2 protein is decreased in both type 1 and type 2 EC.
243 The reason why these data differ from those of Guida et al., is unclear, but could also be due to the source of
244 antibody, since there are reports that not all CB2 receptor antibodies are receptor-specific [18, 19].
245 Nevertheless, we ensured that the antibodies that we used were receptor-specific using known test tissues
246 (spleen and normal endometrium) and appropriate and additional controls (as described above). The
247 significant correlations between transcript and protein expression in the atrophic and EC samples (Figure 2),
248 provide extra support that the observations presented herein are robust and that there is a close-fitting link
249 between CB1/CB2 transcription and translation in the endometrium.

250 A reduction of CB1 and CB2 expression at both the transcript and protein level seemed to be linked to the
251 severity of the clinico-histopathological diagnosis (as determined by H&E diagnosis and clinical
252 presentation), which from a clinical viewpoint may be crucial for patient management. In accordance with the
253 present findings, CB receptor expression has been shown to be reduced in other estrogen-driven cancers, such
254 as colorectal cancer, suggesting that activated CB receptors may prevent some forms of tumour cell growth,
255 through cell cycle arrest, inhibition of cancer neo-angiogenesis, inhibition of metastasis and induction of
256 transformed cell death [23]. Moreover, in patients with colorectal cancer, endocannabinoids and synthetic

cannabinoids inhibited carcinogenesis and induced apoptosis by mechanisms involving both CB receptors. Furthermore, similar results have been documented in other cancers [24] suggesting that CB receptor expression in some estrogen-induced cancers may be protective, and conversely this protection is lost when the receptor is no longer expressed, as has been shown herein. Conversely, CB receptor expression is increased in human oral squamous cell, prostatic and thyroid carcinoma [25, 26, 27, 28], suggesting that different malignant tissues have either a different CB receptor requirement for survival or these tissues have a requirement for a missing co-factor involved in cannabinoid receptor signalling. Nevertheless, the loss of CB1 and CB2 expression has recently been demonstrated to provide a mechanism for endometrial cancer cell survival, supporting the current data [10]; loss of cannabinoid receptor in the endometrium may cause or promote endometrial cancer.

The variability of cannabinoid effects in different tumour models is highly contradictory, which may be a consequence of differential cannabinoid receptor expression or variable experimental technique. It has been hypothesised that cannabinoids are more effective in killing tumours that abundantly express cannabinoid receptors, such as gliomas, but may increase the growth and metastasis or at least inhibit cytotoxicity in other types of tumours, such as breast cancer, that show no or low expression of cannabinoid receptors, with the mechanism possibly being through suppression of an anti-tumour immune response. Several malignancies, such as non-Hodgkin lymphoma [29] and prostate and pancreatic cancer [30], have been shown to have higher CB1 expression that is associated with increased disease severity and poor prognosis, suggesting that CB receptor expression and tumour response to endocannabinoids are likely to be tumour-specific, whereas low cannabinoid receptor expression is associated with increased apoptosis in astrocytoma, whilst no apoptosis occurs in astrocytoma cells with high receptor expression [31]. Additional support for this idea that disease outcome and receptor status may be tumour-specific comes from mouse models, where CB1 knockout, but not CB2 knockout APCmin mice (a model of colorectal cancer), have an increase in the number of intestinal polyps that can be replicated by treatment with CB1 antagonists [32], and also from the *in vitro* studies of Fonseca *et al*. [10] who showed that activation of the CB1 or CB2 receptor in human EC models induces cell death. These data suggest that in the absence of either receptor (as has been demonstrated here *in vivo*), human EC cells are protected from the effects of programmed cell death and can continue to proliferate.

In conclusion, reduced CB1 and CB2 receptor expression at both the transcript and protein level were reduced in both endometrioid and non-endometrioid endometrial carcinoma providing evidence that activated CB receptors may interfere with or protect against endometrial carcinogenesis and may become useful therapeutic targets in the future.

Declaration of conflicts of interest

The authors declare no conflicts of interest related to this work.

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Author contribution

TA designed, performed, analysed the research and wrote the first draft of the manuscript. AHT participated in data analysis and helped to re-draft the manuscript. JCK helped in the preparation of the manuscript, is the chief investigator and the main supervisor of the research.

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Figure Legends

Figure 1. Comparison of CB1 and CB2 expression at the transcript and protein level in type 1 and type 2 endometrial cancer. The levels of CB1 transcript (panel A) and CB2 transcript (panel E) relative to the geometric mean of 3 reference genes in control (atrophic) endometrial tissue are shown together that found in type 1 (endometrioid) and type 2 (non-endometroid) cancer. Data are presented as [median (IQR)] and range. P values were obtained using Kruskal-Wallis ANOVA with Dunn's ad hoc post-test analysis. The H-score values for CB1 staining in the glands and stroma (G+S; panel B) in both types of EC are shown together with H-scores for the glands alone (G; panel C) and stroma alone (S; panel D). Similar data for CB2 are shown in panels, F, G and H, respectively). Data are presented as mean ± SEM; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 one way ANOVA with Dunnett's ad hoc post-test analysis compared to the atrophic control. The numbers of samples used are presented in Table 1.

Figure 2. Comparison of CB1 and CB2 expression at the transcript and protein level in endometrial cancer. The levels of CB1 transcript (panel A) and CB2 transcript (panel E) relative to the geometric mean of 3 reference genes in control (atrophic) endometrial tissue are shown together that found in type 1 (endometrioid) and type 2 (non-endometroid) cancer combined (EC). Data are presented as [median (IQR)] and range; ***p<0.001 Mann-Whitey U-test. The H-score values for CB1 staining in the glands and stroma (G+S; panel B) EC are shown together with H-scores for control (atrophic) tissues (panel B); data are presented as mean ± SEM; **p<0.01; ***p<0.001; one way ANOVA with Dunnett's ad hoc post-test analysis. Spearman correlation between these two measurements is shown in panel C. Corresponding data for CB2 expression are shown in panels, D, E and F, respectively). The numbers of samples used in the different experiments are recorded in Table 1.

Legends for Supplemental files

Supplemental Figure 1. Optimisation and specificity of the CB1 antibodies. The images in panel A show the effect of CB1 antibody dilution on the staining patterns of late-proliferative phase endometria. The different dilution factors are shown below representative images. G=glands, S-stroma. The specificity of antibody binding is shown in panel B where the optimal antibody dilution (1 in 500) was compared with that of non-immune rabbit IgG (Rabbit IgG) used at the same dilution. The data are representative of 3 separate experiments.

Supplemental Figure 2. Optimisation and specificity of the CB2 antibodies. The images in panel A show the effect of CB2 antibody dilution on the staining patterns of late-proliferative phase endometria. The different dilution factors are shown below representative images. G=glands, S=stroma. The specificity of antibody binding is shown in panel B where the optimal antibody dilution (1 in 150) was compared with that of non-immune rabbit IgG (Rabbit IgG) used at the same dilution. In this case, the test tissue used was human spleen. The data are representative of 3 separate experiments.

Supplemental Figure 3. The staining of endometrial tissue with CB1 antibodies. The images A, B and C represent controls for CB1 immunostaining. Non-immune rabbit IgG (Rabbit IgG) was used at 1 in 500 dilution as was the CB1 antibody (all other images). The late proliferative phase endometrial sample (B) was used as a positive control for the assay, and the atrophic (C) as a control for the EC samples. The staining of different grades of Type 1 EC are shown in the middle section of the figure (D to F) whilst that of type 2 EC (serous G and carcinosarcoma H) are in the lower section of the figure. H-score values are shown in panel I, with A=atrophic, G1=grade 1, G2=grade 2, G3=grade 3, S=serous carcinoma and C=carcinosarcoma. The H-score data in panel I are presented as mean ± SEM; *$p<0.05$; **$p<0.01$; ****$p<0.0001$ compared to the atrophic control; ANOVA with Dunnett's ad hoc post-test analysis. The numbers of samples used are recorded in Table 1.

Supplemental Figure 4. The staining of endometrial tissue with CB2 antibodies. The images A, B and C represent controls for CB2 immunostaining. Non-immune rabbit IgG (Rabbit IgG) was used at 1 in 150 dilution as was the CB2 antibody (all other images). The late proliferative phase endometrial sample (B) was

480 used as a positive control for the assay, and the atrophic (C) as a control for the EC samples. The staining of
481 different grades of Type 1 EC are shown in the middle section of the figure (D to F) whilst that of type 2 EC
482 (serous G and carcinosarcoma H) are in the lower section of the figure. H-score values are shown in panel I,
483 with A=atrophic, G1=grade 1, G2=grade 2, G3=grade 3, S=serous carcinoma and C=carcinosarcoma. The H-
484 score data in panel I are presented as mean ± SEM; ****$p<0.0001$ compared to the atrophic control; ANOVA
485 with Dunnett's ad hoc post-test analysis. The numbers of samples used are recorded in Table 1.