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1 **The impact of 27-hydroxycholesterol on endometrial cancer proliferation**

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3 *Running title – 27-hydroxycholesterol in Endometrial Cancer*

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24 **Abstract**

25 Endometrial cancer (EC) is the most common gynaecological malignancy. Obesity is a major
26 risk factor for EC and is associated with elevated cholesterol. 27-Hydroxycholesterol (27HC)
27 is a cholesterol metabolite that functions as an endogenous agonist for Liver X Receptor
28 (LXR) and a selective estrogen receptor modulator (SERM). Exposure to estrogenic ligands
29 increases risk of developing EC however the impact of 27HC on EC is unknown.

30 Samples of stage 1 EC (n=126) were collected from post-menopausal women undergoing
31 hysterectomy. Expression of LXRs (*NR1H3*, LXRα; *NR1H2*, LXRβ) and enzymes required
32 for the synthesis (*CYP27A1*) or breakdown (*CYP7B1*) of 27HC were detected in all grades of
33 EC. Cell lines originating from well-, moderate- and poorly-differentiated endometrial cancers
34 (Ishikawa, RL95, MFE 280 respectively) were used to assess the impact of 27HC or the LXR
35 agonist GW3965 on proliferation or expression of a luciferase reporter gene under the
36 control of LXR- or ER-dependent promoters (LXRE, ERE). Incubation with 27HC or GW3965
37 increased transcription via LXRE in Ishikawa, RL95 and MFE 280 cells ($p<0.01$). 27HC
38 selectively activated ER-dependent transcription ($p<0.001$) in Ishikawa cells and promoted
39 proliferation of both Ishikawa and RL95 cells ($p<0.001$). In MFE 280 cells 27HC did not alter
40 proliferation but selective targeting of LXR with GW3965 significantly reduced cell
41 proliferation ($p<0.0001$).

42 These novel results suggest that 27HC can contribute to risk of EC by promoting
43 proliferation of endometrial cancer epithelial cells and highlight LXR as a potential
44 therapeutic target in the treatment of advanced disease.

45 **Introduction**

46 Endometrial cancer (EC) is the most common gynaecological malignancy and the fourth
47 most common cancer in women in developed countries with incidence increasing in line with
48 rising rates of obesity (reviewed in (Onstad, et al. 2016)). Obesity is a major modifiable risk
49 factor for EC and is thought to contribute to increased risk of malignancy in part due to
50 increased exposure to estrogens which enhance risk of aberrant proliferation within the
51 endometrium (Sanderson, et al. 2017). Obesity is also is associated with an adverse
52 metabolic profile which is postulated to independently increase risk of EC (Trabert, et al.
53 2015).

54 A recent meta-analysis supported a positive association between dietary cholesterol
55 consumption and endometrial cancer risk (Gong, et al. 2016). Notably, obesity also puts
56 individuals at risk of developing an adverse, raised, cholesterol profile. Cholesterol
57 metabolites such as the oxysterol 27-hydroxycholesterol (27HC) have been demonstrated to
58 promote cancer growth and metastasis in studies on breast cancer (Nelson, et al. 2013; Wu,
59 et al. 2013a); providing a plausible mechanistic link between increased adiposity and EC
60 risk. 27HC is a primary metabolite of cholesterol, synthesised by the action of sterol 27-
61 hydroxylase (CYP27A1) and metabolised by 25-hydroxycholesterol 7-α-hydroxylase
62 (CYP7B1; [1]). 27HC acts as an endogenous agonist for the Liver X receptor (LXR), a ligand
63 activated transcription factor involved in the regulation of cholesterol homeostasis. Two
64 isoforms of LXR have been identified; LXRα (encoded by *NR1H3)* which is predominantly
65 expressed in the liver, kidney and small intestine but exhibits low expression in other tissues,
66 and LXRβ (encoded by *NR1H2)* which is ubiquitously expressed. Based largely on studies in
67 breast cancer, LXRs have been proposed as a novel anti-cancer target and the LXR-
68 selective agonists GW3965 and T0901317 are reported to decrease proliferation of LXR-
69 expressing breast cancer cell lines (MCF7, T47D, MDA-MB231) as well as the prostate
70 cancer cell line LNCaP (Kim, et al. 2010; Vedin, et al. 2009). To the best of our knowledge

71 LXR expression has not been reported in human EC tissues and the impact of either 27HC
72 or LXR agonists on the endometrium or endometrial malignancies is not known.

73 In addition to activating LXRs, 27HC can also bind estrogen receptors (ER)
74 (Umetani, et al. 2007) and acts as an endogenous selective estrogen receptor modulator
75 (SERM) (DuSell, et al. 2008). 27HC has diverse impacts and its SERM activity is reported to
76 be both tissue-selective and context-dependent. For example, 27HC acts as a competitive
77 antagonist of ERs expressed in the vasculature and can antagonise E2-mediated endothelial
78 cell migration and re-endothelialisation (Umetani et al. 2007). In contrast, in the absence of
79 E2, 27HC is reported to act as an agonist to ERα (ESR1) to increase cell adhesion and
80 expression of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFA) and
81 interleukin 6 (IL6) (Umetani, et al. 2014) by endothelial cells and macrophages. Notably,
82 27HC is also reported to increase proliferation of ERα-positive breast cancer cell lines and
83 promotes MCF7 tumour xenograft growth in mice by stimulating ER-dependent cell
84 proliferation (Wu et al. 2013a). Given selective LXR agonists have anti-proliferative effects
85 (Vedin et al. 2009), these studies suggest that proliferative effects of 27HC may be mediated
86 via ER and that relative expression of LXR or ER isoforms may define the impact of the
87 ligand.

88 ER isoforms are expressed in EC tissues and ER expression changes with disease
89 progression (Collins, et al. 2009). We have previously reported that ERα is readily detectable
90 in both epithelial and stromal cells in well-differentiated cancers but is significantly reduced in
91 poorly-differentiated cancers. In contrast, expression of ESR2 variants (ERβ1, 2, 5) was
92 readily detected in well, moderate and poorly differentiated stage 1 ECs (Collins et al. 2009).
93 We therefore postulated that 27HC might have distinct effects in EC depending on the
94 bioavailability of ER isoforms present at different stages of disease progression.

95 Obesity and the metabolic syndrome are both associated with an increased risk of
96 developing pre-malignant and malignant endometrial disease (Sanderson et al. 2017) but
97 whether the cholesterol metabolite 27HC has an impact on endometrial cancer
98 risk/progression is not known. In the current study we assessed the expression of the

99 enzymes required for synthesis (CYP27A1) and breakdown (CYP7B1) of 27HC and
100 assessed expression of the cognate receptors LXRα and LXRβ in primary human stage I
101 endometrial adenocarcinomas (n=126) and postmenopausal endometrial controls (n=9). The
102 impact of 27HC and the LXR-selective agonist GW3965 on ERE- and LXRE-dependent
103 expression of a reporter gene, as well as cellular proliferation, was assessed in three
104 endometrial cancer cell lines which phenocopy well-, moderately- and poorly-differentiated
105 stage I endometrial cancers. Our novel findings demonstrate that 27HC can alter responses
106 in endometrial cancer cells and highlight LXR as a potential therapeutic target. Taken
107 together our findings suggest increased exposure to 27HC may increase risk of development
108 and progression of endometrial cancer.

109 **Materials and methods**

110 *Human tissue samples*

111 Endometrial adenocarcinoma tissue was collected from post-menopausal women
112 undergoing total abdominal hysterectomy who had been previously diagnosed to have
113 endometrioid adenocarcinoma of the endometrium; they had received no treatment before
114 surgery (supplementary table 1). Written informed consent was obtained from all subjects
115 prior to surgery, and ethical approval was granted by the Lothian Research Ethics
116 Committee (LREC1999/6/4). Methods were carried out in accordance with NHS Lothian
117 Tissue Governance guidelines. All endometrial cancers were confined to the uterus
118 (International Federation of Obstetrics and Gynaecology, FIGO, stage 1 as described in
119 (Collins et al. 2009)). Diagnosis of adenocarcinoma was confirmed histologically by an
120 experienced gynaecological pathologist and tissues were further graded as well
121 differentiated (G1), moderately differentiated (G2) or poorly differentiated (G3). Samples
122 were anonymised and patient follow-up information was not available. However, survival
123 statistics for stage 1 EC in the UK are reported as 99% 1-year survival and 95.3% 5-year
124 survival (Cancer Research UK; http://www.cancerresearchuk.org/health-professional/cancer-
125 statistics/statistics-by-cancer-type/uterine-cancer/ accessed November 2017) and
126 Information Services Division Scotland figures, which cover the stage 1 EC samples
127 collected in the current study, report 92.9% 1-year survival and 83.2% 5-year survival for all
128 uterine cancers (http://www.isdscotland.org/Health-Topics/Cancer/Cancer-Statistics/Female-
129 Genital-Organ/#uterus accessed November 2017).

130 Postmenopausal controls (n=9) were obtained from women undergoing surgery for non-
131 malignant gynaecological conditions. None of the women were receiving hormonal therapy.
132 A total of 126 EC tissue samples were analysed; 3 samples per grade were assessed for
133 immunohistochemistry and n=30 well differentiated cancers, n=64 moderately differentiated
134 and n=32 poorly differentiated samples were assessed for qPCR studies. A minimum of 10

135 samples at each grade were analysed for each gene, detailed sample numbers are included
136 in supplementary table 2. Tissue for immunohistochemistry was collected in neutral buffered
137 formalin (NBF), RNA extraction samples were collected in RNALater (Qiagen, UK).

138 *Measurement of mRNA*

139 Isolation of mRNAs, preparation of cDNAs, and analysis by qPCR was performed according
140 to standard protocols (Bombail, et al. 2010); samples were quantified by relative standard
141 curve method or by the comparative ΔΔCt method with *CYC* as internal control.
142 Primers/probes are given in supplementary table 3.

143 *Immunohistochemistry*

144 Single antibody immunohistochemistry using 3,3'-diaminobenzidine tetra-hydrochloride
145 (DAB) detection was performed as described previously (Collins et al. 2009). Double
146 immunofluorescence was carried out with antibodies directed against LXR or ERα and the
147 proliferation marker Ki67. Details of antibodies and dilutions are provided in supplementary
148 table 4. Primary antibodies were incubated at 4℃ overnight. Antigen detection was
149 performed using Tyramide signal amplification (Perkin Elmer) system followed by nuclear
150 counterstaining with DAPI (4', 6-Diamidino-2-phenyl-indole dihydrochloride). Negative
151 controls were incubated in the absence of primary antibody but otherwise processed as
152 above; no staining was detected in no primary controls for any of the antibodies used (not
153 shown). Images were captured using a LSM 710 Confocal microscope (Zeiss) at x40
154 magnification.

155 *Cell Cultures*

156 Three endometrial adenocarcinoma cell lines representative of well-, moderately- or poorly-
157 differentiated cancers were used. Ishikawa cells were obtained from the European Collection
158 of Cell Culture (ECACC no 99040201, Wiltshire, UK). This cell line was originally derived
159 from a well-differentiated adenocarcinoma of a 39 year-old woman (Nishida, et al. 1985) and

160 reported to express both ERα and ERβ protein (Johnson, et al. 2007). RL95-2 cells (ATCC® 161 CRL-1671™; hereafter RL95) were originally derived from a Grade 2 moderately 162 differentiated endometrial adenocarcinoma (Way, et al. 1983) and reported to express both 163 ERα and ERβ protein (Li, et al. 2014; Yang, et al. 2008). MFE-280 (ECACC no 98050131) 164 were derived from a recurrent, poorly differentiated, endometrial adenocarcinoma and have 165 low/undetectable expression of ERα and ERβ. Cells were maintained in DMEM/F12 (Sigma) 166 supplemented with 10% FBS, 100U penicillin, streptomycin and 0.25 µg/ml fungizone 167 (Invitrogen, Paisley, UK) at 37℃ in 5% CO_2. Media for RL95 was supplemented with 168 0.005mg/ml Insulin (Sigma). Cells were incubated with 27-hydroxycholesterol (27HC; Tocris 169 Cat. No. 3907) using stocks diluted in ethanol to give final concentrations ranging from 10^{-5}M 170 to 10^{-8}M or GW 3965 hydrochloride (GW; Tocris Cat. No. 2474) using stocks diluted in 171 DMSO to give final concentrations ranging from 10^{-5}M to 10^{-8}M. Some cultures were co-172 incubated with the anti-estrogen fulvestrant (ICI 182,780; Tocris Cat. No. 1047) diluted in 173 DMSO at a final concentration of 10^{-6}M. Appropriate vehicle control incubations were 174 included in all studies. All cell lines were authenticated using the Promega PowerPlex 21 175 system (Eurofins Genomics, Ebersberg, Germany).

176 *Reporter assays*

177 An adenoviral vector containing a 3xERE-tk-luciferase reporter gene was prepared as 178 described previously (Collins et al. 2009). Cells were cultured in DMEM without phenol red 179 and containing charcoal stripped foetal calf serum (CSFCS) for 24 hours before being 180 infected with Ad-ERE-Luc at a MOI of 25. Activation of LXR-dependent signal transduction 181 was assessed according to manufacturer's instructions using reagents from the Cignal LXR 182 Reporter Kit which includes positive and negative controls as well as a luciferase reporter 183 gene under the control of tandem repeats of the LXR transcriptional response element 184 (LXRE) (Qiagen, CCS-0041L).

185 Cells were treated for 24 hours and luciferase activities were determined using 'Bright Glo'
186 reagents (Promega). Luminescence was measured using Fluostar Microplate Reader (BMG
187 labtech) and fold change in luciferase activity calculated relative to vehicle control for each
188 treatment.

189 *Proliferation assays*

190 The impact of treatments on cell proliferation was assessed using CyQUANT® Direct Cell
191 Proliferation Assay (Thermo Fisher, C35011) according to manufacturer's instructions and
192 nuclear fluorescence measured using Novostar Microplate Reader (BMG labtech). For each
193 cell line investigated, cell number was quantified using a standard curve of known cell
194 numbers and fold change in cell number calculated relative to vehicle control for each
195 treatment.

196 *Statistical analysis*

197 Statistical analysis was performed using Graphpad prism. One-way ANOVA was used to
198 determine significance between treatments in data that were normally distributed. Non-
199 parametric testing was utilised where sample sizes were insufficient to confirm normality of
200 data distribution; Kruskal-Wallis test was used to assess differences between treatments.
201 Where data were analysed as fold-change significance was tested using one sample t test
202 and a theoretical mean of 1. Criterion for significance was $p<0.05$. All data are presented as
203 mean ± SEM.

204 **Results**

205 *Enzymes that regulate bioavailability of 27-hydroxycholesterol and its cognate receptor LXR*
206 *are expressed in endometrial cancer.*

207 Messenger RNAs encoded by *CYP7B1* and *CYP27A1* were detected in all cancer grades
208 (Figure 1A and B); expression of *CYP7B1* was significantly lower in poorly-differentiated
209 cancers compared to moderately differentiated cancers ($p<0.05$). Relative expression of

210 *CYP27A1* tended to be higher in poorly differentiated cancers but this was not significant.
211 We next assessed relative expression of mRNAs encoding the LXR receptors known to bind
212 27HC: *NR1H3* (LXRα) and *NR1H2* (LXRβ) were detected in all cancer grades (Figure 1C
213 and D). Expression of *NR1H3* was significantly lower in moderately-differentiated cancers
214 compared to post-menopausal controls ($p<0.01$). Expression of *NR1H2* did not change
215 between sample groups.

216 *Immunolocalisation of LXR and the proliferation marker Ki67 in endometrial cancer tissue*
217 *sections.*

218 The expression of LXR in endometrial cancer tissue sections was assessed by
219 immunohistochemistry using an antibody that detected both isoforms of LXR (mouse anti-
220 LXR; sc-271064). LXR was readily detected in well-, moderately- or poorly-differentiated
221 cancers and was immunolocalised to both stromal and epithelial cells (supplementary figure
222 1). To assess if LXR expression was associated with cell proliferation within endometrial
223 cancer tissue we performed double immunofluorescence staining for both LXR and the
224 proliferation marker Ki67 (Figure 2). In well-differentiated cancers (Figure 2A), nuclear
225 immunoexpression of Ki67 (red staining) was detected which co-localised (yellow arrows)
226 with LXR expression (green staining, note that single channel views show that the intensity
227 of LXR staining varied between cells). Whilst careful evaluation of single channel views
228 confirmed that the majority of LXR-positive cells were also immunopositive for Ki67 some
229 cells were Ki67-negative (white arrows). In contrast, in moderately-differentiated cancers
230 (Figure 2B) both markers were detected but few cells appeared to co-localise (yellow
231 arrows) although LXR-positive cells (white arrows) were found in close association with
232 proliferating cells. In poorly differentiated cancers (Figure 2C), few cells expressed both
233 markers. Ki67-positive cells were clustered in regions with limited LXR expression and no
234 co-expression of LXR and Ki67 was detected. $LXR^{+}Ki67^{-}$ cells (white arrows) were detected
235 close to $Ki67^{+}$ cells. We also assessed the expression of ERα and Ki67 in endometrial
236 cancer tissues (supplementary figure 2) as this receptor is implicated in regulation of

237 proliferation in normal endometrium (Frasor, et al. 2003; Lubahn, et al. 1993). Consistent
238 with our previous study, ERα was not detected in the poorly differentiated cancers (Collins et
239 al. 2009) and immunoexpression of Ki67 was clearly independent of ERα with an increase in
240 abundance of positive nuclei in poor (sample codes 910/2178) as compared to well or
241 moderately differentiated tissue where co-localisation of ERα and Ki67 was readily detected.

242 *27HC activates LXRE- and ERE-dependent transcription in endometrial epithelial cancer*
243 *cells and alters proliferation of endometrial cancer cells*

244 Having demonstrated expression of enzymes and receptors required for 27HC signalling, we
245 extended our observational study by exploring the impact of the ligand on endometrial
246 epithelial cancer cell lines chosen to model well-, moderately- or poorly-differentiated stage I
247 cancers; Ishikawa, RL95 and MFE 280. Protein expression of both LXR isoforms was
248 confirmed by western blot in all cell lines studied (supplementary figure 3A and 3B). We
249 assessed the mRNA expression of LXRs in these cell lines and found that their expression
250 phenocopied that found in tissue samples (Supplementary figure 3). *NR1H3* mRNA
251 expression was significantly decreased in RL95 (moderately-differentiated) cells compared
252 to MFE 280 (poorly-differentiated; $p<0.01$; Supplementary figure 3C). Consistent with tissue
253 mRNA expression patterns *NR1H2* was not different between cell lines (Supplementary
254 figure 3D). Messenger RNAs encoded by both ER genes; ERα *(ESR1)* and ERβ (*ESR2;*
255 ERβ1 specific primers*)* were detected in all of the cell lines (Supplementary figure 4). *ESR1*
256 mRNAs were significantly reduced in RL95 and MFE280 compared to Ishikawa cells
257 (Supplementary figure 4A) consistent with patterns of expression in intact tissue
258 (supplementary figure 2). *ESR2* mRNA was significantly reduced in MFE280 cells compared
259 to Ishikawa (Supplementary figure 4B). As 27HC is both an endogenous agonist for LXR and
260 a SERM, the impact of 27HC on LXRE- and ERE-dependent transcription was investigated
261 in the endometrial cancer cell lines. 27HC significantly increased LXRE-dependent
262 transcription in a dose-dependent manner in all 3 cell lines and was maximally stimulated by
263 10^{-5}M 27HC (Figure 3 A-C). In contrast, 27HC only stimulated ERE-dependent transcription

264 in Ishikawa cells (Figure 3D) at 10^{-8}M (p<0.01) and 10^{-7}M (p<0.0001). The impact of 27HC
265 was abrogated by co-incubation with the anti-estrogen Fulvestrant (ICI 182,780) consistent
266 with ER dependence. In contrast to Ishikawa cells, 27HC had little impact on ERE-
267 dependent transcription in RL95 (Figure 3E) and MFE280 cells (Figure 3F). As 27HC could
268 activate both ERE- and LXRE-promoters, we assessed its impact on cell proliferation (Figure
269 3 G-I). 27HC induced proliferation of Ishikawa cells at concentrations ranging from 10^{-8}M to
270 10^{-6}M (p<0.01) but this was inhibited at the highest concentration (10^{-5}M, p<0.0001). 27HC
271 significantly increased proliferation in RL95 cells at concentrations of 10^{-7}M (p<0.001) or
272 greater. In contrast, 27HC did not alter proliferation of MFE 280 cells at any of the
273 concentrations investigated. Neither RL95 nor MFE280 cell lines expressed *CYP7B1*
274 (supplementary figure 3E and 3F) precluding the potential for in vitro metabolism limiting cell
275 responses to 27HC in these cell lines.

276 *Targeting LXR with the synthetic agonist GW3965 activates LXRE-dependent transcription*
277 *and alters cell proliferation in a cell specific manner.*

278 Incubation of cells with the LXR-selective agonist GW3965 significantly increased LXRE-
279 dependent transcription in a dose-dependent manner (Figure 4) consistent with expression
280 of LXRs in the endometrial cancer cell lines (Supplementary Figure 3). In contrast to 27HC,
281 GW3965 significantly and robustly increased LXRE-dependent transcription at
282 concentrations ≥10^{-8}M in Ishikawa (Figure 4A) and RL95 (Figure 4B) and ≥10^{-7}M in MFE280
283 cells (Figure 4C). Although LXR reporter responses were similar in the different cell lines,
284 proliferation responses were strikingly different. In Ishikawa cells, treatment with GW3965 at
285 concentrations 10^{-8}M (p<0.01) and 10^{-5}M (p<0.01) significantly increased proliferation
286 (Figure 4D). In contrast, GW3965 significantly and robustly decreased cell proliferation at all
287 concentrations investigated in both RL95 (Figure 4E) and MFE 280 cells (Figure 4F).

288 **Discussion**

289 To date, no study has assessed the association between the cholesterol metabolite 27HC
290 and EC. EC incidence rates have increased by ~50% since the early 1990s and
291 approximately 57% of endometrial cancers in the United States have been attributed to
292 being overweight or obese (Cancer Research UK; http://www.cancerresearchuk.org -
293 accessed November 2017, and (Calle and Kaaks 2004)). Although increased exposure to
294 adipose-derived estrogens is believed to increase aberrant proliferation within the
295 endometrium (Zhao, et al. 2016), recent evidence supports an independent role for obesity-
296 associated metabolic factors in modulating EC risk. Notably, both elevated triglycerides and
297 increased dietary cholesterol consumption are reported to be associated with increased EC
298 risk (Gong et al. 2016; Lindemann, et al. 2009). Importantly, concentrations of the
299 cholesterol metabolite 27HC are increased in postmenopausal women (Burkard, et al. 2007)
300 and are associated with increased risk of breast cancer. Several studies have identified that
301 27HC has an adverse impact on breast cancer (Nelson et al. 2013; Wu et al. 2013a) but
302 whether 27HC can affect EC has not previously been investigated.

303 In light of these studies we hypothesised that 27HC signalling could contribute to the
304 aetiology of endometrial cancer and influence disease progression and we investigated this
305 using both archival human tissue as well as cell lines that are derived from different grades
306 of EC. We obtained new evidence for expression of the enzymes required for the both the
307 synthesis (CYP27A1) and breakdown (CYP7B1) of 27HC. As concentrations of *CYP7B1*
308 mRNAs were significantly decreased in poorly- compared to moderately-differentiated
309 cancers and expression of *CYP27A1* did not change significantly across EC grades; we
310 believe this would favour increased bioavailability of 27HC with increasing grade. These
311 findings appear to parallel those reported for ER+ breast cancer where decreased
312 expression of CYP7B1 and increased CYP27A1 has been reported in tumours compared to
313 normal breast tissues (Wu et al. 2013a). Furthermore, we found that the endogenous

314 receptor for 27HC, LXR, was immunolocalised to stage 1 cancers and was expressed
315 throughout the tissue and localised to the nuclei of both stromal and epithelial cells.

316 We sought to establish if 27HC could alter responses in endometrial cancer cells by acting
317 via its cognate receptor, LXR, or via estrogen receptors which are known to regulate
318 endometrial proliferation. 27HC activated LXR-dependent transcription in all cell lines tested.
319 In contrast, we found that 27HC activated ERE-dependent reporter gene expression in well-
320 differentiated cancer cells (Ishikawa; ERα+ERβ+) but not in those from moderately- (RL95;
321 $ER\alpha^{low}ER\beta+$) or poorly-differentiated cancers (MFE280; $ER\alpha^{low}ER\beta^{low}$). However, 27HC
322 increased proliferation of both Ishikawa and RL95 cells but not MFE280 cells consistent with
323 reported ER expression in these cell lines (Johnson et al. 2007; Li et al. 2014; Yang et al.
324 2008). Our immunohistochemistry analysis (supplementary figure 2) supported these in vitro
325 findings. We found that the proliferation marker Ki67 co-localised with ERα in well- and
326 moderately-differentiated cancers consistent with a key role for this receptor in mediating
327 endometrial epithelial cell proliferation (Frasor et al. 2003; Lubahn et al. 1993). In poorly-
328 differentiated cancers, ERα was not detected consistent with previous reports (Collins et al.
329 2009). It has been reported that 27HC, acting as a SERM, can impact on ERα- or ERβ1-
330 dependent regulation of cell function (He and Nelson 2017) and the estrogenic effects of
331 27HC could therefore be mediated via either ER isoform in EC cells. In endometrial
332 endothelial cells, which express ERβ but not ERα, estrogenic effects are mediated via ERβ
333 tethered to Sp1 and not via direct binding to ERE (Greaves, et al. 2013). Furthermore, it has
334 been reported that 27HC promotes proliferation of ERα-positive LNCaP prostate cancer cells
335 via ERβ (Lau, et al. 2000) (Raza, et al. 2017) which may account for the apparent
336 discrepancy between ERE reporter assay and cell proliferation responses in RL95 cells
337 observed in the current study. Taken together, these findings reveal the potential for 27HC
338 generated within the EC tissue microenvironment to influence ER-dependent transcription
339 and proliferation via ERs expressed in early grade stage 1 EC.

340 Although the association between ERs and endometrial proliferation is well recognised,
341 there is limited data investigating the role of LXR in this process. Expression of LXRα and
342 LXRβ mRNA has been previously reported in endometrium and myometrium of mice
343 (Mouzat, et al. 2007) and 27HC is reported to increase mouse uterine weight, consistent with
344 an uterotrophic action, however, whether this was mediated via ER or LXR was not
345 investigated (Wu, et al. 2013b). In mice, targeted ablation of the receptor subtypes revealed
346 that *Lxrα-/-* but not *Lxrβ-/-* females had reduced endometrial areas compared to wildtype
347 mice consistent with a role for LXRα in promoting endometrial growth/proliferation in that
348 species (Mouzat et al. 2007). In the current study we found that LXR co-localised with the
349 proliferation marker Ki67 in well-differentiated but not moderate- or poorly differentiated EC
350 tissues. In vitro assays verified this finding as the synthetic LXR agonist GW3965 had a cell-
351 selective impact on the EC cell lines. In Ishikawa cells GW3965 increased proliferation,
352 whereas in RL95 and MFE280 cells equimolar concentrations of agonist blocked
353 proliferation. Given that LXR expression was detected in all grades of endometrial cancer
354 this may suggest LXR could be an effective therapeutic target in some endometrial cancers,
355 albeit in a grade-dependent context. Indeed, GW3965, is reported to abrogate E2-mediated
356 increases in MCF7 breast cancer cell proliferation and has been proposed as an anti-
357 proliferative ligand in this context (Vedin et al. 2009).

358 LXR classically acts as a heterodimeric partner of retinoid X receptor (RXR). RXR is
359 expressed in the nuclei of endometrial epithelial cells throughout the menstrual cycle
360 (Fukunaka, et al. 2001) as well as in endometrial cancer tissues (Nickkho-Amiry, et al.
361 2012). Interestingly, LXR-RXR functions as a 'permissive' heterodimer and binding of either
362 an LXR agonist or the RXR agonist 9-*cis* retinoic acid activates transcription, while agonism
363 of both dimer partners has an additive effect on activation. Assessment of RXR isoforms in
364 the cell lines used in the current study demonstrated differential expression of RXRs in
365 Ishikawa, RL95 and MFE280 cells which may account for the distinct responses of these cell
366 lines in response to GW3965 treatment (supplementary figure 5). *NR2B1* (RXRα) mRNA

367 expression was greatest in RL95 cells while *NR2B2* (RXRβ) was detected in all cell lines.
368 Notably, mRNA expression of *NR2B3* (RXRγ) was not detected in RL95 cells but was
369 abundant in MFE280 cells. Whether changes in the constitution of the receptor isoforms that
370 contribute to the LXR:RXR heterodimer affect responses requires further investigation,
371 however, previous studies demonstrate that targeting retinoid signalling may affect
372 proliferation of EC cells. Notably, retinoic acid (RA) signalling via retinoic acid receptor
373 (RAR) and RXR is reported to inhibit Ishikawa cell proliferation by inducing cell cycle arrest
374 (Cheng, et al. 2011) and fenretinide, a synthetic derivative of RA, induced apoptosis of
375 Ishikawa cells (Mittal, et al. 2014). These results suggest targeting LXR-dependent signalling
376 with LXR and/or RXR agonists could inhibit proliferation in EC and cancer progression.

377 Changes in the local inflammatory environment that occur during development and
378 progression of endometrial cancer may also increase exposure to 27HC due to infiltration of
379 inflammatory cells. We have previously demonstrated that infiltration of immune cells is
380 increased in endometrial cancer tissues compared to controls. Notably, the numbers of
381 macrophages, neutrophils and dendritic cells were significantly increased in EC tissues
382 (Wallace, et al. 2010) consistent with 27HC-dependent increases in migration of bone
383 marrow-derived $CD11b^+$ cells reported in in vitro assays (Raccosta, et al. 2013). In addition,
384 27HC increases secretion of CCL2 from macrophages which enhances recruitment of
385 monocytes (Kim, et al. 2013) and can also upregulate ER-dependent expression of pro-
386 inflammatory genes (Umetani et al. 2014). Notably, as Cyp27a1 is reported to be abundant
387 in macrophages [2], these cells may also contribute to an increase in 27HC within the
388 tumour microenvironment. In support of this idea, increased 27HC concentrations have been
389 reported in breast cancer tumours (Wu et al. 2013b) and increased concentrations of
390 cholesterol have been reported in tumours of various cancer types although they have not
391 been directly measured in EC. 27HC can also promote secretion of TNFA and IL6 from
392 macrophages and TNFA is reported to increase proliferation of human endometrial glandular
393 epithelial cells (Nair, et al. 2013). Thus, although in the current study we only investigated

the direct impact of 27HC on proliferation of endometrial cancer epithelial cells, 27HC may also exacerbate changes within the tissue microenvironment by modulating inflammatory responses and this merits further investigation in animal models.

Summary

In the current study we provide the first evidence to support a mechanistic link between exposure to elevated cholesterol, biosynthesis of 27HC and EC. Analysis of human stage 1 endometrial adenocarcinomas revealed expression of the key metabolising enzymes of 27HC was altered in EC consistent with increased exposure to 27HC as EC progresses from well- to poorly-differentiated. Although survival rates for EC are high, incidence rates are increasing in line with rates of obesity and a rising incidence in pre- and peri-menopausal women creates unique therapeutic challenges. Based on our novel findings, we propose that exposure to 27HC may influence disease development/progression by activating ER-dependent pathways to increase epithelial cell proliferation. These results suggest strategies that seek to limit exposure to 27HC through lifestyle modification, lipid-lowering drugs such as statins or novel therapeutics that target 27HC synthesis (CYP27A1 inhibitors) may be effective in reducing endometrial proliferation in women at increased risk of developing EC. Taken together our novel findings suggest that altered cholesterol metabolism, and aberrant exposure to 27HC, may contribute to the development and/or progression of endometrial cancer.

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Author contributions

420 Experimental design; DAG, FC & PTKS, experimental procedures; DAG, FC, AEZ, FLC,

421 manuscript preparation; DAG, FC & PTKS.

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520

1 **Figure legends**

2 **Figure 1. 27HC signalling pathway is expressed in endometrial cancer and altered**
3 **with disease severity.** The expression of *CYP7B1, CYP27A1, NR1H3* (LXRα) and *NR1H2*
4 (LXRβ) relative to internal control gene *CYC* was assessed by qPCR in postmenopausal
5 control endometrium (PM Ctrl) and in endometrial cancer tissue homogenates from well-,
6 moderately- and poorly-differentiated endometrial adenocarcinomas. Relative expression of
7 mRNAs encoding *CYP7B1* (**A**) were decreased in poorly differentiated cancers compared to
8 moderately differentiated cancers but *CYP27A1* was not significantly different (**B**). Relative
9 expression of mRNAs encoding *NR1H3* (**C**; LXRα) were significantly decreased in
10 moderately-differentiated cancers compared to post-menopausal control tissues while
11 *NR1H2* (LXRβ) was not significantly different (**D**). *p<0.05, **p<0.01. Kruskal-Wallis test with
12 multiple comparisons. PM, n=9; Well n=12-30; Mod, n=42-64; Poor, n=23-32. All data are
13 presented as mean ± SEM.

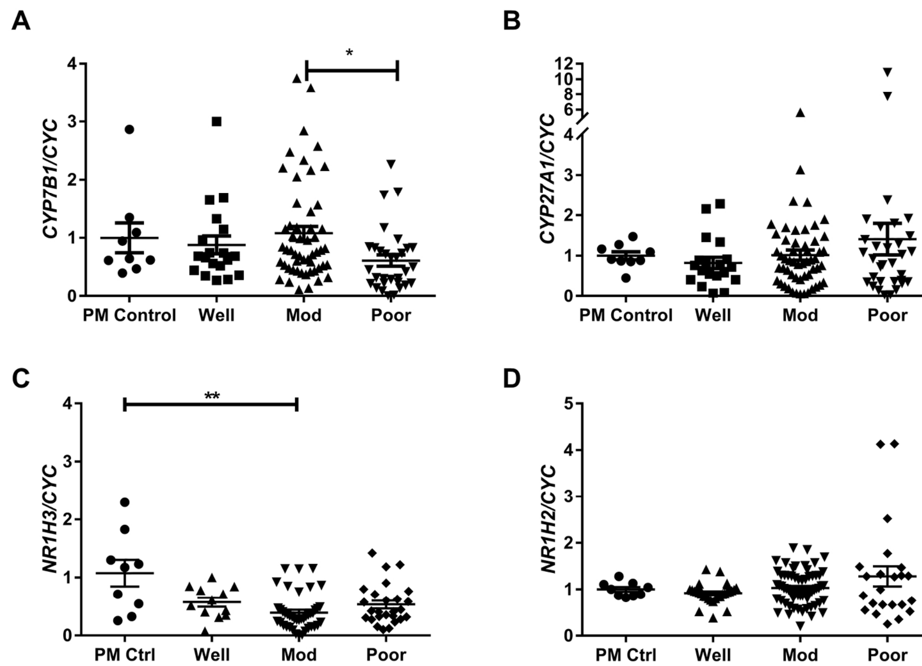
14 **Figure 2. Expression of LXR and the proliferation marker Ki67 in endometrial cancer.**
15 The expression of LXR (antibody identified both isoforms) and the proliferation marker Ki67
16 was assessed by immunohistochemistry in endometrial cancer tissue sections. In well-
17 differentiated cancers (**A**), LXR was expressed throughout the tissue and localised to the
18 nuclei of both stromal and epithelial cells (green staining). Nuclear immunoexpression of
19 Ki67 (red staining) was detected and co-localised with LXR expression (yellow arrows)
20 although some LXR-positive cells did not co-express Ki67 (white arrows). In moderately-
21 differentiated cancers (**B**) both markers were detected but did not appear to co-localise; only
22 few cells expressed both LXR and Ki67 (yellow arrows). Most LXR-positive cells did not co-
23 express Ki67 (white arrows). This was also true of poorly differentiated cancers (**C**), few cells
24 expressed both LXR and Ki67 (yellow arrows) although LXR-positive cells were found in
25 close association with proliferating cells (white arrows). Images representative of at least 3
26 different patients per cancer grade. Nuclear counterstain DAPI (grey). All scale bars 50μM.

27 **Figure 3. 27HC activates LXRE- and ERE-dependent transcription in endometrial**
28 **epithelial cancer cells and alters proliferation.** The cholesterol metabolite 27-
29 hydoxycholesterol (27HC) is the endogenous agonist for LXR and is also classified as
30 selective estrogen receptor modulator. The impact of 27HC on LXRE- (**A-C**) and ERE-
31 dependent (**D-F**) transcription was investigated by luciferase reporter assay in endometrial
32 cancer cell lines; Ishikawa, RL95 and MFE280. 27HC significantly increased LXRE-
33 dependent transcription in a dose-dependent manner in each endometrial cancer cell line.
34 27HC stimulated ERE-dependent transcription only at lower concentrations and was
35 significantly increased by 10^{-8}M 27HC ($p<0.01$) and maximally stimulated by 10^{-7}M 27HC
36 ($p<0.0001$). The 27HC effect was abrogated by co-incubation with the antiestrgoen
37 Fulvestrant (ICI 182,780; ICI) at all concentrations of 27HC (**D**). 27HC did not increase ERE-
38 dependent transcription in RL95 (**E**) and was only increased by 10^{-5}M 27HC ($p<0.05$) in
39 MFE280 cells (**F**). Cell proliferation was assessed by CyQuant® direct proliferation assay in
40 each cell line (**G-I**). Proliferation of Ishikawa cells was increased by 10^{-8}M ($p<0.01$), 10^{-7}M
41 ($p<0.01$) and 10^{-6}M ($p<0.01$) 27HC but decreased by 10^{-5}M 27HC ($p<0.0001$; **G**).
42 Proliferation of RL95 cells was increased by 10^{-7}M ($p<0.001$), 10^{-6}M ($p<0.01$) and 10^{-5}M
43 ($p<0.001$) 27HC (**H**). 27HC did not affect proliferation in MFE280 cells (**I**). *$p<0.05$, **$p<0.01$,
44 *** $p<0.001$, **** $p<0.0001$. One sample t test and a theoretical mean of 1. All data are
45 presented as mean ± SEM.

46 **Figure 4. LXR agonist GW3965 activates LXRE-dependent transcription and alters**
47 **proliferation in endometrial epithelial cancer cells.** The impact of the LXR synthetic
48 agonist GW3965 on LXRE-dependent transcription (**A-C**) and on cell proliferation (**D-F**) was
49 assessed in endometrial cancer cell lines; Ishikawa, RL95 and MFE280. GW3965
50 significantly increased LXRE-dependent transcription in a dose-dependent manner in each
51 endometrial cancer cell line. GW3965 significantly increased LXRE-dependent transcription
52 at all concentrations assessed and was maximally increased by 10^{-6}M GW3965 in Ishikawa
53 cells ($p<0.0001$) (**A**) and RL95 cells ($p<0.01$) (**B**). LXRE-dependent transcription and was not

54 increased by 10^{-8}M GW3965 but maximally increased by 10^{-5}M 27HC ($p<0.001$) in MFE280
55 cells (**C**). Cell proliferation was assessed by CyQuant® direct proliferation assay in each cell
56 line (**D-F**). Proliferation of Ishikawa cells was increased by 10^{-8}M ($p<0.01$) and by 10^{-5}M
57 27HC ($p<0.01$) (**D**). In contrast, proliferation of RL95 cells was decreased by 10^{-8}M
58 ($p<0.001$), 10^{-7}M ($p<0.001$), 10^{-6}M ($p<0.01$) and 10^{-5}M ($p<0.05$) GW3965 (**E**). Proliferation of
59 MFE280 cells was decreased by 10^{-8}M ($p<0.001$), 10^{-7}M ($p<0.0001$), 10^{-6}M ($p<0.0001$) and
60 10^{-5}M ($p<0.05$) GW3965 (**I**). *$p<0.05$, **$p<0.01$, *** $p<0.001$, **** $p<0.0001$. One sample t
61 test and a theoretical mean of 1. All data are presented as mean ± SEM.

62



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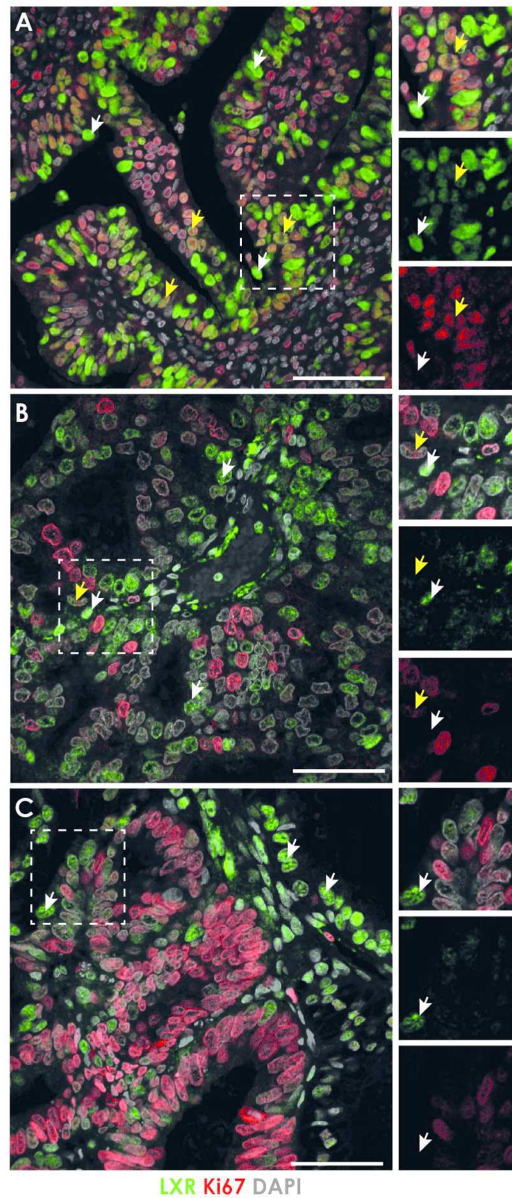

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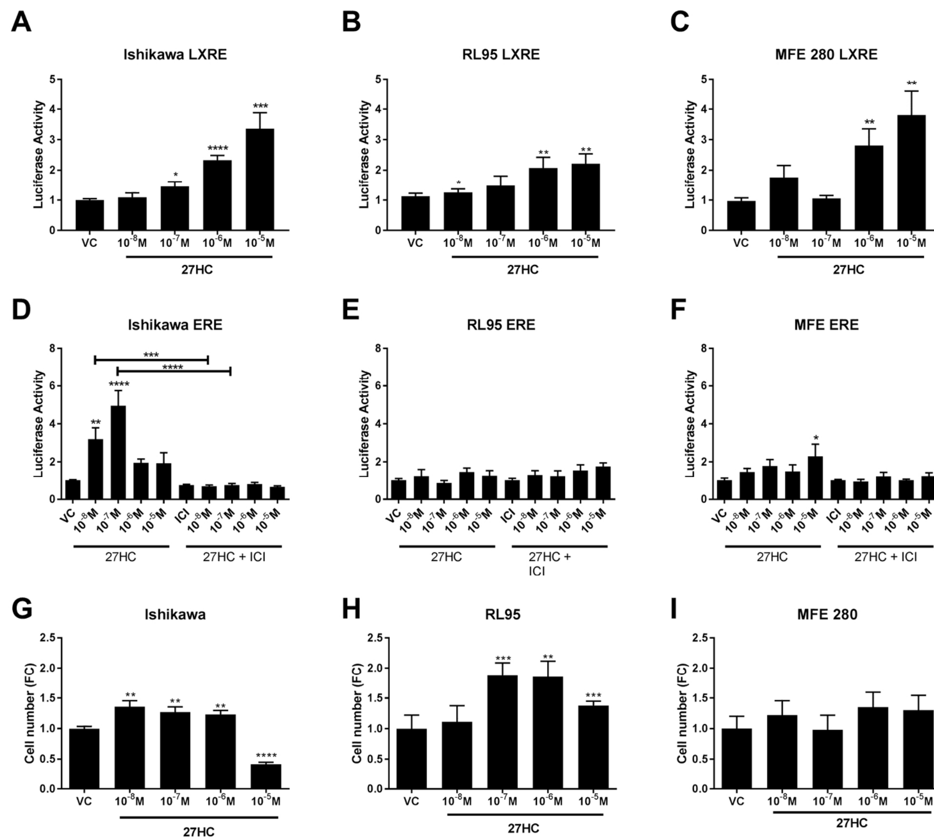

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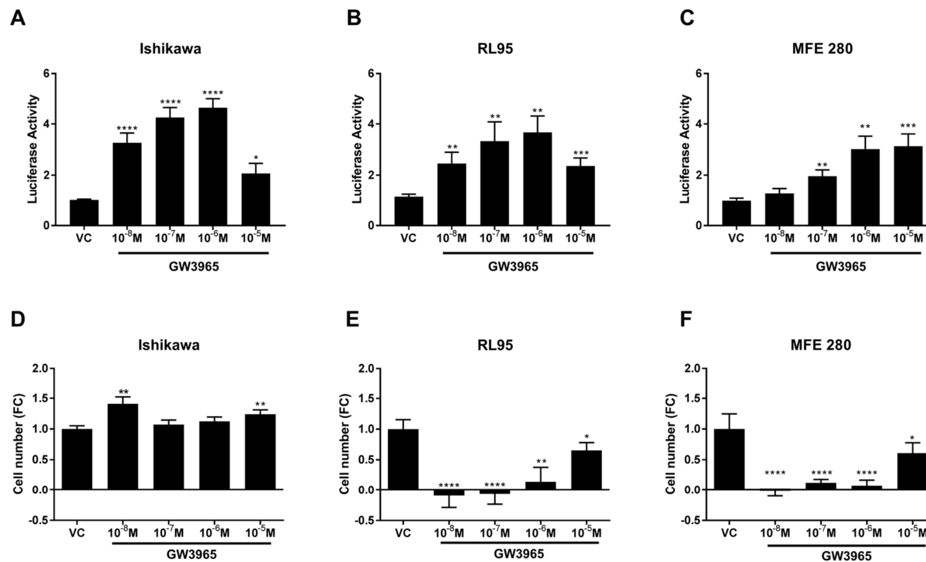

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