# Characterisation of Levonorgestrel Resistant Endometrial Cancer cells

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**Research Article**

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## Abstract

# Background

Endometrial Cancer (EC) is the most common gynaecologic malignancy in the developed world, and incidence is increasing in premenopausal women. The Levonorgestrel Intrauterine System (LNG-IUS) is gaining traction as an alternative treatment for hyperplasia and early-stage EC for women who are unable to undergo surgery. Thirty to 50% of women do not respond to this treatment, making the unknown mechanisms of Levonorgestrel (LNG) resistance a critical obstacle for the conservative management of EC. This study aimed to characterise LNG-IUS treatment resistance in early-stage endometrial cancer in cell line models.

# Method:

LNG resistant endometrial cancer cell lines ($MFE296^{R}$ and $MFE319^{R}$) and cultures from three early stage endometrial cancer patients were developed. The behavioural profile of $MFE296^{R}$ and $MFE319^{R}$ were analysed using proliferation, adhesion, migration (wound healing and transwell) and invasion (spheroid) assays. LNG sensitive cell lines ($MFE296^{S}$ and $MFE319^{S}$) were compared to $LNG^{R}$ cell lines ($MFE296^{R}$ and $MFE319^{R}$). A literature search was conducted to identify possible candidate biomarkers of LNG resistance. RT-qPCR was used to analyse the mRNA expression of 17 candidate biomarkers in $MFE296^{R}$ and $MFE319^{R}$. mRNA expression of the top differentially expressed genes was measured using RT-qPCR in primary cultures.

# Results

LNG resistance did not affect proliferation or invasion in immortalised endometrial cancer cells, however significantly increased transwell migration in $MFE319^{R}$ cells ($p = 0.03$). LNG resistance led to a decrease in cellular adhesion in both $MFE296^{R}$ cells ($p = 0.012$) and $MFE319^{R}$ cells ($p = 0.04$) compared to LNG sensitive clones. mRNA expression of KLF4 and SATB2 was significantly amplified in $MFE296^{R}$ and $MFE319^{R}$ cells compared to their LNG sensitive clones. mRNA expression of KLF4 was significantly upregulated and mRNA expression of ER was significantly downregulated in LNG resistant primary cell lines compared to their LNG sensitive clones.

# Conclusion

LNG resistant cells may be slightly more aggressive and therefore, have more oncogenic potential than their LNG sensitive counterparts. There were significant changes in the mRNA profile of LNG resistant cells compared to LNG sensitive cells, with relative expression of KLF4 and SATB2 significantly

upregulated in resistant immortalised cell lines. This study presents promising preliminary results in biomarker discovery for guiding LNG-IUS treatment of early stage endometrial cancer.

# Background

Endometrial cancer (EC) is the most common gynaecologic malignancy globally contributing to 3.9% of total cancers in women (1). EC is generally diagnosed at earlier stages, with 75% of EC cases being classed as low-grade endometrioid EC (EEC) histological subtypes(2),(3). Currently, the standard treatment for early stage EEC is a hysterectomy and a bilateral salpingo-oophorectomy (4); however, up to 25% of EEC patients are premenopausal, with 5% of these women being under the age of 40 (4),(5). Furthermore, women with a high BMI are more likely to be diagnosed with EEC (6), however, up to 10% of these women are deemed inoperable (7). This data, alongside a shift in global demographics resulting in the rising prevalence of both ageing populations and obesity (8), (9, 10); has led to an increase in the incidence of EEC in inoperable women. This shift is increasing the demand for conservative management options for EEC.

Until recently, systemic progestogen therapy, such as Medroxyprogesterone acetate (MPA) has been used for conservative management of EEC. MPA has proven to be efficacious in the treatment of hormone-sensitive tumours in women where surgery is not a treatment option (11). However, new evidence is shifting the conservative management of EC towards a long-acting reversible contraceptive device, the Levonorgestrel Intra-Uterine System (LNG-IUS). The LNG-IUS is currently used to treat women with abnormal and heavy bleeding (menorrhagia) as levonorgestrel (LNG) (a synthetic form of progesterone) suppresses endometrial proliferation through counteracting the effect of oestrogen (12). The evidence base for the use of the LNG-IUS when treating EEC appears promising, with treatment in this setting yielding response rates between 52% and 67% (13–16). Compared with oral progestogens, the LNG-IUS has a similar disease regression rate and is associated with fewer systemic adverse effects due to being placed locally in the uterus [(17)]. Still, the evidence appears that there is inconsistency in response when using the LNG-IUS for treatment of early stage EC in some women making the unknown mechanisms of LNG resistance a critical obstacle for the conservative management of EC.

Currently, the absence of predictive biomarkers for LNG-IUS treatment of early-stage EC limits the certainty of recommendation (18, 19), with both response and monitoring of treatment depending on invasive biopsies every 3–6 months (4). In recent years, researchers have investigated a variety of approaches for predicting response to conservative treatment of EC, however, few papers investigate predictive biomarkers involved in LNG-IUS resistance explicitly (20), with only 3 looking at the effects of the LNG-IUS in women with EC. Our recent review comments on the current status of predictive biomarkers in LNG-IUS treatment of EC and hyperplasia (20) and highlights the importance of biomarkers in LNG-IUS treatment of EC. Low protein expression of HE4 (14) and progesterone receptor B (21) alongside mRNA expression of *FOXO1* (22) have all been identified as possible predictive biomarkers of non-response to the LNG-IUS specifically. One of the largest-scale genetic based studies done to date on the topic was conducted by Li *et al.*, who observed upregulated mRNA expression of *ANO1, SOX17,*

*CHNL1, DACH1, RUNDC3B, SH3YL1* and *CRISPLD1* in an Ishikawa progesterone resistant cell line (23). This study observes this occurrence in one commercial cell line, and is based on MPA resistant cells, rather than LNG resistant cells Additional research into genes implicated in progesterone resistance is clearly warranted and as currently, there are no predictive biomarkers used clinically in relation to LNG-IUS treatment.

This study aimed to identify differentially expressed genes (DEGs) in LNG resistant cells compared to LNG sensitive cells and to build a behavioural profile of LNG resistant cell lines. These DEGs may then go on to serve as potential predictive biomarkers to be investigated further in patient cohorts.

# Methods

# Cell Culture

Endometrioid EC cell lines MFE296 and MFE319 were cultured as per the supplier's recommendations: MFE296 in Minimum Essential Medium (MEM) (Gibco; Thermo Fisher Scientific #11095-080, CA, USA) containing 10% foetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific #10-091-148, CA, USA) and MFE319 in 1:1 MEM/Roswell Park Memorial Institute Medium (RPMI 1640) (Gibco; Thermo Fisher Scientific #11875-093, CA, USA) containing 20% FBS. All media were supplemented with 100U/ml penicillin/ streptomycin (Gibco; Thermo Fisher Scientific, #15070-063, CA, USA). Cells were grown in 5% $CO_2$ at 37°C. Cells were confirmed negative for mycoplasma.

# Ethics

The current study used Primary cells derived from tissue samples of early-stage EC cultures donated by women as part of the gynaecological cancer tissue bank at Wellington hospital *(Health and Disability Ethics Committees (HDEC) 15/CEN/143 and University of Otago health ethics committee H20/002).* All patients provided written, informed consent prior to donation to the biobank.

# Primary Cell lines

The isolation of adenocarcinoma cells from cancer-associated stromal cells was carried out according to previous studies (24). A section of endometrial cancer tissue (5-20mm) was dissociated for 1 hour using collagenase type I (Sigma-Aldrich #C0130-500MG, NSW Australia) (10mg/mg) in TESCA buffer (50µM TES; 0.36µM CaCl) (Sigma-Aldrich #T1375-100, NSW Australia) diluted to 0.5mg/ml in PBS and DNAse 1 from bovine pancreas (Sigma-Aldrich #DN25, NSW Australia) diluted to 0.1mg/ml in PBS. Cell solution was passed through a 40µm cell strainer to separate cancer-associated stromal cells from adenocarcinoma cells. Cancer-associated stromal cells and adenocarcinoma cells were centrifuged and resuspended separately in Dulbecco's Modified Eagle Medium (DMEM/F12) (Gibco; Thermo Fisher Scientific, CA, USA) medium containing 10% FBS and supplemented with 100U/ml penicillin/ streptomycin. The adenocarcinoma cells were used in the current study. Cells were grown in 5% CO2 at 37°C. Cells were passaged at 80% confluence up to four times.

# Development of resistant cell lines

The MFE319 and MFE296 cell lines were chosen for the current study due to their stability and their definitive EEC phenotypic classification (25). MFE319 and MFE296 cells have been reported as having relatively higher levels of hormone receptor (PR, PR-B and ER) levels, making them a suitable model for studying hormonal responses in cell culture (26). To develop a high-level laboratory model of resistance (27), the $MFE296^{R}$ and $MFE319^{R}$ cells were treated every two days with this concentration of LNG with no escalation. Resistance was then assessed via an $IC_{50}$ and then determined through the observation of fold change. MFE296 and MFE319 cells were trypsinised, counted, and seeded on to a 96 well plate at a concentration of $3 \times 10^5$ cells/mL. Following a 24h incubation at 5% CO2 at 37°C, cells were treated with escalating LNG (Sigma-Aldrich # 797-63-7) dissolved in dimethyl sulfoxide (DMSO, final concentration no greater than 0.001%) (Sigma-Aldrich #D5879, CA, USA) concentrations from 0-500μM and incubated for a further 24h. Plates were then analysed using the Cell Counting Kit-8 (CCK8) (Dojindo #CK04-11) according to manufacturer's instructions. Readings at 450 nm were obtained after 3 hours using the Thermo Scientific ™ Multiskan GO ™ Microplate Spectrophotometer (Thermo Fisher Scientific, MA, USA). Any increase in absorbance indicated an increase in cell density. LNG treatment concentration for resistant clone development was identified as the point of 30% cell viability for each cell line.

LNG-resistant MFE319, MFE296 and primary cell lines were obtained from parental cells via continuous exposure to LNG dissolved in 0.001% DMSO. MFE296 cells were treated with 450μM LNG, MFE319 with 350μM LNG and primary cells with 100μM-200μM LNG. A DMSO control was created for each cell line at a dose of 0.001% dissolved in respective culture media.

A kill curve was use to confirm resistance. Following a LNG treatment period of four months, cells were trypsinised, counted, and seeded on to a 96 well plate at a concentration of $3 \times 10^5$ cells/ml for the LNG sensitive cells and $6 \times 10^5$ cells/ml for the LNG resistant cells of both MFE296 and MFE319 cell lines. Cells were incubated at 5% CO2 at 37°C for 24h and then treated with escalating LNG concentrations from 0-2000μM and incubated for a further 24h. Following incubation plates were analysed using CCK8 according to manufacturer's instructions.

Cell lines from here on will be referred to as $MFE296^{R}$ (LNG resistant), $MFE296^{S}$ (LNG sensitive) and $MFE319^{R}$ (LNG resistant), $MFE319^{S}$ (LNG sensitive).

# Proliferation Assay

Cell proliferation was carried out according to previous studies (28). Cells were seeded in triplicate onto a 96 well plate at a concentration of $3 \times 10^5$ cells/mL ($MFE296^{R}$, $MFE296^{S}$ and $MFE319^{R}$, $MFE319^{S}$) and incubated at 5% CO2 at 37°C for 24 hours. Following incubation plates were analysed using CCK8 according to manufacturer's instructions. Readings were obtained at 0, 24, 48 and 72 hour time points. All absorbance values were normalised to the T0 time point to give the normalised proliferation of each cell line.

# Transwell Migration Assay

Cell migration was measured via the Boyden Chamber assay according to previous studies (28). 6.5mm transwells with 8.0µm pore polycarbonate membrane inserts (Sigma-Aldrich #CLS3422, NSW Australia) were used according to manufacturer's instructions. Cells were seeded in transwell inserts at a concentration of 3 x $10^5$ cells/mL for MFE296$^R$ and MFE296$^S$ and 1 × $10^6$ cells/mL for MFE319$^R$, and MFE319$^S$ and incubated for 48 hours at 5% CO2 at 37°C. Following incubation, cells were washed twice with PBS and fixed with 100% ethanol at room temperature for 20 minutes and stained with 1% crystal violet at room temperature for 15 minutes. Following staining, transwells were washed twice with PBS and non-migrated cells were wiped off using a cotton swab. The membrane was then removed and mounted on a glass slide. Micrographs were taken of four quadrants of the membrane to accurately represent the entire membrane and the cell number was counted using ImageJ (Java Software (29), WI, USA). An average cell count of the four quadrants was used in statistical analysis.

# Wound Migration Assay

Cell migration was measured via a wound healing assay. MFE296 and MFE319 cell lines were plated onto a 6 well plate at a concentration of 1 x $10^6$ cells/mL and incubated at 5% CO2 at 37°C for 24 hours. Following incubation, a 10uL pipette tip was used to create a scratch through the centre of each well. Micrographs of the plates were taken at 0, 24, 48, 72 and 96 hour time points using a 10x objective lens. Wound healing and the percentage of open area was measured using TScratch (CSElab software, ZH, Switzerland) (30).

# Invasion (3D Tumour Spheroid)

Cell invasion was measured using a three-dimensional (3D) tumour spheroid invasion assay and carried out according to previous studies (31). Hanging drop cultures were prepared on a 6 well plate using a cell concentration of 8 x$10^4$ cells/mL for MFE296$^S$ and MFE296$^R$, and 3 x $10^4$ cells/mL for both MFE319$^S$ and MFE319$^R$ and incubated for 96 hours. Following incubation, once spheroids were visible they were embedded into a matrix of 3mg/mL type I collagen (rat tail) (Gibco; Thermo Fisher Scientific #A10483-01, NSW Australia) and 2.7 mg/mL matrigel (Corning Life Sciences #354234, MA, USA) matrix. Micrographs were taken to monitor invasion at 0, 24, 48, 72 and 96 hours following plating using the 20x objective lens. Spheroid invasion was then measured as the total area of spheroids using ImageJ (Java Software) (29).

# 2D Adhesion Assay

Cell adhesion was carried out according to previous studies (32). Adhesion was measured against collagen type I (rat tail) (Gibco; Thermo Fisher Scientific). Tissue culture plates were coated with collagen (10µg/mL) and 3 % bovine serum albumin (BSA) as the negative control, in PBS. Coated plates were incubated for 24 h at 37°C and then rinsed with 80 % ethanol. 3 % BSA in PBS was added to each well and incubated for a further 30 min at 37°C. After rinsing with PBS, concentrations of 5 x $10^5$ cells/mL for all cell lines in serum-free media were added to the coated plates and left to adhere at 37°C for 1 h.

Following incubation, plates were washed 3 times with PBS and fixed with 100 % ethanol before being stained with 0.1 % crystal violet at room temperature for 30 min. Plates were then washed extensively with water to remove excess staining and then left to dry. Once dried, cells were lysed with 50 % acetic acid. Absorbance was measured at 595nm using the Thermo Scientific $^{TM}$ Multiskan GO $^{TM}$ Microplate Spectrophotometer.

*Literature search*

A literature search was carried out to identify a set of key genes involved in progesterone resistance when treating endometrial cancer or endometrial hyperplasia. SCOPUS, PubMed and Google Scholar were searched for the following keywords ("endometrial cancer*" OR "endometrial carcinoma*" OR "endometrial neoplasm*") AND (IUD OR "intra-uterine-device*" OR IUS OR progesterone OR progestin OR Levornogestrel OR "intra-uterine device*" OR "intrauterine system*" OR "intra-uterine system*" OR Mirena) AND ("Biomarkers" OR "Marker" or "Predictive Marker*") AND ("Response")). Articles were critically assessed and genes were selected due to their implication in progesterone resistance in endometrial cancer (EC) and hyperplasia treatment. A review outlining these papers, has been written (20). From this, 13 genes were chosen for analysis: HOTAIR, HE4, ANO1, SOX17, CGNL1, DACH1, RUDC3B, SH3YL1, CRISPLD1, FOXO1, PR-B, ER, and MSX1. A further four genes were selected due to their relationship to EC. MDR1, DKK1, SATB2, CACNA2D3 and KLF4.

*RNA extraction*

RNA extraction was carried out according to previous studies (33). LNG resistant cells and LNG sensitive controls for MFE319, MFE296, GB#13, GB#16 and GB#23 cells were harvested, pelleted, and the RNA from these cells extracted using the zymo *Quick*-RNA kit (Zymo cat# R1057, CA, USA) according to the manufacturer's instructions. RNA quantification (in ng/µL) and purity was assessed using the NanoDrop spectrophotometer (Thermo Fisher Scientific). A 260/280 and 260/230 ratio of ~ 2.0nm was considered optimal "pure" RNA.

# RT-qPCR

Conversion of RNA (1µg) to double-stranded cDNA was carried out using the QuantiTect® RT kit following the manufacturer's instructions (Qiagen #205311). NCBI Primer Blast was used to create a list of potential primers for each of the genes (**supplementary table 1).** qPCR analysis was carried out according to previous studies (33). 25 ng of cDNA, 100 nM of primers and 12.5µL SYBRGreen master mix (Qiagen # 204143) was used in each reaction. RT-qPCR cycling conditions were 95°C for 10mins, (95°C for 15 seconds, 60°C for 30 seconds, 72°C for 40 seconds) for a total of 40 cycles and then 95°C for 60 seconds, followed by melt curve analysis. $C_t$ values were analysed using the ΔCt normalisation method (34) against three housekeeping genes Succinate Dehydrogenase Complex Subunit (SDHA), 90 kDa Heat Shock Protein 1 Beta (HSPCB) and 60S Ribosomal ProteinL13a (RPL13A).

# Statistical analysis

All values are represented as mean ± standard deviation (SD) unless otherwise stated. An *f* test was used to determine variances in standard deviation prior to *t* Test analysis. An unpaired Student's *t* Test was carried out for determination of resistance, transwell migration and adhesion assays to compare values between LNG resistant and LNG sensitive cell lines. An unpaired Student's *t* Test was carried out on the mean of each time point for wound healing, migration and proliferation to give significance at each time point. Technical and biological triplicates (n = 3) were carried out for each experiment. A paired student's *t* Test was performed on ΔCt values for differences in mRNA expression between LNG resistant and LNG sensitive MFE296, MFE319, GB#23, GB#52 and GB#67 cell lines. A p value of $\leq 0.05$ was considered statistically significant (23).

# Results

*Development of LNG resistance MFE296 and MFE319 cell lines*

Resistance was assessed via an $IC_{50}$ and then determined through the observation of fold change. The $IC_{50}$ values of $MFE296^S$ and $MFE296^R$ cells were 250μM and 1800μM respectively, and the fold change increase in resistance from $MFE296^S$ and $MFE296^R$ was 6. The $IC_{50}$ values of $MFE319^S$ and $MFE319^R$ cells were 250μM and 2000μM, respectively, and the fold change increase in resistance from $MFE319^S$ and $MFE319^R$ was 8 **(Supplementary Fig. 1)**.

*LNG resistance impact on cellular proliferation in immortalised cell lines*

There was no significant difference in cell proliferation between LNG resistant cells and LNG sensitive control cells apart from a significant decrease in $MFE319^R$ absorbance and therefore, cell density at the 48 hour time point ($MFE319^S$: 1.535 ± 0.12, $MFE319^R$: 1.23 ± 0.07) ($P<0.05$). Proliferation increased at a similar rate in both cell lines over a period of 72 hours. This observation was conserved in both LNG treated cells and controls. **(Figure 1A-B).** Significance was determined using an unpaired student's *t* test.

*LNG resistance impact on migration in immortalised cell lines*

A significant increase in wound migration was observed in the $MFE296^R$ cell line at the T72 hour time points ($MFE296^S$: 9.33 ± 0.018, $MFE296^R$: 4.13 ± 0.018) ($p= 0.0248$) and T96 hour time points ($MFE296^S$: 6.65 ± 0.013, $MFE296^R$: 1.44 ± 0.010) ($p= 0.0106$) compared to the $MFE296^S$ control represented by a smaller % open area. There was no significant difference in migration at any other time points **(Figure 2A**). There was no significant change in wound migration in the $MFE319^R$ cell line compared to the $MFE319^S$ control **(Figure 2B**). Representative images of wound healing can be seen in **(Figure 2C-D).** There was no significant difference in Boyden chamber transwell migration between $MFE296^R$ cells and $MFE296^S$ cells **(Figure 3A)**. However, a significant increase in transwell migration was observed in the $MFE319^R$ cells compared $MFE319^S$ cells ($MFE319^S$: 210.31 ± 9.24, $MFE296^R$: 254.62 ± 31.77) **(Figure 3B)** ($P =0.03$). Representative images of transwell migration can be seen in **(Figure 3C-D).**

*LNG resistance impact on cellular adhesion in immortalised cell lines*

The adhesive capacity of LNG resistant and LNG sensitive cells was evaluated through the ability of respective cell lines to adhere to collagen in one hour. Adhesion was significantly attenuated in the $MFE296^R$ cells (absorbance: 0.662 ± 0.11) compared to the $MFE296^S$ cells (absorbance: 0.308 ± 0.088) after one hour (p=0.012). **(Figure 4A)**. Adhesion was also attenuated in the $MFE319^R$ cells (absorbance: 0.822 ± 0.18) compared to the $MFE319^S$ cells (absorbance: 0.502 ± 0.06) after one hour (p=0.04). **(Figure 4B)**. BSA served as a negative control for cell adhesion.

*LNG resistance impact on invasion in immortalised cell lines*

Cell invasion rate was measured via a 3D spheroid invasion assay embedded in an ECM matrix of collagen type I and matrigel. Spheroid size ($\mu m^2$) was photographed every 24h for a total of 96h and then measured using Image J. There was no significant difference in spheroid size ($\mu m^2$) in $MFE296^R$ cells compared to $MFE296^S$ **(Figure 5A)** or in $MFE319^R$ compared to $MFE319^S$ cells **(Figure 5B).** Representative images of MFE296 and MFE319 spheroids can be seen in **(Figure 5C-D)**.

*LNG resistant cells express different levels of mRNA to LNG sensitive cells in immortalised cell lines.*

Five DEGs in the MFE296 resistant cell line were identified. ΔCt mRNA expression of KLF4, SATB2, DACH1, CGNL1 and RUNDC3B were significantly upregulated in $MFE296^R$ compared to $MFE296^S$ cells (p = 0.0003, 0.0209, 0.0136, 0.0093, 0.0089 respectively) **(Fig. 6A).** Relative expression of these genes in $MFE296^R$ cells were KLF4: 10.1 ± 0.7, SATB2: 6.3 ± 2.7, DACH: 7.3 ± 0.13, CGNL1: 16.3 ± 7.6 and RUNDC3B: 1.1 ± 0.4. Non-significant data for MFE296 cell lines can be viewed in **(Supplementary table 2).**

Five DEGs were identified in the MFE319 cell line. ΔCt mRNA expression of KLF4, SATB2, ANO1, HE4 and DACH1 were significantly upregulated in $MFE319^R$ compared to $MFE319^S$ cells (p= 0.0203, 0.0235, 0.0023, 0.0171 respectively) **(Figure 6B).** Relative expression of these genes in $MFE319^R$ cells were KLF4: 5.0±1.7, SATB2: 2.6±0.6, ANO1: 4.0±0.5, HE4: 2.0±0.3. Non-significant data for MFE319 cell lines can be viewed in **(Supplementary table 3).**

ΔCt mRNA expression of KLF4 was significantly amplified in primary $LNG^R$ cell lines compared to $LNG^S$ controls (p = 0.0438) **(Fig. 7).** Relative expression of KLF4 in $LNG^R$ primary cells was 1.5 ± 0.2. ΔCt mRNA expression of ER was significantly downregulated in primary $LNG^R$ cell lines compared to $LNG^S$ controls (p = 0.0021) Relative expression of ER in $LNG^R$ primary cells was 0.3 ± 0.02. Non-significant data for primary cell lines can be viewed in **(Supplementary table 4).**

# Discussion

The current study aimed to build a behavioural profile of LNG resistant cell lines and identify significant DEG's in LNG resistant cells compared to LNG sensitive cell lines. LNG resistance did not affect

proliferation or invasion in both MFE296$^{R}$ and MFE319$^{R}$ cell lines, however, it significantly increased transwell migration in MFE319$^{R}$ cells, increased wound healing migration at the 72 and 96 hour time points in MFE296$^{R}$ cells and decreased cellular adhesion to collagen in MFE29$^{R}$ cells and MFE319$^{R}$ cells. Transwell migration in MFE296$^{R}$ cells was unaffected by LNG resistance.

Increased proliferation, migration, invasion and decreased adherence are all implicated in the epithelial-mesenchymal transition (EMT) process. EMT is a physiological process involved in the early stage of carcinogenic dysregulation (35),(36). During this process, cells begin to lose epithelial characteristics and acquire mesenchymal characteristics such as increased motility through the reprogramming of gene expression (35). Acquisition of EMT phenotype has been previously associated with drug resistance, which could make these tumours more likely to metastasise or recur following treatment (37). This is consistent with the results from the current study show that the LNG resistant cells may be slightly more aggressive and therefore, oncogenic potential than their LNG sensitive counterparts. Clinically, this research is warranted as it is the first time the behavioural profile of LNG resistant cells has been investigated. It is vital to understand if LNG resistant cells behave differently as this could lead to alternate treatment pathways for women who are resistant to the LNG-IUS. This further demonstrates a need to identify women that will not respond to the LNG-IUS prior to treatment.

Seventeen potential biomarkers were identified in the literature and mRNA expression was investigated in LNG resistant and LNG sensitive cell lines. Expression of both KLF4 and SATB2 was significantly upregulated in resistant cell lines (MFE296$^{R}$ and MFE319$^{R}$) compared to LNG$^{S}$ controls, suggesting that these genes have the potential to serve as predictive biomarkers for response to the LNG-IUS. In the current study, upregulation of ANO1 in MFE319$^{R}$ cell lines and upregulation of CGNL1 MFE296$^{R}$ cells compared to LNG sensitive controls supports previous findings from Li e*t al.*, (23) where upregulation of these genes were observed in MPA resistant Ishikawa cell lines. The current study did not observe these genetic upregulations across both cell lines, nor did it observe upregulation of DACH1, RUNDC3B, SH3YL1 or CRISPLD1 in LNG resistant cells compared to LNG sensitive controls seen in the Li e*t al.*, (23) study. The current study builds upon the Li *et al.*, study by identifying ANO1 and CGNL1 upregulation in further EEC immortalised cell lines and observing these genetic changes in relation to LNG treatment specifically. The findings of the current study do not support observations made by Orbo *et al.*, and Behrouzi *et al.*, (14, 38) who identified low protein expression of HE4 to be associated with a positive response to the LNG-IUS. In the current study, no significant change in HE4 ΔCt mRNA expression in immortalised or primary LNG resistant cells compared to LNG sensitive controls was detected. However, the current study only investigated mRNA expression of *HE4*, rather than changes in protein expression observed by previous studies (14, 38) and it is not yet clear whether changes in ΔCt mRNA expression of *HE4* directly correlate to HE4 protein expression. The differential mRNA expression observed between the two cell lines and primary cell lines in the current study, alongside the cell lines and patient samples used in previous studies can most likely attributed to tumour heterogeneity. The degree of spatial diversity within an individual's tumour is highly variable (39) with research showing between 0 and 8,000 heterogeneous coding mutations within primary tumours (40). This heterogeneity drives phenotypic variation, thus,

posing a significant challenge to the diagnosis, management and treatment of cancer, alongside contributing to significant challenges when identifying biomarkers to guide clinical decision-making in cancer medicine (41). A panel biomarker approach with clinically validated cut-off points may be more appropriate to address these inter-tumour disparities. However, the current study serves as an exploratory investigation into genes that can be further investigated in a clinical setting.

This is the first time KLF4 and SATB2 have been investigated in the context of predictive biomarkers for EEC treatment and therefore serve as novel potential biomarkers that warrant further investigation. Of note, KLF4 appears to be the most promising potential biomarker due to its increase in expression in LNG resistant cells conserved across both immortalised and primary cell lines. mRNA expression and relative protein expression of these genes should be analysed in further primary EEC samples to determine if they can serve as predictive biomarkers that can be used clinically.

Alongside the two DEGs identified in both MFE296 and MFE319 cell lines, the relative mRNA expression of hormone receptors ER and PR were measured in primary cell lines in the current study. Low expression of PR, both protein and mRNA, has been extensively shown in the literature to predict a negative response to progesterone treatment (21, 22, 42, 43). Contrary to the literature, the current study observed no significant change in mRNA expression of PR in all three primary LNG resistant cells. Research has shown that LNG-IUS treatment can lead to downregulation of nuclear PRs (44) therefore, despite previous research showing PR to have the potential to predict negative response to treatment, it would not make an efficacious predictive biomarker due to its expression being somewhat reliant on treatment (44). Alongside this, the two main subtypes of PR, PR-A and PR-B have been said to have opposing roles in estrogen-induced endometrial proliferation (45). PR-A has been said to inhibit proliferation, while PR-B contributes to it. The two receptor subtypes are also very difficult to differentiate via immunohistochemistry (IHC) and the use of antibodies that recognise both receptor subtypes should be used to evaluate overall PR expression in conditions including endometrial cancer (46). The current study only investigated PR-B expression, therefore it would be beneficial to use RT-qPCR to investigate both subtypes further.

## Strengths And Limitations

Limitations of the current study include the candidate based approach rather than discovery based. The genes investigated in the current study were chosen due to being previously implicated in progesterone resistance or other tumorigenic pathways of EC. A discovery based approach would be to do whole transcriptome sequencing (47) Which could be efficacious in identifying novel targets that have not been previously described in the literature. However, within the confines of this preliminary study, a candidate based approach was most appropriate.

The high-level laboratory model of resistance adopted in the current study was the most appropriate method in this case, however, the level of resistance obtained in the short time frame was on the lower side of resistance (27). It is important to note that there may be differences between induced resistance,

developed in the laboratory, and spontaneous resistance, which occurs in patients that are intrinsically resistant to the LNG-IUS. Because of this, the pathways implicated in acquired resistance after prolonged treatment could differ from pathways that altered resistance in drug naïve cells. This is why it is important to first identify potential markers using a high level laboratory model, and then to validate those findings in women treated with the LNG-IUS where the clinical outcome.

Due to being a 2D monolayer model, these assays do not represent what would occur physiologically, due to the lack of effect of both gravity and other surrounding tissues. This can lead the assays to lack predictivity (48), however, the methods used for the behavioural analysis of resistant cell lines are high throughput and well documented in the literature, allowing us to compare methodology and results to other examples in the literature.

# Conclusion

The need for conservative approaches to endometrial cancer treatment is rising due to the increase in incidence in women who may chose not to have surgery. Despite advances in molecular research, no clinically relevant predictive biomarkers have been established. We have identified six potential biomarkers in immortalised and primary patient cell lines for the use of the LNG-IUS treatment of early stage EC. These markers should go on to be investigated further in patient cohorts to develop clinically relevent tissue and blood based tests.

# Abbreviations

| | |
|---|---|
| **DEG** | Differently Expressed Gene |
| **EC** | Endometrial Cancer |
| **EEC** | Endometrioid endometrial cancer |
| **EMT** | Epithelial-Mesenchymal Transition |
| **ER** | Estrogen Receptor |
| **IHC** | Immunohistochemistry |
| **LNG** | Levonorgestrel |
| **LNG-IUS** | Levonorgestrel Intra Uterine System |
| **MPA** | Medroxyprogesterone Acetate |
| **PR** | Progesterone Receptor |

# Declarations

***Ethics approval and consent to participate***

The current study used Primary cells derived from tissue samples of early-stage EC cultures donated by women as part of the gynaecological cancer tissue bank at Wellington hospital *(Health and Disability Ethics Committees (HDEC) 15/CEN/143 and University of Otago health ethics committee H20/002).* All patients provided written, informed consent prior to donation to the biobank.

### *Consent for publication*

Not applicable.

### *Availability of data and materials*

Not applicable.

### *Competing interests*

The authors declare that they have no competing interests.


### *Funding*

University of Otago research grant to CH.


### *Author's contribution*

MD, under the supervision of CH and KD carried out all in-vitro experiments, analysed and interpreted the data. MD was the major contributor to the preparation of the manuscript. CH made substantial contributions to the conception and design of the study. KD and SF advised on the design of the study and provided manuscript feedback. All authors read and approved the final manuscript.


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# Supplementary Table

**Supplementary Table 1:** List of Primers used in the current study

| GENE | PRIMER | PRODUCT LENGTH | GC% | Tm |
|---|---|---|---|---|
| **CACNA2D3** | F: TGATGTGGTGTGGACCGAAG | 139 | F: 55.00 | F: 59.97 |
| | R: GCCCTTCGATCTGGTTTCGT | | R: 55.00 | R: 60.39 |
| **HOTAIR** | F: CCAGCCCTAGCCTTTGGAAG | 109 | F: 60.00 | F: 60.39 |
| | R: GCTGCCAGTTAGAAAAGCGG | | R: 55.00 | R: 59.83 |
| **SATB2** | F: GGAGAACGACAGCGAGGAA | 139 | F: 57.86 | F: 59.41 |
| | R: CCGATGTATTGCTTTGCCTAGT | | R: 45.45 | R: 58.79 |
| **HE4** | F: GTTCGGCTTCACCCTAGTCTC | 183 | F: 57.14 | F: 60.14 |
| | R: CAGGAACCCTCCTTATCATTGGG | | R: 60.44 | R: 60.44 |
| **DKK1** | F: ACAACTACCAGCCGTACCC | 111 | F: 57.89 | F: 59.02 |
| | R: TGCAGGCGAGACAGATTTGC | | R: 55.00 | R: 61.30 |
| **$KLF_4$** | F: ACCCCACCTTCTTCACCC | 200 | F: 61.11 | F: 58.41 |
| | R: TAAGGTTTCTCACCTGTGTGG | | R: 47.62 | R: 57.51 |
| **ANO1** | F: GAGCCAAAGACATCGGAATCTG | 89 | F: 50.00 | F: 59.39 |
| | R: TGAAGGAGATCACACGAAGGCAT | | R: 47.83 | R: 61.95 |
| **SOX17** | F: AGTGACGACCAGAGCCAGAC | 214 | F: 60.00 | F: 61.53 |
| | R: CCTTAGCCCACACCATGAAA | | R: 50.00 | R: 57.79 |
| **CGNL1** | F: GGCTGAGGAGGAAATCGACA | 142 | F: 55.00 | F: 59.46 |
| | R: ACTCGGCAGCTTCTTCAGTCTTA | | R: 47.83 | R: 61.62 |
| **DACH1** | F: TGCCGCATTCTGTCCCT | 184 | F: 58.82 | F: 58.48 |
| | R: GAGTCTGCTCCATGTTGGTTATT | | R: 43.48 | R: 58.73 |
| **RUNDC3B** | F: TGGATGGCAGTTTTCCTGCT | 113 | F: 50.00 | F: 59.89 |
| | R: ACCACTGCTTCCCAAAGTCC | | R: 55.00 | R: 60.18 |
| **SH3YL1** | F: CCGCAGGTACGCCCTC | 123 | F: 75.00 | F: 59.46 |
| | R: AGGTATAGGGTTATTCATGCTGCCC | | R: 48.00 | R: 62.26 |
| **CRISPLD1** | F: GATATAGGCCCCCGACGTTT | 148 | F: 55.00 | F: 59.32 |
| | R: CCCACCAGTTTCCCCTGTGT | | R: 60.00 | R: 62.01 |
| **PRB** | F: GGTCTACCCGCCCTATCTCA | 195 | F: 60.00 | F: 60.18 |
| | R: TAGTTGTGCTGCCCTTCCAT | | R: 50.00 | R: 59.30 |

| GENE | PRIMER | PRODUCT LENGTH | GC% | Tm |
|---|---|---|---|---|
| ER | F: TGGGAATGATGAAAGGTGGGAT | 129 | F: 45.45 | F: 59.41 |
| | R: GGTTGGCAGCTCTCATGTCT | | R: 55.00 | 60.04 |
| FOXO1 | F: CTGAGGGTTAGTGAGCAGGTTAC | 161 | F: 52.17 | F:60.37 |
| | R: CTGCCAAGTCTGACGAAAGG | | R:55.00 | R: 58.85 |
| RPL13A | F: CCTGGAGGAGAAGAGGAAAGAGA | 126 | F: 52.17 | F: 60.57 |
| | R: TTGAGGACCTCTGTGTATTTGTCAA | | R: 40.00 | R: 60.16 |
| SDHA | F: TGGGAACAAGAGGGCATCTG | 86 | F: 55.00 | F: 59.67 |
| | R: CCACCACTGCATCAAATTCATG | | R: 45.45 | R: 58.48 |
| HSPCB | F: TCTGGGTATCGGAAAGCAAGCC | 80 | F: 54.55 | F: 62.64 |
| | R: GTGCACTTCCTCAGGCATCTTG | | R: 54.55 | R: 61.77 |

**Supplementary Table 2:** (ΔCt) ±SD for non-significant MFE296 data

| | $MFE296^S$ (ΔCt) | $MFE296^R$(ΔCt) |
|---|---|---|
| HOTAIR | N/A | N/A |
| CRISPLD1 | 17.2 ± 4.4 | 15.1 ± 2.5 |
| SOX17 | 17.5 ± 3.8 | 15.7 ± 3.3 |
| ANO1 | 10.9 ± 0.6 | 8.8 ± 1.8 |
| HE4 | 18.6 ± 0.9 | 19.2 ± 1.7 |
| ER | 14.4 ± 5.2 | 15.2 ± 0.1 |
| PR | 12.8 ± 12.4 | 11.7 ± 2.3 |
| SH3YL1 | 17.3 ± 3.2 | 15.7 ± 2.0 |
| FOXO1 | 7 ± 21.1 | 4.1 ± 1.0 |
| DKK1 | 17.5 ± 1.4 | 18.1 ± 0.4 |
| CACNA2D3 | 11.2 ± 12.7 | 13.1 ± 2.2 |
| MSX1 | 7.7 ± 4.6 | 7.2 ± 3.5 |
| MDR1 | 14.7 ± 4.1 | 14.9 ± 3.4 |

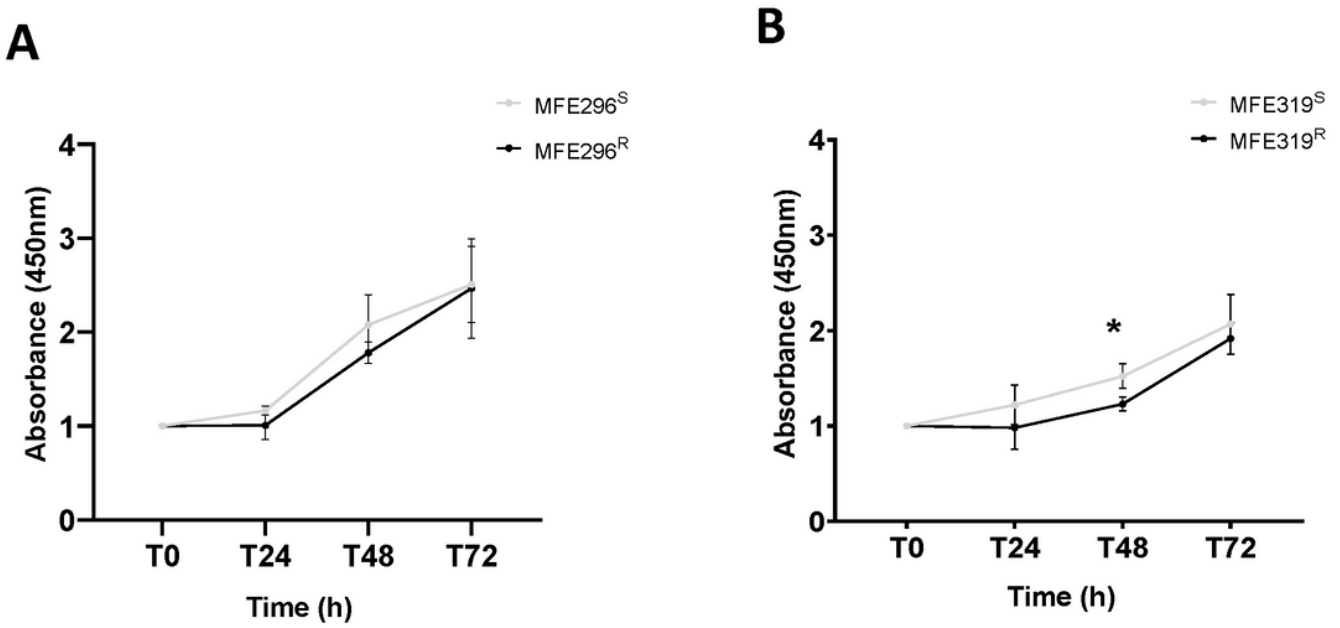
**Supplementary Table 3:** (ΔCt) ±SD for non-significant MFE319 data

| | MFE319$^{S}$ (ΔCt) | MFE319$^{R}$(ΔCt) |
|---|---|---|
| **HOTAIR** | N/A | N/A |
| **CRISPLD1** | 15.2 ± 0.8 | 11.30 ± 2.2 |
| **SOX17** | 14.8 ± 1.1 | 16.6 ± 1.3 |
| **ER** | 10.8 ± 0.3 | 10.8 ± 0.9 |
| **PR** | 11.5 ± 1.25 | 11.2 ± 0.8 |
| **SH3YL1** | 14.3 ± 2.0 | 11.5 ± 1.4 |
| **CGNL1** | 7.4 ± 0.6 | 8.1 ± 0.7 |
| **FOXO1** | 7 ± 21.1 | 3.7 ± 0.7 |
| **RUNDC3B** | 8.5 ± 0.3 | 8.5 ± 2.7 |
| **DKK1** | 11.3 ± 1.4 | 14.1 ± 0.6 |
| **CACNA2D3** | 9.2 ± 3.3 | 11.6 ± 3.9 |
| **MSX1** | 5.8 ± 1.9 | 6.6 ± 6.2 |
| **MDR1** | 13.5 ± 2.0 | 9.8 ± 0.6 |

**Supplementary Table 4:** (ΔCt) ±SD for non-significant Primary cell line data

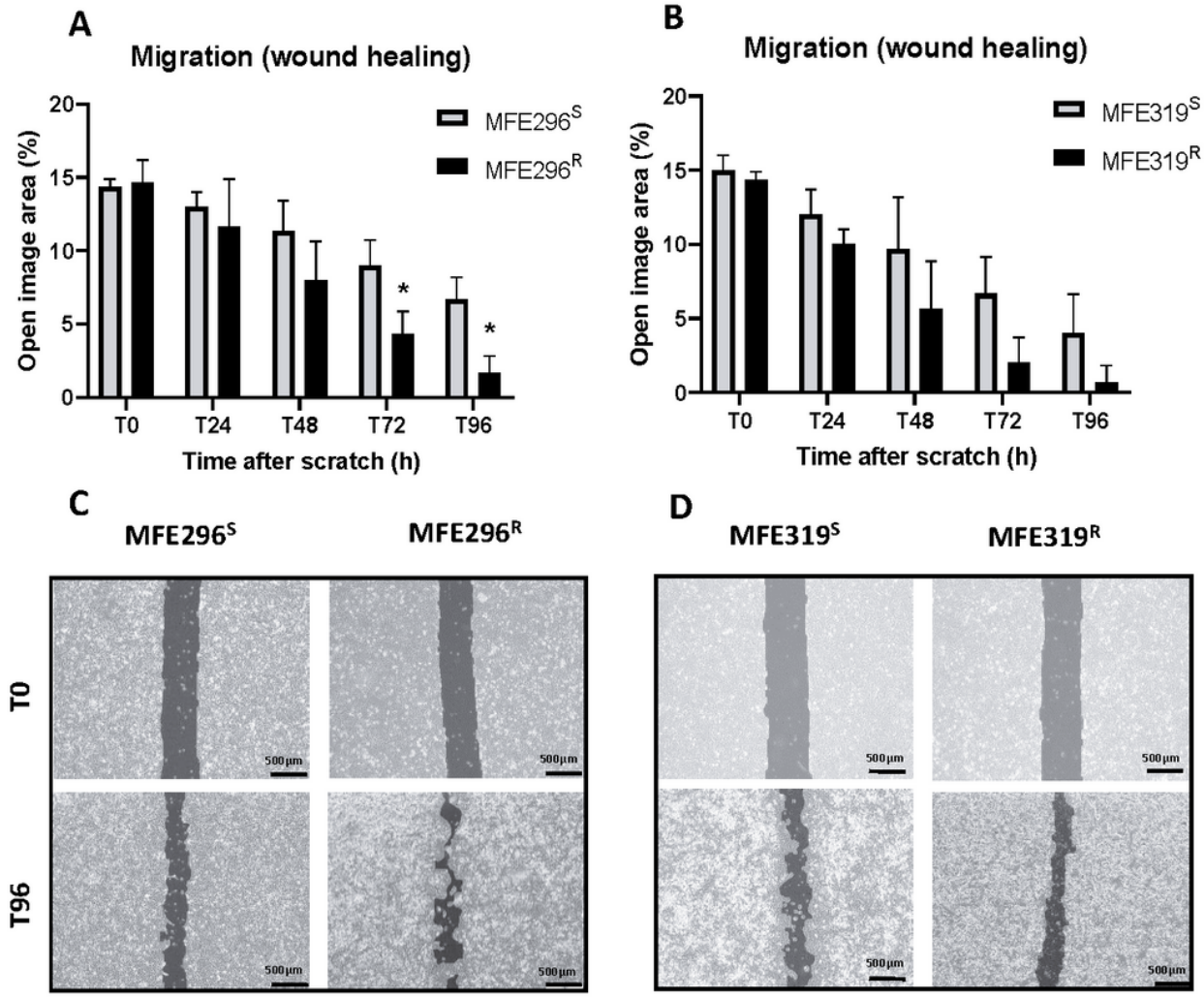
| | LNG$^{S}$ (ΔCt) | LNG$^{R}$(ΔCt) |
|---|---|---|
| **SATB2** | 8.1 ± 1.6 | 6.9 ± 2.6 |
| **SOX17** | 12.7 ±2.9 | 15.1 ± 3.7 |
| **ANO1** | 8.9 ± 1.7 | 14.9 ± 4.5 |
| **HE4** | 8.8 ± 5.0 | 13.6 ± 4.3 |
| **PR** | 7.6 ± 1.8 | 7.0 ± 1.6 |
| **MSX1** | 6.4 ± 1.2 | 4.8 ± 1.6 |
| **MDR1** | 9.0 ± 3.8 | 11.8 ± 1.1 |

# Figures



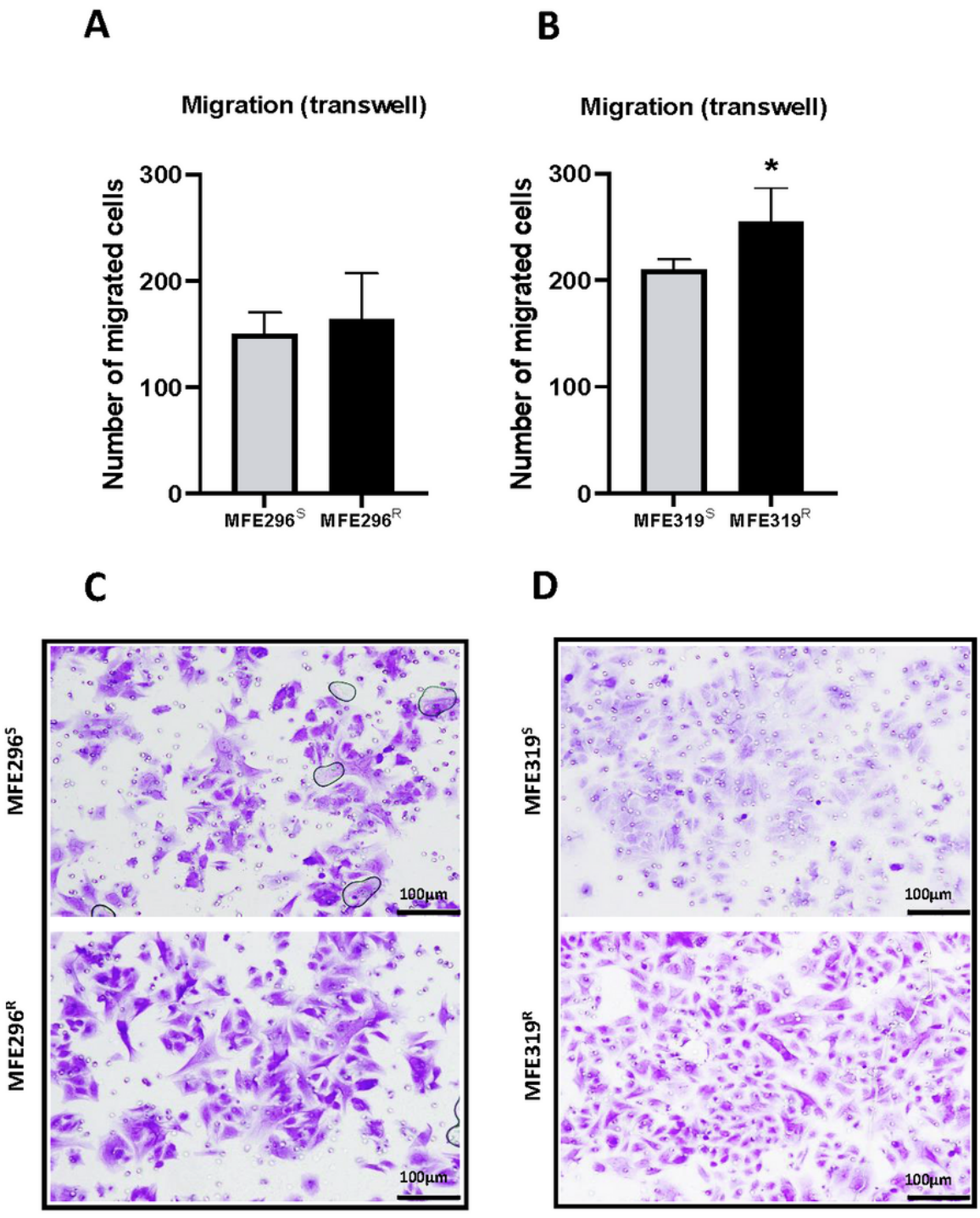

## Figure 1

LNG treated cells proliferate at the same rate as controls in immortalised cell lines Proliferation assay results showing the difference in cell proliferation between A) MFE296R (black) and MFE296S (grey) and B) MFE319R (black) and MFE319S (grey) cell lines respectively. Proliferation rate is displayed as absorbance at 450nm relative to LNG sensitive controls. A) Proliferation rate does not differ between MFE296R and MFE296S cells. B) Proliferation rate significantly decreased in the MFE319R cells at the 48h time point compared to MFE319S cells. Results are expressed as mean ± SD, results are done in triplicate (n=3). Individual groups were analysed using T-test. * P <0.05.




## Figure 2

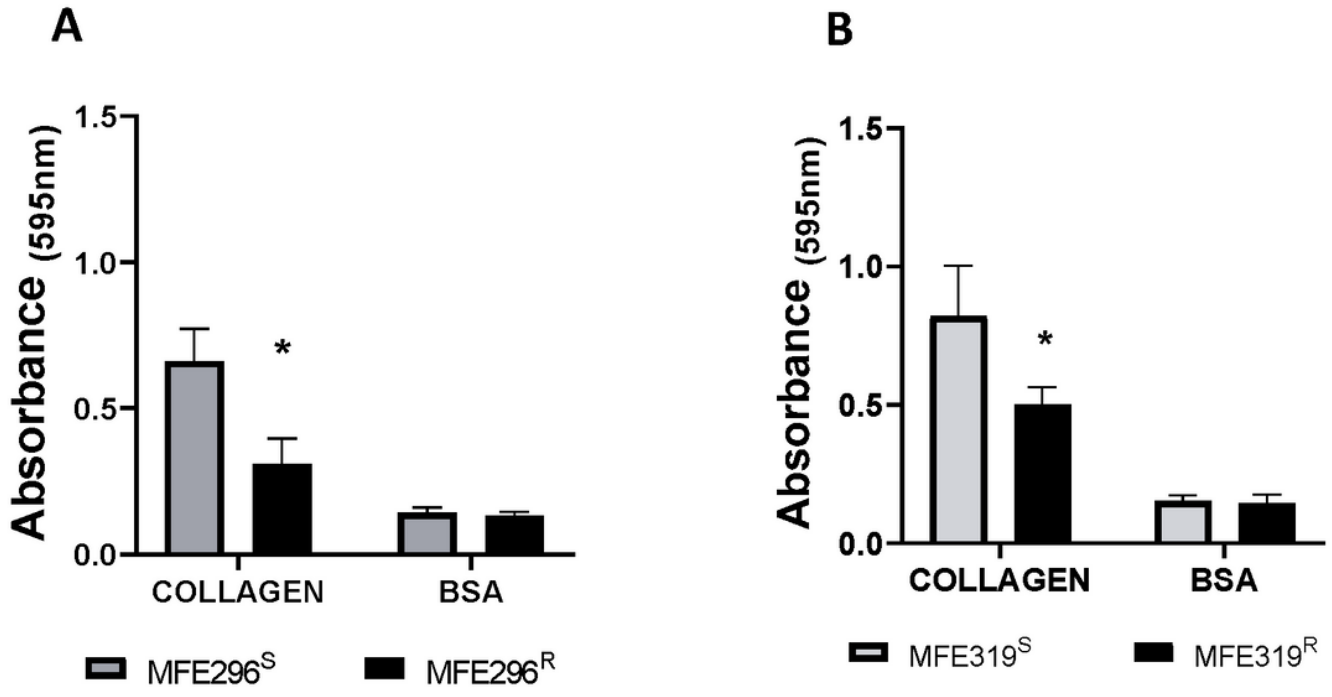
LNG resistance has some effect on migration in immortalised cell lines Wound migration assay results showing the difference between MFE296S (grey) and MFE296R (black) wound migration. Migration significantly increased in the MFE296R cells at the 72h time point compared to MFE296S cells. Wound migration is displayed as % open area. B) Wound migration assay results showing the difference between MFE319S (grey) and MFE319R (black) wound migration. MFE319R and MFE319S cell lines Wound migration significantly decreased in MFE319R cells at the 24h time point. C) Representative images of wound healing in MFE296S, MFE296R cells taken using 4x objective. Yellow lines represent the % open area. D) Representative images of wound healing in MFE319S, MFE319R cells taken using 4x objective. Yellow lines represent the % open area. Results are expressed as mean ± SD, experiments performed in triplicate (n=3). Individual groups were analysed using T-test. * P <0.05.




## Figure 3

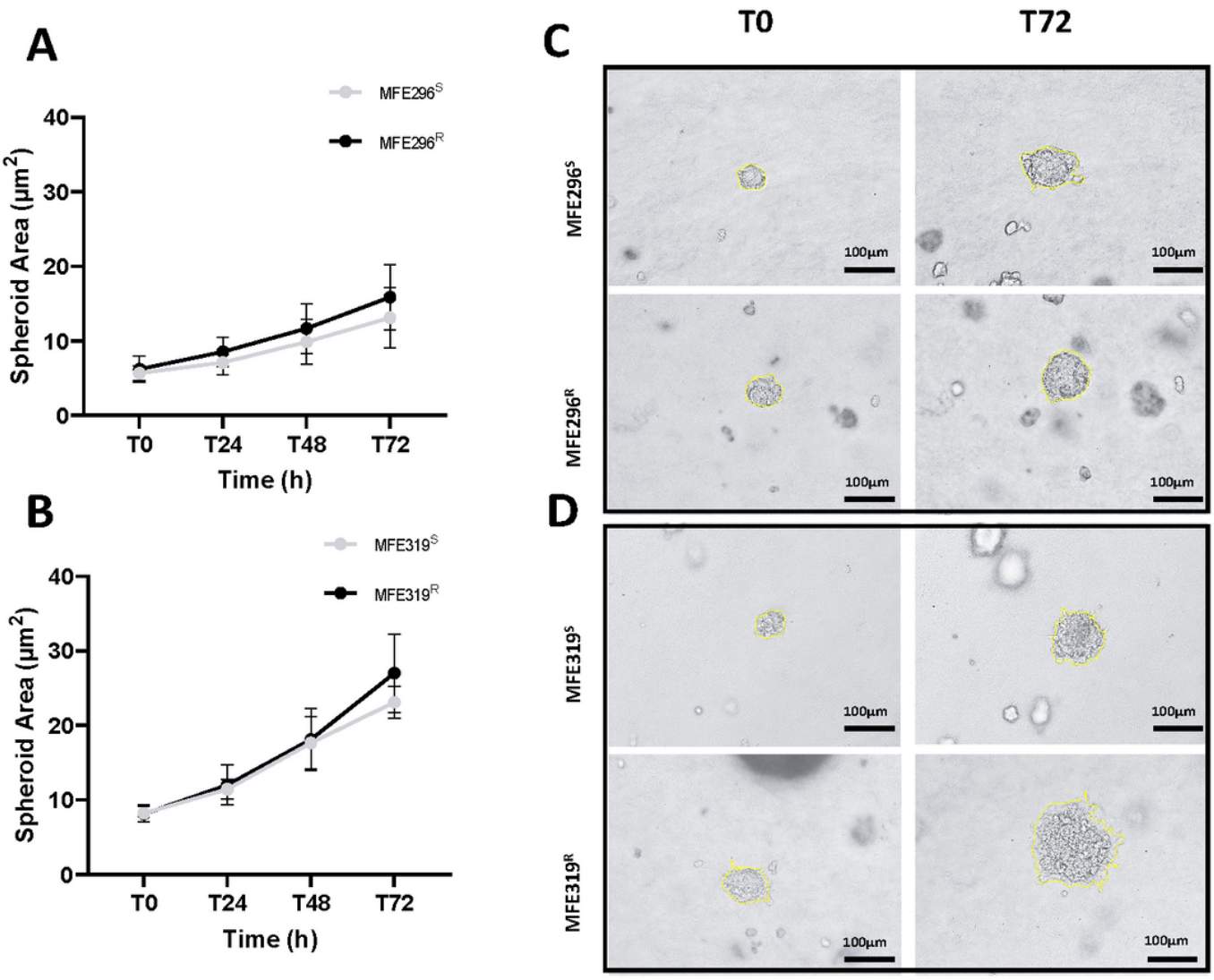
LNG resistance has some effect on migration in immortalised cell lines A) Transwell migration assay results showing the difference in MFE296R (grey) and MFE296S (black). Transwell migration rate does not differ between the MFE296R and MFE296S cells. B) Transwell migration assay results showing the difference in MFE319R (grey) and MFE319S (black) cell lines respectively. Migration significantly increased in the MFE319R cells compared to MFE319S cells. C) Representative images of MFE296S and

MFE296R cell transwell migration taken using 20x objective. D) Representative images of MFE319S and MFE319R cell transwell migration taken using 20x objective Transwell migration is displayed as number of migrated cells on a transwell membrane (average of four quadrant counts). Results are expressed as mean ± SD, experiments performed in triplicate (n=3). Individual groups were analysed using T-test. * P <0.05.



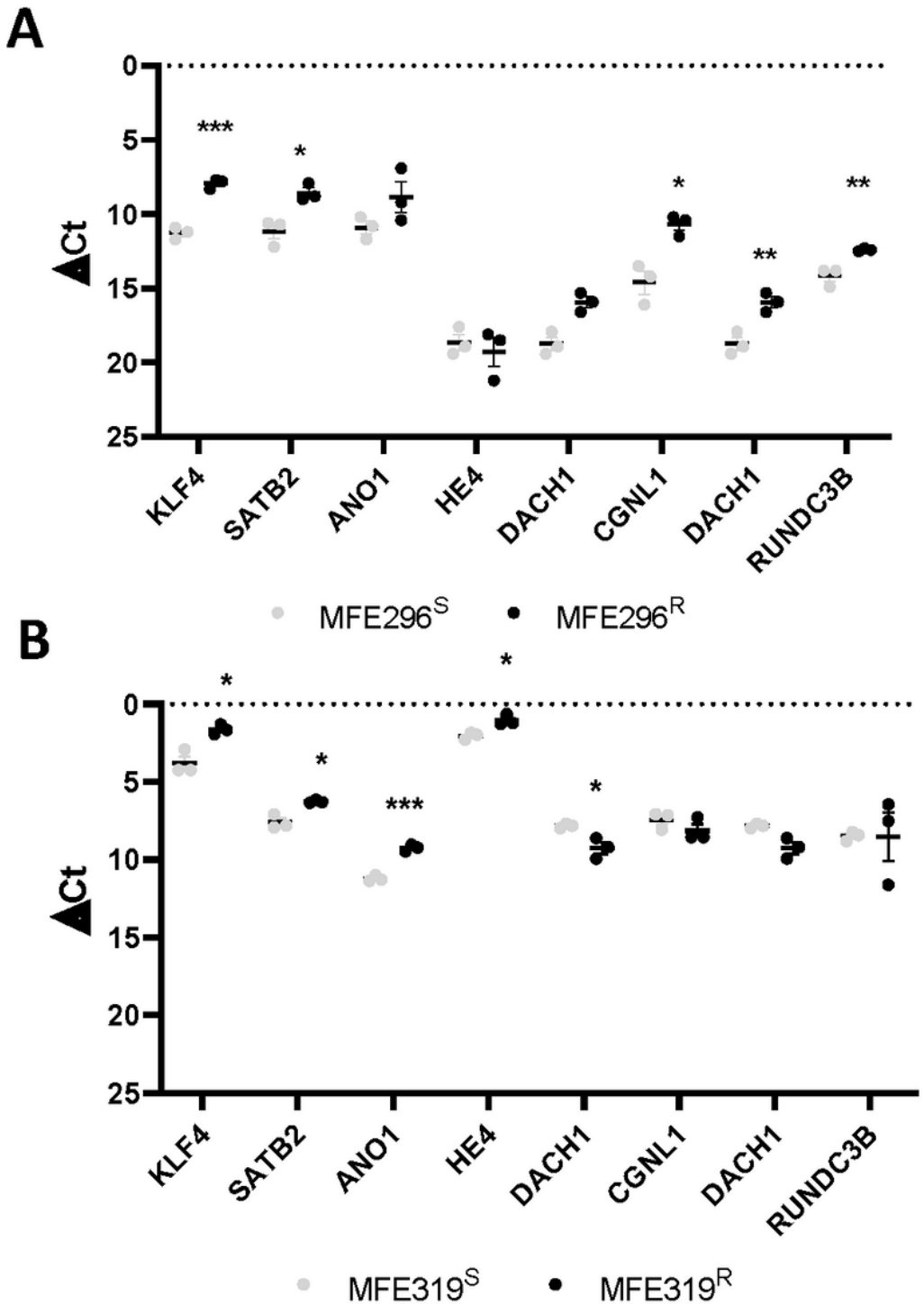

## Figure 4

LNG resistance decreases adhesion in immortalised cell lines Adhesion assay results showing the difference in cell adhesion. A) MFE296S (grey) and MFE296R (black) and B) MFE319S (grey) and MFE319R (black) cell lines respectively. A) Adhesion is significantly decreased in the MFE296R cells compared to MFE296S cells. B) Adhesion is significantly decreased in MFE319R cells compared to MFE319S cells. Adhesion is displayed as Absorbance of crystal violet at 595nm. BSA served as a negative control. Results are expressed as mean ± SD, experiments performed in triplicate (n=3). Individual groups were analysed using T-test. * P <0.05.




## Figure 5

LNG resistance does not affect invasion in immortalised cell lines Spheroid invasion assay results showing no significant difference in cell invasion. A) Average spheroid size (µm2) of MFE296S (grey) and MFE296R (black) over 72 hours. There is no significant change in spheroid size between LNG resistant cells and LNG sensitive cells. B) Average spheroid size (µm2) of MFE319S (grey) and MFE319R (black) over 72 hours. There is no significant change in spheroid size between LNG resistant cells and LNG sensitive cells. C) Representative images of MFE296S and MFE296R spheroids. D) Representative images of MFE319S and MFE319R spheroids. Images taken using the 20x objective. Yellow lines represent the area of the spheroid (µm2). Results are expressed as mean ± SD, experiments performed in triplicate (n=3). Individual groups were analysed using T-test.




## Figure 6

LNG resistant cells express different levels of mRNA to controls in immortalised cell lines. RT-qPCR was performed in triplicate and Ct values were normalised to three different housekeeping genes (SDHA, HSPCB, and RPL13A). A) Significant ΔCt mRNA expression of key genes in the MFE296S (grey) and MFE296R (black) cell lines. B) Significant ΔCt mRNA expression of key genes in the MFE319S (grey) and

MFE319R (black) cell lines SATB2, DACH1, KLF4 and CGNL1 were all significantly upregulated in LNG resistant cells (black) compared to LNG sensitive cells (grey) in MFE296 cells,

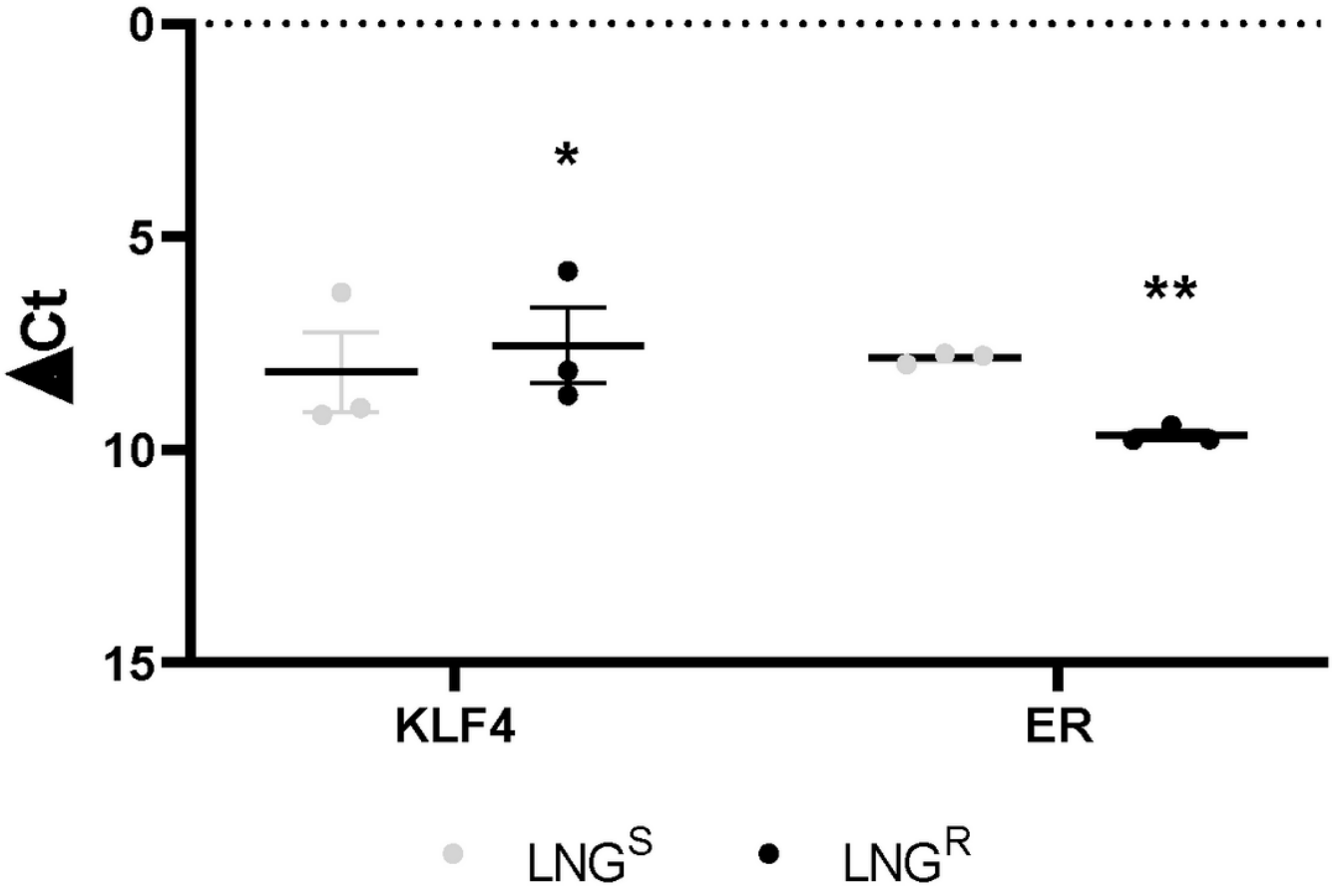



**Figure 7**

LNG resistant cells express different levels of mRNA to controls in primary cell lines. ΔCt mRNA expression of significant key genes in the LNGS (grey) and LNGR (black) primary cell lines. RT-qPCR was performed in triplicate and Ct values were normalised to three different housekeeping genes (SDHA, HSPCB, and RPL13A). mRNA expression of KLF4 was amplified in all 3 primary LNG resistant cell lines compared to LNG sensitive controls, represented by a lower ΔCt. mRNA expression of ER was downregulated amplified in all 3 primary LNG resistant cell lines compared to LNG sensitive controls represented by a higher ΔCt. Results are expressed as mean ± SEM, experiments were performed in triplicate (n=3). Individual groups were analysed using T-test. * P <0.05, **P < 0.01.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SUPPLEMENTARYFIGURE1.pdf