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ORIGINAL RESEARCH

Validation of endogenous control reference genes for normalizing gene expression studies in endometrial carcinoma

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ABSTRACT: Real-time quantitative RT–PCR (qRT–PCR) is a powerful technique used for the relative quantification of target genes, using reference (housekeeping) genes for normalization to ensure the generation of accurate and robust data. A systematic examination of the suitability of endogenous reference genes for gene expression studies in endometrial cancer tissues is absent. The aims of this study were therefore to identify and evaluate from the thirty-two possible reference genes from a TaqMan® array panel their suitability as an internal control gene. The mathematical software packages geNorm qBasePLUS identified Pumilio homolog 1 (Drosophila) (*PUM1*), ubiquitin C (*UBC*), phosphoglycerate kinase (*PGK1*), mitochondrial ribosomal protein L19 (*MRPL19*) and peptidylpropyl isomerase A (cyclophilin A) (*PPIA*) as the best reference gene combination, whilst NormFinder identified *MRPL19* as the best single reference gene, with importin 8 (*IPO8*) and *PPIA* being the best combination of two reference genes. BestKeeper ranked *MRPL19* as the most stably expressed gene. In addition, the study was validated by examining the relative expression of a test gene, which encodes the cannabinoid receptor 1 (*CB1*). A significant difference in *CB1* mRNA expression between malignant and normal endometrium using *MRPL19*, *PPIA*, and *IPO8* in combination was observed. The use of *MRPL19*, *IPO8* and *PPIA* was identified as the best reference gene combination for the normalization of gene expression levels in endometrial carcinoma. This study demonstrates that the arbitrary selection of endogenous control reference genes for normalization in qRT–PCR studies of endometrial carcinoma, without validation, risks the production of inaccurate data and should therefore be discouraged.

Key words: endogenous control reference genes / human / endometrial cancer / quantitative RT–PCR / cannabinoid receptor

Introduction

Real-time quantitative RT–PCR (qRT–PCR) is a very sensitive tool applied widely to the study of molecular factors involved in many biological processes, including the initiation of endometrial cancer and its progression (Burns *et al.*, 2006). It has been used to evaluate and identify several molecular markers associated with the stage of the cancer, its metastatic potential and prognosis (Wong *et al.*, 2008; Porichi *et al.*, 2009). Furthermore, qRT–PCR has increasingly replaced RT–PCR and northern blotting for the study of transcript levels in various cancers because it is highly sensitive, specific, easy to use, fast, reproducible and has a higher throughput (Radonic *et al.*, 2004; Huggett *et al.*, 2005; Mishima *et al.*, 2006; Blazquez *et al.*, 2008). For the results generated with qRT–PCR to be valid, however, sources of error such as differences in the quantity and quality of extracted RNA, the presence of contaminating genomic and operator DNA, and differences in reverse transcription and gene PCR efficiencies must be eliminated (Bustin, 2000, 2002; Romanowski *et al.*, 2007). To ensure consistency and reproducibility of published data the 'Minimum Information for publication of Quantitative real time PCR Experiments' (MIQE) guidelines recommend

that the justification of choice and number of reference genes should be an essential part of all qRT–PCR studies, helping to guarantee the normalization of resulting data. The reference gene ('endogenous control gene') should be one whose expression is stable in all samples, regardless of tissue type, disease state and disease progression and/or treatment (Dheda *et al.*, 2005; Bonefeld *et al.*, 2008). This pivotal normalization step, where a reference gene compensates for any variations in experimental conditions, tissue composition or cellularity, is essential because both the gene of interest and the reference gene are being exposed to the same experimental conditions. A single 'best' reference gene is therefore unlikely in practice, as it has been noted that almost all genes are modified under some conditions (Kubista *et al.*, 2006).

Endometrial cancer remains an important cause of morbidity and mortality. In the UK, data from Cancer Research UK showed that 8500 new cases were diagnosed in 2011 and 1930 women died from it in that year whilst in the USA there were 47 130 new cases with 8010 deaths (American Cancer Society, 2012). The International Agency for Research on Cancer, through the GLOBOCAN series, estimated that there were 287 000 new cases and 74 000 deaths from endometrial malignancies worldwide in 2008 (Ferlay *et al.*, 2010). Despite improved overall survival rates, the incidence of endometrial cancer has risen by 40% and deaths from it by 20% over the past 20 years (Cancer Research UK Cancer Statistics, 2012), primarily because its aetiopathogenesis is complicated and not fully understood. This situation is further compounded by an ageing population and increasing rates and severity of obesity (National Comprehensive Cancer Network Guidelines, 2013). Pathologically, endometrial malignancies can be divided into two types; type I (low grade estrogen-dependent) and type II (the most aggressive estrogen-independent) tumour (Bokhman, 1983). qRT–PCR studies of these cancers are therefore likely to unravel the molecular factors involved in its aetiopathogenesis.

In previous qRT–PCR studies of endometrial cancer, normalization has mostly been performed with seemingly arbitrarily chosen reference genes, such as β-actin (*ACTB*) (Papageorgiou *et al.*, 2009; Guida *et al.*, 2010), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Du *et al.*, 2009; Kashima *et al.*, 2009) or 18S RNA ribosomal unit 1 (*18S*) (Baldinu *et al.*, 2007). Although numerous studies have established the best endogenous references genes in both normal human and animal endometria during the menstrual and oestrous cycles (von Wolff *et al.*, 2000; Walker *et al.*, 2009; Klein *et al.*, 2011; Vestergaard *et al.*, 2011; Gebeh *et al.*, 2012a; Sadek *et al.*, 2012; Jursza *et al.*, 2014), and when studying other gynaecological tissues (von Wolff *et al.*, 2000; Vestergaard *et al.*, 2011; Gebeh *et al.*, 2012a; Sadek *et al.*, 2012), to the best of our knowledge, there have been no studies that have evaluated the suitability of these genes as possible reference genes in qRT–PCR studies on endometrial cancer, despite the recommendations from the MIQE guidelines (Bustin *et al.*, 2009). Although the selection of endogenous control reference genes for microRNA expression studies in human (Torres *et al.*, 2013) and rat (Jurcevic *et al.*, 2013) models of endometrial carcinoma has been reported, none have comprehensively evaluated messenger RNAs (mRNA) in human endometrial cancer of a post-reproductive age cohort. What these numerous studies indicate, however, is that an endogenous reference gene or combination of genes in one species or patient group may not be applicable to a different species or patient group and that the best endogenous reference gene or combination needs empirical assessment and validation. Therefore, the aims of this study were to (i) robustly evaluate, identify and validate the best reference genes for qRT–PCR amongst a large panel of 32 potential normalization genes for the quantification of transcripts in a diverse class of endometrial tissues; normal endometrium (atrophic, secretory, and proliferative) and endometrial cancer types 1 and 2 and then (ii) examine the effect of using each of these reference genes on the analysis of expression in endometrial cancer tissues of a test gene (cannabinoid receptor: *CB1*) in order to highlight any effects that random selection of reference genes might have on data interpretation. The choice of *CB1* as the test gene was made because of our group's interest in evaluation of the endocannabinoid system in cancer including sex steroid hormone-dependent cancers, such as endometrial cancer (Ayakannu *et al.*, 2013). The number of samples taken for each group was also deliberately selected to be small ($n = 3$) to indicate that correctly validated endogenous reference genes are essential for interpretation of these types of data.

Materials and Methods

Ethics statement

The study was approved and conducted according to the guidelines of Leicestershire and Rutland Research Ethics Committee. Volunteers were women undergoing hysterectomy for endometrial carcinoma (study group) or a benign gynaecological condition, such as uterine prolapse (control group) at the University Hospital of Leicester National Health Service Trust, England, and all provided signed written informed consent to take part in the study. Exclusion criteria were those with concurrent or previous hormonal treatment (such as hormone replacement therapy or the levonorgestrel intrauterine system), or currently on prescription or recreational drugs. Women with chronic medical conditions or any other type of cancer were also excluded.

Subjects

The women were categorized according to the histology of the endometrial biopsy obtained at the hysteroscopy prior to the hysterectomy. All the patients had surgery within 2 weeks of diagnosis. The final histopathological diagnosis was divided into type and grade of cancer, according to the International Federation of Gynecology & Obstetrics (FIGO) system (Mutch, 2009; Pecorelli, 2009). A total of 15 endometrial carcinomas were studied: type I grade 1 endometrioid adenocarcinoma ($n = 3$), type I grade 2 endometrioid adenocarcinoma ($n = 3$), type I grade 3 endometrioid adenocarcinoma ($n = 3$), type 2 serous ($n = 3$) and carcinosarcoma ($n = 3$) of endometrial cancer. Normal endometrial tissues samples ($n = 9$) were obtained from volunteers who were undergoing hysterectomies for benign indications (prolapse) and were classified into premenopausal secretory phase ($n = 3$), proliferative phase ($n = 3$) and post-menopausal atrophic ($n = 3$) samples.

Tissue collection

Fresh uteri specimens were immediately transported on ice to the histopathology department where the endometrial biopsies from the hysterectomies were obtained and a consultant gynaecology histopathologist divided the endometrial biopsies into two pieces; one for this study and the other for histological confirmation of diagnosis. The endometrial (cancer and normal tissue) biopsies were washed with phosphate-buffered saline to remove excess blood and immediately stored in RNA*later*® (Life Technologies, Paisley, UK) at −80°C until further processing.

RNA extraction and cDNA synthesis

Endometrial tissues biopsies (100 mg) in lysis/binding buffer (1 ml lysis/binding buffer solution per 100 mg of tissues (miRNA Isolation Kit)) were disrupted and homogenized using a TissueRuptor (Qiagen Crawley, UK) homogenizer at medium speed for 60 s on ice. The tissue was homogenized until all visible 'clumps' were dispersed and total RNA extracted using the *mir*Vana™ miRNA Isolation Kit (Life Technologies) according to the manufacturer's protocol. Total RNA was quantified and its purity determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Detroit, MI, USA) and genomic DNA digested by treating with a TURBO-DNAse free kit (Life Technologies). At this point, the RNA concentration was standardized to 10 μg/100 μl, incubated at 37°C for 30 min, the reaction inactivated with 10 μl of inactivation buffer (supplied in the TURBO-DNase free kit) and the solution centrifuged for 90 s at 10 000*g*. The supernatants containing total cellular RNA (1 μg) were subjected to first strand complementary DNA (cDNA) synthesis using the high capacity cDNA MultiScribe™ Reverse Transcriptase Kit (Life Technologies) according to the manufacturer's protocol; incubation occurred with oligo-dT_{16} as the primer at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and then cooled to 4°C. The cDNA was stored at −20°C.

Quantitative RT–PCR

qRT–PCR experiments were performed using the validated human endogenous control assay TaqMan® Array 96-well plates consisting of 32 reference genes (see Table I; Bustin *et al.*, 2009). Each TaqMan® Gene Expression Assay consisted of a fluorogenic carboxyfluorescein (FAM™) dye-labelled minor groove binder (MGB) probe and two amplification primers (forward and reverse) provided in a pre-formulated 20X mix; 1X final concentrations were 250 nM for the probe and 900 nM for each primer. Each assay had an amplification efficiency of 100 ± 10% (Technologies). The

Table I TaqMan® endogenous control genes used in the study.

Gene ID	Gene name	Accession number	Life Technologies assay ID
RPLP0	Ribosomal protein, large, P0	NM_001002	Hs99999902_m1
ACTB	Actin, beta	NM_001101	Hs99999903_m1
PPIA	Peptidylpropyl isomerase A (cyclophilin A)	NM_021330	Hs99999904_m1
PGK1	Phosphoglycerate kinase	NM_000291	Hs99999906_m1
B2M	Beta-2-microglobulin	NM_004048	Hs99999907_m1
GUSB	Glucuronidase, beta	NM_000181	Hs99999908_m1
HPRT1	Hypoxanthine phosphoribosyl transferase	NM_000194	Hs99999909_m1
TBP	TATA box-binding protein	M34960	Hs99999910_m1
18S	Eukaryotic 18S ribosomal RNA	X03205	Hs99999901_s1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	Hs99999905_m1
TFRC	Transferrin receptor (P90, CD71)	NM_003234	Hs99999911_m1
IPO8	Importin 8	NM_006390	Hs00183533_m1
POLR2A	Polymerase (RNA) II (DNA-directed) polypeptide A	NM_000937	Hs00172178_m1
UBC	Ubiquitin C	NM_021009	Hs00824723_m1
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_003406	Hs00237047_m1
HMBS	Hydroxymethylbilane synthase	NM_000190	Hs00609297_m1
CASC3	Cancer susceptibility candidate 3	NM_007359	Hs00201226_m1
CDKN1A	Cyclin-dependent kinase Inhibitor 1A (p21, Cip1)	NM_000389	Hs00355782_m1
CDKN1B	Cyclin-dependent kinase Inhibitor 1B (p27, Kip1)	NM_004064	Hs00153277_m1
GADD45A	Growth arrest and DNA-damage-include, alpha	NM_001199741	Hs00169255_m1
PUM1	Pumilio homolog 1(Drosophila)	NM_ 001020658	Hs00206469_m1
PSMC4	Proteasome (prosome, Macropain) 26S subunit, ATPase, 4	NM_006503	Hs00197826_m1
EIF2B1	Eukaryotic translation initiation factor 2B, subunit 1 alpha, 26 kDa	NM_001414	Hs00426752_m1
PES1	Pescadillo homolog 1, containing BRCT domain (zebra fish)	NM_001243225	Hs00362795_g1
ABL1	V-abl Abelson murine leukaemia viral oncogene homolog 1	NM_005157	Hs00245445_m1
ELF1	E74-like factor 1 (ets domain transcription factor)	NM_001145353	Hs00152844_m1
MT-ATP6	Mitochondrially encoded ATP synthase 6	NC_012920	Hs02596862_g1
MRPL19	Mitochondrial ribosomal protein L19	NM_014763	Hs00608519_m1
POP4	Processing of precursor 4, ribonuclease P/MRP subunit (*S. cerevisiae*)	NM_006627	Hs00198357_m1
RPL37A	Ribosomal protein L37a	NM_000998	Hs01102345_m1
RPL30	Ribosomal protein L30	NM_000989	Hs00265497_m1
RPS17	Ribosomal protein S17	NM_001021	Hs00734303_g1

TaqMan® gene Expression Assay was purchased from Applied Biosystems by Life Technologies (Paisley, UK). *CB1* (Hs00275634_m1), primers and probes were similarly purchased from Applied Biosystems by Life Technologies (Paisley, UK), as FAM/MGB dye-labelled probe. A reverse transcriptase (RT)-minus control and no template control (NTC) containing DNAse-free water instead of template mRNA were included in each run. No product was synthesized in the NTC and RT-minus controls, confirming the absence of contamination with exogenous DNA. All reactions were performed in the final volume 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 by Life Technologies, Paisley, UK) and the thermal cycler profile was as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. All the reactions for the reference genes were performed in triplicate (biological and technical).

Software determination of reference gene stability

Reference gene expression stability was evaluated using three universally available mathematical software packages: geNorm^{PLUS}, version 2.2, incorporating the updated version of qbase^{PLUS2} (Biogazelle, Zwijnaarde, Belgium) (Hellemans *et al.*, 2007) (available from https://www.biogazelle.com/qbaseplus), NormFinder version 0.953 (Andersen *et al.*, 2004) (available from Aarhus University, Denmark; http://moma.dk/normfinder-software) and BestKeeper (Pfaffl *et al.*, 2004) (available from http://www.gene-quantification.com/bestkeeper.html); accumulated standard deviation data obtained from the NormFinder algorithm were incorporated into GenEx software version 5.3.6.170 (MultiD Analyses AB, Goteborg, Sweden) (http://www.multid.se/contact.php). Details of the statistical methods used by both qbase^{PLUS2} and NormFinder have been described elsewhere (Andersen *et al.*, 2004; Beekman *et al.*, 2011; Pinto *et al.*, 2012).

Gene expression stability analysis

The gene expression (mRNA) stability analysis for the 32 endogenous reference control genes was obtained using the mean qRT–PCR threshold cycle (Ct) value, defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. 0.5 SD above background levels). The Ct values obtained were converted into quantitative relative expression values using the $2^{-\Delta\Delta Ct}$ method (Penna *et al.*, 2011). In addition, the relative expression of *CB1* was normalized against the recommended endogenous control that was obtained from our systematically validated endogenous control selection within the study environment.

Statistical analysis

Statistical analysis of the stability of putative reference genes and ranking was provided by geNorm qBasePLUS, NormFinder and BestKeeper. Expression of *CB1* in the different sample groups and with the different putative normalizing genes were expressed as the mean (SEM) or mean (SD). Student's *t*-test (unpaired) and one-way analysis of variance (ANOVA) followed by appropriate *ad hoc* post analysis were performed using the Prism version 6.00 for Windows (Graph Pad, San Diego, CA, USA) and $P < 0.05$ was considered significant.

Results

Patient characteristics

Patient demographics are shown in Table II. There was no statistically significant difference in either age or BMI of any group when compared with the atrophic uterus (control) group; however, it should be noted that the sample number in each group was only 3. Biopsies were shown to be characteristic of the phase of menstrual cycle or atrophic using the criteria of Noyes *et al.* (1950) or malignant using the updated FIGO criterion (Mutch, 2009; Pecorelli, 2009). All endometrial cancer tissue was shown to consist primarily of proliferating glandular epithelial cells with scant contribution from stromal cells.

Table II Patient characteristics.

Patient group Each group ($n = 3$)	Age in years (mean ± SD)	BMI in kg/m^2 (mean ± SD)
Normal		
Atrophic endometrium	62.33 ± 4.61	26.67 ± 6.42
Secretory phase	46.00 ± 4.35	26.00 ± 1.00
Proliferative phase	47.33 ± 0.57	26.00 ± 1.73
Endometrial carcinoma type 1		
Grade 1	78.00 ± 13.23	31.33 ± 6.65
Grade 2	67.67 ± 11.06	30.67 ± 1.52
Grade 3	72.67 ± 12.06	35.33 ± 6.11
Endometrial carcinoma type 2		
Serous	59.00 ± 3.46	37.67 ± 2.51
Carcinosarcoma	50.00 ± 5.00	36.67 ± 6.42

Age and BMI of women were analysed using one-way analysis of variance with Dunnett's multiple comparison test with atrophic used as the control. None of the data show statistically significant differences among groups.

RNA quality and quantity assessment

The purity of the extracted RNA, as measured with a Nanodrop spectrophotometer, indicated good quality RNA with an absorbance ratio of 2.10 ± 0.31 (OD A_{260}/A_{280} mean ratio ± SD), indicating that all the samples were free from proteins potentially accruing during the RNA extraction step. The absorbance ratio at 260 and 230 nm was 2.19 ± 0.43 (OD A_{260}/A_{230} mean ratio ± SD) indicating that samples were free from contaminants. The average yield of RNA after the extraction was 1.17 ± 0.61 μg/μl (mean ± SD) and ranged between 0.125 and 3.038 μg/μl.

Expression levels of endogenous control reference genes and the *CB1* test gene

Overall comparison of the Ct values in normal endometrium and endometrial cancer demonstrated a wide variation (Table III) in mRNA expression. *18S* showed the highest expression level with a mean Ct value (±SEM) of 13.31 ± 1.23, while hydroxymethylbilane synthase (*HMBS*) showed the lowest expression in the normal endometrium (31.39 ± 0.76). In addition, alpha growth arrest and DNA-damage include (*GADD45A*), TATA box-binding protein (*TBP*) and tyrosine 3-monooxygenase (*YWHAZ*) showed lower expression, with a Ct range of 30.57–30.74 in normal endometrium. The Ct value for *CB1* in normal endometrium was 32.34 ± 0.60. In the endometrial cancer tissues, a mean Ct value of 12.40 ± 0.69 for *18S* was the highest expression level, while 29.97 ± 0.66 for TBP and 29.63 ± 0.67 for *GADD45A* showed lower expression. The expression of *18S* was high in both normal and cancerous endometrium. V-abl Abelson murine leukaemia

Table III Ct values for the 32 normalizing genes.

Gene ID	Normal endometrium Ct value (mean ± SD) $n = 9$	Endometrial cancer Ct value (mean ± SD) $n = 15$	*P*-value
18S	13.31 ± 3.70	12.40 ± 2.70	0.4938
ABL1	27.55 ± 3.00	27.12 ± 1.70	0.6503
ACTB	24.03 ± 3.23	23.73 ± 2.54	0.7706
B2M	23.61 ± 3.20	21.67 ± 2.51	0.1117
CASC3	27.35 ± 3.12	26.02 ± 1.68	0.1854
*CDKN1A**	28.98 ± 2.31	26.92 ± 2.25	0.0429
CDKN1B	27.07 ± 2.76	26.34 ± 2.03	0.4639
EIF2B1	29.58 ± 2.95	27.69 ± 1.76	0.0606
*ELF1**	28.20 ± 3.03	25.64 ± 2.01	0.0205
GADD45A	30.74 ± 2.19	29.63 ± 2.61	0.2971
*GAPDH***	25.90 ± 3.10	22.57 ± 2.35	0.0067
*GUSB**	29.82 ± 2.76	27.26 ± 2.22	0.0203
*HMBS****	31.39 ± 2.31	28.44 ± 1.25	0.0005
HPRT1	29.43 ± 3.32	27.28 ± 2.75	0.1000
*IPO8***	29.43 ± 2.75	26.47 ± 2.04	0.0062
MRPL19	28.14 ± 3.34	25.26 ± 2.77	0.0324
MT-ATP6	24.70 ± 3.98	22.33 ± 3.68	0.1522
*PES1***	28.10 ± 2.14	26.15 ± 1.08	0.0068
*PGK1***	26.65 ± 2.58	24.00 ± 1.76	0.0067
POLR2A	27.38 ± 2.44	26.24 ± 1.18	0.1358
*POP4**	29.45 ± 2.86	27.11 ± 1.76	0.0206
*PPIA**	24.60 ± 2.17	22.33 ± 1.89	0.0132
*PSMC4***	29.19 ± 2.71	26.66 ± 1.64	0.0090
PUM1	27.63 ± 2.69	25.89 ± 1.65	0.0606
RPL30	28.34 ± 4.54	26.20 ± 3.75	0.2235
RPL37A	24.60 ± 3.15	22.75 ± 2.61	0.1340
RPLP0	25.27 ± 3.67	22.81 ± 2.83	0.0785
RPS17	24.53 ± 2.86	22.39 ± 2.27	0.0547
TBP	30.71 ± 2.44	29.97 ± 2.59	0.4965
*TFRC***	29.82 ± 3.02	26.57 ± 2.48	0.0090
*UBC**	25.25 ± 2.26	22.92 ± 1.88	0.0123
*YWHAZ**	30.57 ± 2.50	28.35 ± 2.37	0.0403

The Ct values were obtained from the data output file of the StepOne Plus program. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; unpaired Student's *t*-test endometrial cancer compared with normal endometrium. Exact *P*-values are shown in the final column.

viral oncogene homolog 1 (*ABL1*) showed expression levels (Ct) in the same range for both the normal (27.55 ± 1.00) and malignant tissues (27.12 ± 0.43). Interestingly, the expression level of *CB1* in endometrial cancer was approximately in the same range as for normal endometrium, with a mean Ct value (± SEM) at 32.37 ± 0.63.

Using an unpaired Student's *t*-test, the Ct values of all the 32 potential endogenous control reference genes and *CB1*, between the endometrial cancer and normal endometrial tissues were compared. Statistically significant decreases (in normal endometrium tissues versus endometrial cancer) in the expression levels of the following reference genes were observed: *GAPDH* ($P = 0.0067$), beta glucuronidase (*GUSB*) ($P = 0.0203$), importin 8 (*IP08*) ($P = 0.0062$), *HMBS* ($P = 0.0005$), phosphoglycerate kinase (*PGK1*) ($P = 0.0067$), transferrin receptor (*TFRC*) ($P = 0.009$), ubiquitin C (*UBC*) ($P = 0.0123$), *YWHAZ* ($P = 0.040$), peptidylpropyl isomerase A (*PPIA*) ($P = 0.0132$), cyclin-dependent kinase inhibitor 1A (*CDKN1A*) ($P = 0.0429$), proteasome 26S subunit, ATPase, 4 (*PSMC4*) ($P = 0.009$), Pescadillo homolog 1 (*PES1*) ($P = 0.0068$), E74-like factor 1 (*ELF1*) ($P = 0.0205$) and processing of precursor 4 (*POP4*) ($P = 0.0206$). In contrast, the following reference genes, and the test gene *CB1*, did not differ in normal endometrium versus endometrial cancer tissues; *18S*, hypoxanthine phosphoribosyl transferase (*HPRT10*, *ACTB*, beta-2-macroglobulin (*B2M*), ribosomal protein, large, P0 (*RPLP0*), *TBP*, Polymerase (RNA) II (DNA-directed) polypeptide A (*POLR2A*), cancer susceptibility candidate 3 (*CASC3*), cyclin-dependent kinase inhibitor 1B (*CDKN1B*), *GADD45A*, Pumilio homolog 1 (Drosophila) (*PUM1*), eukaryotic translation initiator factor 2B, subunit 1 alpha (*EIF2B1*), *ABL1*, mitochondrially encoded ATP synthase 6 (*MT-ATP6*), mitochondrial ribosomal protein 19 (*MRPL19*), ribosomal protein 37A (*RPL37A*), ribosomal protein L30 (*RPL30*), ribosomal protein S17 (*RPS17*).

Analysis of reference gene stability

The geNormPLUS (q base^{PLUS2}) algorithm

The geNorm^{PLUS} (q base^{PLUS2}) algorithm determines the medium reference target stability measure (*M*), as the average pair-wise variation of each reference gene in relation to all the other reference genes enabling the elimination of the least stable gene. This is followed by recalculation of the *M* values resulting in ranking of the most stable genes, i.e. the lower the *M* value, the higher the gene stability. The software indicates that a good stable reference gene should have an average geNorm *M* value ≤1.0 in a heterogeneous set of samples (Hellemans *et al.*, 2007). Using geNorm^{PLUS} (q base^{PLUS2}) the rank order of the reference genes evaluated (from least stable to most stable) was ribosomal protein L30 (*RPL30*)> *18S*> mitochondrially encoded ATP synthase 6 (*MT-ATP6*)> beta actin (*ACTB*)> TATA box-binding protein (*TBP*)> ribosomal protein, large, P0 (*RPLP0*)> hypoxanthine phosphoribosyl transferase (*HPRT1*)> *TFRC*> *CDKN1A*> *PES1*> *POLR2A*> HMBS> *GADD45A*> *GAPDH*> *RPL37A*> *ABL1*> *B2M*> *RPS17*> *CDKN1B*> *POP4*> *CASC3*> *EIF2B1*> *PSMC4*> *GUSB*> *YWHAZ*> *ELF1*> importin 8 (*IPO8*)> *PUM1*> *UBC*> *PGK1*> *MRPL19*> *PPIA* (Fig. 1). Analysis of the rank order indicated that *MRPL19* and *PPIA* ($M \leq 1.0$) were the two most stable genes in normal and cancer endometrium samples, and the two least stable genes were *RPL30* and *18S*. The commonly used β-actin (*ACTB*) and *GAPDH* reference genes were in the least stable ($M \geq 1.0$) category, with *M*-values of 1.387 and 1.150, respectively.

Furthermore, the geNorm^{PLUS} (q base^{PLUS2}) algorithm was used to calculate the minimum number of genes required for a reliable normalization factor, using the pair-wise variation V_n/V_{n+1} between two sequential normalization factors (NF) NF_n and NF_{n+1}. In addition, this software defines a pair-wise variation of 0.15 as the optimal cut off value, below which the inclusion of additional reference genes is unnecessary (Fig. 2) (Hellemans *et al.*, 2007). Gene stability analysis revealed that the optimal number of reference targets was five genes (geNorm $V <$ 0.15 when comparing a normalization factor based on the five or six most stable targets). geNorm^{PLUS} (qbase^{PLUS2}) thus predicted that the

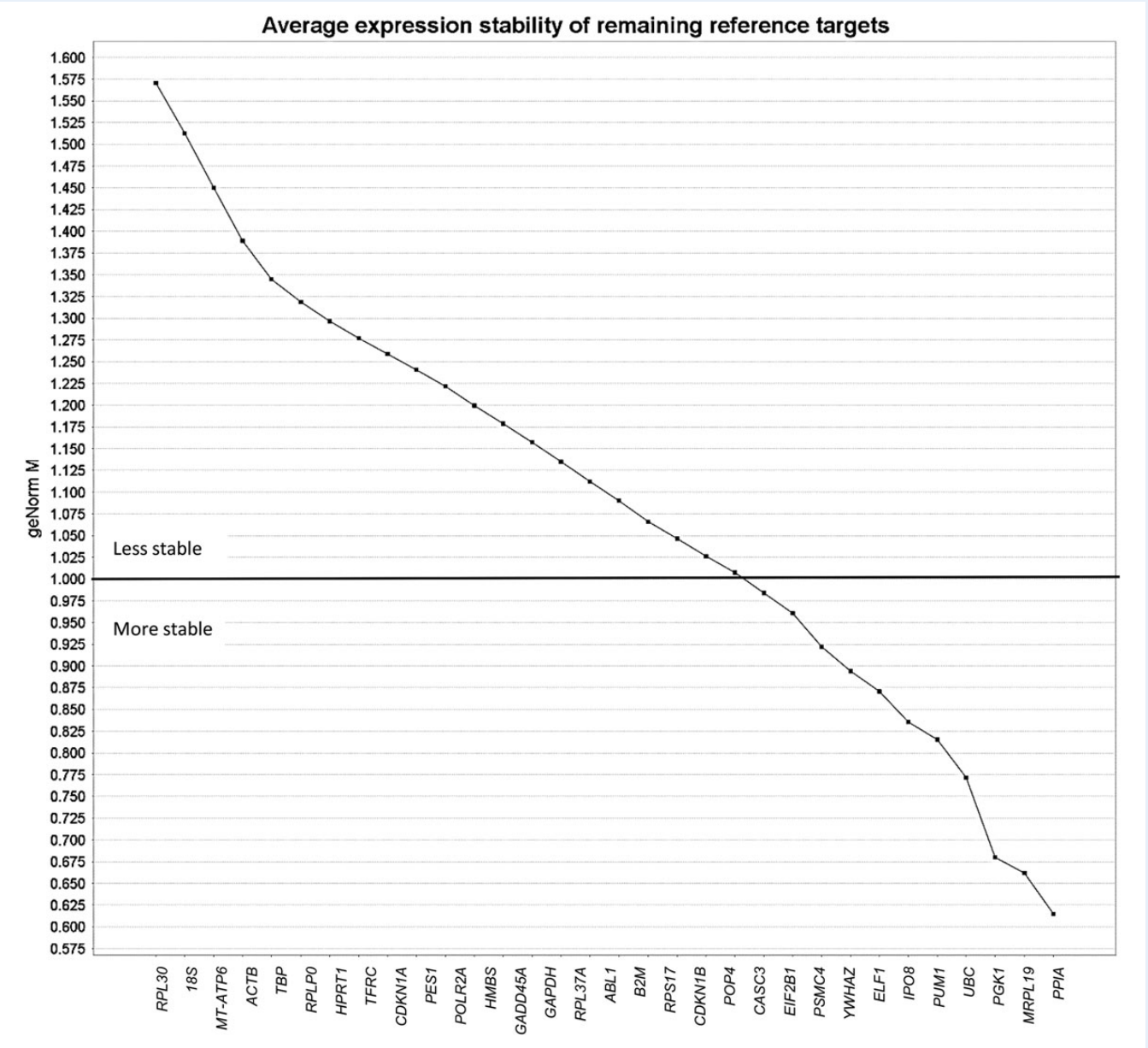

Figure 1 geNorm analyses of reference genes. Medium reference target stability values (average geNorm $M \leq 1.0$) are shown. Average expression stability values (M, y-axis) of 32 reference genes (x-axis) and the associated ranking from least to most stable expression (left to right). Results are presented according to the output file obtained from qBasePLUS2 software. The $M \leq 1.0$ of average expression stability indicates more stable expression, using both normal ($n = 9$) and malignant ($n = 15$) endometrial samples. The gene names are: 18S RNA ribosomal unit 1 (*18S*), V-abl Abelson murine leukaemia viral oncogene homolog 1 (*ABL1*), beta actin (*ACTB*), beta 2-macroglobulin (*B2M*), cancer susceptibility candidate 3 (*CASC3*), cyclin-dependent kinase inhibitor 1A (*CDKN1A*), cyclin-dependent kinase inhibitor 1B (*CDKN1B*), eukaryotic translation initiator factor 2B, subunit 1 alpha (*EIF2B1*), E74-like factor 1 (*ELF1*), alpha growth arrest and DNA-damage include (*GADD45A*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta glucuronidase (*GUSB*), hydroxymethylbilane synthase (*HMBS*), hypoxanthine phosphoribosyl transferase (*HPRT1*), importin 8 (*IPO8*), mitochondrial ribosomal protein L19 (*MRPL19*), mitochondrially encoded ATP synthase 6 (*MT-ATP6*), Pescadillo homolog 1 (*PES1*), phosphoglycerate kinase (*PGK1*), Polymerase (RNA) II (DNA-directed) polypeptide A (*POLR2A*), processing of precursor 4 (*POP4*), peptidylpropyl isomerase A (cyclophilin A) (*PPIA*), proteasome 26S subunit, ATPase, 4 (*PSMC4*), Pumilio homolog 1 (Drosophila) (*PUM1*), ribosomal protein L30 (*RPL30*), ribosomal protein 37A (*RPL37A*), ribosomal protein, large, P0 (*RPLP0*), ribosomal protein S17 (*RPS17*), TATA box-binding protein (*TBP*), transferrin receptor (*TFRC*), ubiquitin C (*UBC*) and tyrosine 3-monooxygenase (*YWHAZ*).

optimal normalization factor would be the geometric mean of the reference targets *PUM1*, *UBC*, *PGK1*, *MRPL19* and *PPIA* when based on the five most stable genes, or *IPO8*, *PUM1*, *UBC*, *PGK1*, *MRPL19* and *PPIA* when based on the six most stable reference gene targets.

Normfinder algorithm

NormFinder uses a mathematical model which takes into consideration the intergroup and intragroup expression variations (stability) and then ranks them in order; the lower the stability value the better the reference

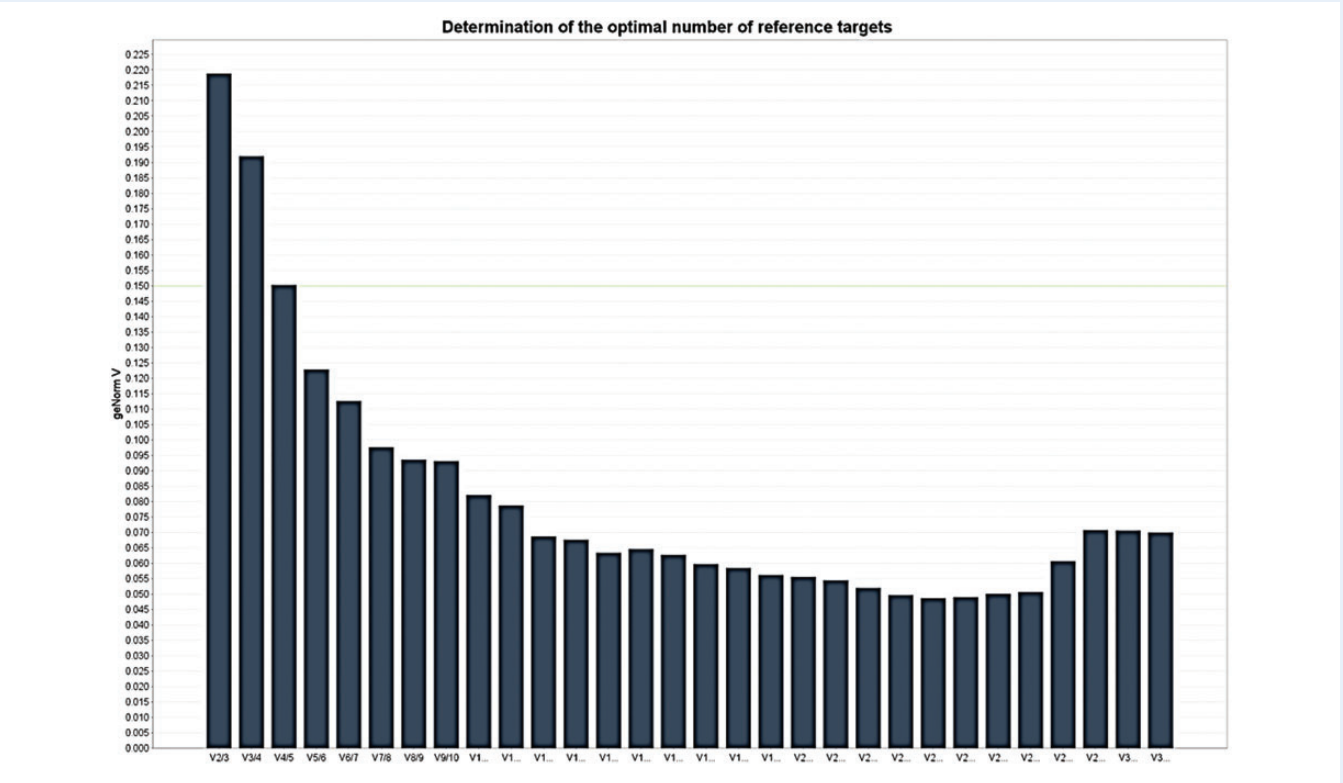

Figure 2 geNorm^{PLUS} analysis of 32 reference genes. Results are presented according to the output file obtained from qBase^{PLUS2}. Determination of the optimal number of reference genes for normalization depends on pair-wise variation (*V*) analyses, using both normal ($n = 9$) and malignant ($n = 15$) endometrial samples. The *x*-axis represents the incremental combination of reference genes from V2/3 to V31/32. The most stable combination with the least number of genes is V4/5 and V5/6. The threshold at 0.15 geNorm V is indicated with a darker horizontal line.

gene or reference gene combination (Normfinder, 2005). NormFinder selected mitochondrial ribosomal protein L19 (*MRPL19*) with a stability value of 0.5353 as the single most stable gene and indicated that the best combination of two genes was importin 8 (*IPO8*) and cyclophilin A (*PPIA*) with a stability value of 0.3824 (Fig. 3).

Bestkeeper algorithm

BestKeeper is a widely available software package used to generate the best stable reference genes, but has a limitation in that it can only work with 10 genes. Therefore, following the geNorm and NormFinder analyses for the most stable gene expression, the best 10 candidate reference control genes (*MRPL19*, *ELF1*, *PPIA*, *PUM1*, *YWHAZ*, *PGK1*, *GUSB*, *IP08*, *UBC* and *EIF2B1*) were chosen for assessment using the BestKeeper algorithm. Since BestKeeper calculates SD and the coefficient of variance (CV) based on raw Ct values, the average Ct values for each gene were calculated and imported to the Microsoft Excel-based BestKeeper application (Pfaffl *et al.*, 2004), where a SD <1 indicates putative stably expressed genes. The software estimates the BestKeeper index (BI) as the geometric mean of the stable control genes' Ct values and then creates pair-wise correlations between each gene and the BI. Reference genes with the highest Pearson correlation coefficient ($r \sim 1$) and probability (P) <0.05 (Table IV) are then considered to be most stable.

The best correlation between one endogenous reference control gene and the BI was obtained for *MRPL19* ($r = 0.98$), followed by *ELF1*, *PPIA*, *PUM1* and *YWHAZ*, *PGK1*, *GUSB*, *IPO8*, *UBC* and *EIF2B1*. BestKeeper analyses revealed that *MRPL19* (SD 1.66) was the gene with the lowest overall variation, followed by *PUM1* (SD 1.73), *IPO8* (SD 1.79) and *PPIA* (SD 1.81), whereas *GUSB* was the highest (SD 2.21) (Table IV).

The effect of using individual reference genes to normalize data for genes of interest

The expression levels of *CB1* in the malignant endometrial tissue (study group) compared with the normal endometrium (control group) following normalization with each of the 32 potential reference genes are shown in Figs 4 and 5. The data indicate that relative *CB1* mRNA expression levels were unchanged, decreased or increased, depending on the reference gene selected. The relative expression, normalized by geometric means of individual endogenous control reference genes (*CDKN1A*, *PES1*, *PGK1*, *POP4*, *PPIA*, *PSMC4*, *PUM1*, *GAPDH*, *GUSB*, *HMBS*, *RPS17*, *TFRC*, *UBC*, *YWHAZ*, *ELF1*, *MRPL19* and *IP08)* showed statistically significant ($P < 0.05$) down-regulation of *CB1* in the endometrial cancer tissues. In contrast, when *CB1* was normalized against the endogenous control reference genes *MT-ATP6*, *CASC3*, *CDKN1B*, *POLR2A*, *GADD45A*, *HPRT1*, *18S*, *ACTB*, *ABL1*, *B2M*, *EIF2B1*, *RPL37A*, *RPLP0*, *TBP* or *RPL30* the difference between normal and malignant tissue was not statistically significant. The mean Ct values for CB1 expression ($\pm$SD) were 30.26 ± 0.26 for normal endometrium ($n = 9$) and 34.37 ± 2.60 for the endometrial cancer samples ($n = 15$).

Expression stability of putative reference genes recommended by the three software packages

In order to test the effects of using combinations of reference genes on the gene expression profiles, the relative expression of *CB1* mRNA in the endometrial cancer tissues (study group) and the normal endometrium (control group) was calculated using the recommended combinations of genes from the three software packages, geNorm, NormFinder and BestKeeper. The relative expression of *CB1* using *PUM1*, *UBC*, *PGK1*, *MRPL19* and *PPIA* as the best reference gene combinations recommended by geNorm-qBasePLUS, revealed statistically significant ($P = 0.0067$) down-regulation of *CB1* in malignant tissue (Fig. 6A).

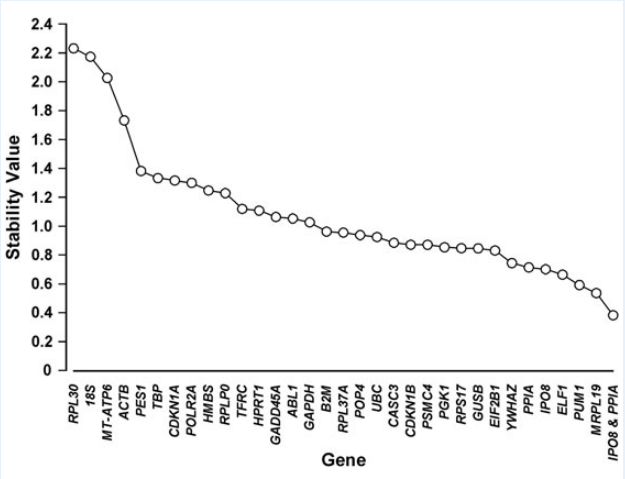

Figure 3 Stability values for the 32 endogenous 'housekeeping genes' as ranked by NormFinder. High expression stability is shown by a low stability value as an evaluation of the combined intergroup and intragroup variations of the individual reference genes, thus the most stable genes are to the right. NormFinder also calculated that most stable gene combination was importin 8 (*IPO8*) and cyclophilin A (*PPIA*). Both normal ($n = 9$) and malignant ($n = 15$) endometrial samples were used.

CB1 expression was also significantly ($P = 0.0027$) down-regulated in malignancy when normalized against the single best gene (*MRPL19*), as recommended by NormFinder and BestKeeper (Fig. 6B). Furthermore, using the best two-gene combination recommended by NormFinder (*IPO8* and *PPIA*), *CB1* expression was also significantly ($P = 0.0067$) down-regulated in the malignant samples (Fig. 6C). Interestingly, the statistically significant values following the normalization recommended by geNorm and Normfinder (two best combination genes) were similar. The normalized *CB1* expression data using the genes recommended by the three software packages are displayed in Fig. 6.

The effect of using three endogenous control reference genes (*MRPL19*, *IPO8* and *PPIA*) for normalization

Finally, when the expression levels of *MRPL19*, *PPIA* and *IPO8* were combined and evaluated as the endogenous control for normalization of *CB1* transcript levels (Fig. 6D), the data showed that there was a statistically significant ($P = 0.0004$) decrease in *CB1* expression in the endometrial cancer tissues when compared with the normal endometrium (control group). This is further illustrated in Fig. 6A–D where the *P*-values for the difference in *CB1* expression depended on the number of endogenous control genes and type of endogenous control used, with the expression of *CB1* transcripts being most significantly ($P < 0.0001$) down-regulated when normalized against the geometric mean of *MRPL19*, *IPO8* and *PPIA*. The *P*-value of this normalization manipulation was lower than any other combination of endogenous control genes, suggesting that these three genes provide the best normalizer of gene expression for studies of human endometrial cancer, when normal endometrial tissue is used as the control.

Discussion

qRT–PCR is a robust and sensitive technique often used to determine small changes in gene expression profiles in diverse tissues. It is especially useful when tissue samples are scarce (as for a less common form of cancer), small or difficult to obtain because the malignant tissue is small or intermingled with other non-malignant tissues, as in the case of some endometrial cancers or normal endometrial biopsies.

Table IV BestKeeper output.

Measurements	*MRPL19*	*PPIA*	*PUM1*	*ELF1*	*IPO8*	*YWHAZ*	*EIF2B1*	*GUSB*	*PGK1*	*UBC*
N	24	24	24	24	24	24	24	24	24	24
Geo Mean Ct	27.57	23.08	26.46	26.47	27.57	29.07	28.30	28.10	24.93	23.69
Ar Mean Ct	27.64	23.18	26.55	26.60	27.66	29.18	28.39	28.22	25.04	23.79
Min Ct	24.96	19.79	22.85	22.92	24.19	25.53	24.87	23.64	21.54	19.47
Max Ct	32.44	27.95	31.90	33.42	33.92	34.36	34.93	33.94	30.81	28.59
SD Ct	1.66	1.81	1.73	2.11	1.79	2.15	1.80	2.21	1.88	1.83
CV % Ct	6.02	7.81	6.53	7.95	6.49	7.36	6.34	7.82	7.53	7.71
Coeff. of corr (*r*)	0.979	0.972	0.970	0.978	0.962	0.970	0.933	0.963	0.965	0.959
P-value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

N, number of samples; Geo Mean Ct, geometric mean of Ct values; Ar Mean Ct, arithmetic mean of the Ct values; Min and Max Ct, extreme values of the Ct; SD Ct, standard deviation of the Ct values; CV % Ct, coefficient of variance expressed as percentage on the Ct level; Coeff. of corr (*r*), coefficient of correlation (*r*).

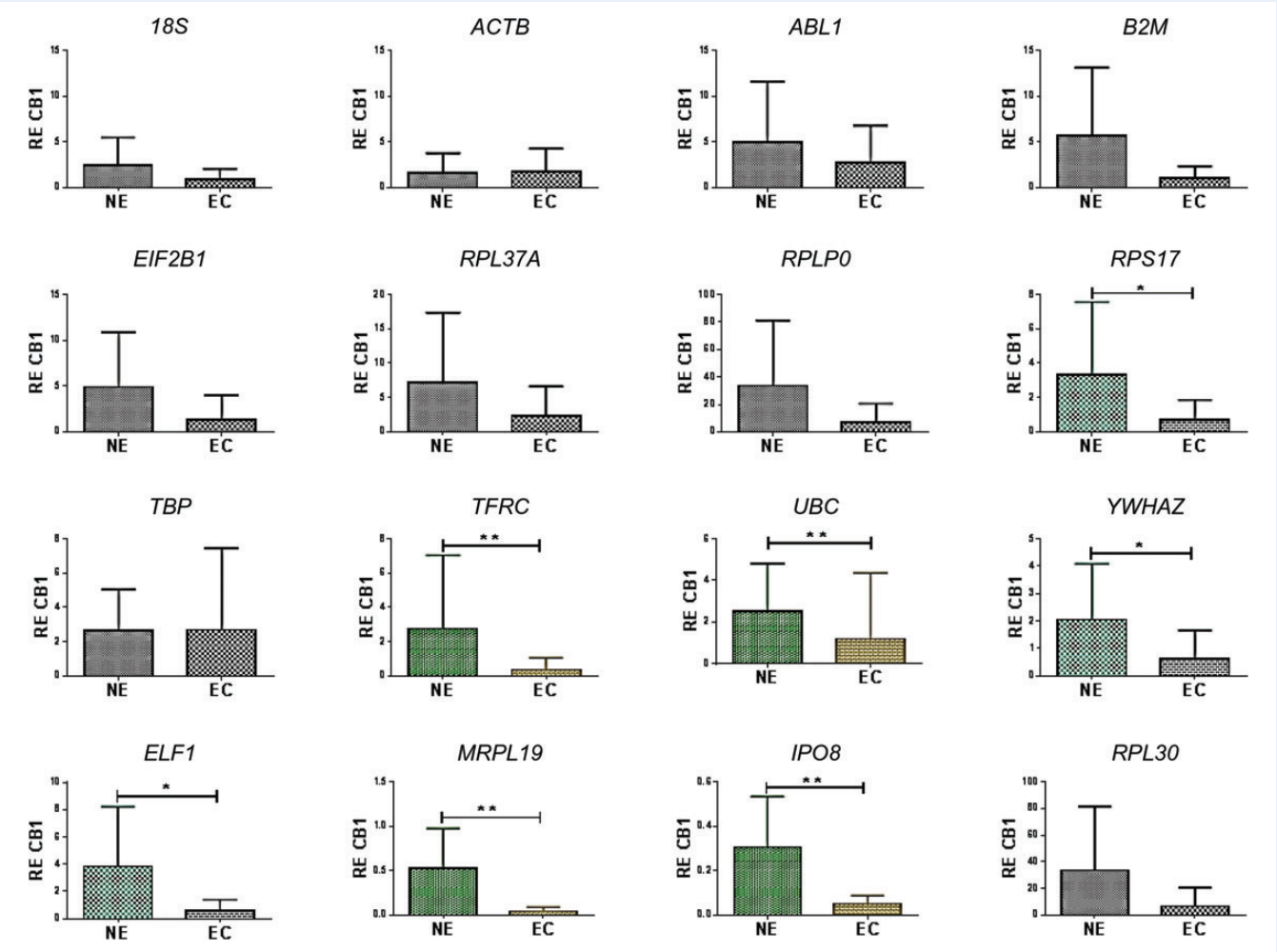

Figure 4 The relative expression of cannabinoid receptor 1 (*CB1*) in endometrial carcinoma using the first set of 16 endogenous controls reference genes as an internal control. Samples used for analysis were from the endometrial carcinoma (EC; Type 1 and 2) and normal endometrium (NE; atrophic, secretory and proliferative) used as the control ($n = 3$ in all cases). Relative expression of *CB1* was statistically significantly lower ($P < 0.05$) when ribosomal protein S17 (*RPS17*), tyrosine 3 monooxygenase (*YWHAZ*) or E74-like factor 1 (*ELF1*); ($P < 0.001$) when transferrin receptor (*TFRC*), ubiquitin C (*UBC*), mitochondrial ribosomal protein 19 (*MRPL19*) or importin 8 (*IPO8*), were used as the single internal control reference gene. Unpaired Student's *t*-test was used for the statistical analysis. The non-colour graphs using eukaryotic 18S ribosomal RNA (*18S*), beta actin (*ACTB*), v-abl Abelson murine leukaemia viral oncogene homolog 1 (*ABL1*), beta-2-macroglobulin (*B2M*), eukaryotic translation initiator factor 2B, subunit 1 alpha (*EIF2B1*), ribosomal protein 37A (*RPL37A*), ribosomal protein, large, P0 (*RPLP0*), TATA box-binding protein (*TBP*) or ribosomal protein L30 (*RPL30*) as the internal control gene did not reach statistical significance.

However, to generate valid data from such studies, the correct endogenous control reference gene(s) for normalization of data must be found, since failure to do so may lead to erroneous conclusions. The use of the correct endogenous control reference gene(s) for normalization in qRT–PCR experiments has been the subject of vigorous debate (Thellin *et al.*, 1999), with the advantages and disadvantages discussed (Bustin, 2002), even given the MIQE guidelines (Bustin *et al.*, 2009). Several studies have shown differences in the stability of reference genes in a variety of tissues (Hoogewijs *et al.*, 2008) and also in the same tissues under different conditions (Nelson, 2004), therefore, defining the experimental conditions is important for the appropriate selection of the correct reference genes. In this study, normal endometrial tissues showed wide variations in housekeeping gene Ct values ranging from 13.31 to 31.39, with the highest expression of *18S* and the lowest expression of *HMBS*. Overall levels of Ct values in endometrial cancer revealed that *18S* was also the most abundant, whilst *TBP* and *GADD45A* showed lowest expression. This observation is expected because *18S* is a ribosomal RNA (rRNA) whereas all other genes used are transcribed to produce mRNA. This discourages many from using *18S* as an endogenous reference to normalize gene expression data, as the proportion of rRNA to mRNA is very high (rRNA more than 80% of total versus 1–4% mRNA), resulting in a false sense of security when using *18S*. However, as this study found, *18S* was the least stable endogenous reference gene, indicating that the rRNA machinery could be dramatically affected in endometrial cancer. Additionally, a wide variation in Ct values was observed among the normal and cancer endometrial samples for all the individual putative reference genes. These wide variations may be due to the effect of cancer on the modulation of

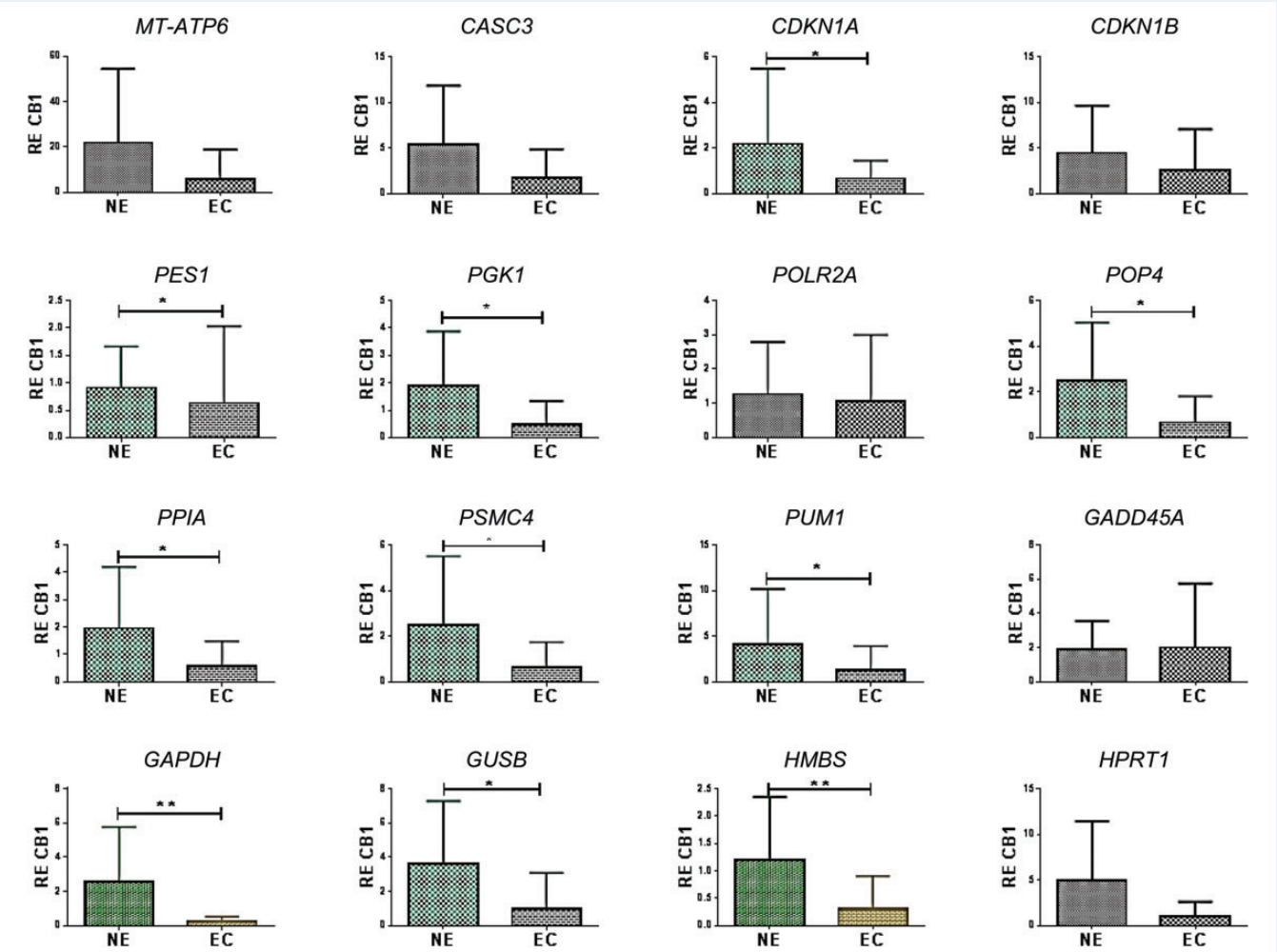

Figure 5 The relative expression of *CB1* in endometrial carcinoma using the second set of 16 endogenous controls reference genes as an internal control. Details of the analyses are the same as that described in the legend to Fig. 4. Relative expression of *CB1* was significantly lower ($P < 0.05$) when cyclin-dependent kinase inhibitor 1A (*CDKN1A*), Pescadillo homolog 1 (*PES1*), phosphoglycerate kinase (*PGK1*), processing of precursor 4 (*POP4*), peptidylpropyl isomerase A (*PPIA*), proteasome 26S subunit (*PSMC4*), Pumilio homolog 1 (*PUM1*) or beta glucuronidase (*GUSB*); ($P < 0.001$) when glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or hydroxymethylbilane synthase (*HMBS*) were used as the single internal control reference gene. Unpaired Student's *t*-test was used for the statistical analysis. The non-colour graphs using mitochondrially encoded ATP synthase 6 (*MT-ATP6*), cancer susceptibility candidate 3 (*CASC3*), cyclin-dependent kinase inhibitor 1B (*CDKN1B*), polymerase (RNA) II polypeptide A (*POLR2A*), alpha growth arrest and DNA-damage-include (*GADD45A*) and hypoxanthine phosphoribosyl transferase (*HPRT1*) as the internal control gene did not reach statistical significance.

gene expressions of the 32 potential reference genes that have diverse cellular functions. These data therefore support the recommendation that arbitrary selection of a single reference gene is not appropriate (MIQE guidelines (Bustin *et al.*, 2009)) and should be avoided, because these reference genes may be differentially regulated and thus increase the risk of producing false data. Furthermore, the normalizing gene Ct values should be close to that of the target gene (Dheda *et al.*, 2004).

The three software programs employed here use different mathematical models for the identification of the most stable reference gene, either alone or in combination with others (Pfaffl *et al.*, 2004; Normfinder, 2005; Hellemans *et al.*, 2007). Interestingly, there were only minor variations noted in the ranking of the individual reference genes and whilst geNorm indicated that the optimal reference target selection was achieved using 5 or 6 of the most stable reference genes (*IPO8*, *PUM1*, *UBC*, *PGK1*, *MRPL19* and *PPIA*), NormFinder identified *MRPL19* as the single most stable gene, while the best combination of two genes was *IPO8* and *PPIA*. The data from BestKeeper were similar to that generated by NormFinder, indicating that MRPL19 was the best single stable reference gene, followed by *ELF1* and *PPIA*. Thus, from the analysis of all three software packages, *MRPL19* and *PPIA* emerged as the most stable normalizer genes. Although the overall ranking of the 32 reference genes using geNorm and Normfinder was different, the worst genes were the same. The results of the NormFinder analysis identified RPL30 and 18S as the least stable genes, which was similar to the geNorm software results.

The MIQE guidelines state that improved normalization is possible when the use of one reference gene is changed to that of multiple endogenous control reference genes, because it is unlikely that a single

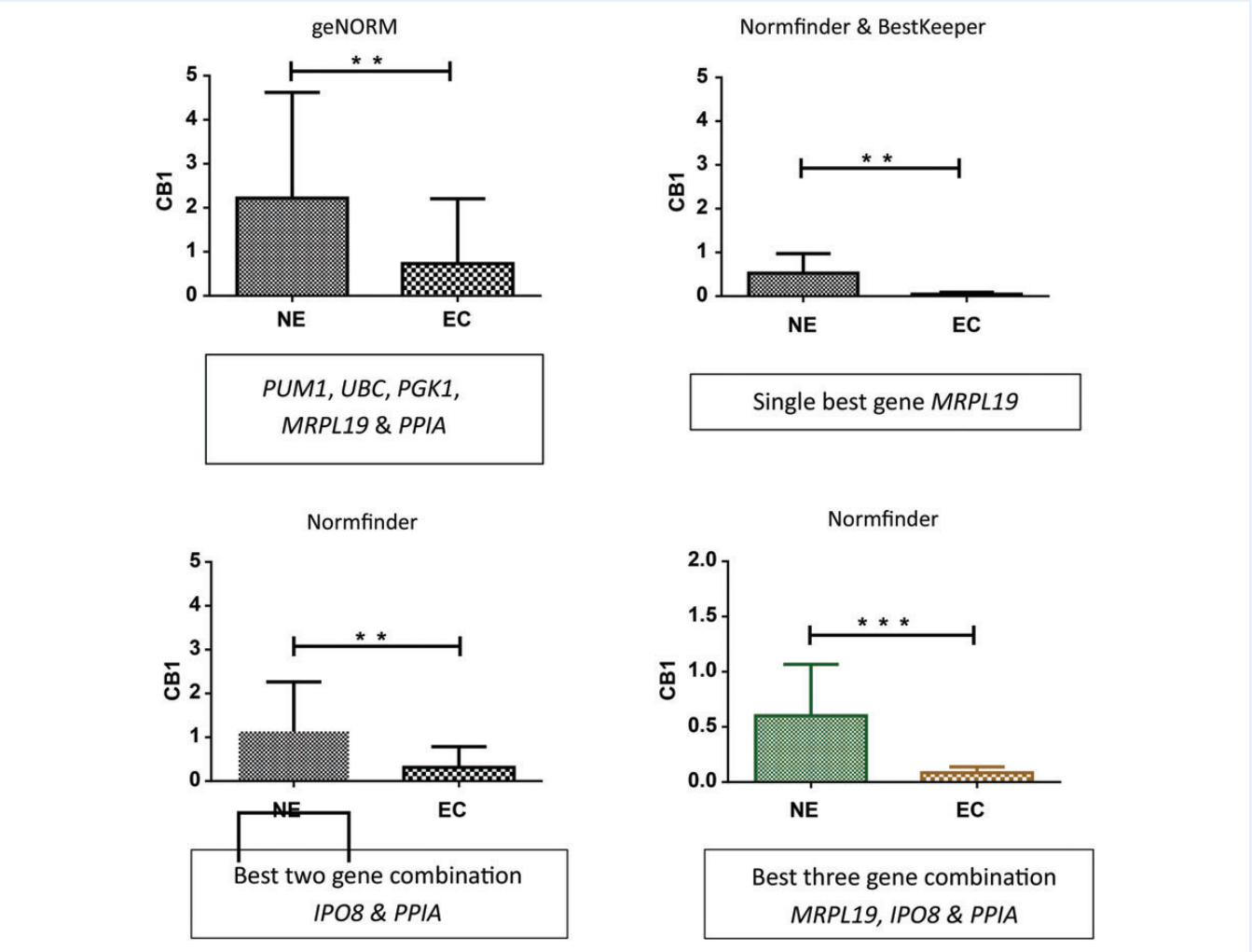

Figure 6 Relative *CB1* expressions in endometrial carcinoma using the indicated combinations of genes for normalization from the three software packages. Samples from the endometrial carcinoma group (EC; Type 1 and 2) were compared with the normal endometrium group (NE; atrophic, secretory and proliferative phases) ($n = 3$ in all cases). Data are presented as the mean ± SD; $^{**}P < 0.001$; $^{***}P < 0.0001$, unpaired Student's *t*-test. The data above each graph indicate the software used and the box below each graph indicates the predicted best gene or gene combinations.

reference gene would provide an ideal endogenous control (Bustin *et al.*, 2009). Similarly, the optimal normalization factor calculated by using a combination of genes would thus produce more accurate data, as this is more likely to minimize gene-to-gene and sample-to-sample variation. To evaluate the normalization effects of various reference genes on gene expression profiles, the expression of *CB1* mRNA was determined using each of the 32 reference genes as the endogenous control. *CB1* was selected because it is expressed in endometrial cancer (Guida *et al.*, 2010) and is known to be regulated at the transcript (Gebeh *et al.*, 2012b) and protein level throughout the menstrual cycle (Taylor *et al.*, 2010) and because our research group has a specific interest in the regulation of mammalian reproduction and sex steroid hormone-dependent cancers by the endocannabinoid system.

The results for the endometrial cancer samples indicated that *CB1* mRNA level appeared either unchanged, up-regulated or down-regulated compared with normal endometrial samples depending on the individual reference gene selected; supporting our original assertion that selection of the correct reference gene or combination of reference genes is critical to obtaining the correct expression profile. Indeed, in other tissues, such as the testis (Svingen *et al.*, 2014) and Fallopian tube (Gebeh *et al.*, 2012a), the numerous different cell types can dramatically alter gene expression profiles. Thus, the selection of endogenous reference genes and more importantly, how these should be combined, becomes a critical first step in studies involving gene expression profiling of dynamic tissues, such as those of the reproductive tract or those affected by rapid proliferation, such as malignant tissues. To the best of our knowledge, this is the first study that has systematically assessed the most commonly used endogenous control reference genes for normalization of qRT–PCR investigations in malignant and normal endometrial samples.

In summary, a TaqMan® gene Expression Assay panel of 32 endogenous control reference genes, which represent different gene families and functional classes for use as reference genes to normalize the qRT–PCR data, were evaluated either alone or with all three commonly available

software packages (geNorm, NormFinder and BestKeeper) to ascertain the best endogenous control reference genes. The study environment consisted of multiple patient groups and in addition had within group variation. This was demonstrated by the average Ct values (Table III) and by the BestKeeper analysis (Table IV), where there was a significant difference in the average Ct values between normal and malignant tissue and the SD of all best endogenous reference genes were greater than 1, when an SD <1 was considered to be a good indicator, when using BestKeeper software. Therefore, taking into account this factor, we discovered that the NormFinder software package was the most robust method of evaluating reference gene stability because it takes into consideration both the inter- and intra-variability during stabilization assessment, where BestKeeper does not. Using NormFinder, *MRPL19* was revealed to be the best single reference gene and that *PPIA* and *IP08* are the best two-gene combination. To validate the best stable endogenous control to use in our study environment, *MRPL19*, *PPIA* and *IP08* were combined and used to normalize the expression of our gene of interest (CB1) and showed that *CB1* expression is reduced in endometrial cancer. It is thus our recommendation that when comparing gene expression in normal and malignant endometrial tissues by qRT–PCR, the most reliable normalization is achieved when using a combination of *MRPL19*, *PPIA* and *IPO8* as the endogenous reference genes.

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Authors' roles

T.A. designed, performed, and analysed the research and wrote the paper. A.H.T. participated in data analysis and helped to draft the manuscript. J.M.W. supervised the research. L.B. helped in FIGO classification and provided the biopsy sample for the analysis. D.G.L. helped to provide lab space to perform total RNA. J.M. helped to teach T.A. how to process the tissues into cDNA. Q.D. helped in providing the tissue samples. E.L.M. helped in providing tissue samples and helped in the construction of the manuscript. J.C.K. helped in the preparation of the manuscript, is the chief investigator and the main supervisor of the research.

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Conflict of interest

None declared.

References

American Cancer Society. *Cancer Facts and Figures 2012*. American Cancer Society, Atlanta, GA, USA, 2012. http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2012/ (12 May 2015, date last accessed).

Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004;**64**:5245–5250.

Ayakannu T, Taylor AH, Marczylo TH, Willets JM, Konje JC. The endocannabinoid system and sex steroid hormone-dependent cancers. *Int J Endocrinol* 2013;**2013**:259676.

Baldinu P, Cossu A, Manca A, Satta MP, Sini MC, Palomba G, Dessole S, Cherchi P, Mara L, Tanda F *et al.* CASC2a gene is down-regulated in endometrial cancer. *Anticancer Res* 2007;**27**:235–243.

Beekman L, Tohver T, Dardari R, Leguillette R. Evaluation of suitable reference genes for gene expression studies in bronchoalveolar lavage cells from horses with inflammatory airway disease. *BMC Mol Biol* 2011; **12**:5.

Blazquez C, Salazar M, Carracedo A, Lorente M, Egia A, Gonzalez-Feria L, Haro A, Velasco G, Guzman M. Cannabinoids inhibit glioma cell invasion by down-regulating matrix metalloproteinase-2 expression. *Cancer Res* 2008;**68**:1945–1952.

Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol* 1983;**15**:10–17.

Bonefeld BE, Elfving B, Wegener G. Reference genes for normalization: a study of rat brain tissue. *Synapse* 2008;**62**:302–309.

Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, Penfold ME, Sunshine MJ, Littman DR, Kuo CJ *et al.* A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med* 2006;**203**:2201–2213.

Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000; **25**:169–193.

Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* 2002;**29**:23–39.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009;**55**:611–622.

Cancer Research UK. Cancer Statistics 2012. www.cancerresearchuk.org/cancer-info/cancerstats/keyfacts/uterine-cancer-key-facts/ (12 June 2015, date last accessed).

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques* 2004;**37**:112–119.

Dheda K, Huggett JF, Chang JS, Kim LU, Bustin SA, Johnson MA, Rook GA, Zumla A. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Anal Biochem* 2005;**344**:141–143.

Du XL, Jiang T, Sheng XG, Gao R, Li QS. Inhibition of osteopontin suppresses in vitro and in vivo angiogenesis in endometrial cancer. *Gynecol Oncol* 2009; **115**:371–376.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;**127**:2893–2917.

Gebeh A, Marczylo EL, Amoako AA, Willets JM, Konje JC. Variation in stability of endogenous reference genes in Fallopian tubes and endometrium from healthy and ectopic pregnant women. *Int J Mol Sci* 2012a;**13**: 2810–2826.

Gebeh AK, Willets JM, Marczylo EL, Taylor AH, Konje JC. Ectopic pregnancy is associated with high anandamide levels and aberrant expression of FAAH and CB1 in fallopian tubes. *J Clin Endocrinol Metab* 2012b; **97**:2827–2835.

GenEx software. version 5.3.6.170 edn, Göteborg, Sweden, 2011.

Guida M, Ligresti A, De Filippis D, D'Amico A, Petrosino S, Cipriano M, Bifulco G, Simonetti S, Orlando P, Insabato L *et al.* The levels of the

endocannabinoid receptor CB2 and its ligand 2-arachidonoylglycerol are elevated in endometrial carcinoma. *Endocrinology* 2010;**151**:921–928.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 2007;**8**:R19.

Hoogewijs D, Houthoofd K, Matthijssens F, Vandesompele J, Vanfleteren JR. Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in C. elegans. *BMC Mol Biol* 2008;**9**:9.

Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun* 2005;**6**:279–284.

Jurcevic S, Olsson B, Klinga-Levan K. Validation of suitable endogenous control genes for quantitative PCR analysis of microRNA gene expression in a rat model of endometrial cancer. *Cancer Cell Int* 2013;**13**:45.

Jursza E, Skarzyński DJ, Siemieniuch MJ. Validation of reference genes in the feline endometrium. *Reprod Biol* 2014;**14**:302–306.

Kashima H, Shiozawa T, Miyamoto T, Suzuki A, Uchikawa J, Kurai M, Konishi I. Autocrine stimulation of IGF1 in estrogen-induced growth of endometrial carcinoma cells: involvement of the mitogen-activated protein kinase pathway followed by up-regulation of cyclin D1 and cyclin E. *Endocr Relat Cancer* 2009;**16**:113–122.

Klein C, Ruttlant J, Troedsson MHT. Expression stability of putative reference genes in equine endometrial, testicular and conceptus tissues. *BMC Res Notes* 2011;**4**:120.

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K, Sindelka R, Sjoback R, Sjogreen B, Strombom L *et al.* The real-time polymerase chain reaction. *Mol Aspects Med* 2006;**27**:95–125.

Life Technologies. Amplification Efficiency of TaqMan® Gene Expression Assays. Life Technologies Application Note: lifetechnologies.com.

Mishima K, Kato Y, Kaneko MK, Nishikawa R, Hirose T, Matsutani M. Increased expression of podoplanin in malignant astrocytic tumors as a novel molecular marker of malignant progression. *Acta Neuropathol* 2006;**111**:483–488.

Mutch DG. The new FIGO staging system for cancers of the vulva, cervix, endometrium, and sarcomas. *Gynecol Oncol* 2009;**115**:325–328.

National Comprehensive Cancer Network. *NCCN Guidelines Uterine Neoplasms*. Version 1.2014, 2013. http://intervalolibre.files.wordpress.com/2012/06/nccn-c3a1ncer-de-endometrio-y-sarcomas-uterinos-2014.pdf.

NormFinder. version 0.953 edn, Aarhus University Hospital: Aarhus, Denmark, 2005.

Nelson PS. Predicting prostate cancer behavior using transcript profiles. *J Urol* 2004;**172**:S28–S32.

Noyes R, Hertig A, Rock J. Dating the endometrial biopsy. *Fertil Steril* 1950;**1**:3–25.

Papageorgiou I, Nicholls PK, Wang F, Lackmann M, Makanji Y, Salamonsen LA, Robertson DM, Harrison CA. Expression of nodal signalling components in cycling human endometrium and in endometrial cancer. *Reprod Biol Endocrinol* 2009;**7**:122.

Pecorelli S. Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. *Int J Gynaecol Obstet* 2009;**105**:103–104.

Penna I, Vella S, Gigoni A, Russo C, Cancedda R, Pagano A. Selection of candidate housekeeping genes for normalization in human postmortem brain samples. *Int J Mol Sci* 2011;**12**:5461–5470.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. *Biotechnol Lett* 2004;**26**:509–515.

Pinto F, Pacheco CC, Ferreira D, Moradas-Ferreira P, Tamagnini P. Selection of suitable reference genes for RT-qPCR analyses in cyanobacteria. *PLoS One* 2012;**7**:e34983.

Porichi O, Nikolaidou ME, Apostolaki A, Tserkezoglou A, Arnogiannaki N, Kassanos D, Margaritis L, Panotopoulou E. BCL-2, BAX and P53 expression profiles in endometrial carcinoma as studied by real-time PCR and immunohistochemistry. *Anticancer Res* 2009;**29**:3977–3982.

Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* 2004;**313**:856–862.

Romanowski T, Markiewicz A, Bednarz N, Bielawski KP. Housekeeping genes as a reference in quantitative real-time RT-PCR. *Postepy Hig Med Dosw* 2007;**61**:500–510.

Sadek KH, Cagampang FR, Bruce KD, Shreeve N, Macklon N, Cheong Y. Variation in stability of housekeeping genes in endometrium of healthy and polycystic ovarian syndrome women. *Hum Reprod* 2012;**27**:251–256.

Svingen T, Jørgensen A, Rajpert-De Meyts E. Validation of endogenous normalizing genes for expression analyses in adult human testis and germ cell neoplasms. *Mol Hum Reprod* 2014;**20**:709–718.

Taylor AH, Abbas MS, Habiba MA, Konje JC. Histomorphometric evaluation of cannabinoid receptor and anandamide modulating enzyme expression in the human endometrium through the menstrual cycle. *Histochem Cell Biol* 2010;**133**:557–565.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E. Housekeeping genes as internal standards: use and limits. *J Biotechnol* 1999;**75**:291–295.

Torres A, Torres K, Wdowiak P, Paszkowski T, Maciejewski R. Selection and validation of endogenous controls for microRNA expression studies in endometrioid endometrial cancer tissues. *Gynecol Oncol* 2013;**130**:588–594.

Vestergaard AL, Knudsen UB, Munk T, Rosbach H, Martensen PM. Transcriptional expression of type-I interferon response genes and stability of housekeeping genes in the human endometrium and endometriosis. *Mol Hum Reprod* 2011;**17**:243–254.

von Wolff M, Thaler CJ, Strowitzki T, Broome J, Stolz W, Tabibzadeh S. Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion. *Mol Hum Reprod* 2000;**6**:627–634.

Walker CG, Meier S, Mitchell MD, Roche JR, Littlejohn M. Evaluation of real-time PCR endogenous control genes for analysis of gene expression in bovine endometrium. *BMC Mol Biol* 2009;**10**:100.

Wong JY, Huggins GS, Debidda M, Munshi NC, De Vivo I. Dichloroacetate induces apoptosis in endometrial cancer cells. *Gynecol Oncol* 2008;**109**:394–402.