

# Loss of GPER identifies new targets for therapy among a subgroup of ER $\alpha$ -positive endometrial cancer patients with poor outcome

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**BACKGROUND:** The G protein-coupled oestrogen receptor, GPER, has been suggested as an alternative oestrogen receptor. Our purpose was to investigate the potential of GPER as a prognostic and predictive marker in endometrial carcinoma and to search for new drug candidates to improve treatment of aggressive disease.

**MATERIALS AND METHODS:** A total of 767 primary endometrial carcinomas derived from three patient series, including an external dataset, were studied for protein and mRNA expression levels to investigate and validate if GPER loss identifies poor prognosis and new targets for therapy in endometrial carcinoma. Gene expression levels, according to ER $\alpha$ /GPER status, were used to search the connectivity map database for small molecular inhibitors with potential for treatment of metastatic disease for receptor status subgroups.

**RESULTS:** Loss of GPER protein is significantly correlated with low GPER mRNA, high FIGO stage, non-endometrioid histology, high grade, aneuploidy and ER $\alpha$  loss (all *P*-values  $\leq 0.05$ ). Loss of GPER among ER $\alpha$ -positive patients identifies a subgroup with poor prognosis that until now has been unrecognised, with reduced 5-year survival from 93% to 76% (*P* = 0.003). Additional loss of GPER from primary to metastatic lesion counterparts further supports that loss of GPER is associated with disease progression.

**CONCLUSION:** These results support that GPER status adds clinically relevant information to ER $\alpha$  status in endometrial carcinoma and suggest a potential for new inhibitors in the treatment of metastatic endometrial cancers with ER $\alpha$  expression and GPER loss.

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In industrialised countries, endometrial cancer is the most common pelvic gynaecologic malignancy, with a 2–3% lifetime risk. Hormone receptor status in patients with endometrial cancer is an established prognostic marker (Engelsen *et al*, 2008), where loss of hormone receptors ER $\alpha$  or PR predicts poor survival. Oestrogen receptor  $\alpha$  and PR statuses have also been reported to predict response to anti-hormonal therapy in metastatic endometrial cancer (Singh *et al*, 2007). Still, in contrast to breast cancer treatment, hormone receptor status has not yet routinely been implemented in the clinics to improve and individualise endometrial cancer therapy. Thus, a more systematic approach to tailored treatment based on receptor status is needed in endometrial cancer.

The discovery of the G protein-coupled receptor GPER (formerly GPR30) as an alternative oestrogen receptor (Carmeci *et al*, 1997; Filardo *et al*, 2000) has added complexity to this task. GPER is a seven-transmembrane receptor that binds 17 $\beta$ -estradiol but differs from the classic oestrogen receptors (ER $\alpha$  and ER $\beta$ ) by

eliciting nongenomic, rapid signalling as well as genomic transcriptional events of oestrogen. The GPER has been ascribed roles in the immune, cardiovascular, endocrine and reproductive system, and dysregulation of its expression has also been reported in cancer (Maggiolini and Picard, 2010; Prossnitz and Barton, 2011).

The ER modulator tamoxifen and the ER inhibitor fulvestrant are used in the treatment of metastatic endometrial cancer, with modest response rates (Decruze and Green, 2007). Interestingly, these ER modulators also act as GPER agonists (Filardo *et al*, 2000, 2002; Thomas *et al*, 2005) and the risk of unwanted side effects from treatment is present. This highlights the need for improved definition of drug targets and molecular subgroups in addition to the ER status. Such knowledge will be particularly important to promote biomarker restricted phase I/II trials.

In the present study we hypothesised that GPER loss defines an aggressive subgroup among ER $\alpha$ -positive patients with molecular alterations reflected in distinct transcriptional signatures, indicating new targets for therapy. We report for the first time to the best of our knowledge, a link between GPER loss and poor prognosis in ER $\alpha$ -positive endometrial carcinomas. Also, drugs targeting HDACs are identified through a search in the small molecule database Connectivity Map as promising to treat metastatic disease in ER $\alpha$ -positive tumours with GPER loss.

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## MATERIALS AND METHODS

### Patient series and tumour specimens

We have studied a total of 767 endometrial cancer patients from three independent patient series with primary tumour tissue available. Two population based patient series with formalin-fixed paraffin-embedded tissue (FFPE) from primary tumours were used for immunohistochemical staining as primary investigation ( $n=182$ ) and prospective validation series ( $n=474$ ). For patients with advanced or recurrent disease in the prospective series, FFPE biopsies were collected from metastatic tissue in parallel when available, and mounted in tissue microarrays (TMAs;  $n=78$ ). From the prospective validation set, two series with freshly frozen primary tumour tissue were used for mRNA studies, including a microarray set ( $n=76$ ) and one series used for validation of candidate genes by qPCR ( $n=161$ ). Freshly frozen tissue was collected in parallel with FFPE. One publicly available external microarray validation set was also investigated ( $n=111$ ).

For the two independent population-based patient cohorts with FFPE tissue available, tissues were mounted in TMA. These patients were diagnosed with endometrial carcinoma in Hordaland County (Norway) during the periods 1981–1990 (primary investigation set) and 2001–2009 (prospective validation set). For patients in the primary investigation set, tumour tissue was retrospectively collected and patients were staged according to FIGO 1988 criteria, whereas tumour tissue from patients in the prospective validation series was included prospectively and staged according to FIGO 2009 criteria. Tissue microarrays were generated as previously described and validated in several studies (Stefansson *et al*, 2004). Briefly, the area of highest tumour grade was identified on H&E-stained slides, and three tissue cylinders were punched out from the selected areas of the donor block and mounted into a recipient paraffin block using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA). The majority of the selected samples were verified by frozen sections to contain >80% malignant epithelial component (minimum purity 50%). Age at diagnosis, FIGO stage, histological subtype and grade, treatment and follow-up were registered as previously reported (Salvesen *et al*, 1999; Trovik *et al*, 2011).

A publicly available endometrial carcinoma gene expression data set was obtained from the Expression Project for Oncology (expO: <http://www.intgen.org/expo/>). Clinico-pathologic data as well as GEO accession numbers for these 111 tumours have been described (Salvesen *et al*, 2009). The GEO data sets were run at Affymetrix U133+2 arrays (Santa Clara, CA, USA). To construct transcript level probe sets, individual probes were sequence-matched against Aceview (NCB135) (Carter *et al*, 2006; Salvesen *et al*, 2009).

### Immunohistochemistry

Tissue microarrays sections (5  $\mu$ m) were dewaxed with xylene, rehydrated in ethanol before microwave antigen retrieval, and stained for GPER expression using ab12564 and ab12563 (Abcam, Cambridge, UK) and for ER $\alpha$  expression using anti-ER $\alpha$  M7047 (Dako, Copenhagen, Denmark). The stainings were recorded as previously described (Salvesen *et al*, 2000; Engelsen *et al*, 2008). Briefly, a semiquantitative and subjective grading was used, and a staining index was calculated as a product of staining intensity (0–3) and area of positive tumour cells ( $1 \leq 10\%$ ,  $2 = 10–50\%$  and  $3 \geq 50\%$ ). Evaluation of the staining was carried out blinded for patient characteristics and outcome. In subsequent statistical analyses, staining index was categorised in quartiles considering the frequency distribution for each marker, the size of the subgroups and the number of events in each category. Groups with similar survival were merged. To estimate reproducibility of this scoring, two independent observers scored random TMA slides (95 patients for ER $\alpha$  and 81 for GPER) and inter-observer

Kappa values were calculated to be 0.82 for ER $\alpha$  and 0.80 for GPER. For patients with multiple metastases available, expression level was defined by any metastatic lesion demonstrating the loss of expression for ER $\alpha$  and/or GPER in cases of heterogeneity.

### Real-time quantitative PCR assays

cDNA was synthesised from 1  $\mu$ g RNA by the High capacity RNA to cDNA kit (Applied Biosystems, Carlsbad, CA, USA). Gene expression of GPER was determined using the two equally good TaqMan gene expression assays GPER-Hs00173506\_m1 (data not shown) and GPER-Hs01922715\_s1 (Applied Biosystems), and all samples were run on microfluidic cards with GAPDH-Hs99999905\_m1 as endogenous control. Assays were chosen using the UMapIt Microarray-to-TaqMan Assays Mapping Tool to ensure that the selected assays corresponded to the Agilent probes for microarray. Microfluidic cards were run as previously reported (Salvesen *et al*, 2009) and as described by the manufacturer. All samples were run in triplicates and analysed in RQ manager (Applied Biosystem software), using the DDC<sub>t</sub>-based method for calculation of relative quantification (RQ) values (Livak and Schmittgen, 2001). Outliers and runs with standard deviation > 0.5 among triplicates were excluded before analysis.

### Oligonucleotide DNA microarray analyses

RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and hybridised to Agilent Whole Human Genome Microarrays 44k (Cat.no. G4112F), according to the manufacturer's instructions. Arrays were scanned using the Agilent Microarray Scanner Bundle. Raw data were imported and analysed in J-Express software (Molmine, Bergen, Norway). Mean spot signal was used as intensity measure, and expression data were normalised using median over entire array. Connectivity Map (<http://www.broadinstitute.org/cmap/>) queries were performed independently for our microarray data and the external dataset using differentially expressed genes. Differentially expressed genes for ER $\alpha$  expressing tumours according to GPER status (ER $\alpha$ + / GPER+ vs ER $\alpha$ + / GPER-) were identified using the Feature subset selection (FSS) method ( $P < 0.05$ , fold > 1.5) from 59 (internal set) and 58 (external set) ER $\alpha$ + patients. The presented Connectivity Map results represent the top-ranked instances of the same perturbation made in the given cell line (Lamb *et al*, 2006).

### Statistical analysis

Statistical analyses were performed using PASW statistics 18.0 software for Mac (IBM, Armonk, NY, USA). Probability of < 0.05 was considered statistically significant. Groups were compared using Pearson chi-square test or Fisher's exact test, when appropriate, for categorical variables. Mann–Whitney *U* test and linear regression were used to test correlations for continuous variables. Univariate survival analyses of time to recurrence (recurrence free survival) or death due to endometrial carcinoma (disease specific survival) were performed using the Kaplan–Meier (product-limit) method. Entry date was the date of primary surgery. Patients who died from other causes were censored at the date of death. Differences in survival between groups were estimated by the log-rank (Mantel Cox) test. Variables were visually examined by a log-minus-log plot to check the assumptions about proportionality over time for inclusion in the multivariate proportional hazards regression models (Cox analyses). Unadjusted and adjusted hazard ratios were calculated as measures of effect. Significance of change in protein expression from primary tumours to corresponding metastatic lesions was evaluated using Fisher's exact and Wilcoxon signed rank tests. All *P*-values were two-sided.

## RESULTS

## Low GPER expression identifies aggressive endometrial cancers

The GPER protein expression was seen both in the stroma and in the glandular tissue. Only staining in glandular tissue was scored systematically, and protein expression was mainly cytoplasmic (Figure 1C). Loss of GPER expression, as defined by the lower quartile, was highly significantly associated with reduced disease-specific survival in the primary investigation set ( $n=182$ , Figure 1A). To further investigate the clinical phenotype related to GPER expression, we examined the larger prospectively collected validation series of 474 endometrial carcinoma patients. Low expression of GPER was significantly associated with high age, high FIGO stage, non-endometrioid histology, high grade, presence of lymph node metastasis, aneuploidy, ER $\alpha$  loss and poor prognosis (Figure 1B, Table 1) confirming the link between GPER loss and aggressive phenotype. In addition, proliferation activity was significantly higher in GPER-negative compared with -positive tumours measured by mitotic count (median 17 compared with 10, respectively,  $P=0.006$ , Mann–Withney  $U$  test). The poor survival associated with GPER loss was also seen for the subgroup of endometrioid tumours only ( $n=391$ ), with an 80% 5-year survival compared with 94% for tumours expressing GPER ( $P=0.001$ , Supplementary Figure 1A). When including GPER and ER $\alpha$  in multivariate Cox regression analysis, GPER and ER $\alpha$  were both found to be independent predictors of poor survival with hazard ratios (HR) of 1.9 for GPER ( $P=0.03$ , 95% CI: 1.1–3.3) and 3.1 for ER $\alpha$  ( $P<0.001$ , 95% CI: 1.8–5.4).

To explore to what extent the protein level was reflected in mRNA expression, GPER gene expression data were investigated in 76 tumour specimens analysed by microarray and validated by qPCR in 161 patients independent from the microarray series. Low GPER mRNA expression was confirmed to predict poor disease-specific survival (Figures 2A and C) and was significantly associated with the loss of GPER protein expression (Figures 2B and D). In both data sets, mRNA levels for GPER and ESR1 were significantly correlated ( $P<0.001$ , Pearson  $r(s)$  0.3 and 0.6). Low GPER mRNA expression also correlated with grade 3 in all examined mRNA datasets (Supplementary Table 1).

Loss of GPER among ER $\alpha$ -positive patients identifies poor prognosis

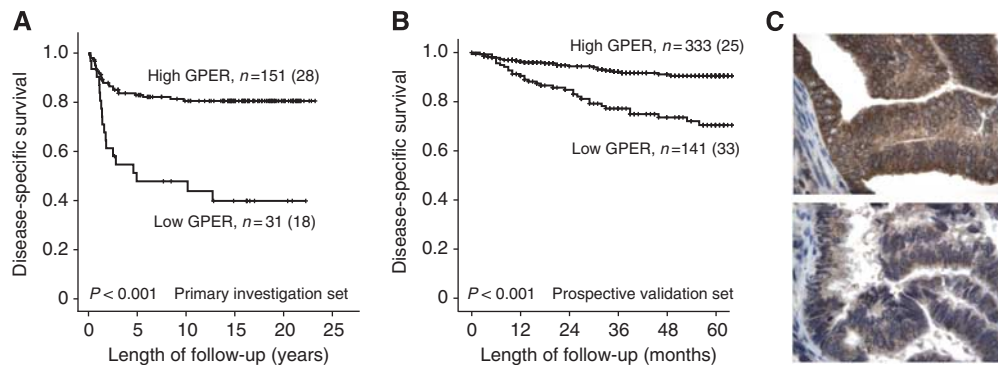
As GPER is suggested as an alternative oestrogen receptor, we further investigated a possible link between expression levels of GPER and ERs in the larger prospective validation series. As expected, loss of ER $\alpha$  was consistently associated with markers for

poor prognosis (Supplementary Table 2) as well as poor survival ( $P<0.001$ ; Figure 3A). ER $\alpha$  loss also predicted poor survival in the subgroup of endometrioid tumours only ( $P=0.001$ , Supplementary Figure 1B). ER $\beta$  mRNA levels by microarray and qPCR were neither correlated with phenotype nor ER $\alpha$  expression in tumour (data not shown). When combining the ER $\alpha$  and GPER protein expression data, we find that GPER loss within the ER $\alpha$ -positive subgroup of tumours significantly correlates with high FIGO stage, lymph node metastasis, non-endometrioid histology, high grade and aneuploidy (Table 2). Interestingly, combining the data also improved the prediction of outcome: ER $\alpha$ /GPER double positive was the most favourable group, whereas ER $\alpha$ /GPER double

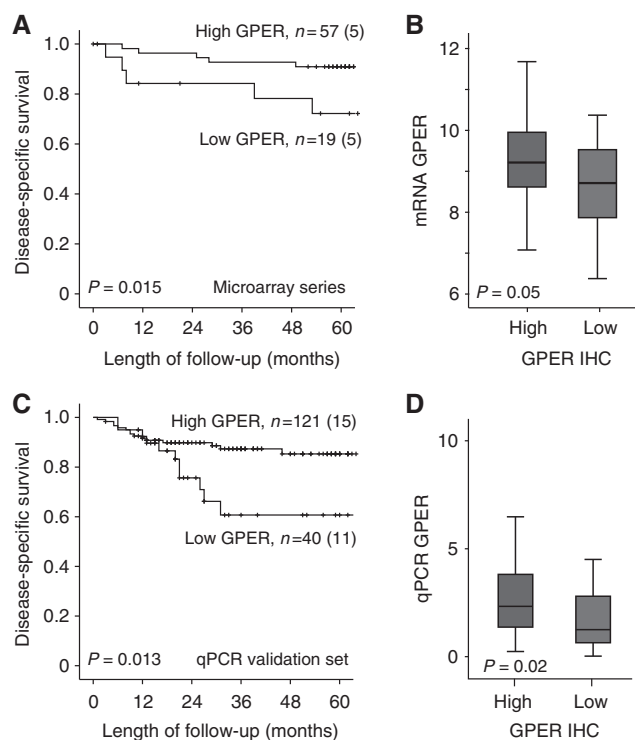
**Table 1** GPER protein expression in prospectively collected tumours related to clinico-pathologic factors in 474 patients with endometrial carcinoma

Variable	GPER <sup>a</sup>		P-value
	High, n (%)	Low, n (%)	
Age (years)			0.05
<66	187 (74)	65 (26)	
≥66	146 (66)	76 (34)	
FIGO-09 stage			<0.001
I–II	295 (74)	103 (26)	
III–IV	38 (50)	38 (50)	
Histologic type			<0.001
Endometrioid	302 (77)	89 (23)	
Non-endometrioid	31 (37)	52 (63)	
Histologic grade			<0.001
Grade 1/2	265 (81)	62 (19)	
Grade 3	68 (46)	79 (54)	
Metastatic nodes			<0.001
Negative	250 (73)	95 (27)	
Positive	21 (48)	23 (52)	
Ploidy			<0.001
Diploid	191 (74)	66 (26)	
Aneuploid	31 (48)	33 (51)	
ER $\alpha$			<0.001
Positive	292 (80)	71 (20)	
Negative	41 (37)	70 (63)	

Abbreviations: ER $\alpha$  = oestrogen receptor  $\alpha$ ; GPER = G protein-coupled oestrogen receptor. <sup>a</sup>Low = index 0–3 (lowest quartile), high = index 4–9.



**Figure 1** Loss of GPER expression in tumour is significantly associated with reduced survival for endometrial carcinomas. Kaplan–Meier curves for disease specific survival by expression of GPER protein in (A) a primary investigation set ( $n=182$ ), and (B) a prospectively collected validation series ( $n=474$ ). Representative examples of GPER expression (C); high expression in the upper panel compared with low cytoplasmic expression in the lower panel.

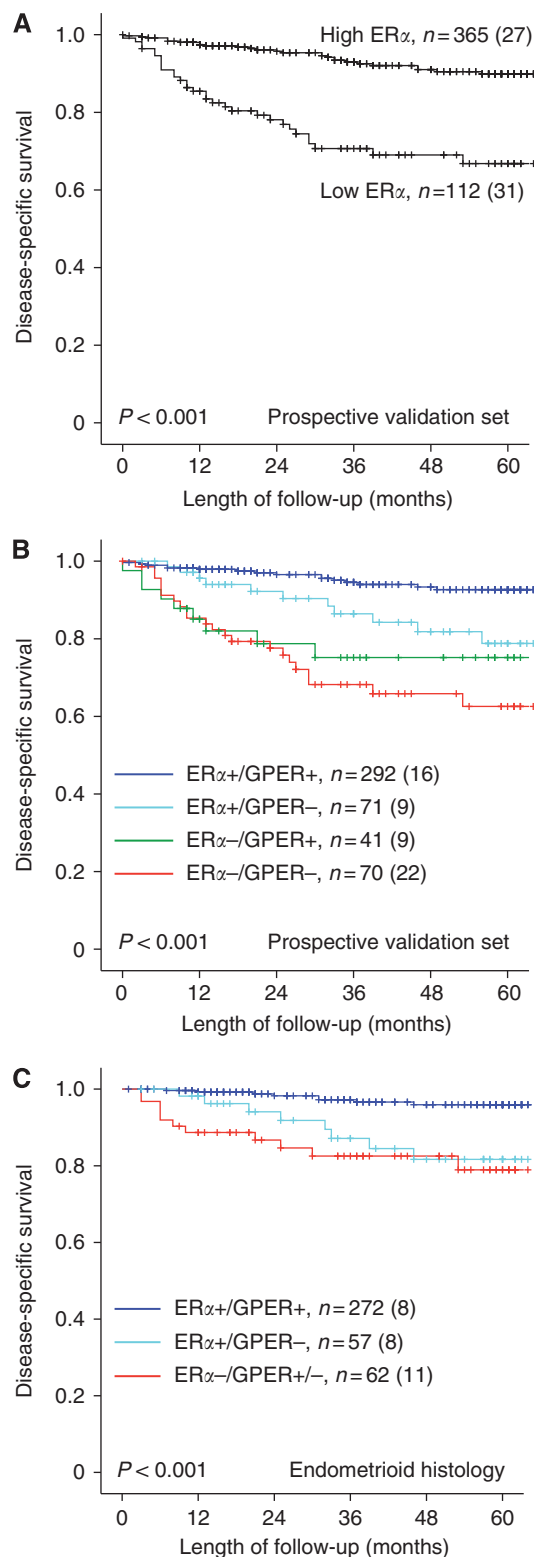


**Figure 2** Low mRNA GPER expression predicted poor disease-specific survival (A) and was significantly correlated with low GPER protein expression (B) in the microarray series ( $n=76$ ). qPCR analysis of GPER mRNA expression in a series independent from the microarray set ( $n=161$ ) validated the poor outcome associated with low GPER mRNA (C) and significantly correlated with low GPER protein expression (D). Vertical bars on the survival curves represent censored cases.

negative was most unfavourable ( $P<0.001$ , Figure 3B). Among the ER $\alpha$ -positive patients, normal GPER expression was associated with a 17% higher 5-year disease-specific survival compared with patients with loss of GPER ( $P=0.01$ ; Figure 3B). As intact expression of ER $\alpha$  is regarded to be one of the hallmarks of endometrioid histology, we also investigated ER $\alpha$ /GPER expression for this subgroup, confirming the loss of GPER as a factor predicting poor prognosis for endometrioid tumours ( $P=0.002$ , Figure 3C). Cox multivariate analysis of patients with tumour confined to the uterus, endometrioid histology and intact expression of ER $\alpha$  showed an independent prognostic impact of GPER with a HR of 7.3 (95% CI: 1.8–29.6,  $P=0.006$ ) adjusted for age and grade.

### Loss of GPER from primary to metastatic lesions is common for ER $\alpha$ expressing primary tumours

Consistent with the pattern of poor survival for patients with receptor loss, we find a significantly higher proportion of metastatic lesions compared with primary lesions, with loss of ER $\alpha$  and GPER (Figures 4A and B). To evaluate to what extent the metastatic lesions showed receptor loss not present in the primary lesions, we further analysed the ER $\alpha$ /GPER status in metastatic lesions from ER $\alpha$ -positive primary tumours ( $n=34$ ). For ER $\alpha$ /GPER-positive primary tumours, 91% of the metastases lost at least one receptor and 61% lost both receptors compared with their primary lesion counterparts (Figure 4C,  $P<0.001$ , Wilcoxon signed rank test). In the ER $\alpha$ + /GPER- subgroup of primary tumours, 45% of the metastases developed loss of ER $\alpha$  not present in the primary lesions (Figure 4D, Wilcoxon signed Rank test;  $P<0.03$ ).



**Figure 3** Disease-specific survival according to ER $\alpha$  and GPER expression. ER $\alpha$  loss (A) is associated with poor disease-specific survival in endometrial cancer patients. When combining ER $\alpha$  and GPER staining, loss of GPER expression identifies a subgroup among ER $\alpha$ -positive patients with poor survival, for all endometrial carcinomas (B) and for the subgroup of endometrioid tumours only (C).

## Loss of GPER indicates new targets for therapy amongst ER $\alpha$ -positive tumours

The gene expression profiles for ER $\alpha$ -positive tumours were classified in two groups based on GPER status. To investigate potential targets for treating metastatic ER $\alpha$ -positive tumours with loss of GPER expression, we sought to identify new potential drugs using gene lists generated from the ER-positive tumours with and

**Table 2** Loss of GPER in ER $\alpha$ -positive tumours

Variable	GPER		P-value
	High, n (%)	Low, n (%)	
Age (years)			0.4
< 66	164 (82)	36 (18)	
≥ 66	128 (78)	35 (22)	
FIGO-09 stage			0.005
I–II	268 (82)	57 (18)	
III–IV	24 (63)	14 (37)	
Histologic type			0.001
Endometrioid	272 (83)	57 (17)	
Non-endometrioid	20 (59)	14 (41)	
Histologic grade			0.001
Grade 1/2	244 (84)	47 (16)	
Grade 3	48 (67)	24 (33)	
Metastatic nodes			0.04 <sup>a</sup>
Negative	226 (82)	51 (18)	
Positive	13 (62)	8 (38)	
Ploidy			0.02
Diploid	175 (83)	36 (17)	
Aneuploid	23 (66)	12 (34)	

Abbreviations: ER $\alpha$  = oestrogen receptor  $\alpha$ ; GPER = G protein-coupled oestrogen receptor. <sup>a</sup>Fisher's exact test. GPER protein expression related to clinico-pathologic variables in 363 patients with ER $\alpha$ -positive endometrial carcinomas.

without expression of GPER. When querying a database of small molecular inhibitors (Connectivity Map) using expression data from our microarray set, two HDAC inhibitors and the PI3K-inhibitor Wortmannin were the top-ranked compounds significantly ( $P < 0.01$ ) anti-correlated with the ER $\alpha$ + /GPER- gene signature (Table 3). When analysing the external data set, three HDAC inhibitors, Tricostatin A, Valproic acid and Vorinostat, were all among the four top-ranked compounds (Supplementary Table 3). Taken together, this supports a potential for HDAC inhibitors in the treatment of metastatic ER $\alpha$ -positive endometrial carcinomas with loss of GPER expression.

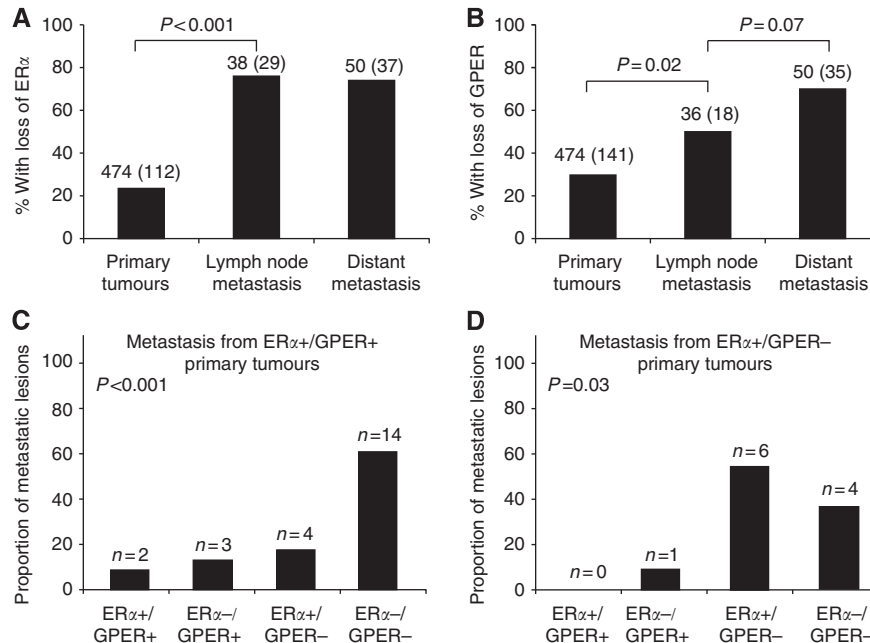
## DISCUSSION

Evaluation of hormone receptor status in endometrial cancer biopsies could potentially improve the selection of patients benefiting from endocrine therapy, as is the case in the management of breast

**Table 3** Connectivity Map identifies HDAC inhibitors to be most significantly anti-correlated with the gene signature defined by GPER loss within the ER $\alpha$ -positive subgroup

Rank	Name of compound	Cell line	n <sup>a</sup>	P-value <sup>b</sup>	Known function
1	Vorinostat	HL60	3	<0.001	HDAC inhibitor
2	Wortmannin	MCF7	10	<0.001	PI3K-inhibitor
3	Valproic acid	HL60	14	0.001	HDAC inhibitor
4	Sulfacetamide	PC3	2	0.001	Antibiotic
5	BAS-012416453	MCF7	2	0.002	
6	Ciclopirox	MCF7	2	0.005	Antifungal
7	Wortmannin	PC3	2	0.006	PI3K-inhibitor
8	Econazole	MCF7	2	0.009	Ca <sup>2+</sup> modulator

Abbreviations: ER $\alpha$  = oestrogen receptor; GPER = G protein-coupled oestrogen receptor; HDAC = histone deacetylase; PI3K = phosphoinositide 3-kinase. <sup>a</sup>Number of active instances <sup>b</sup>P-value as described (Lamb et al, 2006). Compounds are ranked according to significance ( $P$ -value) as anti-correlated with the gene signature from ER $\alpha$ -positive subgroup with GPER loss.



**Figure 4** Loss of ER $\alpha$  and GPER in metastatic lesions. The proportion of samples with loss of ER $\alpha$  (A) and GPER (B) expression is lowest in primary tumours and highest in metastatic lesions. Numbers indicate total number of patients investigated, with number of patients with loss of expression in parenthesis. Distribution of ER $\alpha$ /GPER protein expression in corresponding metastatic lesions derived from ER $\alpha$ + /GPER+ primary tumours ( $n = 23$ ; C) and ER $\alpha$ + /GPER- primary tumours ( $n = 11$ ; D);  $n$  = number of patients in each subgroup. We demonstrate a significant increase in the proportion of metastatic lesions with protein loss compared with their primary tumour counterparts (Wilcoxon signed rank test).

cancer patients (Chen and Colditz, 2007). Only a few clinical trials in endometrial cancer are stratified according to hormone receptor status, aiming to enrich for ER $\alpha$ -positive tumours (Decruze and Green, 2007). Treatment stratification based on ER $\alpha$  positivity is based on the assumption that this is the most important target for oestrogen in cancer tissue (Thomas and Gustafsson, 2011). However, although expression of ER $\alpha$  predicts response to anti-hormonal therapy like tamoxifen in breast cancer, non-responders to tamoxifen may also express ER $\alpha$ , while unexpected responses are seen for patients with ER $\alpha$  loss (EBCTCG Early Breast Cancer Trialists' Collaborative Group, 2005). However, alternative receptors and targets for oestrogen might mediate unknown or even undesired effects of treatment targeting hormone receptors. We have previously investigated the expression of ER $\beta$  in endometrial cancer but failed to demonstrate any significant correlation with ER $\alpha$  expression or survival (Engelsen *et al*, 2008). Confirming this, mRNA levels of ER $\beta$  was neither correlated with phenotype nor ER $\alpha$  expression in the present study. Thus, exploring expression levels of GPER in oestrogen-dependent tissue may be important to improve our prediction of response to anti-hormonal treatment. Also, GPER is emerging as a relevant drug target as both agonists (G1) (Bologa *et al*, 2006) and antagonists (G15, G36) (Dennis *et al*, 2009, 2011) have been developed. These drugs are at present not included in the connectivity map database for testing.

In the present study of endometrial carcinomas, being the largest and most comprehensive study of GPER expression in clinically annotated samples, we find that loss of GPER protein predicts poor survival in endometrial cancer patients. We confirmed this in several independent patient series and are, to the best of our knowledge, the first group to validate this also at the mRNA level. We utilised a highly robust scoring system to define expression (Aas *et al*, 1996). The GPER has already been suggested as a prognostic marker in cancer, however, with strikingly different results. In breast (Filardo *et al*, 2006), ovarian (Smith *et al*, 2009) and endometrial cancers (Smith *et al*, 2007), high expression of GPER has previously been related to aggressive phenotypes. However, the relatively small sample size of 45 ovarian and 46 endometrial carcinomas previously studied, enrichment for high stage cases (Smith *et al*, 2007) and the use of different antibodies and staining cut off (median) for estimation of GPER status (Filardo *et al*, 2006; Smith *et al*, 2007, 2009) may explain the discrepancy. Additionally, none of these reports utilised quantitative assays in parallel to detect GPER. The validation of our findings, both at the mRNA level and for two different GPER antibodies and in several independent datasets, supports that the role of GPER in the endometrium should be reconsidered. When analysing ER $\alpha$  and GPER protein levels in primary tumours and their metastatic counterparts, we found significant change towards loss of both ER $\alpha$  and GPER expression in metastases, further suggesting that loss of both receptors may be important for disease progression.

Interestingly, a recent report investigated the role of GPER in breast tumourigenesis, comparing mRNA levels of GPER from tumour tissue samples with paired normal breast tissue (Poola *et al*, 2008). They found that GPER is downregulated in breast cancer at the mRNA level and that low GPER mRNA predicted lymph node metastasis. Like in the present study, investigation of 1250 breast cancer patients from five independent patient cohorts (Ariazi *et al*, 2010) reports mRNA levels for ER $\alpha$  and GPER to be correlated. Also, GPER was found to inhibit proliferation in ER $\alpha$ -positive breast cancer cells in line with our findings that loss of GPER protein

expression is associated with high proliferation and poor prognosis. This suggests that GPER suppresses growth in the endometrium. However, it seems puzzling that a majority of ER $\alpha$ -positive cancers are enriched for expression of GPER both in our datasets and in breast cancer (Ariazi *et al*, 2010), indicating that intact expression of GPER reduces the aggressiveness of the disease, but is not enough to prevent tumour development. This may be explained by the dual role of GPER on the MAPK pathway (Filardo *et al*, 2002), where two opposing G-protein signalling pathways (PKA and EGFR) are activated by oestrogen binding to GPER. In support of this, in *in vivo* studies, GPER has recently been shown to regulate homeostasis in uterine epithelia in mice by inhibiting ER $\alpha$ -driven growth (Gao *et al*, 2011), where activation of GPER inhibits ERK1/2 and prevents ER $\alpha$  phosphorylation (Ser118), rendering ER $\alpha$  inactive.

The available treatment for patients with oestrogen-dependent tumours aims at targeting ER $\alpha$ . As we identified a subgroup of ER $\alpha$ -positive patients with the loss of GPER expression related to aggressive disease, we explored alternative drug targets for this subgroup of patients through a search in the small molecular inhibitor database connectivity map. Three different HDAC inhibitors were among the top-ranked drugs identified as promising agents for the treatment of metastatic ER $\alpha$ -positive endometrial cancers with GPER loss. In line with this, HDAC inhibitors have been associated with a transcriptional downregulation of ER $\alpha$  and its response genes (Thomas and Munster, 2009), as well as downregulation of aromatase in ER $\alpha$ -positive breast cancer cells (Chen *et al*, 2010). In endometrial cancer cell lines, HDAC inhibitors have been reported to be anti-proliferative (Takai *et al*, 2004) and have been suggested to be beneficial in combination with chemotherapy in treatment of metastatic endometrial cancer (Takai and Narahara, 2010). Taken together, this suggests that the ER $\alpha$ /GPER status may be potential predictors of response to HDAC inhibitors in metastatic endometrial cancers, and needs further study.

In summary, loss of GPER predicts poor survival and is more common in metastatic lesions compared with primary lesions in ER $\alpha$ -positive endometrial cancers. Whether GPER in addition to ER $\alpha$  status is relevant for predicting response to anti-hormonal therapy needs further studies. In addition, our data support HDAC inhibitors as promising drug candidates for treating metastatic disease, with GPER loss among ER $\alpha$ -positive endometrial cancers.

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## Conflict of interest

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on British Journal of Cancer website (<http://www.nature.com/bjc>)

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