Hyperglycaemia-induced chemoresistance in breast cancer cells: role of the estrogen receptor

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Abstract

Breast cancer patients with diabetes respond less well to chemotherapy; in keeping with this we determined previously that hyperglycaemia-induced chemoresistance in estrogen receptor (ERα) positive breast cancer cells and showed that this was mediated by fatty acid synthase (FASN). More recent evidence suggests that the effect of metabolic syndrome and diabetes is not the same for all subtypes of breast cancer with inferior disease-free survival and worse overall survival only found in women with ERα positive breast cancer and not for other subtypes. Here we examined the impact of hyperglycaemia on ERα negative breast cancer cells and further investigated the mechanism underlying chemoresistance in ERα with a view to identifying strategies to alleviate hyperglycaemia-induced chemoresistance. We found that hyperglycaemia-induced chemoresistance was only observed in ERα breast cancer cells and was dependent upon the expression of ERα as chemoresistance was negated when the ERα was silenced. Hyperglycaemia-induced an increase in activation and nuclear localisation of the ERα that was downstream of FASN and dependent on the activation of MAPK. We found that fulvestrant successfully negated the hyperglycaemia-induced chemoresistance, whereas tamoxifen had no effect. In summary our data suggests that the ERα may be a predictive marker of poor response to chemotherapy in breast cancer patients with diabetes. It further indicates that anti-estrogens could be an effective adjuvant to chemotherapy in such patients and indicates the importance for the personalised management of breast cancer patients with diabetes highlighting the need for clinical trials of tailored chemotherapy for diabetic patients diagnosed with ERα positive breast cancers.

Key Words

- breast cancer
- estrogen receptor
- hyperglycaemia
- chemoresistance

Introduction

Breast cancer is the most common malignancy in women with a lifetime risk of one in eight. In all Western societies women present with breast cancer are increasingly likely to also suffer from co-morbid conditions such as diabetes and obesity due to the increasingly high prevalence of these conditions in the general population. In a study of over a thousand women treated for breast cancer at MD Anderson Cancer Centre in Houston, 30% were found to be obese and a further 32% overweight (Litton et al. 2008). In addition the prevalence of metabolic syndrome in
patients with breast cancer has been reported to be between 39 and 50% (Healy et al. 2010, Stebbing et al. 2012).

For all cancers, the co-morbidity of type 2 diabetes has been reported to be associated with poor prognosis and reduced survival: with a mean survival period of 10.4 years for those with diabetes and 14.3 years for those without (Currie et al. 2012). In women with breast cancer, having metabolic syndrome was also associated with more aggressive tumour characteristics (Healy et al. 2010) and being obese confers worse overall survival (Litton et al. 2008).

Evidence also suggests that the effect of metabolic syndrome and diabetes is not the same for all subtypes of breast cancer. In a study of women involved in three large trials, inferior disease-free survival and worse overall survival was only identified in women with estrogen receptor (ERa) positive breast cancer and not for other subtypes (Sparano et al. 2012). A recent study found that hyperglycaemia was the only feature associated with metabolic syndrome that was associated with disease progression following chemotherapy in a cohort of women with breast cancer (73% of whom had ERa positive tumors; Stebbing et al. 2012).

In an attempt to identify the mechanisms underlying these in vivo clinical observations we previously investigated the impact of raised glucose levels on chemo-sensitivity of ERa positive breast cancer cells. We found that hyperglycaemia-induced chemoresistance in these cells, but not in non-malignant breast epithelial cells, and showed that this was mediated by increased activity of the enzyme fatty acid synthase (FASN), which synthesises fatty acids (with glucose as the main substrate) (Zeng et al. 2010). In keeping with the current clinical data (Stebbing et al. 2012), we found that hyperglycaemia-induced chemoresistance was observed in ERa positive but not in ERa negative breast cancer cells. We further determined that a functional ERa was required to mediate the hyperglycaemia-induced chemoresistance and finally that anti-estrogens may be an effective adjuvant to chemotherapy in breast cancer patients with diabetes.

Materials and methods

Reagents and antibodies

All chemicals were purchased from Sigma. All siRNAs and the transfection reagent, HiPerFect were purchased from Qiagen.

Cell culture

The human breast cancer cell lines MCF7, T47D, MDA-MB-231 and Hs578T were purchased from ATCC (Molsheim, France) that authenticates using short tandem repeat DNA profiles and the cells were used for a maximum of ten passages. They were maintained as described before (McIntosh et al. 2010, Zeng et al. 2010).

Dosing protocols

Cells were seeded in normal (5 mM) glucose-containing growth media for 24 h and then switched to either high (25 mM) a or normal (5 mM) glucose-containing serum free media for a further 48 h with or without a MAPK inhibitor, UO126 (30 µM) or for 24 h prior to dosing with chemotherapy drugs doxorubicin (0–40 µM), paclitaxel (0–300 µM) or C2-ceramide (0–30 µM) in the presence or absence of target siRNA to the ERα, FASN or non-silencing (ns) siRNA (as described previously (Zeng et al. 2010, Foulstone et al. 2013)), or tamoxifen (1 µM) or fulvestrant (100 nM). We used two siRNAs to silence both ERα and FASN: the second siRNA we used was FASN: the second siRNA we used was

Cell viability

This was determined by Trypan blue dye exclusion assay as outlined before (McIntosh et al. 2010, Zeng et al. 2010). We confirmed apoptotic cell death by assessing the cleavage of poly (ADP-ribose) polymerase (PARP) using western immunoblotting as described previously (Thomas et al. 2009).

Cell fractionation assay

Cytoplasmic and nuclear fractions were separated with NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Waltham, MA, USA; cat #78835), following the manufacturers’ instructions. Protein content of fractionated proteins and whole cell lysates were assessed using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) and then run on either 8/12% SDS-PAGE and transferred to a Hybond-C nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) as previously described (Zeng et al. 2010). Membranes were probed with p-ER (1:500), p-MAPK (1:1000), MAPK (1:500), tubulin (1:5000), lamin (1:1000), GAPDH
(1:5000), β-actin (1:10 000), FASN (1:1000) and ERz (1:750), PARP (1:1000), following the manufacturers’ instructions. Tubulin and lamin were used as markers for identifying cytoplasmic and nuclear cell fractions respectively. Secondary antibodies conjugated to per-oxidise were used: anti-mouse for p-ERz (1:1500), tubulin (1:5000), lamin (1:2000), GAPDH (1:5000), FASN (1:5000), ERz (1:1500), PARP (1:2000) and β-actin (1:10 000). Chemiluminescence was detected using the ChemiDoc-IT Imaging (UVP, Bio-Rad) and analysed using Vision Works Analysis Software (UVP, Inc., Upland, CA, USA).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay (ChIP) was performed using the Imprint Immunoprecipitation Kit (cat #CHP1) from Sigma–Aldrich. Briefly, after DNA–protein crosslinking with formaldehyde, samples were sonicated and immunoprecipitated with a ChIP grade anti-ERz antibody (Millipore, Watford, Hertfordshire, UK; cat #17-603). Anti-RNA polymerase II and mouse IgG supplied with the kit were used as positive and negative controls respectively. 5% input DNA was used for quantification. PCR (HotStarTaq Plus PCR Kit from Qiagen) was performed with purified DNA. The following primers were used for cyclin D1 (CCND1): forward (–1039) AACAAAAACCAATTAGGAACCTT, reverse (–770) ATTTCTTCATCCTTGTCTTCT (as reported in Zheng et al. (2013)) After 38 cycles, PCR products were detected by 2% agarose gel electrophoresis, stained with Midori Green (Nippon Genetics, Dueren, Germany; #MG04) and analysed by Bio-Plex Imaging System from Bio-Rad (serial no. 731BR01508).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described previously (Gill et al. 1997, Perks et al. 2003). Briefly, cells were seeded into 96-well plates at 7500 (MCF7) or 22 500 (T47D and MDA-MB-231) cells and treated with the chemotherapeutic drugs, doxorubicin, paclitaxel and ceramide for 3 h at 37 °C. The reaction was stopped by the addition of 50 μl stop solution (0.1 M HCl + 10% Triton-100) at room temperature for 20 min. The absorbance at 590 nm was measured using an ELISA plate reader.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay

Cells were incubated with an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (2 mg/ml, Promega, cat #G1118)/phenazine methosulfate (PMS) (0.92 mg/ml, Sigma P9625) solution at a ratio of 20:1 for 3 h at 37 °C. 200 μl of cell medium was used to measure the absorbance at 490 nm with an ELISA plate reader. The cells remain viable for further analysis using this assay as opposed to the MTT assay and so we used the MTS assay for experiments in which we also wished to assess cell number and viability.

Statistical analysis

Data were analysed with SPSS 12.0.1 for Windows using one-way ANOVA followed by least significant difference post-hoc test. A statistically significant difference was considered to be present at \( P < 0.05 \).

Results

Hyperglycaemia-induced chemoresistance is only observed in ERz positive breast cancer cells

We demonstrated previously that ERz positive breast cancer cells, MCF7 and T47D, were resistant to cell death induced by chemotherapeutics (doxorubicin, paclitaxel and ceramide) when exposed to high levels of glucose (Zeng et al. 2010). With MCF7 cells Fig. 1A confirms these previous data and Fig. 1B also shows that the MTT assay also indicates the differential effects induced by altered levels of glucose: increasing doses of C2 reduced metabolic activity more effectively in normal compared to high glucose conditions. Using both the MTT assay and cell counting in this study we found that in contrast to the ERz positive breast cancer cells, ERz negative Hs578T (Fig. 1C and D) and MDA-MB-231 (Fig. 1E and F) cells exhibited no chemoresistance following exposure to doxorubicin, paclitaxel or ceramide in high compared to normal glucose conditions. With Hs578T cells ceramide was able to induce cell death at 10 μM and not at the lower doses as demonstrated by the induction of PARP cleavage (insert Fig. 1C), this confirms that any differential effects in response to ceramide in relation to the levels of glucose at the lower doses of ceramide as observed in the dose–response (Fig. 1C) were not related to the induction of cell death. We characterised the growth of the cells when exposed to normal and high levels of glucose and found that basal cell growth of ERz positive and negative cells was unaffected over 48 h by
changes in the levels of glucose (Supplementary Figure 2A and D, see section on supplementary data given at the end of this article). Having observed chemoresistance in high glucose in the ERα positive breast cancer cells, we chose MCF7 cells and assessed changes in glucose uptake and expression of the key glucose transporters 1 (GLUT1) and GLUT12. We found that there was a 1.1-fold increase in glucose uptake in 25 mM glucose that was associated with an increase in expression of GLUT1 (P<0.05) and GLUT12 (P<0.05). Clearly other GLUTs are likely to play a role (Supplementary Figure 3A and C).

Silencing the ERα alleviates chemoresistance in hyperglycaemic conditions in ERα positive breast cancer cells

Having observed that hyperglycaemia only induced chemoresistance in ERα positive breast cancer cells, we next investigated if the presence of the ERα was required. With MCF7 cells, we found that in the presence of the ns siRNA, hyperglycaemic conditions reduced the ability of doxorubicin to induce cell death compared to euglycaemic conditions (from 31 to 23%; P=0.05; Fig. 2A) whereas with the ERα silenced, the hyperglycaemia-induced chemoresistance was negated (Fig. 2A). Similarly with T47D cells (Fig. 2B), in the presence of the ns siRNA, hyperglycaemic conditions reduced the ability of doxorubicin to induce cell death compared to euglycaemic conditions (from 24 to 18%; P<0.05; Fig. 2B) whereas with the ERα silenced, the hyperglycaemia-induced chemoresistance was negated (Fig. 2B). With MCF7 and T47D cells we also showed that in the presence of the ns siRNA that high glucose reduced the ability of doxorubicin to decrease metabolic activity (from 0.43- to 0.26-fold and from 0.37- to 0.06-fold; P=0.05 and P<0.05 respectively; Fig. 2C and D) and that

Figure 1

Hyperglycaemia-induced chemoresistance is only observed in ERα positive breast cancer cells. (A) Using Trypan blue dye exclusion method, the percentage cell death was assessed in the ERα positive MCF7 cells treated with chemotherapeutics (doxorubicin (DOX) 1 μM, P≤0.01; paclitaxel (Pac) 1 μM, P=0.01 and ceramide (C2) 12 μM, P=0.01) in 5 and 25 mM glucose for 24 h (n=3 experiments). Changes in metabolic activity of the MCF7 cells in 5 and 25 mM glucose in response to ceramide (0–20 μM) was examined using an MTT assay (B) (n=3 experiments, *P<0.05). The metabolic activity of the ERα negative Hs578T cells in 5 and 25 mM glucose in response to DOX (0–20 μM), C2 (0–30 μM) and Pac (0–300 μM) was examined with MTT assay (C) (n=3 experiments). Insert shows ceramide-induced PARP cleavage (85 kDa fragment) by western blotting (n=3 experiments). Percentage of cell death triggered by these drugs in 5 and 25 mM glucose was assessed by Trypan blue dye exclusion assay (D) (n=3 experiments). The change in metabolic activity of another ERα negative cell line, MDA-MB-231 in 5 and 25 mM glucose exposed to the above drugs is shown in (E) and percentage of cell death in (F) (n=3 experiments).
Silencing the ERα alleviates chemoresistance in hyperglycaemic conditions in ERα positive breast cancer cells, compared to the non-silencing (ns) siRNA control. Percentage of cell death induced by doxorubicin (DOX, 1 μM in MCF7 and 5 μM in T47D cells) was assessed in MCF7 (A) or T47D (B) cells with or without ERα knocked down with 20 nM siRNA in 5 or 25 mM glucose

![Figure 2](image)

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Hyperglycaemia increases phosphorylation and nuclear localization of ERα

Using cellular fractionation followed by western blotting, we examined alterations in the localisation and phosphorylation of ERα. With MCF7 cells (Fig. 3A and B), following exposure to high levels of glucose, we observed a shift in the localisation of the ERα with the ratio of ERα in cytoplasmic and nuclear part reduced from 1.9 to 0.8 (P<0.05). As ERα nuclear localisation is concomitant with an increase in ERα phosphorylation, we assessed changes in one of the key ERα phosphorylation sites: ser118. We observed that high levels of glucose increased total levels of ERα ser118 phosphorylation and that as anticipated there was a 1.6-fold increase in ERα ser118 phosphorylation in the nucleus (P<0.01). Similarly with T47D cells (Fig. 3A and B) we observed a shift in the localisation of the ERα with the ratio of ERα in cytoplasmic and nuclear part reduced from 1.4 to 1.1 (P<0.05) concomitant with a 1.4-fold increase ERα ser118 phosphorylation in the nucleus (P<0.05).

Hyperglycaemia increases nuclear ERα binding to a target gene, CCND1

As a further confirmation of the increased nuclear localisation of the ERα, we chose to assess alterations in the association of ERα with one of its known target genes, CCND1. Using ChIP assay we found that high levels of glucose caused a 2.7-fold increase (P<0.05) with MCF7 cells and an 11.7-fold increase (P<0.05) with T47D cells (Fig. 3C and D) in the association of ERα with the CCND1 gene.
The hyperglycaemia-induced increase in ERα ser118 phosphorylation is downstream of FASN

We had shown previously in ERα positive breast cancer cells that hyperglycaemia-induced chemoresistance was dependent upon FASN (Zeng et al. 2010). Having now demonstrated a role for the ERα in hyperglycaemia-induced chemoresistance, we silenced FASN using siRNA in both MCF7 (Fig. 4A) and T47D (Fig. 4B) cells to determine any impact on the ERα and levels of ERα ser118 phosphorylation. We found that silencing FASN reduced levels of the ERα in both normal and high glucose and reduced the ability of hyperglycaemia to activate ERα ser118 phosphorylation (Fig. 4C) suggesting that FASN is acting upstream of the ERα.

FASN regulates p-ERα (ser118) levels via the MAPK pathway

Having observed that the effect of hyperglycaemia on the phosphorylation of the ERα was downstream of FASN, we next wished to elucidate the mechanism through which FASN regulated p-ERα ser118 levels. With MCF7 cells, we found that in the presence of the ns FASN siRNA, hyperglycaemic conditions increased p-MAPK levels (×1.8-fold increase; \( P<0.01 \)) compared to euglycaemic conditions and that this was negated when FASN was silenced (Fig. 5A and B). To determine whether MAPK signalling pathway was required for the hyperglycaemia-induced increase in ERα ser118 phosphorylation, we used U0126, a MAPK inhibitor. As illustrated in Fig. 5C and D, treatment with U0126 completely blocked the ability of hyperglycaemia to activate ERα ser118 phosphorylation. Taken together, these results indicate that FASN regulates p-ERα (ser118) levels via activation of MAPK.

Fulvestrant blocks but tamoxifen has no effect on hyperglycaemia-induced chemoresistance

Having shown that silencing the ERα using siRNA negated chemoresistance induced by high glucose, we then assessed the effects of blocking the ER in a more clinically relevant manner by using two anti-estrogens, fulvestrant (selective ER down regulator) and tamoxifen (selective ER modulator). We first ensured we were using effective doses of each of the drugs.

With Figs 6A, B and 7A, B we show that fulvestrant (respectively) effectively blocked estrogen-induced ERα ser118 phosphorylation in both MCF7 and T47D respectively (\( n=3 \) experiments). The densitometry measurements from the western blot are shown in (B, \( P<0.05 \)). Chromatin immunoprecipitation assay (ChIP) was used to examine the changes in association of the ERα with one of its target genes, CCND1 (C). 5% Input was used as a quantification control (\( n=3 \) experiments). The relative enrichment of the CCND1 gene bound to ERα was quantified in (D, \( P<0.05 \)).
T47D cell lines. We also confirmed in both cell lines that fulvestrant down-regulates the ERα and tamoxifen stabilises it as reported in the literature (Supplementary Figure 3B and C). Having identified effective doses of both drugs we investigated if either fulvestrant or tamoxifen would negate hyperglycaemia-induced chemoresistance as we had observed when the ER was artificially silenced using siRNA. Figure 6C and D indicate that hyperglycaemia-induced resistance to doxorubicin-induced cell death (18 to 11%, $P < 0.01$ and 13 to 7% respectively) was

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Figure 4
The hyperglycaemia-induced increase in ERα (ser118) phosphorylation is downstream of fatty acid synthase (FASN). Using western blotting, the ERα and p-ERα (ser118) abundance were assessed in MCF7 (A) or T47D (B) cells, with or without FASN knocked down with 20 nM siRNA ($n = 3$ experiments). β-actin was probed as a loading control. Relative fold changes of p-ERα (ser118) against total ERα were measured (C, $P < 0.05$).

Figure 5
FASN regulates p-ERα (ser118) levels via activation of MAPK. Using western blotting, the abundance of MAPK and p-MAPK were assessed in MCF7 cells with or without FASN silenced with 20 nM siRNA in 5 or 25 mM glucose ($n = 3$ experiments) (A). Relative fold changes of p-MAPK against total MAPK were measured in (B, $P < 0.05$). Effect of MAPK inhibition with U0126 on p-ERα (ser118) was assessed in MCF7 cells treated with or without U0126 (30 μM) in 5 or 25 mM glucose for 48 h ($n = 3$ experiments) (C). The densitometry measurements from the western blot are shown in (D, $P < 0.05$).
Discussion

The current report has determined that hyperglycaemia-induced chemoresistance only occurs in breast cancer cell lines that possess a functional ERα and has identified that the ERα is key in mediating this hyperglycaemia-induced chemoresistance. We believe that our novel data may explain important clinical observations: in a study of women involved in three large trials, inferior disease-free and overall survival was only found in women with ER positive breast cancer and not for other subtypes (Sparano et al. 2012). In addition, that hyperglycaemia was the only feature associated with metabolic syndrome that was linked with disease progression following chemotherapy in a cohort of women with breast cancer (73% of whom had ER positive tumors; Stebbing et al. 2012). It has been reported that ERs are important regulators of components of the glycolytic pathway and contribute to the Warburg effect in cancer cells (Cai et al. 2012). Our data indicate control (n = 3 experiments). Percentage of cell death induced by doxorubicin (DOX, 1 μM in MCF7 and 5 μM in T47D cells) was assessed in MCF7 (C) or T47D (D) cells with or without 24 h pre-treatment of 100 nM Ful in 5 or 25 mM glucose (n = 3 experiments).
that the ERα is also an important determinant of how metabolic conditions specifically affect breast cancer cells and their response to chemotherapy. We clearly observed that silencing the ERα in ERα positive breast cancer cells negated hyperglycaemia-induced chemoresistance; consistent with these findings we determined that exposing ERα negative breast cancer cells to different levels of glucose did not influence chemo-sensitivity. The ERα has been linked to chemoresistance previously: Tokuda et al. (2012) showed that silencing the ERα in MCF7 breast cancer cells enhanced sensitivity to paclitaxel. This study was only performed under hyperglycaemic conditions and in that context their results were consistent with our findings.

As the ERα can be located at numerous sites within the cell we assessed if exposure to high glucose impacted on the localisation of the ERα. We found that hyperglycaemia increased the relative amounts of ERα in the nucleus compared to the cytoplasm. The ERα needs to be phosphorylated to translocate to the nucleus: in keeping with the ERα localisation data we observed a significant increase in phosphorylation of ERα at ser118. To corroborate these findings we assessed the level of ERα binding to one of its known target genes, CCND1 when exposed to different levels of glucose: as anticipated based on the localisation and phosphorylation data, we found an increase in association of the ERα with the CCND1 gene when ERα positive breast cancer cells were exposed to high levels of glucose. Ross-Innes et al. (2012) assessed the dynamics of ERα binding to DNA in clinical breast cancer samples and found that differential ERα binding was associated with clinical outcome in breast cancer: ERα bound to different sites and with different affinities depending on the stage. In light of these novel findings our data may suggest that breast cancer patients with altered metabolism may have tumour cells with altered ERα/DNA binding patterns that may contribute to chemoresistance.

We showed previously that hyperglycaemia-induced chemoresistance was dependent upon FASN (Zeng et al. 2010). Associations between the ERα and FASN have been identified in breast cancer cells previously (Lupu & Menendez 2006). We delineated that our new data, showing the important role of the ERα, was related to our previously defined signalling pathway. Our data suggests that the effect of hyperglycaemia on the phosphorylation of the ERα is downstream of FASN as silencing FASN reduced activation of the ERα: in addition that the increased phosphorylation of the ERα was mediated by MAPK activation downstream of FASN.

We then blocked the ER in a more clinically relevant manner using two anti-estrogens, fulvestrant and tamoxifen to assess their effectiveness in alleviating the resistance induced by hyperglycaemic conditions. We found that fulvestrant successfully negated the hyperglycaemia-induced chemoresistance, whereas tamoxifen had no effect. We believe this relates to how these two drugs act: fulvestrant degrades the ERα whereas tamoxifen stabilises the protein. The mechanism by which fulvestrant acts would be most comparable to our experiments where we artificially silenced the ERα with siRNA.

In summary our data provides a mechanism to support the clinical studies indicating that women with ERα positive breast cancer who also have diabetes respond less well to chemotherapy: it suggests that the ERα may be a predictive marker of poor response to chemotherapy in breast cancer patients with diabetes. It further indicates that certain classes of anti-estrogen therapy may prove effective adjuvants to chemotherapy in such patients but the specific type of anti-estrogen needs to be considered carefully. Our data indicates the potential importance and benefit of personalised medical therapy in the management of breast cancer, highlighting the need for clinical trials of tailored chemotherapy for diabetic patients diagnosed with ERα positive breast cancers.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-15-0507.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by The European Foundation for the Study of Diabetes (EFSD).

Author contribution statement
L Zeng, H A Zielinska and A Arshad performed the research experiments. C M Perkins wrote the manuscript, contributed to the design, obtained the funding and supervised the study. J M P Holly contributed to the design, supervision and to the writing of the paper. A Bahl and J P Shield also contributed to writing the paper and provided invaluable advice regarding the potential clinical applications of the work.

Acknowledgements
We thank Lindsay Durant for contributing to this work.

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-15-0507
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Printed in Great Britain
Published by Bioscientifica Ltd.

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Received in final form 3 November 2015
Accepted 1 December 2015
Made available online as an Accepted Preprint
7 December 2015