Obesity and menopause modify the epigenomic profile of breast cancer

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Abstract

Obesity is a high risk factor for breast cancer. This relationship could be marked by a specific methylome. The current work was aimed to explore the impact of obesity and menopausal status on variation in breast cancer methylomes. Data from Infinium 450K array-based methylomes of 64 breast tumors were coupled with information on BMI and menopausal status. Additionally, DNA methylation results were validated in 18 non-tumor and 81 tumor breast samples. Breast tumors arising in either pre- or postmenopausal women stratified by BMI or menopausal status alone were not associated with a specific DNA methylation pattern. Intriguingly, the DNA methylation pattern identified in association with the high-risk group (postmenopausal women with high BMI (>25) and premenopausal women with normal or low BMI <25) exclusively characterized by hypermethylation of 1287 CpG sites as compared with the low-risk group. These CpG sites included the promoter region of fourteen protein-coding genes of which CpG methylation over the ZNF577 promoter region represents the top scoring associated event. In an independent cohort, the ZNF577 promoter methylation remained statistically significant in association with the high-risk group. Additionally, the impact of ZNF577 promoter methylation on mRNA expression levels was demonstrated in breast cancer cell lines after treatment with a demethylating agent (5-azacytidine).

In conclusion, the epigenome of breast tumors is affected by a complex interaction between BMI and menopausal status. The ZNF577 methylation quantification is clearly relevant for the development of novel biomarkers of precision therapy in breast cancer.
Introduction

The prevalence of obesity and its associated co-disorders is rapidly increasing worldwide. Nowadays, these diseases are considered to be pandemic non-communicable diseases and are a major challenge for the health care system (Seidell & Halberstadt 2015). Breast cancer, the leading cause of cancer diagnosis in women, is among the co-disorders associated with obesity. Overweight or obesity is a known risk factor for breast cancer (Lorincz & Sukumar 2006), especially in postmenopausal females (Crujeiras et al. 2011, van Gemert et al. 2015). In contrast, being overweight or obese has no effect, or even a small protective effect, in premenopausal women. For every 5-unit increase in body mass index (BMI), the risk of developing postmenopausal breast cancer increases by 12%, and elevated BMI is associated with a protective effect against the development of breast cancer in premenopausal women (Renehan et al. 2008). Notably, despite epidemiological and clinical evidence, the mechanisms that underlie the association between obesity and breast cancer risk are not completely understood. Obesity-induced inflammation promoted by adipose tissue dysfunction was proposed as an important link between obesity and cancer (Crujeiras et al. 2013, Howe et al. 2013).

Together with an imbalance in energy homeostasis, obesity is characterized by a state of chronic low-grade inflammation that promotes oxidative stress due to dysfunction in adipose tissue and alterations in adipocyte-derived hormone secretion and cytokine synthesis (Zou & Shao 2008). Hormonal and inflammatory mediators can induce and maintain epigenetic regulation (Milagro et al. 2013, Yara et al. 2013). Frequent epigenetic modifications, such as DNA hypo- and hypermethylation and histone modifications, have been observed in several types of tumors, including breast cancer (Heyn et al. 2013, Stefansson & Esteller 2013). This DNA methylation pattern is associated with an increased risk of cancer disease, a poor prognosis and a decreased likelihood of relapse-free survival (Hill et al. 2011). On the other hand, DNA methylation have also been found to be associated with obesity itself in a genome-wide DNA methylation analysis (Dick et al. 2014) and in analysis of specific genes (Milagro et al. 2012). Therefore, obesity could contribute to malignancies of the breast by altering the microenvironment such that it favors epigenetic changes and increases the susceptibility for inducing and maintaining a disease state (Crujeiras & Casanueva 2015). Because epigenetic modifications are dynamic, reversible and change in response to dietary patterns, physical activity and weight loss, epigenetic markers related to obesity may represent therapeutic targets for the prevention of obesity-related disorders, including breast cancer. In this regard, previous studies have recently evaluated the associations between body mass and the methylation of a single gene or a panel of cancer-related genes in breast tumors (Tao et al. 2011, Naushad et al. 2014, Hair et al. 2015, McCullough et al. 2015). After these pioneer studies, examining the epigenome-wide association of breast cancer that depends on adiposity and the menopausal status could add novel insight to the epigenetic regulation of the obesity and breast cancer relationship.

The aim of the current work was to explore the effects of obesity and menopausal status on the DNA methylation profile of breast tumors using a genome-wide DNA methylation approach and to identify an epigenetic signature of breast cancer taking into account the adiposity and menopausal state.

Subjects and methods

Study participants

A total of one hundred forty-five women diagnosed with histologically confirmed invasive breast cancer and eighteen healthy women were included in the current study (Table 1 and Fig. 1).

The discovery cohort was composed of breast tumor tissues (frozen tissues; n=64) obtained from the Department of Pathology, University Hospital, Iceland. Patient information associated with the breast cancer samples was obtained from the population-based Icelandic Cancer Registry. Informed consent was obtained from all patients, and all analyses were performed in accordance with the Icelandic Data Protection Commission (200650307) and Bioethics Committee (VSNb2006050001/03-16)

The validation cohort consisted of normal breast (n=18) and breast cancer (n=81) human paraffin-embedded (FFPE) tissue blocks from the BioBank Complejo Hospitalario Universitario de Santiago (CHUS) (PT13/0010/0068), integrated in the Spanish National Biobanks Network and they were processed following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees (ref 2009/076).

Pre-diagnosis body weight, height, age and menopausal status were retrieved from medical records for all participants in the study. BMI was calculated as weight
in kg divided by the squared height in meters and was further categorized using the World Health Organization (WHO) criteria: normal/under-weight, BMI < 25 kg/m²; overweight, 25 ≤ BMI < 30 kg/m² and obese, BMI ≥ 30 kg/m² (WHO, 2000). Then, patients were classified considering overweight and obese patients in the same group, as obese (BMI > 25 kg/m²) and non-obese (BMI ≤ 25 kg/m²) to evaluate the effect of excess body weight.

DNA preparation and bisulfite conversion

DNA from fresh-frozen (FF) tissue samples was isolated using a standard phenol-chloroform/proteinase-k protocol, whereas paraffin samples (FFPE) (10 sections of 14 μm) were processed using the E.Z.N.A. FFPE DNA kit (Omega Bio-Tek, Norcross, GA, USA), with a xylene wash to remove the paraffin. For each sample of tumor tissue, subsequent sections were stained with hematoxylin and eosin for histologic confirmation of the presence (>50%) of tumor cells. The obtained DNA was treated with RNase A for 1 h at 45°C. All DNA samples were quantified using the fluorometric method (Quan-iT PicoGreen DsDNA Assay, Life Technologies) and were assessed for purity using a NanoDrop (Thermo Scientific) with 260/280 and 260/230 ratio measurements. The integrity of the FF DNA was verified by electrophoresis in a 1.3% agarose gel. FF DNA (600 ng) and FFPE DNA (300 ng) were processed using the EZ-96 DNA Methylation kit (Zymo Research Corp, Irvine, CA, USA) following the manufacturer’s recommendations for Infinium assays.

DNA methylation analysis

**Infinium Human Methylation 450 BeadChip array** Microarray-based DNA methylation analysis was conducted with the Infinium Human Methylation 450 BeadChip (450k array). DNA quality checks, bisulfite modification, hybridization, data normalization, statistical filtering and β value calculations were performed as described elsewhere (Sandoval et al., 2011). High-quality DNA samples obtained from breast tumors (discovery cohort, n=64) were selected for bisulfite conversion (Zymo Research; EZ-96 DNA Methylation Kit) and
hybridization to Infinium Human Methylation 450 BeadChips (Illumina) following Illumina’s Infinium HD methylation protocol. The DNA concentration of the quality control sample standards was measured using the PicoGreen method (Invitrogen), coupled with assessments of DNA purity based on the A260/A280 ratio (ranging between 1.75 and 1.95) and the A260/A230 ratio (ranging between 2.00 and 2.20). DNA fragmentation or RNA contamination was analyzed by 1% agarose gel electrophoresis. The Infinium Human Methylation 450 BeadChip provides coverage of >450,000 CpG sites targeting nearly all RefSeq genes (>99%) (Sandoval et al. 2011). The chips were designed to cover coding and non-coding genes without bias against those lacking CpG islands. The design further aimed to cover not only promoter regulatory regions but also CpGs across gene regions to include 5’-untranslated regions (5’ UTRs), the first exons, gene bodies and 3’-untranslated regions (3’-UTRs).

A total of 600 ng from high-quality DNA samples was bisulfite converted. Whole-genome amplification and hybridization were then carried out on the BeadChip, followed by single-base extension and analysis on the HiScan SQ module (Illumina) to assess cytosine methylation states. The annotation of CGIs used the following categorization: (1) shore, for each of the 2-kb sequences flanking a CGI; (2) shelf, for each of the 2-kb sequences next to a shore; and (3) open sea, for DNA not included in any of the previous sequences or in CGIs (Heyn et al. 2012). Transcription start site 200 or transcription start site 1500 indicates the region either 200 or 1500 bp from the transcription start site, respectively.

**Pyrosequencing analysis**  Pyrosequencing was used to assess selected markers in the validation cohort (n=98 FFPE breast tumors and n=19 normal FFPE mammary glands) as previously described (Heyn et al. 2012). The primer sequences used in this analysis were designed using Qiagen’s PyroMark Assay Design 2.0 software to hybridize to CpG-free sites to ensure methylation-independent amplification (details and primer sequences are available in the Supplementary Table 1, see section on supplementary data given at the end of this article). Briefly, PCR was performed using standard conditions with biotinylated primers, and the PyroMark Vacuum Prep Tool (Biotage, Uppsala, Sweden) was used to prepare single-stranded PCR products according to the manufacturer’s instructions. Pyrosequencing reactions and methylation quantification were performed in PyroMark Q96 System, version 2.0.6 (Qiagen) using appropriate reagents and recommended protocols.

**Bisulfite genomic sequencing** Methyl Primer Express v1.0 software was used to identify CpG islands and to design specific primers for the ZNF577 methylation analysis (Supplementary Table 1). DNA methylation status was determined using bisulfite genomic sequencing of multiple clones in DNA samples that were previously treated with sodium bisulfite (EZ DNA Methylation Gold Kit, Zymo Research).

**Cell lines and treatments for functional analysis**

Representative human breast cancer cell lines (MCF7, Hs578T, MDA-MB-468 and MDA-MB-134IV) and normal epithelial breast cell line (MCF10A) from American Type Culture Collection (Manassas, VA) were used to evaluate the DNA methylation and gene expression of ZNF577. Cells were cultured in DMEM containing 10% FBS, penicillin and streptomycin at 37°C and 5% CO₂. To restore the expression of DNA-methylated ZNF577, the MCF7 cells were treated with the DNA demethylating agent 5-aza-2′-deoxycytidine (A3656, Sigma) at 5 µM for 72h. All the cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and were used with passage lower than 15. ATCC authenticates the cell lines routinely following a very strict test that includes short tandem repeat (STR) profiling, karyotyping and cytochrome C oxidase I (COI) testing.

**Expression assays by qRT-PCR and RT-PCR**

RNA from the cell culture assays was extracted using the Thermo Scientific GeneJet RNA Purification Kit (Thermo Scientific, K0739). The RNA concentrations were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with 2 µg of total RNA. qRT-PCR was performed using TaqMan Universal PCR Master Mix, TaqMan Probes (Applied Biosystems) and the StepOnePlus Real-Time PCR System (Applied Biosystems). All experiments were performed in duplicate, and gene expression levels were normalized to those of the housekeeping genes ACTB or GAPDH, depending on the sample. The fold change in gene expression was calculated using the 2−ΔΔCt relative quantitation method according to the manufacturer’s guidelines.
DNA methylation in obesity-related breast cancer

(Applied Biosystems), and data are reported as the geometric mean (S.E.M.).

**Statistical analyses**

The methylation level of each cytosine was expressed as a \( \beta \) value, which was calculated as the fluorescence intensity ratio of the methylated to the unmethylated versions of the probes. \( \beta \) values ranged between 0 (unmethylated) and 1 (completely methylated) according to a combination of the Cy3 and Cy5 fluorescence intensities. Color balance adjustment and quantile normalization were performed to normalize the samples between the two color channels. \( \beta \) values with a detection \( P \) value \( >0.01 \) are considered to fall below the minimum intensity and threshold, and these values were consequently removed from further analysis. Approximately 96% of CpG islands were covered, along with regions proximal to CpG islands (‘CpG shores’) and the more distal CpG shelves. Additionally, probes that were localized to the sex chromosomes and those considered to be single nucleotide polymorphisms (SNPs) were filtered out.

Samples were classified in the discovery and validation cohorts according to adiposity and menopausal status. The genome-wide DNA methylation patterns were first compared for tumors from obese and normal-weight patients. Then, the samples were categorized into two risk groups that combined adiposity and menopausal status, based on the epidemiological data that reported a higher risk of breast cancer in obese postmenopausal women compared to obese premenopausal women (Crujeiras et al. 2011). The high-risk group was composed of samples from obese postmenopausal and normal-weight premenopausal women, and the low-risk group was composed of obese premenopausal and normal-weight postmenopausal women.

To identify consistent patterns of differentially methylated CpG sites between obese and normal-weight samples and between the high-risk group and group B, we carried out a univariate permutation-based approach using the SAMr package for R. By this analysis, false discovery rates (FDR) below 5% were considered statistically significant. In addition, we applied a threshold for the significant sites using mean differences between groups, with a minimum change of \( \pm 0.10 \) in \( \beta \) values. Hierarchical cluster analysis of the significant CpGs was performed using the heatmap function in R.

To estimate the enrichment in biological processes, a hypergeometric test was performed on biological processes defined by gene ontology (GO) (Falcon & Gentleman 2007). This analysis detects significant over-representation of GO terms in one of the sets (list of selected genes) with respect to the other for the entire genome. GO terms with an adjusted \( P \) value \( <0.05 \) were considered significant.

Additionally, the diagnostic efficiency (percent of correctly classified) of the candidate genes differentially methylated was calculated as the percent of agreement by using the receiver-operating characteristic (ROC) curve analysis. These results are often interpreted as negligible efficiency (<20%), minimal (20–40%), moderate (41–60%), good (61–80%) and excellent (>80%).

**Results**

**Characteristics of patients**

Among the 64 patients with primary breast cancer included in the discovery cohort, 60.9% were classified as overweight/obese, 39.1% were normal weight and 51.6% were postmenopausal. In the validation cohort, 71.4% patients were classified as overweight/obese, 28.6% were normal weight and 61.2% were postmenopausal. The clinical characteristics of the discovery and validation cohorts are shown in Table 1.

**DNA methylation analysis by adiposity and menopausal state**

DNA methylation profiling in human breast tumors involving the analysis of approximately 450,000 CpGs were analyzed with respect to BMI and menopausal status. Firstly, tumors arising in obese or overweight (BMI >25) women were compared with those arising in normal weight women (BMI <25). This analysis revealed no statistically significant differences following adjustment for multiple hypothesis testing. Secondly, no significant differences were detected between tumors arising in premenopausal women when compared with tumors arising in postmenopausal women.

Epidemiological studies have consistently identified high BMI (>25) in postmenopausal women as a risk factor for developing breast cancer (Crujeiras et al. 2011). This observation gives reason to specifically determine whether the DNA methylation profiles of tumors arising in these highest-risk women (BMI >25 and postmenopausal) differ from the breast tumors from postmenopausal normal-weight (BMI <25) women in the cohort. However, this analysis did not reveal any significantly associated CpGs after adjustment for multiple hypothesis testing.
Another high-risk group defined on the basis of BMI and menopausal status includes premenopausal women with normal or low BMI (BMI <25). Similarly, tumors arising in this risk group, i.e. in premenopausal women with normal or low BMI, did not differ significantly with respect to normal-weight postmenopausal women. However, when combining together tumors from these two high-risk groups, i.e. postmenopausal women having BMI >25 together with premenopausal women having BMI <25, we detected statistically significant differences involving 1,287 CpGs with FDR <5% corresponding to 624 unique genes (see detailed list in Supplementary Table 2). The differentially methylated CpGs are exclusively characterized as changes towards CpG hypermethylation occurring during tumor development in the high-risk group (Fig. 2A). Notably, these differentially methylated CpG sites between the high- and low-risk groups were mainly located in promoters and CpG islands (Fig. 2B), suggesting the existence of a different methylator phenotype between both risk groups.

The CpG sites representing promoter regions (TSS1500, TSS200, 5' UTR and 1st exon) at CpG islands were selected to investigate biological relevance. This selection yielded 215 CpGs representing 138 unique genes (Supplementary Table 3). The CpGs with the greatest change in DNA methylation (>15%) are listed as representatives of the 215 CpG sites in Table 2.

Additionally, a gene ontology (GO) analysis was performed to determine whether some molecular functions or biological processes were significantly associated with these differentially methylated genes represented by CpGs located in promoter regions and CpG islands. Thus, among the significantly enriched biological processes, the top overrepresented categories were related to the regulation of transcription, signal transduction, the regulation of cell differentiation and proliferation, cell migration and cell cycle regulation (Fig. 2C). Most of the genes (76%) regulated by methylation belonged to a network significantly enriched in protein interactions ($P < 0.001$), according to STRING analysis (Supplementary Fig. 1).

To identify potentially novel signatures of DNA methylation in the high-risk group, those genes with more than 4 CpG sites located within CpG islands and promoters and a difference in β-values ≥10% were selected (Supplementary Table 3). With these criteria, C17orf64, DUOX1/DUOXA1, ELOVL3, EVX1, POU4F1, TLX3, TOX2 and YOD1 were represented by 4 sites; IQSEC1 and

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**Figure 2**
Characteristics of the candidate differentially methylated CpGs. (A) Supervised clustering of the 1287 CpGs found differentially methylated between the high-risk and low-risk groups identified by 450k array analysis. (B) Genomic distribution of the differentially methylated CpG sites and their respective location regarding the broader CpG context and the gene region. Data represent DMCPGs in a region divided by the total DMCPGs (1282). (C) Summary of the gene ontology (GO) analysis of the biological process categories for differentially methylated genes represented by CpG sites located in promoter regions and islands.
Table 2 The forty-two highest differentially methylated CpGs between high- and low-risk groups in promoter and island. Bold denotes genes with more than 4 differentially methylated CpG sites located within CpG islands and promoters and a difference in p-values ≥ 10%.

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LYPD5 were represented by 5 sites, and PRKCDBP and RASSF1, by 6 sites. Relevantly, ZNF577 was the most-represented gene, with 7 CpGs that were differentially methylated in promoter region and island with high statistical significance.

Validation of candidate genes in an independent cohort

We used pyrosequencing, a technique that is most feasible for studies of patients in hospitals, to evaluate the DNA methylation levels for RASSF1 and ZNF577 in an independent cohort of paraffin samples from breast cancer patients and cancer-free donors (validation cohort; N = 81 breast tumors and N = 18 non-tumoral breast tissues; Table 1).

We confirmed significant higher levels of methylation for RASSF1 in tumor samples compared with non-tumoral donors (Supplementary Fig. 2A). When the methylation levels were analyzed in breast tumors according to the
adiposity and menopausal state, no differences were observed in RASSF1 (Supplementary Fig. 2B and C). Regarding ZNF577, the epigenetic regulation in breast tumors has not been previously described. Therefore, first the methylation levels were evaluated by comparing tumor with non-tumoral samples. A significant increase in methylation levels was observed for ZNF577 in tumor compared with non-tumoral samples in both, our cohort (Fig. 3A) and an independent cohort from The Cancer Genome Atlas; TCGA (Supplementary Fig. 3). When the methylation levels were analyzed in breast tumors according to adiposity and menopausal state, ZNF577 exhibited higher methylation levels in postmenopausal obese and premenopausal normal-weight women than in the other cohort groups (Fig. 3B). In fact, the statistical differences observed in the Infinium Human Methylation 450 BeadChip array between the high-risk group and low-risk group were corroborated by pyrosequencing, with statistical significance in ZNF577 methylation levels (Fig. 3C). Notably, the methylation levels of the promoter region of ZNF577 (Fig. 4A) assessed in different breast tumor cell lines by bisulfite genomic sequencing analysis (Fig. 4B) and by the Infinium Human Methylation 450 BeadChip array (Fig. 4C) were inversely correlated with the transcript levels of ZNF577 (Fig. 4D). Moreover, after treating breast cancer cell lines that exhibited ZNF577 hypermethylation with the demethylating agent 5-aza-2′-deoxycytidine, the expression of this gene was significantly restored (Fig. 4E), indicating that DNA methylation has a functional role in the transcriptional control of ZNF577.

Receiver-operating characteristic (ROC) curves were used to evaluate the ability of ZNF577 methylation levels to discriminate tumor from non-tumor as well as high-risk from low-risk patients. The area under the ROC curve (AUC) of ZNF577 methylation levels was 0.93 ($P < 0.001$) for breast tumor detection (Fig. 5A) and 0.69 ($P=0.008$) to discriminate the high-risk from low-risk group (Fig. 5B).

Discussion

Epigenetic modifications of the genome mediate the response of the organism to environmental pressure. There is a growing body of evidence that demonstrates a relevant role for epigenetic marks in susceptibility

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**Figure 3**

Methylation analysis of ZNF577 by pyrosequencing. (A) Methylation level differences between tumor and non-tumor breast tissues. (B) Methylation levels in non-tumor breast tissues from obese patients, depending on the menopausal state. (C) Methylation levels in breast tumor tissues, depending on obesity and menopausal state. (D) Methylation levels in breast tumor tissues in the high-risk and low-risk groups. Statistical analysis was evaluated using Student $t$ test and significance symbols correspond to a. $P<0.05$ vs non-tumor, premenopausal obesity or premenopausal normal-weight, respectively; b. $P<0.05$ vs premenopausal overweight/obesity; c. $P<0.05$ vs postmenopausal normal-weight.
to obesity and its co-diseases. The results presented herein demonstrate that the impact of obesity on the breast cancer features could be mediated by a specific alteration of the methylation profile, particularly in cases of postmenopausal breast cancer. The epigenome-wide analysis identified 138 genes that exhibited a differential methylation in the promoter region and island and they comprised an epigenetic signature of high-risk breast cancer by including obesity-related postmenopausal and normal weight premenopausal women. Among the identified signature genes, ZNF577 was the most relevant and its differential methylation levels were validated in an independent cohort. In addition, we demonstrate that ZNF577 gene expression is epigenetically regulated. The methylation levels of ZNF577 vary depending on the type of breast cancer cell line. This fact is in agreement with the heterogeneity of primary tumors of breast cancer, which is associated with clinical outcome of disease. Therefore, understanding the function of the methylation pattern of ZNF577 in response to therapy could be relevant to improving management of the disease taking into account adiposity and menopausal state.

To the best of our knowledge, the current study used the largest cohort to highlight the impact of obesity on primary breast tumor methylation profiles and to identify genome-wide high-resolution DNA methylation patterns that depend on menopausal status. Prior research has reported an association between obesity and methylation in tumor tissue from women diagnosed with breast cancer. These studies were focused on individual genes (Tao et al. 2011, Naushad et al. 2014) or on a panel of cancer-related genes (Hair et al. 2015, McCullough et al. 2015). For example, Hair and coworkers (Hair et al. 2015) demonstrated that obesity is associated with the degree of methylation in some cancer-relevant genes. Although the candidate genes that were differentially methylated in the former study were not validated in an independent cohort, and the functional relevance of the identified candidate genes was not evaluated, the results supposed new and relevant evidence on the epigenetic regulation of cancer-related genes induced by obesity in breast tumors. The current work adds new information to this issue by performing a comprehensive genome-wide analysis of the DNA methylation that interrogates 485,000 CpG sites. In the current work, we first compared the breast

Figure 4
DNA methylation-associated silencing of ZNF577 breast cancer. (A) Schematic representation of ZNF577 genomic loci and CpG island. (B) Bisulfite genomic sequencing analysis of ZNF577 CpG island in human breast cancer cell lines and normal breast and lymphocytes as tissue controls. Location of bisulfite genomic sequencing PCR primers (black arrows), CpGs (vertical lines) and the transcriptional start site (grey arrow) are shown. Ten single clones are represented for each sample. Presence of unmethylated or methylated CpGs is indicated by white or black squares, respectively. Red circles represent the location of CpGs detected by the 450k methylation array. (C) ZNF577 methylation levels of breast cancer cell lines analyzed by 450k methylation array. (D) ZNF577 expression levels in methylated or unmethylated human breast cancer cell lines and in normal breast as control by RT-PCR and qRT-PCR. Values were determined by qRT-PCR in triplicates and are expressed as mean (n=3). (E) Restored ZNF577 expression after treatment with DNA demethylating agent 5-aza-2’-deoxycytidine (AZA) in ZNF577 CpG island methylated MCF7 cell line. Values were determined in triplicate by RT-PCT and qRT-PCR of three independent experiments and are expressed as mean ± s.e.m (n=3).
tumors from obese vs normal-weight women, and we did not find statistically differences after adjusting for multiple comparisons. Then, we compared the obese with normal-weight samples grouping for premenopausal or postmenopausal state. Again, no differences were observed after adjusting for multiple comparisons. This lack of statistical significance could be due to the sample size yielded from the classification of the samples into 4 groups. Under this situation, the breast cancer patients were further classified into two groups according to their adiposity and menopausal status, considering that the effect of obesity on breast cancer is modulated by menopausal status (Crujeiras et al. 2011). In agreement with our working hypothesis, these two groups were differentiated by their DNA methylation profile. The CpGs that were differentially methylated were mainly hypermethylated in the high-risk group, and these CpGs were located in genes involved in several pathways related to carcinogenesis. Some genes previously reported to be differentially methylated in cancer were found among the highest differentially methylated CpGs in promoter regions and CpG islands. For example, among the genes represented by more than 4 CpGs, high methylation levels were observed for TOX2 (Tessema et al. 2012), RASSF1 (Conway et al. 2014), PRKCDBP (Li et al. 2015) and POU4F1 (Faryna et al. 2012). Other genes with increased methylation in the high-risk group were also previously reported as hypermethylated in cancer, such as IQSEC1 (Dmitriev et al. 2012). The dual NADPH oxidases were proposed as potential targets for cancer therapy because their overexpression in many tissues is associated with pre-malignant conditions (Roy et al. 2015). However, in the current work, increased methylation levels were found in CpGs associated with the dual oxidases DUOX/DUOX1, similar to that observed in lung cancer (Luxen et al. 2008). On the other hand, genes associated with lipid metabolism and obesity features were observed such as ELOVL3 (Zadra vec et al. 2010). Thus, the DNA methylation of the identified genes could represent an epigenetic signature that could be relevant in the personalized management of the breast cancer associated to obesity.

Additionally, we selected RASSF1 and ZNF577 for further validation in an independent cohort among the identified differentially methylated gene signatures of high-risk breast cancer depending on adiposity and menopausal state. RASSF1 a gene characteristically methylated in breast cancer (Conway et al. 2014) and was represented by 5 CpG sites in the current work and ZNF577 was the most represented gene in the differential methylated profile of high-risk group with the highest statistical differences between the groups. According to previous work, RASSF1 exhibited high methylation levels in tumor breast than non-tumor breast tissue. However, the differential methylation pattern in tumor breast tissue according to adiposity and menopausal state was not validated in our independent cohort. Importantly, in this study, increased methylation of ZNF577 was demonstrated for the first time in breast tumors but not in non-malignant breast tissue. Moreover, the methylation profile of ZNF577 observed in obesity-related postmenopausal breast tumors was observed in an independent cohort. Accordingly, the ZNF577 CpG island was previously found to be methylated in 85% to 100% of squamous cell carcinomas of the lung (Rauch et al. 2012), and in polycythemia vera, aberrant methylation of ZNF577 was correlated with differential expression of the ZNF577 gene (Barrio et al. 2011). Likewise, a previous
Zinc finger proteins regulate gene transcription. Although the functional role of ZNF577 is currently unknown, individual ZNFs, and even some clusters of ZNFs, are often hypermethylated and silenced in different types of tumors, suggesting that the current findings represent a commonly disrupted epigenetic pathway in cancer progression. For example, such a disrupted epigenetic mechanism was found in a model of arsenite-transformed prostate epithelia (Severson et al. 2013). Several studies have identified individual ZNFs as potential tumor suppressors that regulate cellular proliferation by inhibiting MAPK signaling and repressing various oncogenes (Severson et al. 2013). Previous research suggests that oxidative stress could be a possible mechanism by which ZNFs are hypermethylated during malignant transformation (Severson et al. 2013) because oxidative stress disrupts the CTCF/PARP-1 interaction, leading to increases in DNA methylation and other repressive epigenetic marks at corresponding genomic loci (Severson et al. 2013). Similarly, the impact of obesity on the epigenetic regulation of breast tumors could be mediated by oxidative stress (Crujeiras et al. 2013). However, additional studies are required to understand the functional role of ZNF577 in breast cancer biology as well as its utility in the personalized treatment of obese patients with breast cancer. In this line, the ZNF132, also identified in the current work as differentially methylated, was discovered among the epigenetic markers of an epi-subtype of the Luminal-B subtype of breast cancer (Stefansson et al. 2015). These results evidenced a potential role of the ZNFs for identifying tumor subtypes regardless of their specific function on carcinogenesis. Relevantly, the differential pattern of ZNF577 methylation levels found in cell lines from different breast cancer subtypes is in agreement with a potential role of ZNF577 in the heterogeneity of these tumors that could determine the response to anticancer therapies.

The strength and novelty of the current work are represented by the epigenome-wide analysis of a large discovery cohort that included data on BMI and menopausal state and the validation of the identified genes in an independent cohort. This study was performed in breast tumor samples; however, the use of FFPE tissues in the validation cohort, in contrast to FF tissues in the discovery cohort, could condition the validation of other epigenetic markers.

In conclusion, the current findings demonstrate the potential of methylation profiles to distinguish subtypes of breast cancer that depend on adiposity and menopausal state. The epigenome of breast tumors is affected by a complex interaction between BMI and menopausal status. Thus, patients with breast cancer associated with the described DNA methylation markers might also receive DNA demethylation agents as co-adjuvant therapy to the standard therapeutic protocol. Although further studies are needed, ZNF577 may be a biomarker of the obesity and menopausal state effect on breast cancer biology and a suitable therapeutic target in precision medicine.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-16-0565.

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.

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Author’s contribution statement
The authors’ responsibilities were as follows-A B C, F F C and M E: Project conception and leadership of overall research plan, writing the first draft of the manuscript and primary responsibility for the final content of the manuscript. A B C, A D L, O A S, J S: Performing the bioinformatics and statistical analysis. A B C and A D L: Bisulfite genomic sequencing and pyrosequencing assessment. M S: Conducting the microarray-based DNA methylation. M C C and A B C: Performing the functional and gene expression analysis. J C, R L-L, J J G, T L, O E: Sampling and clinical data collection. A D L, O A S, J S, M M-G, F J T: Interpretation of the data and critical revision of the manuscript. M E and F F C acquired funding for this study. All authors: Involvement in the writing of the manuscript and approving the final version of this article. None of the authors had a conflict of interest regarding the manuscript.

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