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ORIGINAL ARTICLE

Secreted factors derived from obese visceral adipose tissue regulate the expression of breast malignant transformation genes

AB Crujeiras1,2,8, B Cabia1,2,8, MC Carreira1,2, M Amil1,2, J Cueva1, S Andrade1,2, LM Seoane6,2, M Pardo5,2, A Sueiro1, J Baltar6, T Morais7, MP Monteiro7, R Lopez-Lopez7 and FF Casanueva1,2

BACKGROUND/OBJECTIVES: Obese adipose tissue, especially the visceral depot, exhibits altered production of several molecules that could have a role on the initiation/promotion of breast cancer development. The aim of this work was to evaluate the effect of excess adipose tissue and its secreted factors on the expression of genes involved in the early steps of tumor promotion on the mammary gland.

SUBJECTS AND METHODS: Carcinogenesis-related gene expression was evaluated in mammary gland tissue from female diet-induced obese (DIO) Sprague-Dawley rats and circulating leukocytes isolated from a group of breast cancer diagnosed and non-diagnosed obese women and compared with their normal weight counterparts. In addition, the human non-tumoral mammary epithelial cell line MCF10A was treated in vitro with the visceral (retroperitoneal adipose tissue (RPAT)) or subcutaneous adipose tissue (SAT) secretome and with rising concentrations of the lipid peroxidation by-product 4-hydroxynonenal (4-HNE).

RESULTS: DIO rats were classified as susceptible to DIO (DIO-S) or partially resistant to DIO (DIO-R) according to the maximum fat mass gain of the lean group as a cut-off. As compared with lean and DIO-R, the DIO-S group showed a higher fat mass and lower lean mass. The anatomical characteristic of DIO-S was correlated with differential expression of cellular proliferation (ALDH3A1 and MYC) and antioxidant and DNA protection (GSTM2, SIRT1), and tumor suppression (TP53, PTEN, TGFβ1) genes. Remarkably, this carcinogenesis-related gene expression pattern was reproduced in MCF10A treated with the RPAT secretome from DIO-S rats and with the lipid peroxidation by-product 4-HNE. Moreover, this pattern was also detected in leukocytes from obese women compared with normal weight women without evidence of breast cancer.

CONCLUSIONS: Lipid peroxides secreted by the obese visceral adipose tissue could be among the relevant factors that promote changes involved in the early steps of tumor development in mammary gland. These changes can be detected even before histological alterations and in circulating leukocytes.

International Journal of Obesity accepted article preview 7 October 2015; doi:10.1038/ijo.2015.208

INTRODUCTION

Obesity has become a major health problem in recent years with 1.46 billion overweight and 502 million obese adults worldwide.1 Cancer has become well recognized among the comorbidities associated with this increase in the obesity rates.2,3 Breast cancer is one of the most common tumors among women in both the United States and Europe and is a leading cause of death in women. Obesity is a known risk factor for breast cancer,2,4,5 especially for postmenopausal breast.6 Furthermore, the prognosis is poorer for obese premenopausal and postmenopausal women following breast cancer diagnosis.7–9 However, despite the available epidemiological data, the mechanisms that underlie the association between obesity and breast cancer risk are not fully understood to date.

A number of hypotheses have been proposed to explain this link,2,3 including the high production of several factors, such as adipokines and inflammatory cytokines, by excess adipose tissue in the obese individuals.5,10,11 In obesity, adipose tissue becomes dysfunctional, which results in metabolic and inflammatory changes that increase oxidative stress and the production of reactive oxygen species. These species are carcinogenic, as they lead to increased DNA damage.12–14 In fact, the role of adipocytes as a major constituent of the tumor stroma has recently been examined in the context of breast cancer development.
Specifically, the paracrine effect of dysfunctional obese adipose tissue-related factors has been examined.\textsuperscript{13,16} These factors secreted from obese adipose tissue could act in a paracrine manner to regulate malignant transformation and cancer progression in the most susceptible tissues, such as the breast.

Another proposed mechanism favoring cancer initiation and/or progression in obese patients is the effect of the chronic hyperinsulinemia induced by the insulin resistance in obesity.\textsuperscript{17,18} However, the clinical relevance of this pro-cancer effect of insulin in diabetic/obese patients is still unclear.\textsuperscript{18} Of note, experimental evidences suggest that the consequent hyperinsulinemia triggered by the insulin resistance aggravates the increased oxidative stress promoted early in the accumulated fat of obese individuals.\textsuperscript{13}

Cancer is a complex disease with several differentiated phases, that is, initiation, promotion and progression. The expression levels of proto-oncogenes (activation) and tumor-suppressor genes (inactivation) change during the initiation phase.\textsuperscript{19} The expression levels of genes belonging to both groups have been determined to be altered in breast cancer.\textsuperscript{20} We hypothesized that obesity-related factors may regulate the expression of representative genes involved in proliferation and known to be altered in breast cancer.\textsuperscript{21–23} as well as genes that protect from oxidative stress,\textsuperscript{29} DNA damage and tumor suppression\textsuperscript{24–26} to create a microenvironment that permits malignant transformation. This effect could be detected even in the absence of a macroscopical tumor challenge.

Thus, the objective of this study was to evaluate the association between excess body adiposity and the regulation of genes involved in the early steps of tumor promotion by focusing on mammary gland tissue. Furthermore, this study examined the effect of oxidative stress generated by adipose tissue in obesity, particularly lipid peroxidation by-products, on breast cancer susceptibility in obese individuals.

MATERIALS AND METHODS

Animals

Three-week-old female Sprague–Dawley rats were kept in cages in air-conditioned rooms (22–24°C) under a controlled (12:12 h) light–dark cycle. After a 3-day acclimation period, the rats were randomized into a standard and obese female individuals (diabetic/obese patients is still unclear.\textsuperscript{18} Of note, experimental evidences suggest that the consequent hyperinsulinemia triggered by the insulin resistance aggravates the increased oxidative stress promoted early in the accumulated fat of obese individuals.\textsuperscript{13}

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At the end of the study, the animals were killed by decapitation. The trunk blood was collected and immediately centrifuged at 3500 r.p.m. and 4°C for 15 min, and the plasma was stored at −80°C for biochemical measurements. The tissues were isolated, weighed and stored. The mammary gland tissue was fixed in 10% buffered formalin for 24 h and embedded in paraffin following routine procedures. A fraction of the subcutaneous and retroperitoneal adipose tissue (RPAT) was frozen on dry ice and stored at −80°C, whereas the other fraction was cultured with the corresponding medium for the secretome analysis. The experimental protocol was approved by the Animal Care Committee of Santiago de Compostela University (USC) in accordance with the European Union standards for the care and use of experimental animals.

Patients and blood samples

A group of healthy normal weight (n = 10; 28.2 ± 3.5 years, 21.4 ± 1.6 kg m−2) and obese female individuals (n = 8; 40.0 ± 13.7 years; 38.8 ± 2.8 kg m−2) were recruited for our study. All participants were in apparent good health, as determined by their medical history, physical examination, and routine biochemical and hematologic laboratory tests. The participants reported not to have used supplemental vitamins, minerals or regular prescription medications during the previous 3 months. In addition, female normal weight (n = 33; 49.5 ± 9.4 years, 23.4 ± 1.9 kg m−2) and obese (n = 48; 59.0 ± 10.7 years, 31.6 ± 4.1 kg m−2) patients diagnosed with breast cancer were recruited at the Clinic Hospital of Santiago de Compostela.

Blood samples were obtained after overnight fasting, and the plasma was separated by centrifugation at 3000 r.p.m. and 4°C for 15 min. The plasma samples were then frozen at −80°C for subsequent analyses. The peripheral blood mononuclear cells were isolated by differential centrifugation using Polytron preps (Axis Shield PoC AS, Oslo, Norway). The cell pellet was re-suspended in Trizol reagent (Invitrogen, Carlsbad, CA, USA) and immediately frozen at −80°C until the RNA was extracted.\textsuperscript{30} All participants provided informed consent, and this consent and the study protocol were approved by the Institutional Review Boards of the participating institution (comité ético de investigación clínica (CEIC) de Galicia (Ref2009/076).

Chemicals and cell lines

The human mammary epithelial cell line MCF10A was generously provided by Dr Manel Esteller from the Instituto de Investigacion de Bellvitge (IDIBELL, Barcelona, Spain). The cells were maintained in Dulbecco’s modified Eagle’s medium (F12 medium supplemented with 5% fetal bovine serum (Lanza, Iberica S.A., Barcelona, Spain), 10 μg ml−1 insulin (Sigma Aldrich, St Louis, MO, USA), 100 ng ml−1 cholaer toxin (Sigma), 0.5 μg ml−1 hydrocortisone (Sigma) and 20 ng ml−1 epidermal growth factor (Sigma). The rat mammary tumor cell line LA7 (no. CRL-2283), obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), was cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 20 μg HEPES, 50 μg hydrocortisone and 5 μg ml−1 insulin. The cells were maintained in medium supplemented with 100 μl−1 penicillin and 100 μg ml−1 streptomycin (Lonza) in 5% CO2 humidified atmosphere at 37°C. The reagent 4-hydroxyxoroneal (4-HNE) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Adipose tissues and secretomes processing

The rat and human subcutaneous adipose tissue from the skin of the hips (subcutaneous adipose tissue (SAT)) and the visceral fat located inside the peritoneal cavity around the internal organs (RPAT), as well as the rat muscle tissues were processed for secretome collection based on a previously optimized protocol.\textsuperscript{31} The secretomes were immediately aliquoted and frozen at −80°C for subsequent analysis (details are available in the Supplementary Methods).

Biochemical measurements

The lipid peroxide concentrations were determined in the rat secretomes from subcutaneous and visceral adipose tissue, as well as in plasma samples from obese women. The formation of the lipid peroxidation by-products malondialdehyde (MDA) and 4-HNE were evaluated using a commercially available colorimetric assay kit (Biotech LPO-586 assay; OXIS International, Portland, OR, USA), and the colorimetric reactions were read at corresponding wavelengths (Versamax ELISA Microplate Reader, Molecular Devices, CA, USA).

RNA isolation and quantitative real-time PCR

The RNA from formalin-fixed, paraffin-embedded mammary gland was extracted using the RNeasy FFPE Kit (Qiagen Iberia SL, Madrid, Spain) according to a modified protocol.\textsuperscript{32} The postgenomic and complementary DNA (cDNA) was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with 2 μg of total RNA.
Quantitative real-time PCR was performed using TaqMan Universal PCR Master Mix, TaqMan Probes (Applied Biosystems) and the Step OnePlus Real-Time PCR System (Applied Biosystems). All experiments were performed in duplicate, and the gene expression levels were normalized with the housekeeping genes ACTB for the animal model breast tissue and GAPDH for the cell lines and women leukocytes. The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ relative quantitation method according to the manufacturer’s guidelines (Applied Biosystems), and data are reported as the geometric mean (s.e.m.).

Cell proliferation assay

Cell proliferation was estimated using the Cell Proliferation Reagent WST1 (Roche, Basel, Switzerland). Briefly, $5 \times 10^3$ MCF10A or LA7 cells were seeded into a 96-well plate and incubated overnight for attachment. The complete medium was then replaced with serum-free medium to allow for cell cycle synchronization, and treatment was added after 5 h. The cells were incubated with SAT and RPAT adipose tissue secretome from obese (DIO-sensitive (DIO-S)) and lean rats for 24 h at 1% and 5% concentrations or with a varied concentrations of 4-HNE (0.5, 1, 2.5 and 5 μM), vehicle (ethanol) for 48 h. Specific cell line serum-free culture medium was considered as a control. The treatments were performed in quadruplicate in at least three independent experiments and normalized against the control.

Gene expression assays in treated cell lines

For the gene expression assay, MCF10A cells were seeded at $5 \times 10^4$ cells per well in six-well plates and incubated overnight. The total medium was then replaced with serum-free medium for 5 h to allow for cell cycle synchronization. The cells were treated with the secretomes from the SAT and RPAT adipose tissue of lean or obese rats at a concentration of 1% and then incubated for 24 h or they were incubated in six-well plates with 1, 2.5 and 5 μM of 4-HNE, vehicle (ethanol) for 48 h. Specific cell line serum-free culture media was considered as a control. The expression levels of the target genes were normalized to the expression levels of the housekeeping gene GAPDH (TaqMan, Applied Biosystems) and expressed relative to the average value of the control.

Western blotting

For the immunoblot assay, one proliferative/oncogene and one tumor suppressor gene were selected from the studied genes taking into account their consistent obesity-related regulation in overall experiments, in vivo and in vitro. Thus, after secretome or 4-HNE treatment for 24 or 48 h, respectively, MCF10A cells were processed according to previous reports. Primary anti-PTEN and anti-ALDH3A1 antibodies were purchased from Santa Cruz (Paso Robles, CA, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Life Technologies Ltd (Paisley, UK). The protein expression was analyzed using the ImageJ Software (National Institutes of Health, Bethesda, MD, USA) and normalized against the GAPDH loading control (arbitrary units).

Statistical analysis

The statistical analysis was performed using the SPSS version 17.0 software (SPSS, Chicago, IL, USA) for Windows XP (Microsoft, Redmond, WA, USA). The sample size for the animals and patients studies was estimated after accounting for the differences in body weight (main variable) and was calculated for an α = 0.05 and a power of 80%. Thus, to detect differences, the sample size was established at a minimum of eight individuals per group. The normal distribution was explored through the Kolmogorov–Smirnov and Shapiro–Wilks tests. Accordingly, differences between the animal model groups were detected by applying a univariate analysis of variance test using the Tukey’s test for post hoc comparisons or Student’s t-test as applicable. Differences between treatments in cell lines were evaluated by means of Mann–Whitney U-test in 4–6 independent experiments. Differences between normal weight and obese patients were detected by means of an analysis of covariance test adjusting for age. The data show the mean (s.e.), and $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Differential response to high-fat diet in Sprague–Dawley rats

As a group, DIO rats gained more weight (343.1 ± 9.5%) and fat mass (44.2 ± 11.4%) with respect to baseline values (T0) after 10 weeks of being fed a high-fat diet than their lean counterparts, which were fed a standard diet (301.1 ± 12.5%; $P < 0.05$ and $-0.80 \pm 0.65$%; $P < 0.001$, respectively). Owing to their sedentary state, the animals experienced an overall loss in lean mass, which was greater in the DIO group ($-13.15 \pm 0.91\%$) than in the lean group ($-8.37 \pm 1.15\%; P < 0.01$). Moreover, the mass of the RPAT was significantly higher in DIO (3.29 ± 0.21 g) than in the lean group (2.22 ± 0.19 g, $P < 0.01$). These differences were independent of food intake because the chow-fed lean group food intake (13.79 ± 0.50 g day$^{-1}$) was significantly higher than that of the DIO group (9.85 ± 0.23 g day$^{-1}$; $P < 0.001$).

Sprague–Dawley rats are usually resistant to DIO. Thus, the DIO group was subdivided into two groups according to the percent fat mass gain with respect to fat mass at baseline (T0: Figure 1a). The classification was performed to consider the maximum value of fat mass gain for the lean group (58.35%). Under this condition, individuals with a fat mass gain higher than this cut-off were classified as susceptible to DIO (DIO-S, mean fat mass gain = 118.1 ± 9.2%), whereas individuals with a fat mass gain ≤ 58.35% were classified as partially resistant to DIO (DIO-R, mean fat mass gain = 33.3 ± 11.3%).

Although the food intake and body weight were similar between both DIO subgroups after 10 weeks of high-fat diet feeding, DIO-S rats presented significant differences in body weight gain with respect to T0 compared with lean rats, whereas DIO-R rats did not exhibit significant changes with respect to the lean group (Table 1). The fat mass and lean mass differed between DIO-R and DIO-S rats. Importantly, the DIO-S group showed a higher fat mass and lower lean mass than the DIO-R and lean groups (Table 1). These differences were also reflected in the higher RPAT weight in the DIO-S with respect to both DIO-R and lean rats (Table 1).

High adiposity regulates the expression of genes related to carcinogenesis in mammary tissue of DIO rats

The mammary gland of DIO-S group showed a significant increase in the expression of genes related to cellular proliferation with higher fold changes in ALDH3A1 and MYC gene expression than the lean group ($P < 0.05$; Figure 1b). Interestingly, these increases in the ALDH3A1 and MYC transcript levels were also significantly higher than those observed in the DIO-R group (Figure 1b). The BIRC5 gene expression did not significantly differ between DIO-S rats and lean rats ($P > 0.05$).

In addition, higher adiposity correlated with the modified transcription of genes related pathways that protect from oncogenesis. Thus, SIRT1, PTEN and TGFB1 were downregulated, whereas GSTM2 and TP53 were upregulated in the DIO-S group with respect to the lean group (Figure 1b). SIRT6 tended to be downregulated, although this difference was not significant.

Remarkably, the expression levels of these genes did not significantly differ between the DIO-R and lean groups, except for SIRT1 and ALDH3A1; both of these genes were downregulated with respect to the lean group (Figure 1b). Therefore, we further studied the effect of factors secreted by adipose tissue on the regulation of carcinogenic genes.

RPAT secretome from DIO-S rats increases the proliferation of the human mammary epithelial cell line MCF10A and modulates the expression of carcinogenesis-related genes

The RPAT secretome from obese rats induced a significant increase in cell proliferation with respect to lean secretome treatment (Figure 2a). However, treatment with the DIO or lean
secretome obtained from the SAT or soleus and plantaris muscle did not induce significant differences in cell proliferation (Figure 2a). These results were consistent for secretome concentrations of 1% and 5% (data not shown). Regarding human adipose tissue secretome, although no effect was observed when cells were exposed to 1% secretome from the patients RPAT, the

Figure 1. Differential expression profile of carcinogenesis-related genes in the mammary gland of rats feed a high-fat diet for 10 weeks. (a) The experimental protocol consisted on feeding 3-week-old female Sprague–Dawley rats with either a high-fat diet or chow diet for 10 weeks. DIO group was subdivided into two groups (DIO-S or DIO-R) according to the maximum value of fat mass gain for the lean group. (b) Mammary gland gene expression analysis of a number of genes involved in early steps of breast carcinogenesis, such as proliferation and oncogenesis, antioxidant protection, DNA damage and tumor suppression. The gene expression levels were normalized with the housekeeping gene ACTB. The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method of relative quantification above the expression levels of lean group and data are reported as the geometric mean (s.e.m.). Statistical differences were evaluated by Student’s t-test (*P < 0.05 vs lean; **P < 0.05 vs lean; #P < 0.05 vs DIO-R).

Table 1. Differences in body weight and body composition between lean, DIO-R or DIO-S rats

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<th>Lean (n=11)</th>
<th>DIO-R (n=8)</th>
<th>DIO-S (n=12)</th>
<th>ANOVA P-value</th>
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<td>Food intake (g day$^{-1}$)</td>
<td>13.79 ± 0.50</td>
<td>9.12 ± 0.39$^*$</td>
<td>9.85 ± 0.23$^*$</td>
<td>P &lt; 0.01</td>
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<td>Body weight T10 (g)</td>
<td>228.4 ± 4.7</td>
<td>257.5 ± 5.5$^*$</td>
<td>238.2 ± 4.5</td>
<td>P = 0.003</td>
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<td>Fat mass T10 (g)</td>
<td>15.59 ± 1.71</td>
<td>24.52 ± 2.01$^*$</td>
<td>32.47 ± 1.64$^*$</td>
<td>P &lt; 0.001</td>
</tr>
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<td>Lean T10 (% of body weight)</td>
<td>6.76 ± 0.64</td>
<td>9.58 ± 0.75$^*$</td>
<td>13.61 ± 0.62$^*$</td>
<td>P &lt; 0.001</td>
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<tr>
<td>Lean T10 (g)</td>
<td>172.2 ± 3.3</td>
<td>185.7 ± 3.9$^*$</td>
<td>166.1 ± 3.2$^*$</td>
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<td>Lean T10 (% of body weight)</td>
<td>74.94 ± 0.82</td>
<td>72.10 ± 0.96</td>
<td>70.21 ± 0.80$^*$</td>
<td>P = 0.001</td>
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<tr>
<td>Body weight gain (% respect to T0)</td>
<td>301.1 ± 12.5</td>
<td>339.7 ± 14.7</td>
<td>345.4 ± 12.0$^*$</td>
<td>P = 0.039</td>
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<td>Fat mass gain (% respect to T0)</td>
<td>−0.80 ± 9.65</td>
<td>33.53 ± 11.30</td>
<td>118.1 ± 9.2$^*$</td>
<td>P &lt; 0.001</td>
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<td>Lean gain (% respect to T0)</td>
<td>−8.37 ± 1.15</td>
<td>−11.45 ± 1.36</td>
<td>−14.29 ± 1.11$^*$</td>
<td>P &lt; 0.001</td>
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<td>RPAT (g)</td>
<td>2.22 ± 0.21</td>
<td>2.72 ± 0.24</td>
<td>3.71 ± 0.21$^*$</td>
<td>P &lt; 0.001</td>
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<td>BAT (g)</td>
<td>0.33 ± 0.03</td>
<td>0.40 ± 0.03</td>
<td>0.44 ± 0.03$^*$</td>
<td>P = 0.047</td>
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<td>Liver (g)</td>
<td>7.27 ± 0.22</td>
<td>7.62 ± 0.25</td>
<td>6.91 ± 0.21</td>
<td>P = 0.110</td>
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<td>Spleen (g)</td>
<td>0.77 ± 0.03</td>
<td>0.76 ± 0.04</td>
<td>0.66 ± 0.03$^*$</td>
<td>P = 0.026</td>
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<td>Kidney (g)</td>
<td>1.60 ± 0.04</td>
<td>1.73 ± 0.05</td>
<td>1.61 ± 0.04</td>
<td>P = 0.070</td>
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Abbreviations: ANOVA, analysis of variance; BAT, brown adipose tissue; DIO-R, diet-induced obesity-resistant SD rats; DIO-S, diet-induced obesity-sensitive SD rats; Lean, chow-fed SD rats; SD, Sprague–Dawley; RPAT, retroperitoneal adipose tissue. Significant differences were analyzed by ANOVA test using the Tukey’s test for post hoc comparisons. *P < 0.05 vs lean; **P < 0.05 vs lean; #P < 0.05 vs DIO-R.
results from the animal model were reproduced when the MCF10A cells were exposed for 24 h to 5% secretome obtained from lean, DIO-R and DIO-S rat adipose tissue (RPAT and SAT) and muscle (soleus and plantaris as secretome control) at 1% of concentration for 24 h. (b) Cell proliferation analysis of the MCF10A after treatment with RPAT and SAT secretome from lean or obese patients at 1% and 5% of concentration for 24 h. (c) Gene expression analysis of MCF10A cells after SAT secretome treatment at 1% for 24 h from lean or obese (DIO-S) rats. (d) Gene expression analysis of MCF10A cells after RPAT secretome treatment at 1% for 24 h from lean or obese (DIO-S) rats. The expression of ALDH3A1 was markedly upregulated after the treatment of MCF10A cells with the RPAT secretome from lean and DIO-S rats compared with treatment with the RPAT lean secretome (P = 0.03; Figure 2d). Conversely, treatment with the RPAT secretome from obese rats downregulated the antioxidant gene SIRT1 (P = 0.001). In addition, genes related to tumor suppression, PTEN and TGFβ, were also downregulated in cells treated with the RPAT secretome from lean rats (P < 0.05).

The observed changes in the transcript levels were also observed in the protein expression of two of the selected genes

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The ALDH3A1 and PTEN levels did not differ between SAT treatments (Figure 2e). Interestingly, ALDH3A1 tended to be upregulated and PTEN tended to be downregulated in MCF10A cells treated with obese RPAT secretome compared with cells treated with lean RPAT secretome, although these differences were not significant (Figure 2f).

Lipid peroxidation by-products induces proliferation and modulates the expression of carcinogenesis-related genes in MCF10A cells

As predicted by the working hypothesis and according to previous reports,36,37 the level of lipid peroxidation by-products (malondialdehyde and 4-HNE) was significantly higher in the RPAT secretome obtained from obese DIO-S rats than in that obtained from the lean and DIO-R rats (Figure 3a). The SAT secretome did not show differences between groups. Moreover, an elevation in lipid peroxidation by-products in the plasma from obese patients was found, and this difference was significant with respect to normal weight subjects (Figure 3a).

Treating MCF10A cells with 0.5, 1, 2.5 and 5 μM 4-HNE, one of the most extensively studied lipid peroxidation by-products, for 48 h produced dose-dependent increases in proliferation (Figure 3b). Moreover, treatment with 5 μM 4-HNE for 48 h increased the expression of the proliferative genes BIRC5 (1.99 ± 0.06, \( P < 0.05 \)) and ALDH3A1 (1.80 ± 0.06, \( P < 0.05 \)) and decreased the expression of the antioxidant gene GSTM2 (0.40 ± 0.10, \( P < 0.05 \)) and the tumor-suppressor genes SIRT6 (0.87 ± 0.03, \( P < 0.05 \)), PTEN (0.68 ± 0.08, \( P < 0.05 \)) and TGFB (0.91 ± 0.00; \( P < 0.05 \)), respectively, were downregulated (Figure 3c).

Interestingly, the protein expression of ALDH3A1 increased, and this difference reached significance for concentrations of 2.5 and 5 μM (Figure 3d); the PTEN protein levels decreased in a dose-dependent manner, and these differences were significant for 4-HNE concentrations 1, 2.5 and, especially, 5 μM (Figure 3e).

RPAT secretome from DIO-S or 4-HNE induces proliferation of the rat breast cancer cell line LA7

To delineate the possible role that products generated by the obese adipose tissue could have in cancer cell proliferation, the LA7 rat breast cancer cell line was exposed to 1% secretome obtained from lean or obese (DIO-S) rat SAT or RPAT for 24 h (Figure 4a). The obese secretome from SAT tended to produce a decrease in cell proliferation with respect to the lean secretome, although this difference was not significant (Figure 4a). On the contrary, the proliferation of LA7 cells exposed to obese RPAT

(\textit{ALDH3A1} and \textit{PTEN}). The \textit{ALDH3A1} and \textit{PTEN} levels did not differ between SAT treatments (Figure 2e). Interestingly, \textit{ALDH3A1} tended to be upregulated and \textit{PTEN} tended to be downregulated in MCF10A cells treated with obese RPAT secretome compared with cells treated with lean RPAT secretome, although these differences were not significant (Figure 2f).
A concentration of 5 μtissue. According to previous hypotheses, this work demonstrates for the first time that excess adipose tissue occurs concomitantly with a dysregulation of genes involved in cancer development mechanisms, such as cellular proliferation, antioxidant protection and tumor suppression. This effect was observed before the manifestation of a detectable tumor mass in breast cancer patients. According to previous hypotheses, this work demonstrates that products secreted by obese adipose tissue, especially the visceral depot, can induce the proliferation of breast epithelial cells, generating a microenvironment that alters the expression of carcinogenesis driver genes. This effect could be attributed to lipid peroxidation, which is increased in obesity, as demonstrated by the induction of cell proliferation and gene expression regulation in a similar manner to that observed in the DIO animal model.

The findings of this study were first observed in a model of DIO in Sprague-Dawley rats because this model has previously been established to study obesity and increased adipose tissue because of its similarity to the symptoms observed in humans. Usually, DIO rats are subdivided into sensitive or resistant to obesity according to body weight gain after high-fat diet consumption. In this study, we focused on fat mass gain as a cut-off point, which was measured with an Echo Magnetic Resonance Imaging system. This approach is the gold standard to study the effect of excess fat mass and was used to classify the DIO group as DIO-S and DIO-R. This subclassification of DIO rats enables the effects of high-fat diet versus excess adiposity per se to be discerned. In fact, body composition differences were observed between both DIO groups, despite the same feeding protocol.

Under these conditions, the upregulation of ALDH3A1 and MYC, genes elevated in breast cancer7,12, suggest that the mitogenic capacity of cells from the mammary gland of DIO-S rats could be occurring. ALDH3A1 is also involved on the detoxification of lipid peroxidation by-products,9 thus, its upregulation may indicate increased oxidative stress and DNA damage in DIO-S mammary tissue. Supporting this hypothesis, the gene expression of the antioxidant enzyme GSTM225 and the expression of the DNA repair protein TP5346 were also increased. In addition, the potential increase in oxidative stress associated to obesity could be responsible for the downregulation of the SIRT1, PTEN and TGFβ gene expression levels in our model. These genes are tumor suppressors, they can be regulated by reactive oxygen species41,42 and are downregulated in breast cancer41 or involved in the defense against the first steps of neoplastic change.27,43

As a high-fat diet has previously been described to be able to induce carcinogenesis,44 the results observed in DIO rats could be due to the effect of a high-fat diet. However, the gene expression profile observed in DIO-S animals was significantly different from those of DIO-R and lean rats for most of the studied genes, whereas very few differences were observed between the DIO-R and lean group. Therefore, the regulation of the carcinogenesis-related genes appears to be induced by obesity per se, rather than by a high-fat diet. Interestingly, the low adipose tissue content observed in DIO-R rats was accompanied by higher lean mass levels than those in DIO-S rats. Recent studies show that higher levels of lean (muscle) mass in obese individuals correlate with a better metabolic profile.45 Skeletal muscle is an organ that...
produces molecules known as myokines, which have been proposed to protect from illnesses associated with physical inactivity, and the inhibitory effect of exercise on mammary cancer cell proliferation may be partly mediated by some of these molecules. These findings indicate that factors secreted by dysfunctional obese adipose tissue could have carcinogenic properties. The enhancement in the mitogenic capacity of breast cells induced by the obese RPAT secretome correlated with the differential regulation of studied genes, reinforce this hypothesis. These effects were not observed when cells were treated with SAT neither muscle secretome, suggesting that the susceptibility to breast cancer promoted by obesity is mediated by factors secreted by the visceral adipose tissue depot, the most prejudicial one regarding pathophysiological alterations associated with obesity.

Adipose tissue in obese individuals generates an altered secretion of diverse molecules, leading to metabolic and inflammatory changes that increase oxidative stress over the long term, which has been proposed to be the link between obesity and its comorbidities. Indeed, lipid peroxidation is increased in breast cancer, and these levels decrease after a dietary treatment to lose weight. In keeping with this, a proliferative effect of 4-HNE on the mammary epithelial cell line has been observed in the current work. Therefore, although other factors associated with dysfunctional adipose tissue should not be discarded, the oxidative stress generated during obesity could be a relevant factor involved in the alteration of mechanisms that protect from abnormal cell proliferation and thus have a role in the protection against the first steps of tumor formation.

Strikingly, the regulation of the studied genes associated with carcinogenesis observed in the animal model was reproduced in leukocytes from obese women. Both tumor transformation promoter and tumor protective pathways were upregulated in obese women without evidence of breast cancer. In contrast, only genes related to cellular proliferation and oncogenes were upregulated in obese female breast cancer patients with respect to normal weight breast cancer patients. Moreover, antioxidant protection and tumor suppression genes were downregulated. These results suggest that a compensatory response could be occurring in tumor-free obese women to counteract the cellular damage induced by obesity-related factors. When these mechanisms are overcome, tumoral transformation ensues.

In conclusion, the current findings demonstrate for the first time that obesity may promote a dysregulation of carcinogenesis-related genes, even before the presence of a tumor lesion. This effect does not depend on high-fat diet but appears to be triggered by factors secreted by visceral adipose tissue depots, and lipid peroxidation products could be among these adipose tissue-secreted carcinogenic factors. These results indicate that long-term obesity could trigger the development of breast cancer. Importantly, this potential susceptibility to carcinogenic transformation associated with obesity can be detected in peripheral blood cells instead of tissue biopsies. Further studies are needed to confirm these findings.

Figure 5. Expression of carcinogenesis-related genes in leukocytes from normal weight and obese women with and without breast cancer. Gene expression analysis was carried out in leukocytes from normal weight or obese patients both tumor-free (a) or diagnosed with breast cancer (b). The gene expression levels were normalized with the housekeeping gene GAPDH. The fold change in gene expression was calculated using the 2^−ΔΔCt method of relative quantification above the expression levels of normal weight group and data are reported as the geometric mean (s.e.m.). Statistical differences were evaluated by analysis of covariance (ANCOVA) adjusted by age (*P < 0.05 vs lean; #P < 0.10 vs lean).
warranted to evaluate whether the susceptibility to breast cancer transformation triggered by obesity can be reverted and prevented by therapy to reduce adiposity.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This work was supported by grants from the Fondo de Investigacion Sanitaria, INTRASALUD programme (PI10/02464), PI14/01012 research projects, and CIBERobn (CB06/003), Instituto de Salud Carlos III (ISCIII), and the Health Department of the Government Xunta de Galicia (GR2014/034), Spain as well as Fundacion Lilly and Fundacion Mapfre. B Cabia was funded by a Santiago de Compostela University (USC)-Campus Vida predoctoral contract (ref. 011–020). AB Crujeiras was funded by the ISCIII through a research contract ‘Sara Borell’ (C03/00363). We thank Patricia Vivaño for their excellent technical support in develop the hepatoxinol and eosin staining and Dr Tomas Garcia-Caballero for his advise and encouragement in this study, as well as the patients who voluntarily took part in this study.

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Supplementary Information accompanies this paper on International Journal of Obesity website (http://www.nature.com/ijo)